Detergents profoundly affect inhibitor potencies against both cyclo-oxygenase isoforms

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The sensitivity of Coxs (cyclo-oxygenases) to inhibition is known to be highly dependent on assay conditions. In the present study, the inhibitor sensitivities of purified Cox-1 and -2 were determined in a colorimetric assay using the reducing agent *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. With the detergent genapol X-100 (2 mM) present, the potencies of nimesulide, ibuprofen, flufenamic acid, niflumic acid and naproxen were increased over 100-fold against Cox-2 and titration curve shapes changed, so that maximal inhibition now approached 100 %. Indomethacin, diclofenac and flosulide were not changed in potency. Similar effects of genapol were observed with inhibitors of Cox-1. DuP-697 and two analogues became more than 10-fold less potent against Cox-2 with genapol present. Tween-20, Triton X-100 and phosphatidylcholine, but not octylglucoside, gave qualitatively similar effects as genapol. Similar detergent-dependent changes in inhibitor potency were also observed using a [^{14}C]arachidonic acid HPLC assay. The increases in potency of ibuprofen, flufenamic acid, isoxicam and niflumic acid towards Cox-2 and ibuprofen towards Cox-1 were accompanied by a change from time-independent to time-dependent inhibition. The interactions of Cox inhibitors has been described in terms of multiple binding step mechanisms. The genapol-dependent increase in inhibitor potency for ketoprofen was associated with an increase in the rate constant for the conversion of the initial enzyme–inhibitor complex to a second, more tightly bound form. The loss of potency for some inhibitors is probably due to inhibitor partitioning into detergent micelles. The present study identifies detergents as another factor that must be considered when determining inhibitor potencies against both Cox isoforms.

Key words: cyclo-oxygenase, detergent, genapol X-100, non-steroidal anti-inflammatory drug (NSAID), prostaglandin H synthase.

INTRODUCTION

Coxs (cyclo-oxygenases) catalyse both the oxygenation of arachidonic acid to form PG (prostaglandin) G_2 (oxygenase reaction) and its subsequent reduction to PGH_2 (peroxidase reaction). PGH_2 is the common precursor to other PGs, thromboxane and prostacyclin, these reactions being catalysed by cell-specific PG isomerases [1–3].

Interest in the scientific community on Cox research was renewed with the discovery of a second isoform called Cox-2. In contrast to the originally discovered constitutively expressed Cox-1, which plays a housekeeping role in maintaining homoeostasis, Cox-2 is induced in certain tissues by a number of stimuli and plays an important role in inflammation, pain and fever [4,5]. Classical NSAIDs (non-steroidal anti-inflammatory drugs) such as indomethacin and flurbiprofen, which are used to alleviate pain, inflammation and fever, inhibit both Cox-1 and Cox-2 with approximately equal potencies. However, inhibition of Cox-1 causes the unwanted gastric side effects common to these agents. The new selective Cox-2 inhibitors have similar anti-inflammatory activities to non-selective Cox inhibitors, but are gastric sparing [6,7].

The search for novel selective Cox-2 inhibitors has, at least initially, involved screening large numbers of compounds against both Cox-1 and Cox-2. In general, either microsomal or purified enzymes have been employed, in part because they are more amenable to a high-throughput type of assay, as compared with cell-based Cox assays. However, IC_{50} values obtained using isolated enzymes for a given compound have often differed widely between laboratories [8]. In addition, inhibitory potencies in whole-cell Cox assays are generally >10-fold higher than in isolated enzyme systems. A number of factors that can affect NSAID inhibitory potencies have been identified, including peroxide concentration [9–12], identity of reducing agent co-substrate [13], substrate concentration and the length of preincubation time between enzyme and inhibitor. The latter factor is due to the time-dependent nature of many, but not all, Cox inhibitors [3,14].

Isolated Cox enzyme activity has been measured directly by PG formation (generally PGE_2, which is the major non-enzymic decomposition product of PGH_2) via immunoassay or HPLC with a radiolabelled substrate, or by oxygen uptake. The technique most applicable to a high-throughput type of assay is to follow co-substrate oxidation during the peroxidase reaction (reduction of PGG_2 to PGH_2), using a chromogenic or fluorogenic reducing agent [15,16]. Although this is an indirect method, the oxidation of the peroxidase co-substrate TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine) to form a blue compound (ε_{610} 14 000 $M^{-1} \cdot cm^{-1}$) has been shown to accurately reflect the rate of conversion of arachidonic acid to PGH_2 [17,18].

In another report on this coupled colorimetric TMPD assay using purified Cox [19], the authors used a 96-well plate format and a reaction buffer containing Tween-20. We adapted this assay for our own use and changed the detergent to genapol X-100. However, when validating this assay using Cox-2 we noticed large differences in the potencies of a number of inhibitors compared with other assays being used. These differences in Cox-2 inhibitor potencies were traced to the presence of genapol, although further investigation showed that this was not a genapol-specific

Abbreviations used: Cox, cyclo-oxygenase; PG, prostaglandin; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; cmc, critical micelle concentration; NSAID, non-steroidal anti-inflammatory drug.

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phenomenon. This report describes how the presence of detergents or phospholipid can alter the inhibitor sensitivities of both purified Cox-1 and Cox-2. The source of this effect for many of the inhibitors tested appears to be due to an interaction of detergent monomers with the Cox protein leading in some cases to a change in the kinetic constants for inhibitor binding, resulting in an increase in potency. For a smaller number of inhibitors, a loss of potency was observed that can be attributed to the partition of the inhibitors into detergent micelles. These effects have, in some cases, been noted in the literature before, but they have not been the subject of a systematic investigation to uncover the mechanism.

MATERIALS AND METHODS

Materials

Arachidonic acid (peroxide-free), ibuprofen, indomethacin, naproxen and ovine Cox-1 were purchased from Cayman Chemical Co. Meclofenamic acid, niflumic acid, flufenamic acid, ketoprofen, carprofen, isoxicam, piroxicam, zomepirac and TMPD were purchased from Sigma. [^{14}C]Arachidonic acid was from NEN-DuPont and [^{3}H]*RS*-ibuprofen was from American Radiolabelled Chemicals. Genapol X-100 (protein grade) was from Calbiochem and Triton X-100 and Tween-20 were from Pierce. The Medicinal Chemistry Department at Merck Frosst Canada synthesized all other inhibitors used in this study. Recombinant human Cox-1 and Cox-2 were expressed in Sf9 cells and purified as described previously [20–22].

