Full Papers

Development of a Radiochemical Cyclooxygenase-1 and -2 in Vitro Assay for Identification of Natural Products as Inhibitors of Prostaglandin Biosynthesis

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A radiochemical enzyme assay for studying cyclooxygenase (COX)-catalyzed prostaglandin biosynthesis in vitro was optimized with respect to both COX-1 and COX-2 activity. The assay can be used to assess the relative selectivity of plant-derived inhibitors on COX-1 and COX-2. Assay conditions were optimized for both enzymes with respect to concentration of cofactors (*l*-epinephrine, reduced glutathione, and hematin), activation time (enzyme and cofactors), reaction time, and pH. Moreover, the kinetic parameters, K_m and $K_{cat.}$, of both enzymes were estimated. Five COX inhibitors were used to validate the assay, indomethacin, aspirin, naproxen, ibuprofen, and the arylsulfonamide NS-398, all with different COX selectivity and time dependency. Time-dependent inhibition was determined by comparing the inhibition, with and without preincubation of enzyme and inhibitor. Two flavonoids, (+)-catechin and quercitrin, were examined with respect to inhibition of COX-catalyzed prostaglandin biosynthesis. (+)-Catechin showed equal inhibitory effects on the two enzymes. Quercitrin was found to be inactive toward both COX-1- and COX-2-catalyzed prostaglandin biosynthesis. The optimization procedure resulted in a considerable reduction of the amount of enzyme required for adequate prostglandin biosynthesis and a reliable method suited to evaluate natural products on inhibition of COX-2-catalyzed prostaglandin biosynthesis, as well as on COX-1.

The enzyme cyclooxygenase-1 (COX-1, prostaglandin endoperoxide H synthase-1, PGHS-1), a catalyst of prostaglandin synthesis, has been extensively used as a tool for studying the antiinflammatory effects of plant extracts and plant-derived compounds.[1−4] In our medicinal plant research program, prostaglandin biosynthesis inhibition has been evaluated using an in vitro assay originally described by White and Glassman.[5] The assay has been employed for evaluation of plant extracts[6−8] as well as in bioactivity-directed fractionation, leading to the characterization of several new inhibitors of COX-1-catalyzed prostaglandin biosynthesis.[9−11]

With the discovery of the isoenzyme COX-2, whose production is induced by inflammation mediators,[12] interest in cyclooxygenase inhibitors has grown. The adverse effects observed with traditional nonsteroidal antiinflammatory drugs (NSAIDs), such as aspirin and indomethacin, are believed to stem from an inhibition of constitutive COX-1 activity, and it is hypothesized that selective COX-2 inhibitors exhibit an improved safety profile.[13] Since almost all clinically approved NSAIDs preferentially inhibit COX-1 over COX-2 (with the exception of meloxicam and nimesulide), the search for selective inhibitors of the COX-2 isoenzyme is considered important.[12] An overview of the selectivity of different COX inhibitors in relation to the bioassays used has been reported by Battistini et al.[14]

Until now, very few compounds of natural origin have been shown to possess COX-2 inhibitory effects. These include the sesterterpenes, manoalide and scalaradial, of marine origin,[15] the diterpene derivative, akendo 3,[16] and resveratrol, a *trans*-stilbene, although the latter compound was found to inhibit COX-1 with some selectivity.[17]

The plant kingdom is hitherto a practically unexplored resource of potential selective COX-2 inhibitors, which motivated us to further develop a COX-1 radiochemical enzyme assay, based on the White and Glassman assay,[5] for simultaneous use against the COX-2 enzyme, to assess relative COX-2/COX-1 inhibitory activities of extracts and pure compounds of natural origin. The White and Glassman assay has been used, with some modification, by other groups[18−21] and was employed recently in a COX-2 sheep placental enzyme preparation.[22] We describe here an improved assay, focused on optimized conditions for inhibition studies of COX-1 and COX-2. The influence exerted by the following parameters on prostaglandin synthesis cata-

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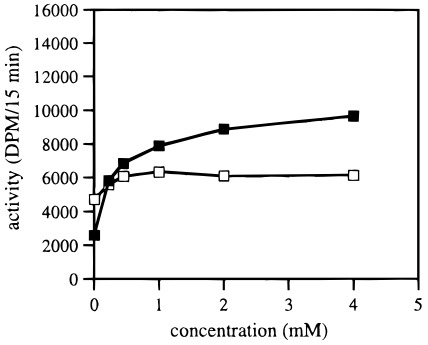

Figure 1. Effect of *l*-epinephrine on COX-1 (■) and COX-2 (□) catalyzed prostaglandin biosynthesis, measured after 15 min reaction. Each point represents the mean ± SEM (n = 5−6).