TMPD spectrophotometric Cox assay

The enzymic activity of the purified Cox was measured using a chromogenic assay based on the oxidation of TMPD during the reduction of PGG_2 to PGH_2 [19] in 96-well plates (Nunc, Maxisorp, catalogue no. 439454). The assay mixture (180 μl) contained 100 mM sodium phosphate, pH 6.5, 1 μM haematin, 1 mg/ml gelatin and approx. 40 nM purified Cox-1 or 20 nM purified Cox-2. The assay was also performed in the presence of the detergent genapol X-100 at a final concentration of 2 mM. For assays in the absence of genapol, the arachidonic acid and TMPD solution was prepared in 50 % aqueous ethanol. The mixture was preincubated at room temperature (22 °C) with test compound in DMSO (4 μl) for 15 min before initiation of the enzymic reaction by the addition of 20 μl of a solution of 1 mM arachidonic acid and 1 mM TMPD in assay buffer (without enzyme or haematin). For assays performed in the absence of a preincubation, the reaction was initiated by the addition of enzyme (180 μl) to arachidonic acid/TMPD solution (20 μl) and inhibitor (4 μl). The enzyme activity was measured by estimation of the initial velocity of TMPD oxidation over the first 36 s of the reaction as followed by the increase in absorbancy at 610 nm (V_{max}; Molecular Devices). Control experiments using H_2O_2 (0.4 mM) as substrate showed that inhibitors did not affect the peroxidase activity of Cox-2 under the assay conditions. A low rate of non-enzymic oxidation was observed in the absence of Cox and was subtracted before the calculation of the percentage of inhibition. IC_{50} values were obtained from fitting the data to a four-parameter logistical model of the graph of log dose against percentage inhibition or by interpolation of the data using Kaleidagraph software. All reported IC_{50} values are the results of averages of independent experiments conducted at least twice.

The kinetic constants for the inhibition of Cox-2 by ketoprofen, indomethacin and biaryl A were determined using the above TMPD spectrophotometric assay in the presence and absence of 2 mM genapol. Reactions were either initiated with enzyme, or inhibitor and Cox-2 were preincubated for 5 s to 15 min and the reaction initiated with substrate. The observed first-order rate constants (k_{obs}) for the time-dependent loss of Cox-2 activity at each inhibitor concentration were obtained by fitting the data to the equation $y = a + b^{*}\exp(-k_{obs}{}^{*}t)$ using Kaleidagraph software. The values of the kinetic constants for inhibitor binding were obtained by fitting k_{obs} and inhibitor concentrations (I) using the equation:

$$k_{obs} = \frac{k_2[I]}{K_D + [I]} + k_{-2} \quad (1)$$

where $k_{\pm1}$ and $k_{\pm2}$ are defined in Scheme 1 and $K_D = k_{-1}/k_1$ [23].

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EI^{*}$$

Scheme 1 Two-step kinetic mechanism for Cox inhibition

[^{14}C]Arachidonic acid HPLC assay

The assay was performed in exactly the same manner as the TMPD assay above except that [^{14}C]arachidonic acid (0.005 μCi) was used as substrate, the final enzyme concentration was approx. 250 nM, the 96-well plates were from Nunc (polypropylene, catalogue no. 442587) and the total assay volume was 50 μl. After 1 min the reaction was stopped by the addition of 5 μl of 1 M HCl and 50 μl of acetonitrile. Substrate conversion was determined by injection of 50 μl of each reaction mixture on to a Nova-Pak C_{18} HPLC column (3.9 mm × 150 mm; Waters) which was developed with acetonitrile/water/acetic acid (85:15:0.1, by vol.) at 2 ml/min. Arachidonic acid metabolites and arachidonic acid eluted at 0.6–1.1 min and 2.2–2.6 min respectively, and were quantified on a Packard Flo-one Radiochromatography detector. The data reported are averages from duplicate experiments.

Determination of inhibitor partitioning into genapol micelles

An HR 10/10 Fast Desalting column (Amersham Biosciences) was equilibrated with 20 mM potassium phosphate pH 6.5, 200 mM KCl and 2 mM genapol X-100 at a flow rate of 2 ml/min. Injections of 100 μl of inhibitor in the mobile phase were performed on a Waters 600E system with detection by UV/visible absorption or radiochromatography detector in the cases of arachidonic acid and ibuprofen. Acetone eluted at 6.3 ml. Partitioning was also determined by ultrafiltration [24]. Solutions (500 μl) of 10 μM ketoprofen and indomethacin were prepared in 100 mM sodium phosphate, pH 6.5, containing 0–2 mM genapol and were centrifuged in pre-rinsed Microcon-30 ultrafiltration devices (Amicon) to approx. 100 μl. Aliquots of the filtrate and retentate were analysed for inhibitor concentration on a Nova-Pak C_{18} HPLC column (3.9 mm × 150 mm), developed with acetonitrile/water/acetic acid (60:40:0.1, by vol.) at 2 ml/min. Ketoprofen and indomethacin eluted at 0.97 and 1.38 min respectively.

RESULTS

Effects of detergents on inhibitor potencies towards Cox-2

Our adaptation of a previously described [19] 96-well plate format Cox TMPD colorimetric assay of Cox-2 inhibition involved replacing the detergent Tween-20 (0.8 mM) with the poly(oxyethylene) type genapol X-100 (2 mM). Genapol had been

Table 1 Titrations of inhibitor potency with human Cox-2 using the TMPD assay in the presence and absence of 2 mM genapol and a 15 min preincubation period

The results are means from at least two titrations. IC_{50} ratio = IC_{50} without genapol/IC_{50} with 2 mM genapol.

Compound	IC_{50} (μM)		IC_{50} ratio
	No genapol	+2 mM genapol	
Nimesulide	49	0.024	2021
Ibuprofen	> 100	0.052	> 1923
Niflumic acid	> 100	0.21	> 476
Flufenamic acid	62	0.22	282
Naproxen	13	0.048	260
Isoxicam	> 100	1.4	> 71
Piroxicam	26	1.1	23
Ketoprofen	0.54	0.042	13
Thioflosulide	4.4	0.43	10
Carprofen	0.18	0.027	7
Flurbiprofen	0.058	0.01	6
Zomepirac	0.13	0.037	4
NS-398	0.44	0.15	3
Diclofenac	0.064	0.034	2
Indomethacin	0.68	0.38	2
Flosulide	1.5	0.83	2
Meclofenamic acid	0.15	0.1	2
Etodolac	4.7	3.2	1
DuP-697	0.035	0.36	0.1
Biaryl B	3.1	48	0.06
Biaryl A	0.099	3.2	0.03

previously used in-house in a homovanillic acid fluorometric Cox assay because it did not interfere at the required wavelengths [22]. The modified assay employed a 15 min preincubation of approx. 20 nM purified Cox-2 with inhibitor, prior to the initiation of the reaction by the addition of 100 μM of the substrate arachidonic acid. The velocity was measured over the first 36 s of the reaction as the activity of purified Cox declines rapidly ($t_{1/2}$, approx. 40 s) during catalysis due to turnover inactivation [1]. A number of Cox inhibitors were tested and the IC_{50} values compared with those obtained in other Cox-2 enzyme assays and values from the literature. It was apparent that a number of inhibitors were many fold more potent in the TMPD assay than in similar isolated-enzyme assays from which detergent was absent. When the TMPD assays were repeated in the same manner, but in the absence of detergent, a number of large differences in potency were observed (Table 1). The magnitude of IC_{50} ratios (IC_{50} no detergent/IC_{50} with detergent) ranged from >1900 for ibuprofen to 0.03 for biaryl A (Table 1, Figure 1). In some cases (e.g. nimesulide and flurbiprofen) the potencies obtained with genapol present may be underestimates as the Cox-2 concentration in the assay was approx. 20 nM.

The effect of genapol was not restricted to inhibitor potency alone. In the absence of detergent the titration curves of a number of compounds, including ibuprofen (Figure 2A), ketoprofen (Figure 2A), or carprofen, nimesulide and thioflosulide (results not shown) were of a biphasic nature, showing plateaux of inhibition at intermediate concentrations that ranged from approx. 20 to 80%. Very high concentrations (30–100 μM) of inhibitor gave a further increase in inhibition. When these compounds were titrated in the presence of 2 mM genapol normal sigmoidal curves (Hill coefficients close to 1) with maximal inhibition reaching close to 100% were obtained (Figure 2). In some cases the increase in inhibition plateau with genapol present resulted in a large decrease in IC_{50} value (e.g. ibuprofen), whereas in other cases (e.g. flurbiprofen and carprofen), the increase in plateau

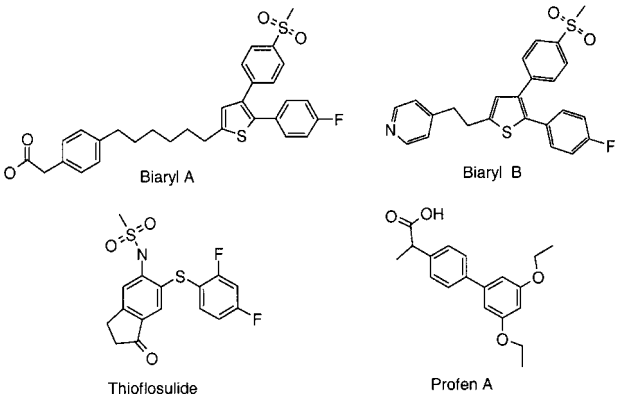


Figure 1 Structures of non-standard Cox inhibitors used in this study

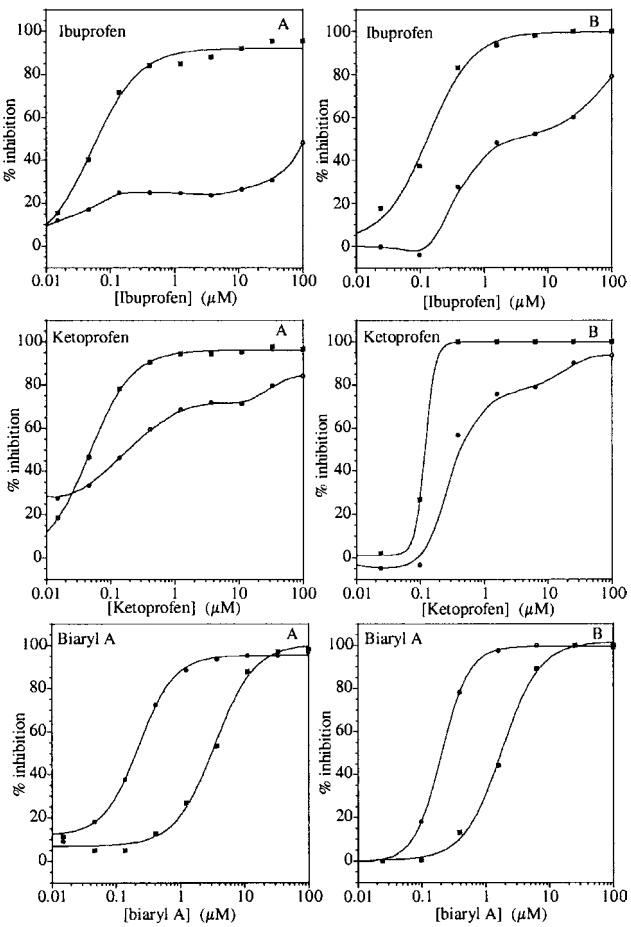


Figure 2 Titration curves of Cox-2 inhibition with ibuprofen, ketoprofen, and biaryl A

Titrations were performed using either the TMPD assay (**A**) or [^{14}C]arachidonic acid assay (**B**) as described in the Materials and methods section using a 15 min preincubation of Cox-2 with inhibitor. Titrations: ■, +2 mM genapol; ●, no genapol.

value from approx. 60 to 100% inhibition resulted in only small shifts (5-fold) in the measured IC_{50} value. This phenomenon of a plateau of Cox inhibition at values of less that 100% has been observed previously [25]. A plateau of approx. 50% inhibition of Cox-2 activity at 1–100 μM ibuprofen was reported using a similar TMPD assay (no detergent) to that employed here [26]

and SC-560 gave maximal 80 and 60 % inhibition of Cox-2 as measured by oxygen electrode [27] and PGE_2 immunoassay [28] respectively. Similar partial titrations were also observed with naproxen, nimesulide, piroxicam and thioflosulide in whole-cell Cox-2 assays [29]. The titrations of inhibitors that became less potent with genapol, such as biaryl A (Figure 2), were both of a normal sigmoidal type, as were those that were not significantly shifted, such as indomethacin, NS-398 and meclofenamic acid (results not shown). The presence of genapol in this TMPD Cox-2 assay also did not significantly shift the potencies of the Cox-2-selective inhibitors rofecoxib and etoricoxib [30,31].