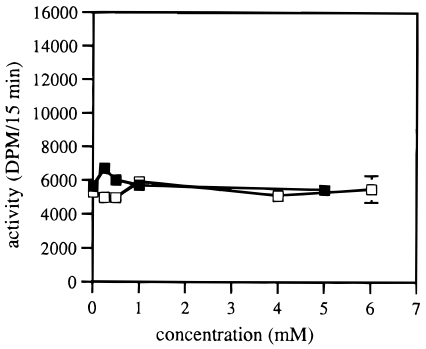

Figure 2. Effect of reduced glutathione on COX-1 (■) and COX-2 (□) catalyzed prostaglandin biosynthesis, measured after 15 min reaction. Each point represents the mean ± SEM (n = 3−6).

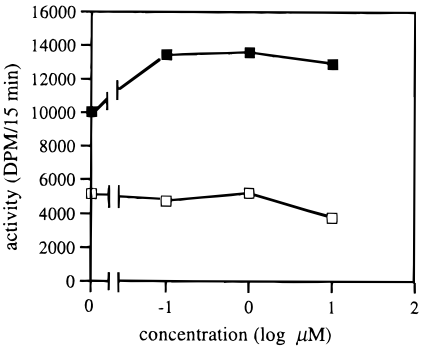

Figure 3. Effect of hematin concentration (log μM) on COX-1 (■) and COX-2 (□) catalyzed prostaglandin biosynthesis, measured after 15 min reaction. Each point represents the mean ± SEM (n = 3−6).

lyzed by either enzyme was studied: concentration of cofactors (*l*-epinephrine, reduced glutathione and hematin), activation time of enzyme and cofactors, reaction time, and the pH in the range 7.6−8.4. The kinetic parameters (K_m and $K_{cat.}$) were also estimated. The optimized COX-1 and COX-2 assay was validated using five known COX inhibitors, aspirin, ibuprofen, indomethacin, naproxen, and the arylsulfonamide, NS-398, all exhibiting different properties concerning enzyme selectivity and time-dependency. The inhibitors were assayed with and without preincubation (enzyme and inhibitor) in order to account for time-dependent inhibition. Further, two common flavonoids, (+)-catechin and quercitrin, of previously known activity against COX-1-catalyzed prostaglandin biosynthesis, were evaluated in the newly optimized assay.

Results and Discussion

A radiochemical enzyme assay has been optimized and validated for assessing antiinflammatory activity, in terms of reduced COX-1- as well as COX-2-catalyzed prostaglandin biosynthesis in vitro. The optimization was performed with reference to output in prostaglandin biosynthesis per amount of enzyme at a given substrate concentration. Throughout the optimization procedure the aim was to select conditions suitable for both enzymes, rendering a uniform assay, thus facilitating comparison of activities of extracts and test compounds on the two different enzymes.

The prerequisite of cofactors for efficient conversion of fatty acid substrate to the prostaglandin products by COX-1 and COX-2 has been documented in several studies.[23−26] As prostaglandin E_2 (PGE_2) is an important mediator of inflammation,[27] there is specific interest in ensuring high yields of this prostaglandin in the assay. Formation of PGE_2 (and of $PGF_{2\alpha}$, PGD_2, and malondialdehyde) has been shown to increase in the presence of *l*-epinephrine.[23] Figure 1 shows the increased COX-1 and COX-2 activity (expressed as the obtained radioactivity in disintegrations per minute, DPM/15 min reaction time) with increased *l*-epinephrine concentration, reaching a plateau at about 4 mM for COX-1 and 1 mM for COX-2. COX-1 was more sensitive to *l*-epinephrine than COX-2; the 4-fold increase in *l*-epinephrine concentration from 0.45 to 1.95 mM yielded a 30% increase in COX-1 enzyme activity, while the COX-2 activity was not affected. Further measurements were performed with 1.95 mM of *l*-epinephrine, yielding a maximal prostaglandin production for both enzymes.