This detergent effect was not specific to genapol. Qualitatively similar results to those obtained in Table 1 and Figure 2 were observed with eight representative inhibitors (ibuprofen, flufenamic acid, DuP-697, biaryl A, indomethacin, meclofenamic acid, ketoprofen amd nimesulide) using the Cox-2 TMPD assay in the presence of 7 mM Triton X-100 and 0.8 mM Tween-20 (results not shown). However, the presence of up to 3 mM octylglucoside had no significant effect on the potencies of the same inhibitors. Cox-2 inhibitor titrations were also performed in the presence of 12 μg/ml phosphatidylcholine. In this case, a 15 min preincubation of Cox-2 with phosphatidylcholine in the absence of inhibitor resulted in an 87 % decrease in Cox activity. Consequently, titrations with phosphatidylcholine employed a 1 min preincubation of enzyme with inhibitor prior to the addition of substrate. The resulting IC_{50} values for ibuprofen and flufenamic acid were 0.25 and 0.3 μM respectively. Under identical conditions, but in the absence of phosphatidylcholine (no detergent) IC_{50} values of $>100\,\mu$M and 75 μM, respectively, were obtained. Finally, Sf9 microsomes expressing recombinant Cox-2 were titrated with ibuprofen using the TMPD assay in the presence and absence of genapol. Under these conditions, the detergent caused the solubilization of the Cox-2 (results not shown). The presence of genapol again resulted in an increase in potency (IC_{50}) of ibuprofen from >100 to 0.045 μM. Thus the shifts in Cox-2 inhibitor potency are not restricted to genapol, but are also caused by other non-ionic detergents as well as phospholipid.

Titrations of four representative compounds were also performed in an identical manner to the TMPD assay, except that [^{14}C]arachidonic acid was used as substrate and Cox-2 activity was quantified after a 1 min reaction time by HPLC with radiometric detection. The resulting IC_{50} values and titration curve shapes (Figure 2B) were very similar to those obtained using the TMPD assay (Figure 2A). For ibuprofen, in the absence of genapol, the TMPD assay showed a plateau of approx. 20 % inhibition between 0.5 and 50 μM (IC_{50} >100 μM) whereas the [^{14}C]arachidonic acid assay showed approx. 55 % inhibition (IC_{50}, approx. 2 μM) over this concentration range, but only reached 75 % inhibition at 100 μM. In the presence of genapol both assay methods for ibuprofen showed normal sigmoidal titration curves and gave IC_{50} values of 50–200 nM (Figure 2). The slightly higher IC_{50} value of 200 nM for the [^{14}C]arachidonic acid assay is probably due to the higher Cox-2 concentration (250 nM) which was required to obtain approx. 50 % substrate conversion in control reactions. Ketoprofen also produced the same biphasic titration curves in the absence of genapol for both TMPD and [^{14}C]arachidonic acid Cox-2 assays, showing plateaus of inhibition of approx. 70 % and giving IC_{50} values of 0.5 and 0.35 μM respectively (Figure 2). With genapol present, both Cox-2 assays gave normal sigmoidal titration curves with IC_{50} values of approx. 0.04 and 0.1 μM respectively. Normal sigmoidal shaped titrations were obtained using both assay methods, in the presence and absence of genapol, for biaryl A (Figure 2) and indomethacin (results not shown). The IC_{50} values for indomethacin were approx. the same with both assay types (0.3 μM) and were unaffected by detergent.

Table 2 Titrations of inhibitor potency with ovine Cox-1 using the TMPD assay in the presence and absence of 2 mM genapol and a 15 min preincubation period

The results are means from at least two titrations. IC_{50} ratio = IC_{50} without genapol/IC_{50} with 2 mM genapol.

Compound	IC_{50} (μM)		IC_{50} ratio
	No genapol	+ 2 mM genapol	
Ibuprofen	92	0.14	664
Flufenamic acid	81	5.7	14
Flurbiprofen	0.022	0.012	2
Ketoprofen	0.019	0.012	2
Indomethacin	0.012	0.0098	1
Valdecoxib	> 100*	12*	> 8
Profen A	36	7.7	5
NS-398	> 100	63	> 1
DuP-697	19	17	1

* Titration performed with 10 μM arachidonic acid.

For biaryl A, both assay methods gave similar IC_{50} values, but inhibitor potency was decreased approx. 10-fold in the presence of genapol (Figure 2). These results show that the genapol-induced changes in potency are not artefacts of the assay method and that the TMPD assay accurately reflects the potency of inhibitors as measured by the inhibition of production of PGH_2.

The effect of genapol on the time dependency of inhibition of Cox-2 by each inhibitor shown in Table 1 was determined using the TMPD assay. Reactions with no preincubation were initiated by addition of enzyme to a mixture of substrate and inhibitor, whereas those reactions with a 15 min preincubation of enzyme and inhibitor were initiated by substrate. Ibuprofen and niflumic acid gave IC_{50} values >100 μM at all preincubation times in the absence of detergent. Ibuprofen is generally described as being a time-independent inhibitor of both Cox isoforms [14,32]. Isoxicam and flufenamic acid [22] also showed time-independent behaviour against Cox-2 in the absence of genapol, as observed previously. The remainder of the inhibitors presented in Table 1 showed time dependency (defined as a greater than 5-fold increase in potency between 0 and 15 min preincubation) in the absence of genapol. In contrast, all inhibitors tested (including ibuprofen, niflumic acid, isoxicam and flufenamic acid), showed time-dependent behaviour against Cox-2 in the presence of genapol. These four inhibitors gave IC_{50} values of 10.4, 12.1, >100 and 19.9 μM with no preincubation and 0.052, 0.21, 1.4 and 0.22 μM respectively with a 15 min preincubation.

Effects of genapol on inhibitor potencies towards Cox-1

Experiments were also conducted with Cox-1 to determine if the effect of genapol was specific to the Cox-2 isoform. As only a limited amount of purified human Cox-1 was available, purified ovine Cox-1 was also used. Both Cox-1 enzymes showed qualitatively similar results when compared. As was observed with Cox-2, ibuprofen and flufenamic acid were very weak inhibitors of Cox-1 using the TMPD assay in the absence of genapol (15 min preincubation) giving IC_{50} values of 92 and 81 μM respectively (Table 2). In the presence of genapol IC_{50} values decreased to 0.14 and 5.7 μM respectively. Further experiments revealed that ibuprofen was a time-independent inhibitor in the absence of genapol, but became time dependent when genapol was included (IC_{50} value shifting from 3.0 to 0.14 μM on preincubation). Interestingly, ibuprofen was also

Table 3 Titrations of inhibitor potency with human Cox-2 using the TMPD assay in the presence of increasing concentrations of genapol

Cox-2 was preincubated for 15 min with inhibitor prior to the initiation of the reaction with substrate. The data represent means from duplicate titrations.

	Cox-2 IC_{50} (μM)								
Genapol (mM)...	0	0.05	0.1	0.25	0.5	1	2	4	8
NS-398	0.45	0.3	0.27	0.2	0.16	0.15	0.18	0.17	0.26
Indomethacin	1.19	0.67	0.63	0.44	0.37	0.35	0.75	0.4	0.62
Ibuprofen	> 100	0.25	0.12	0.07	0.05	0.04	0.03	0.03	0.02
Flufenamic acid	> 100	3.4	3.3	0.49	0.89	0.47	0.38	0.15	0.12
Niflumic acid	> 100	0.34	0.49	0.08	0.09	0.08	0.13	0.08	0.09
Ketoprofen	0.30	0.11	0.13	0.073	0.047	0.032	0.021	0.026	0.019
Nimesulide	14	0.058	0.053	0.048	0.030	0.027	0.023	0.030	0.043
DuP-697	0.04	0.02	0.04	0.05	0.1	0.18	0.31	0.42	0.88
Biaryl A	0.09	0.06	0.1	0.41	1.6	1.6	3.1	3.8	7.1
Biaryl B	2.1	1.7	3.0	6.1	8.8	14	45	56	122

reported as showing time dependency towards both Cox-1 and Cox-2 in assays carried out in the presence of the non-ionic detergent tyloxapol [33]. However, flufenamic acid remained a time-independent inhibitor of Cox-1 in the presence of genapol (IC_{50} value not shifting from 6 ± 1 μM on preincubation).

The effect of genapol on the inhibition of Cox-1 by the time-dependent Cox-1 and Cox-2 inhibitors flurbiprofen, indomethacin and ketoprofen was determined using the TMPD assay. Time-dependent inhibition was observed in the presence and absence of genapol for all three inhibitors and their potencies were not significantly increased with genapol present (Table 2). The major effect of genapol on Cox-2 with flurbiprofen and ketoprofen (exemplified by ketoprofen; Figure 2) was to change the titration curve shapes from biphasic to sigmoidal. However, for Cox-1 with both inhibitors, the titration curves at 0 and 15 min preincubation, with or without genapol, were of a normal sigmoidal shape with maximal inhibition approaching 100 % (results not shown).

The Cox-2-selective inhibitors profen A, [34], DuP-697 [19], valdecoxib [27] and NS-398 [19,22] all showed time-independent inhibition against Cox-1 when tested in the TMPD assay, either in the presence or absence of genapol. However, the potencies of valdecoxib and profen A were increased significantly in the presence of genapol. For valdecoxib, complete titrations were only obtained when the substrate concentration was reduced from 100 to 10 μM, confirming the competitive nature of this inhibition.

Investigations into the mechanism of detergent-dependent shifts in potencies

To gain an insight into the causes of these detergent-induced shifts of inhibitor potency, selected inhibitors were titrated against Cox-2 in the presence of a range (0–8 mM) of genapol concentrations. The results (Table 3) show that the inhibitors could be grouped into distinct categories depending on the effect of genapol on their potencies. Inhibitors such as NS-398 and indomethacin, which were not shifted in potency between 0 and 2 mM genapol, showed no effect over the entire range of detergent concentrations tested. Ibuprofen, flufenamic acid, niflumic acid and nimesulide all showed considerable increases in potency at the lowest concentration of genapol tested (0.05 mM). The changes in titration curve shape, described above for a subset of compounds, also occurred at the lowest genapol dose. No further increases in potency were observed at genapol concentrations above 0.25 mM, which is close to the literature value of the cmc (critical micelle concentration) value of genapol X-100 of 0.15 mM, indicating that the increase in inhibitor potency is due to the detergent monomers rather than micelles. The potencies of DuP-697, biaryl A and biaryl B showed no effect of genapol concentrations up to 0.1 mM. Above 0.25 mM genapol the IC_{50} values for each inhibitor increased linearly with detergent concentration, results consistent with the partition of the inhibitor away from the enzyme into detergent micelles.

The concentration of detergent in the final assay mix, carried over from the enzyme purification buffer, was 8 μM Triton X-100 for Cox-1 and 0.3 mM octylglucoside for Cox-2. The cmc of a two-detergent mix approximates the lower of the two cmc values of the unmixed detergents. Therefore, as the cmc values of both Triton X-100 and octylglucoside are higher than that of genapol (Figure 3), the cmc of the resulting mixed micelle would be the same as that of genapol.

The substrate K_m was measured for Cox-2 in the presence and absence of genapol to determine whether the detergent-dependent shifts could be related to a change in this kinetic parameter. The apparent K_m of arachidonic acid for Cox-2 was shifted from 11 μM with no detergent to 33 μM in the presence of 2 mM genapol. Higher concentrations showed a linear increase in apparent K_m value to 109 μM at 10 mM genapol, consistent with the partition of the substrate into detergent micelles. As IC_{50} is related to substrate concentration by the equation $IC_{50} = (1 + [S]/K_m) \times K_i$, the small change in K_m cannot account for the large increases in inhibitory potencies observed for some compounds.

The time dependency of inhibition of Cox-2 by ketoprofen, indomethacin and biaryl A were investigated in more detail. Each inhibitor was preincubated with Cox-2 for 0–15 min prior to the initiation of the reaction by substrate. The activity of Cox-2 in the presence of each inhibitor decreased in a first-order fashion, although for ketoprofen with no genapol a decay to a clearly non-zero residual activity was observed (Figure 4). For indomethacin (results not shown), biaryl A (Figure 4) and ketoprofen with genapol present, maximal inhibition approached 100 %. The time-dependent inhibition of Cox has been modelled to a two-step reversible mechanism (Scheme 1) [23]. Plotting k_{obs}, the observed first-order rate constant for the onset of enzyme inhibition, versus inhibitor concentration allows (see the Materials and methods section) the calculation of the K_D value for the initial EI complex, as well as estimations of k_2 and k_{-2}. Examination of these calculated kinetic constants (Table 4) shows that the k_2 value for ketoprofen is increased 13-fold by genapol and the K_D for biaryl A is increased by 500-fold. The other constants for these inhibitors were not significantly affected. Similarly, only small changes in the kinetic constants for indomethacin were determined, consistent with the 2-fold increase in its potency with genapol present (Table 1).