From earlier studies,[25,26,28] it is apparent that reduced glutathione is needed for PGE_2 production, as its presence enhances PGE_2 formation at the expense of other prostaglandins and hydroxy fatty acids. In the present assay, no clear-cut benefit could be implicated from a change in reduced glutathione concentration, in the interval 0−6 mM (Figure 2). The concentration of 0.49 mM, according to the original method, was therefore conserved.

Hematin is a prostethic group, essential for both catalytic activities (cyclooxygenase, as well as peroxidase activity) of the enzymes.[29] Moreover, heme has been reported to stimulate PGE_2 synthesis.[26] Figure 3 demonstrates the effect of hematin on prostaglandin biosynthesis by COX-1 and COX-2. The prostaglandin production catalyzed by COX-1 was increased by 35%, after addition of 1 μM hematin, whereas the COX-2-catalyzed synthesis was essentially unaffected. The results may reflect differences between the enzyme preparations used, since the prostethic group can be lost during the purification. Further assays were performed with 1 μM hematin, based on the present results and previous studies.[30] A change in pH between 7.6 and 8.4 had no effect on prostaglandin synthesis by either enzyme (results not shown). The results are in line with earlier reports.[31] Also, the PGE_2 formation has been reported to be optimal near pH 8.[23,26]

Figure 4 depicts the levels of COX-1- and COX-2-derived prostaglandin production obtained as a function

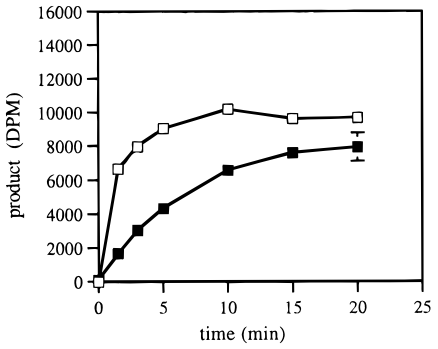

Figure 4. Time course of COX-1 (■) and COX-2 (□) catalyzed prostaglandin biosynthesis by reaction time (37 °C). Each point represents the mean ± SEM (n = 6−12).

of reaction time (0−30 min). To obtain a repeatable assay (where many samples can be tested each time) the reaction time was increased from 10 to 15 min. At this time, the activity of COX-1 had nearly leveled, while the activity of COX-2 was at a steady level. A 15 min activation time of enzyme and cofactors (*l*-epinephrine and reduced glutathione) was also included in the original assay. However, since no significant changes in prostaglandin production could be observed for either enzyme when the activation time was varied from 1 to 20 min (results not shown), this time period was decreased to 5 min.

COX inhibitors have been categorized into time-dependent and time-independent inhibitors, according to their kinetic behavior toward the enzymes.[12] Prolonged incubation of enzyme and inhibitor will increase the inhibitory effect of a time-dependent inhibitor, while the effect of a time-independent inhibitor will remain almost unaffected. On the basis of previously reported kinetic studies of COX-1 and COX-2 inhibition by various NSAIDs,[30,32] the assay was performed either with 10 min of preincubation or without preincubation of enzyme and inhibitor.

Since our aim was to obtain an antiinflammatory bioassay for assaying plant extracts and naturally occurring compounds likely to exhibit different types of kinetic behavior and degrees of selectivity, the assay was evaluated using known inhibitors (Chart 1) selected with reference to time-dependency and enzyme selectivity revealed in previous studies. Indomethacin and aspirin were chosen due to their time-dependent COX-1-selective behavior,[30,32] while naproxen is a time-independent inhibitor with weak COX-1 selectivity.[33] Ibuprofen is time-independent, exhibiting equipotency toward the two enzymes,[32] while NS-398 is a COX-2-selective inhibitor, acting time-dependently with COX-2.[34] Two time-dependent inhibitors (indomethacin and aspirin) and one time-independent (ibuprofen) were selected for investigations both with and without preincubation to verify the influence of preincubation time on inhibitory effect in the assay.