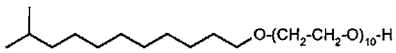
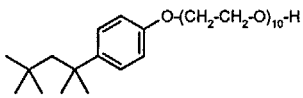
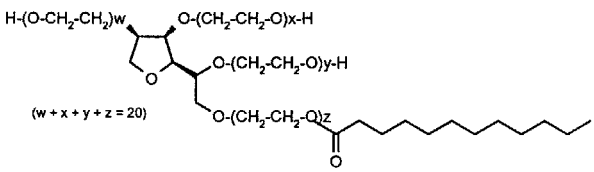
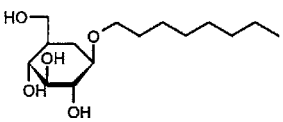
Detergent	cmc (mM)[a]	
Genapol X-100	0.15	
Triton X-100	0.2-0.9	
Tween-20	0.059	
Octylglucoside	20-25	

Figure 3 The structures and cmc of detergents used in this study

Values of cmc are from the Calbiochem website (http://www.calbiochem.com/SharedImages/TechnicalLiterature/1_Cb0603_Detergents_Buffers.pdf).

Direct measurements using gel-filtration chromatography were used to determine whether the Cox inhibitors and arachidonic acid partition into genapol micelles. The classical method for determining macromolecule–ligand dissociation constants [35] in which a gel-filtration column is equilibrated with ligand and the macromolecule (in this case genapol micelles) injected was not appropriate as most Cox inhibitors interacted strongly with the column matrix. An alternative procedure, in which a fast desalting column (molecular-mass cut-off, 5 kDa) was equilibrated with buffer containing 2 mM genapol and the individual inhibitors injected gave qualitative results. Compounds that partition strongly into the genapol micelles (molecular mass, 56 kDa) would be expected to elute in the column void volume (2.8 ml) while those not interacting with the micelles should appear in the total column volume (6.3 ml). This method was a rapid way of demonstrating an interaction with the detergent micelles and obviated the need for detergent-induced absorption or fluorescence shifts for binding information. Arachidonic acid, DuP-697, biaryl A and biaryl B all eluted in volumes of 2.4–3.0 ml, which corresponds to the column exclusion volume, indicating a strong partitioning into the micelles. Flufenamic and niflumic acid eluted near the total column volume (5.2–5.5 ml), indicating little or no interaction with the micelles. The remaining inhibitors tested [NS-398 (7.8 ml), ibuprofen (8.6 ml), nimesulide (10.4 ml), ketoprofen (11.5 ml), carprofen (11.5 ml) and indomethacin (12.1 ml)] interacted strongly with the resin and were significantly retarded. The interpretation of the data with these compounds is therefore obscured, but would suggest, at best, a weak interaction with the detergent micelles. An ultrafiltration method [24] was also used to determine the partition coefficient of ketoprofen and indomethacin into genapol micelles under the conditions of the TMPD assay. The K_D values obtained of 1.2 and 3.6 mM respectively indicate a weak interaction with the micelles.

As it has been recently demonstrated that Cox inhibitor potencies can depend on peroxide concentration [10,12], the possibility was considered that the effect of genapol on inhibitor potency could be due to the presence of a peroxide contaminant. However, this explanation was ruled unlikely by experiments performed with sodium borohydride-reduced genapol [36] which showed no change in Cox-2 IC_{50} for ibuprofen, ketoprofen, indomethacin and biaryl A as compared with untreated genapol.

DISCUSSION

The effect of detergents on enzyme properties and inhibitor potencies is not a phenomenon unique to Cox [37–40]. The introduction of a micellar environment allows for the possibility of partitioning of substrate, enzyme or inhibitor into the micelle, as well as the potential for the specific interaction of detergent monomers with each componant. For example, sub-cmc concentrations of Tween-20 significantly reduced the potency of darbufelone towards Cox-2, but octylglucoside had no effect, leading to the hypothesis that Tween monomers induced an aggregation of several darbufelone molecules [18]. The present work shows that the potencies of many Cox inhibitors towards purified Cox-1 and Cox-2 are dramatically affected by the presence of the non-ionic detergent genapol (Tables 1 and 2). For many inhibitors which became more potent, the shift was associated with a change in titration curve shape (Figure 2) and for several, a change from time-independent to time-dependent inhibition was observed. Another subset of apparently more hydrophobic inhibitors became less potent with genapol present.

The mechanism by which detergents such as genapol increase inhibitor potency and alter titration curve shape appears to depend on the mechanism of inhibition of Cox by each inhibitor. The