(+)-Catechin and quercitrin, two flavonoids (Chart 1), were also investigated. The COX-1 inhibitory effect of the flavan-3-ol, (+)-catechin, has been well studied.[35,36] Following bioactivity-guided fractionation by the COX-1-catalyzed prostaglandin biosynthesis assay, (+)-catechin has been isolated from the Samoan medicinal plant *C. pentandra* (L.) Gaertner.[11] The flavone rhamnoside, quercitrin, one of the most common flavonoids

Chart 1. Structures of Five Synthetic and Two Flavonoid Reference Compounds

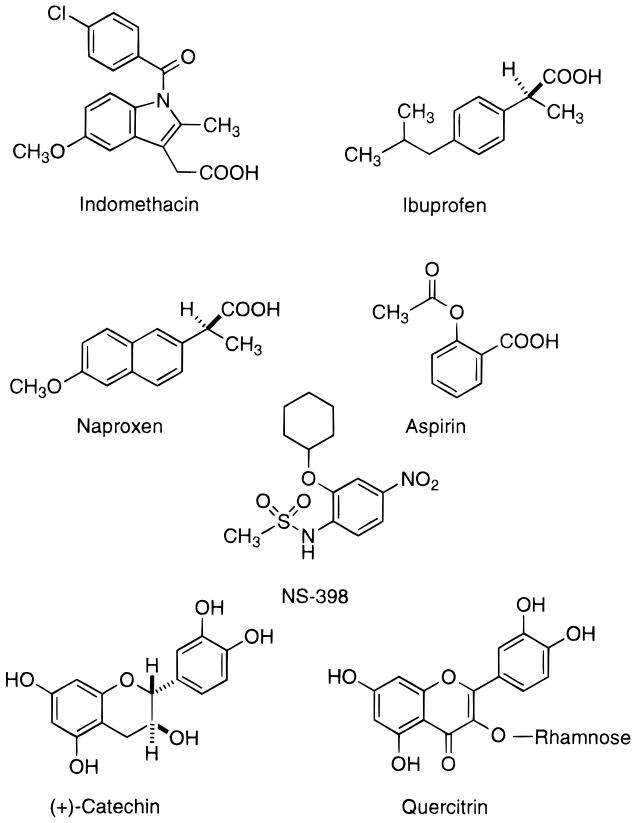

Table 1. IC_{50} Values (μM) and COX-2/COX-1 Ratios of Five Synthetic and Two Flavonoid Reference Compounds

	preincubation					
	0 min			10 min		
	IC_{50} (μM)		COX-2/	IC_{50} (μM)		COX-2/
	COX-1	COX-2	COX-1	COX-1	COX-2	COX-1
indomethacin	3.3	1710	518	1.4	164	117
ibuprofen	13	370	28	18	1030	57
naproxen				11	1015	96
aspirin	3770	>5000	>1	342	>5000	>15
NS-398				>100[a]	53	<0.5
(+)-catechin	183	546	3	279	5910	21
quercitrin	>2000	>2000		>2000	>2000	

[a] Maximum dissolved concentration.

occurring in plants,[37] stimulates COX-1 activity at high substrate concentrations[38] and is inactive at low substrate concentrations in vitro[36] but possesses antiinflammatory activity in vivo.[39] It was thus speculated that quercitrin might exhibit a COX-2 inhibitory effect.

Dose−response curves for indomethacin, ibuprofen, naproxen, aspirin, NS-398, and (+)-catechin on COX-1- and COX-2-catalyzed prostaglandin biosynthesis, with 10 min of preincubation of enzyme and inhibitor, are shown in Figure 5. For indomethacin, ibuprofen, aspirin, and (+)-catechin the inhibition graphs also include curves obtained without preincubation. IC_{50} values (μM), as well as the calculated selectivity ratios (COX-2/COX-1) for all seven compounds investigated, are summarized in Table 1. Indomethacin was a time-dependent inhibitor for both enzymes, exhibiting higher potency after preincubation than without. The time-dependency was especially notable with COX-2, IC_{50} 1710 μM compared to 164 μM at 0 and 10 min prein-