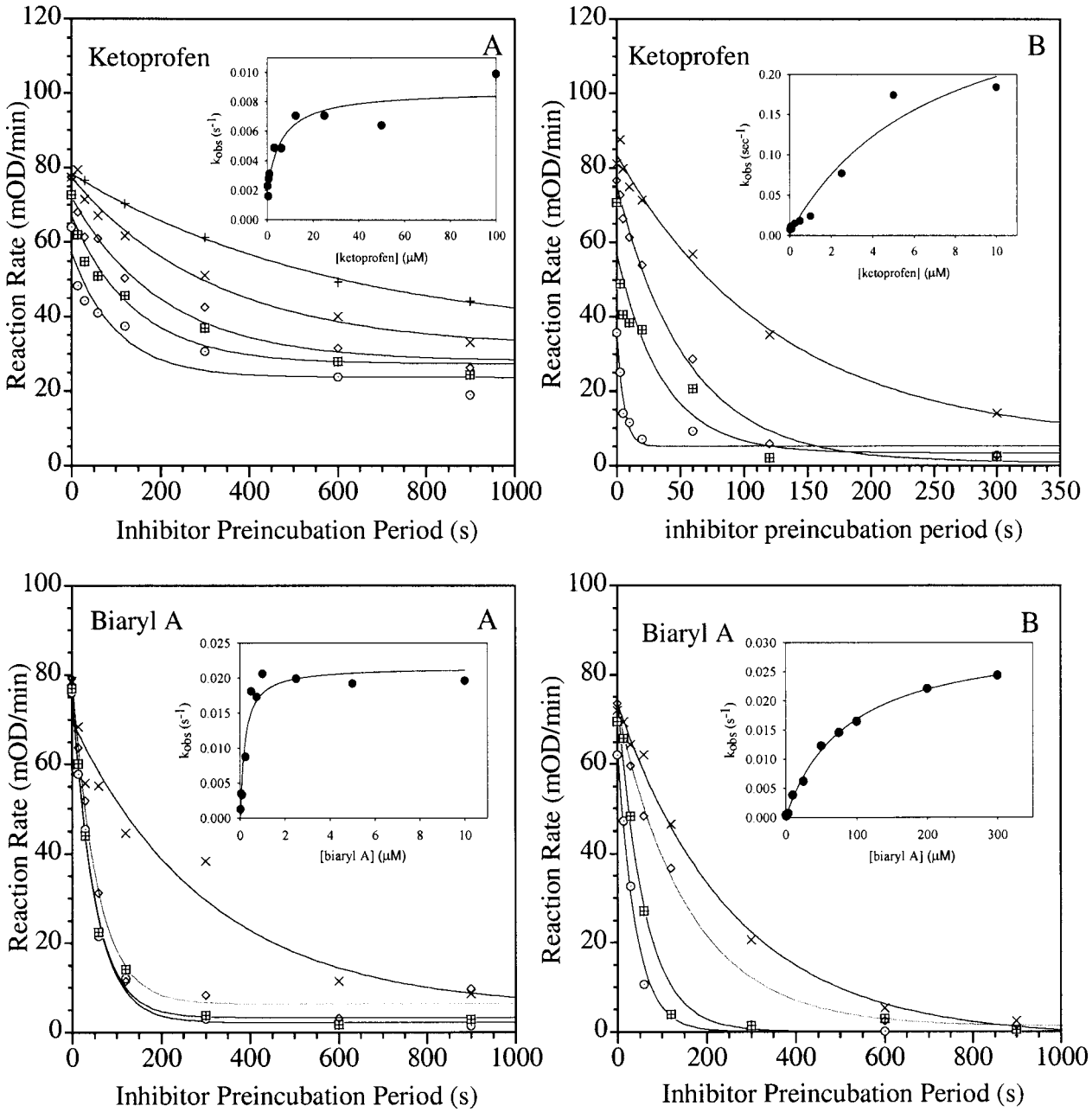


Figure 4 Time-dependent loss of Cox-2 activity in the absence (A) and presence (B) of 2 mM genapol

Ketoprofen and biaryl A were preincubated with Cox-2 for 5 s to 900 s prior to the initiation of the reaction with substrate. The remaining percentage enzyme activity at each preincubation time was fitted to a first-order equation (see Materials and methods section) to obtain the observed first-order rate constant for the onset of inhibition (k_{obs}). Each data point represents a single experiment. The concentrations plotted are as follows. Ketoprofen, no genapol, 100 (⊙), 25 (⊞), 6 (◇), 1 (×) and 0.25 (+) μM; + genapol, 10 (⊙), 2.5 (⊞), 0.25 (◇) and 0.1 (×) μM. Biaryl A, no genapol, 10 (⊙), 2.5 (⊞), 0.5 (◇) and 0.1 (×) μM; plus genapol, 300 (⊙), 100 (⊞), 25 (◇) and 10 (+) μM. Higher concentrations gave faster onset of inhibition. The insets show the plot of k_{obs} versus inhibitor concentration. The line represents the fit to an equation defining a two-step equilibrium (Scheme 1) as described in the Materials and methods section. The derived kinetic constants are shown in Table 4.

NSAIDs ibuprofen and flufenamic acid have been described as simple competitive, time-independent inhibitors of both Cox-1 and Cox-2. Others, such as indomethacin and flurbiprofen are non-covalent time-dependent inhibitors, consistent with a two-step mechanism of enzyme inhibition involving an initial rapidly reversible interaction with the enzyme, followed by the slow formation of a more tightly bound complex (Scheme 1). As the off rates of these inhibitors are generally slow compared with the rate of enzyme turnover inactivation, these inhibitors can appear effectively irreversible and non-competitive. Cox-2-selective inhibitors such as rofecoxib, celecoxib, DuP-697 and NS-398 behave in a time-dependent manner towards Cox-2, but show time-independent inhibition towards Cox-1 [19,22,26,30], whereas non-selective inhibitors show the same mechanism of inhibition towards both isoforms (either time-independent or time-dependent). More recent studies have shown that the binding of the biarylheterocyclic Cox-2-selective inhibitors SC-299 and celecoxib with Cox-2 occur via three kinetically observable

Table 4 Kinetic constants for inhibition of Cox-2 by ketoprofen, indomethacin and biaryl A determined using the coupled TMPD assay in the presence and absence of genapol

Data were fit to a two-step equilibrium mechanism (Scheme 1) as described in Materials and methods section. All values represent the means ± S.E.M. from three independent experiments.

	No genapol	+ 2 mM genapol
Ketoprofen		
K_D (μM)	6 ± 2	7 ± 3
k_2 (s^{-1})	0.02 ± 0.007	0.26 ± 0.06
k_{-2} (s^{-1})	0.002 ± 0.001	0.005 ± 0.002
Indomethacin		
K_D (μM)	65 ± 13	152 ± 39
k_2 (s^{-1})	0.08 ± 0.024	0.3 ± 0.1
k_{-2} (s^{-1})	0.0025 ± 0.001	0.004 ± 0.002
Biaryl A		
K_D (μM)	0.2 ± 0.06	107 ± 26
k_2 (s^{-1})	0.02 ± 0.004	0.034 ± 0.001
k_{-2} (s^{-1})	− 0.0016 ± 0.0006	0.000 ± 0.0004

steps [27,41]. Furthermore, fluorescence quenching experiments with SC-299 are consistent with a two-step relatively rapid equilibrium binding to Cox-1 [41]. Similarly, a two-step rapidly reversible interaction of Cox-2 with the Cox-1-selective inhibitor SC-560 has also been observed [27]. These results suggest that other apparently rapidly reversible, time-independent Cox inhibitors may also bind via a two-step mechanism. Clearly, depending on the absolute values of the rate constants for the second equilibrium step, time dependency may or may not be experimentally observable. A mathematical model of this two-step mechanism of inhibition (Scheme 1) showed that as the inhibitor concentration and preincubation time tend to infinity, the percentage of enzyme in the EI* state is defined by $[k_2/(k_2 + k_{-2})] \times 100$ [42]. For inhibitors with off rates (k_{-2}) that are significant compared with their on rates (k_2), Cox activity will approach a non-zero value even after prolonged preincubation times [23,42]. This will result in titration curves with a maximal plateau of less than 100 % inhibition at high inhibitor concentrations. The titrations in the present experiments were initiated, following an enzyme–inhibitor preincubation, by approx. $10 \times K_m$ concentration of the substrate. The enzyme activity measured therefore reflects the concentration of both E and EI states, as the high substrate concentration effectively competes with the inhibitor. At very high inhibitor concentrations the further increase in inhibition observed for ibuprofen and ketoprofen in the absence of genapol (Figure 2) may reflect additional competitive inhibition.