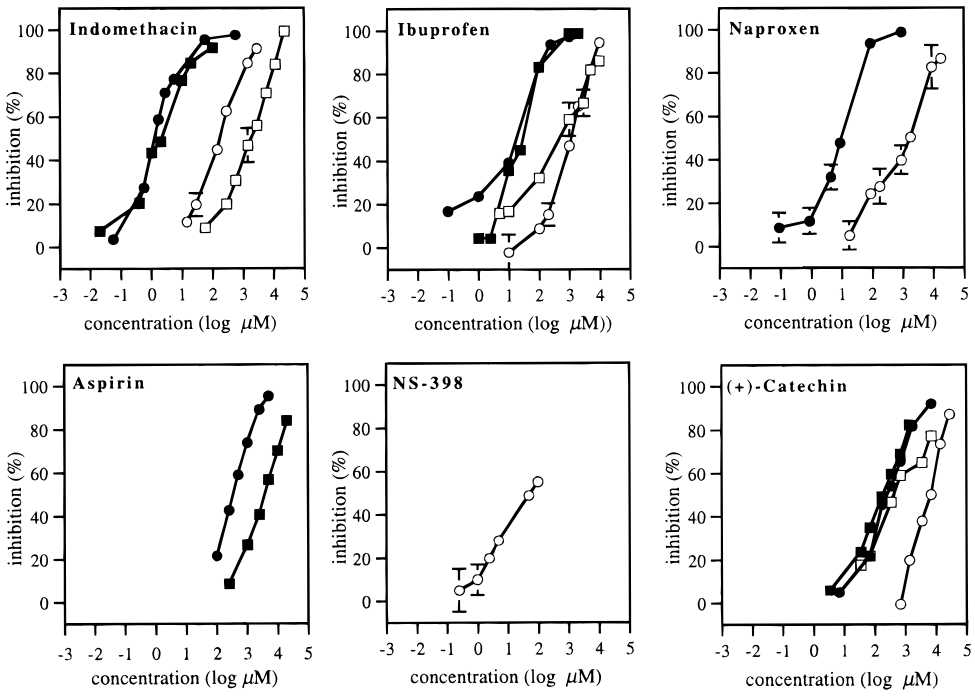

Figure 5. Inhibitory effects of reference compounds; indomethacin, ibuprofen, aspirin, naproxen, and NS-398 together with the natural product (+)-catechin on COX-1 (filled) and COX-2 (unfilled) catalyzed prostaglandin biosynthesis, without preincubation of enzyme and inhibitor (squares) and after 10 min of preincubation (circles). Naproxen and NS-398 were measured at 10 min preincubation only. Each point represents the mean ± SEM ($n = 5-18$).

cubation, respectively. Indomethacin showed a pronounced COX-1 selectivity, which was reduced by preincubation as the effect toward COX-2 had increased in relation to that of COX-1 (ratio 518 and 117, respectively). These results are in good agreement with earlier studies.[32,40]

Ibuprofen showed time-independent inhibition of both enzymes, with a selectivity toward COX-1. Ibuprofen has been found to be equipotent by a number of investigators.[32,41] Our results are, however, similar to those of Mitchell et al.,[40] reporting a COX-2/COX-1 ratio of 46. When comparing ibuprofen to indomethacin inhibition of COX-1 activity by ibuprofen with no preincubation was weaker (IC_{50} 13 μM) than that by indomethacin but stronger for COX-2 (IC_{50} 370 μM). However, since indomethacin is a time-dependent inhibitor, ibuprofen became relatively less efficient, compared to indomethacin, after 10 min preincubation.

Naproxen exhibited a selective COX-1 ratio (96) close to that of indomethacin, but with activities of the same order of magnitude as found for ibuprofen (IC_{50} 11 μM and 1015 μM on COX-1 and COX-2, respectively). Earlier studies, however, have classified naproxen as either weakly COX-2 selective,[40] equiselective,[41] or somewhat COX-1 selective.[33]

Aspirin exhibited a marked time-dependency for COX-1 (IC_{50} value of 3770 μM without preincubation, as compared to 342 μM with preincubation), which agrees with the results of Johnson et al.[30] The inhibitory effects exerted by aspirin on COX-2 were weaker (IC_{50} value > 5 mM).

NS-398 inhibited COX-2 in a selective manner (ratio < 0.5), corroborating earlier results,[34,42] with a potency (IC_{50} 53 μM) somewhat more potent than indomethacin, assayed after 10 min of preincubation. The maximum dissolved concentration of NS-398 in solvents possible to the assay was 100 μM, and at this concentration the compound inhibited COX-1-catalyzed prostaglandin biosynthesis by 9 ± 5%.

(+)-Catechin exhibited equipotency toward the two enzymes with no preincubation (ratio 3). The COX-1 inhibitory effect after 10 min preincubation (IC_{50} of 279 μM) was of the same order of magnitude as that without preincubation (IC_{50} of 183 μM), but for COX-2 the inhibitory effect was less. The IC_{50} values reported in the literature for (+)-catechin have varied considerably (40 μM − 943 μM).[35,36] Judging from the rather similar IC_{50} values, obtained with and without preincubation, this variability does not seem to be linked to a pronounced time-dependency of (+)-catechin toward the COX-1 enzyme, but rather on other differences in assay conditions from one determination to another.

Quercitrin was found to be inactive toward both enzymes, tested at concentrations up to 2 mM. The possibility that the antiinflammatory effect observed with this compound in vivo[39] may to some extent be derived from a COX-2 inhibitory action can however not be excluded, since results obtained in vitro do not necessarily reflect the situation in vivo.[12,43]

In this study, two different enzyme sources and preparations of different degrees of purity are employed; COX-1 was a microsomal fraction purified from bovine seminal vesicles[30] and COX-2 was a commercially available enzyme, purified from sheep placenta. Enzymes originating from different species (guinea pig, sheep, cow, or mouse) show similar IC_{50} values for various NSAIDs.[27,44] The different degrees of purity of the enzyme preparations, however, might influence the results and increase the possibility of discrepancies in the assay. The microsomal COX-1 preparation was found to contain 44% protein. This can be compared to earlier reports of a protein content of 60−70% of

microsomes purified by a similar procedure.[25,44] The K_m values of COX-1 and COX-2 enzymes for arachidonic acid were determined as 8.8 ± 2.2 μM and 9.7 ± 2.7 μM, respectively, which is in accordance with the data reported for the same enzyme source for COX-1[44] and for COX-2.[30] $K_{cat.}$ was estimated to 192 DPM/μg·s of COX-1 and 280 DPM/μg·s of COX-2. As a result of the optimization procedure, the amount of COX-1 and COX-2 enzyme required per assay for reaching an adequate level of prostaglandin production [control (vehicle) vs blank] could be reduced from 20 to 30 μg to 7−8 μg and from 0.8 μg to 0.3 μg, respectively.

Direct comparison of IC_{50} values between different investigations are of marginal value, since assay conditions vary substantially between different laboratories. Reports indicate inhibitors to be more potent in intact cells than against purified enzymes or enzymes originating from broken cells.[40,41] The safest means of comparison, however, is the rank order of relative selectivity (expressed as the ratio of IC_{50} values, COX-2/COX-1), with all values being obtained by the same method under the same assay conditions.[13,27] When examining the relative selectivity rank order, irrespective of preincubation time, it was headed by NS-398 and followed in order by (+)-catechin, ibuprofen, naproxen, and indomethacin. This is in agreement with investigations in the literature.[30,40,41]

IC_{50} values obtained in this study were in general higher in magnitude than those previously reported for the synthetic drugs in question. IC_{50} values of indomethacin and aspirin obtained with the unoptimized assay and those determined by the optimized COX-1 assay were compared. The earlier IC_{50} value of indomethacin (1.1 μM) was found to be of the same magnitude as after 10 min of preincubation (1.4 μM) in the optimized assay, and the IC_{50} value of aspirin (1050 μM) was of an order between the values obtained without preincubation (3770 μM) and with 10 min preincubation (342 μM) in the optimized assay. According to kinetic studies performed by Laneuville et al.,[32] indomethacin showed markedly increased COX-1 inhibition with every additional minute of preincubation until 5 min (37 °C). In the case of aspirin, as shown by Johnson et al.,[30] maximum inhibition was not reached until 10 min of preincubation time (37 °C), and inhibition continued to increase slightly with another 20 min of preincubation. On the basis of these results and the literature reports, the importance of a defined preincubation time for highly time-dependent inhibitors, such as indomethacin and aspirin, is apparent. Preincubation time, however, is not the sole parameter differentiating the White and Glassman assay and the present optimized assay. The increase in *l*-epinephrine concentration and the addition of hematin may be acting in concert with the prolonged incubation time to impair the effects, the differences in earlier and present IC_{50} values, observed. Further studies are necessary to clarify this matter.

The COX enzymes house two activities: a cyclooxygenase and a peroxidase activity. The present method does not distinguish between these, and as a result, observed inhibition is not a pure cyclooxygenase inhibitory activity but might also reflect degrees of peroxidase inhibitory activity of the compounds or plant extracts tested. However, other studies have shown NSAIDs to specifically inhibit the cyclooxygenase activity of the enzymes.[45,46] The specific activities of natural products in this respect cannot be elucidated using the optimized assay.

Studies of the influence of inhibitors and cofactors on the consecutive production of individual prostaglandins, especially PGE_2, using natural products as inhibitors, are in progress.