The detergent-dependent changes in the shape of the titration curves resulting in an increase in the intermediate plateau of percentage inhibition can therefore be explained by changes in the relative values of k_2 and k_{-2} for the second step of inhibitor binding (Scheme 1). In the case of ketoprofen (Figure 4, Table 4) genapol caused a 13-fold increase in k_2 value and plots of Cox activity versus time tended towards an asymptote close to zero activity. Changes in k_2 or k_{-2} may also result in increased inhibitor potency as the overall dissociation constant (K_D^*) is defined as [43]:

$$K^*_D = K_D \frac{k_{-2}}{k_{-2} + k_2} \quad (2)$$

Such detergent-induced changes in the kinetic constants for a second binding step may therefore account for the large increase in potencies and change in time dependency of other inhibitors such as flufenamic and niflumic acid.

The decreased potency for a subset of inhibitors against Cox-2 in the presence of genapol (Table 1) is likely to be caused by partitioning of the inhibitor into micelles resulting in the reduction in concentration of free inhibitor available to the enzyme. No effect of detergent on inhibitor potency against Cox-2 was observed below the cmc value for this series of compounds, whereas concentrations of detergent above the cmc resulted in a linear increase in IC_{50} values (Table 3). In addition, gel-filtration experiments suggest a strong partitioning of DuP-697, biaryl A and biaryl B into genapol micelles. The fate of Cox-2 in the presence of genapol is unknown, but even if both enzyme and inhibitor partition in the micelle, the effect of addition of further detergent will be to dilute the surface mole fraction of inhibitor resulting in a lower effective concentration [44]. Finally, the K_D value for the interaction of Cox-2 and biaryl A (Table 4) increased 500-fold with detergent present in the absence of apparent major changes in the values of k_2 and k_{-2}.

The molecular mechanism of time-dependent inhibition of Cox has been suggested to involve hydrogen-bonding networks at the mouth of the channel leading to the Cox active site, one network (ovine Cox-1 numbering) involving Arg-513, Glu-524 and Tyr-355 and the other Arg-120, Glu-524 and Tyr-355 [45,46]. It was proposed that the first of these two networks represents a relaxed form of the enzyme that is open to substrate and inhibitors and the second represents a closed, tightened form, the formation of the tightened network representing the time-dependent step in inhibitor binding. Comparison of the inhibition of wild-type Cox-2 with a Cox-2 Tyr-355 → Phe mutant by indomethacin, flurbiprofen and RS-57067 showed that both k_2 and k_{-2} (Scheme 1) rate constants were significantly affected. Of note, the magnitudes of these changes were compound-dependent [46]. The interactions of these inhibitors with these two hydrogen-binding networks or Tyr-355 therefore lead to different effects on k_2 and k_{-2}. Another study showed that mutation of Arg-120, Glu-524 and Tyr-355 affected the percentage residual activity following prolonged incubation of Cox-2 with an indomethacin ester [23]. In the present study, the effect of genapol on inhibitor sensitivity was not a general effect on all inhibitors. The crystal structure of Cox-1 [47], which was crystallized in the presence of octylglucoside, shows a detergent molecule in close contact with the hydrophobic mouth of the Cox channel, in close proximity to where these two hydrogen-bonding networks are proposed to occur. Although octylglucoside does not significantly affect the potency of any of the inhibitors tested, it is possible that other types of detergent or lipophilic molecule could interact with these networks, either directly or indirectly, and so alter the rates of isomerization between relaxed and tightened forms, hence changing both inhibitor time dependency and potency. Alternatively, the detergents may act in a non-specific manner, binding to the enzyme or the inhibitors, or preventing the interaction of the enzyme with the plastic surfaces in which these assays were performed. Genapol, Tween-20 and Triton X-100 all contain the same poly(oxyethylene) tail that could provide a hydrophilic surface to the plastic, once bound via their hydrophobic head group. In any event, the presence of detergents profoundly affects the interactions of Cox with a subset of inhibitors.

It is well understood that Cox inhibition is highly sensitive to assay conditions [8] and that IC_{50} values cannot be compared between assays. A review of literature IC_{50} values for flurbiprofen against Cox-2 revealed a range of 0.003–7 μM [48]. We have identified another factor that should be considered when performing Cox inhibitor assays, namely detergent concentration. The membrane solubilization and purification of Cox requires detergent and here it was shown that 50 μM genapol can cause shifts in inhibitor potency (Table 3). Therefore, depending on

the dilution factor of enzyme into the assay mixture, the final concentration of detergent carried through could significantly affect inhibitor potency.

It is not clear whether the effects of detergent and phosphatidylcholine described here could somehow be related to the discrepancies often observed between whole-cell and microsomal and purified Cox assays. Nimesulide is an extremely weak inhibitor of purified Cox-2 in the absence of genapol (Table 1) but is a potent inhibitor (IC_{50}, 9 nM) of whole-cell Cox-2 [29] and also purified Cox-2 with genapol present (Table 1). In contrast, ibuprofen, the potency of which is increased >1900-fold by detergent to 0.05 μM (Table 1) is a relatively weak (IC_{50}, 0.67 μM) inhibitor of whole-cell Cox-2 [29]. Similarly, indomethacin is not shifted in potency against Cox-2 by detergent (IC_{50}, 0.5 μM), but is a potent (IC_{50}, 26 nM) whole-cell inhibitor of Cox-2 [29]. Thus there does not appear to be a common trend in detergent-dependent potency changes with purified Cox and activity in a whole-cell assay, although potency in the latter assay will certainly be complicated by effects such as inhibitor cellular permeability and peroxide concentration.

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