The amount of enzyme required for sufficient prostaglandin biosynthesis was reduced after the optimization, and a defined incubation time of enzyme and inhibitor was introduced into the assay. We believe the method to be suited for assessing COX-2/COX-1 selectivity ratios of plant extracts and pure compounds in the plant-based search for selective COX-2 inhibitory natural products.

Experimental Section

Enzymes. Purified cyclooxygenase-2 (prostaglandin endoperoxide H synthase-2) from sheep placental cotyledons (Cayman Chemical Co., Ann Arbor, MI) was used. The source of cyclooxygenase-1 (prostaglandin endoperoxide H synthase-1) was microsomes prepared from bovine seminal vesicles according to Takeguchi et al.,[25] with minor modifications. The bovine seminal vesicles, obtained from newly slaughtered bulls (Farmek, Uppsala, Sweden), were placed immediately on ice and trimmed free from fat and connective tissue. Two volumes of 0.1 M Tris−HCl buffer (pH 8.0) were added, and the seminal vesicles were gently homogenized by cutting. The homogenate was centrifuged for 10 min at 4 °C in a Sorvall Super-Speed Ultracentrifuge model SS 38 at 12000*g*. The supernatant was filtered through double layers of cheesecloth and centrifuged (48000*g*) at 4 °C for 60 min. After the supernatant had been siphoned off, the precipitate was suspended in a minimal amount of Tris−HCl and immediately frozen at −80 °C in 20 μL portions. The protein content of the preparation was determined using a modified micro-Bradford procedure.[47] Data-fitting of the kinetics of both enzymes was performed using GraFit computer software.[48]

Materials. Compounds and their sources were as follows: [1-^{14}C]arachidonic acid (Amersham, Stockholm, Sweden), *l*-epinephrine, ibuprofen (Apoteksbolaget, Stockholm, Sweden), hematin [Fe^{3+}-protoporphyrin IX] (Chemicon, Stockholm, Sweden), arachidonic acid, aspirin [acetylsalicylic acid], (+)-catechin, indomethacin, naproxen, prostaglandins E_2 and $F_{2\alpha}$, quercitrin, reduced glutathione (Sigma, Stockholm, Sweden), NS-398 [*N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide] (SMS-Gruppen, Helsingborg, Sweden). Indomethacin and ibuprofen were dissolved in water with addition of 2 M NaOH (about 0.5% at an iodomethacin concentration of 10 mg/mL in water), naproxen and NS-398 were dissolved in 20% DMSO, and aspirin, (+)-catechin, and quercitrin were dissolved in 20% DMSO or 10% EtOH. IC_{50} values were obtained by linear regression analysis. For column chromatography, silica gel 60 [particle size 0.063−0.2 mm] (Merck, Darmstadt, Germany) was used. Solvents and materials were of analytical grade.

COX-1- and COX-2-Catalyzed Prostaglandin Biosynthesis Assay in Vitro. Experiments were performed according to White and Glassman,[5] with modification. In brief, 10 μL of enzyme [COX-1 (0.7−0.8 μg protein), COX-2 (3.0 units, 0.3 μg protein)] was activated with 50 μL of cofactor solution [*l*-epinephrine (1.3 mg/mL), reduced glutathione (0.3 mg/mL), and hematin (1.3 mg/mL) in oxygen-free Tris−HCl buffer (pH 8.0), with the cofactors occurring in final concentrations in the reaction mixture of 1.95 mM, 0.49 mM, and 1 μM, respectively, on ice for 5 min. For measurements with preincubation of enzyme and inhibitor, the enzyme solution (60 μL) was added to test solutions or vehicle (20 μL) in 0.2 mL eight-strip test tubes and preincubated 10 min on ice. Prior to incubation (37 °C), 20 μL of [1-^{14}C]arachidonic acid (30 μM, 17 Ci/mol) was added. When measuring without preincubation of enzyme and inhibitor, test solution and [1-^{14}C]arachidonic acid were added before the enzyme solution. Samples were incubated for 15 min at 37 °C, whereafter the reaction was terminated by addition of 10 μL of 2 M HCl and 5 μL of carrier solution (PGE_2 and $PGF_{2\alpha}$, 0,2 μg/mL of each in EtOH). The unmetabolized arachidonic acid was separated from the prostaglandin products by column chromatography and eluted with *n*-hexane−dioxane−glacial acetic acid (70:30:1). The prostaglandin products were then eluted with EtOAc−MeOH (85:15), and the samples were counted in a Packard scintillation spectrometer.

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