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Screening of Ubiquitous Plant Constituents for COX-2 Inhibition with a Scintillation Proximity Based Assay

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A rapid semi-homogeneous cyclooxygenase-2 (COX-2) enzymatic assay using scintillation proximity assay (SPA) technology was developed, and 49 ubiquitous plant secondary metabolites were screened for inhibition of COX-2-catalyzed prostaglandin E_2 (PGE_2) biosynthesis. Assay conditions were optimized with respect to reaction time, amount of antibody, radiolabeled PGE_2, and SPA beads, and the kinetic parameter, K_m, was estimated. The assay was validated with two natural triterpenoids, ursolic and oleanolic acid, known to inhibit COX-2, as well as with four synthetic COX inhibitors, NS-398, rofecoxib, indomethacin, and aspirin. Plant metabolites of different biosynthetic origin representing several substance classes, including alkaloids, anthraquinones, flavonoids, phenylpropanes, steroids, and terpenes, were screened for inhibition of COX-2-catalyzed PGE_2 production. Of these 49 plant metabolites, eugenol, pyrogallol, and cinnamaldehyde (with IC_{50} values of 129, 144, and 245 μM, respectively) were found to inhibit COX-2. This study showed that a COX-2-catalyzed PGE_2 assay using SPA is suitable for screening natural compounds with respect to COX-2 inhibition.

The enzyme cyclooxygenase-2 (COX-2), which catalyzes prostaglandin (PG) biosynthesis, has become an important target for the discovery and development of new antiinflammatory drugs. Selective COX-2 inhibitors, such as celecoxib and rofecoxib, have an improved safety profile compared with traditional nonsteroidal antiinflammatory drugs (NSAIDs) that inhibit both COX-1 and COX-2.[1] Although both COX isoenzymes catalyze PG biosynthesis, they are expressed differently: Cyclooxygenase-1 is induced in nearly every cell and produces PGs with basic physiological effects, whereas COX-2 is preferentially induced in cells by inflammatory mediators and produces PGs that contribute to inflammation. Recent studies suggest that inhibition of COX-2 may be an important strategy for prevention or treatment of various forms of cancer and Alzheimer's disease.[2]

The search for natural compounds that act as COX-2 inhibitors is rapidly progressing. In our review, we report that by 1999 over 300 plant extracts and about 30 compounds of natural origin have been evaluated for inhibition of COX-2 enzymatic activity.[3] Among the COX-2 inhibitors summarized in the review, we note that several of these commonly occur in plants, including catechin, quercetin, isoquercetin, kampherol, rosmarinic acid, oleanolic acid, and ursolic acid. As part of our search for new COX-2 inhibitory compounds, we have subjected plant extracts to bioassay-directed fractionation guided by a COX-2-catalyzed PG biosynthesis assay (in vitro). This strategy resulted in isolation of the triterpenoids ursolic and oleanolic acid and the fatty acids linoleic and α-linolenic acid as COX-2 inhibitory principles in *Plantago major* L.[4,5] These compounds are ubiquitous in plants.

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When using bioassay-guided fractionation, re-isolation of the same active principles is a risk. In addition, active compounds, occurring at high concentrations, can mask other active compounds occurring at lower concentrations. An increase in knowledge regarding the ways that common plant constituents affect COX-2 makes adjustment of the separation method possible, to avoid repeated isolation of active compounds (i.e., dereplication).[6] This increases the likelihood of finding new natural COX-2 inhibitors. Therefore, in this study, plant metabolites of different biosynthetic origin, which represent a variety of substance classes (alkaloids, anthraquinones, flavonoids, phenylpropanes, steroids, and terpenes), were selected for evaluation of inhibition of COX-2-catalyzed PGE_2 biosynthesis.

The development of more sensitive separation and detection techniques has led to rapid expansion of the number of compounds isolated from plants.[7] This development, in combination with the use of combinatorial chemistry (likewise producing large numbers of new compounds), emphasizes the need of rapid screening systems for measuring biological activity. A homogeneous assay system that is possible to automate could achieve this, increasing the throughput of compounds and plant extracts used to investigate COX-2 inhibition.[8]

A variety of in vitro methodologies have been used to assess selective COX-2 inhibitors of both natural and synthetic origin.[3,9] Two principal strategies have been employed. One involves examining the effect of inhibitors on mRNA and protein levels, along with the effect of enzymatic activity, using cell-based assays. The other strategy involves identifying inhibitors that affect the isolated enzyme. The latter can be done either continuously, using methods such as the oxygraph system, or noncontinuously, using a stop-time assay to detect the produced PGs through methods such as radioimmunoassay (RIA), enzyme immunoassay (EIA), HPLC, or radiotracer.[10−13]

The scintillation proximity assay (SPA), a development of the radiotracer method,[14] is a radioisotopic assay technique that is commonly used in a variety of enzyme, cellular, and receptor-binding assays.[15−17] The SPA technique has also been applied for complex mixtures such as plant extracts without significantly affecting the assay system.[18] Instead of a RIA method for measuring prostacyclin (PGI_2), SPA has been used for this measurement.[19] Though not designed for complex systems, due to a prepurification process, a SPA method for measuring PGE_2 has been available through Amersham. In high-throughput screening, the SPA technique has the advantage of allowing a homogeneous approach to biochemical assay systems.[8] The technique is based on immobilization of radiolabeled molecules on the surface of scintillant-containing microspheres. Only β-particles emitted from immobilized isotopes are in close proximity to the scintillation core and, thereby, are capable of producing light emission that is detectable with a β-scintillator counter.[8,17] Hardly any light is detected from radioisotopes free in the solution, due to the longer distance between these β-particles and the scintillation core; therefore, no separation of free and bound radioisotopes is necessary. This makes the method rapid, easy to handle, and amenable for automation, compared with conventional immunoassays, such as the RIA or EIA.[17] Automation of the latter immunoassays is often disregarded because they include time-consuming centrifugation or washing steps for separation of bound and free ligands.

This paper describes the development and validation of a COX-2 assay using the SPA technique for rapid investigation of COX-2-catalyzed PGE_2 biosynthesis inhibitors. The assay has been used in the screening of 49 ubiquitous natural compounds, from several substance classes, with the aim of increasing our knowledge about common plant constituents that affect COX-2.

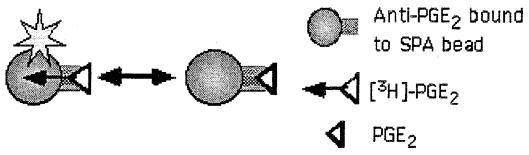

Figure 1. Radiolabeled PGE_2 binds to anti-PGE_2 on the SPA bead, emitting light, whereas no light is emitted when unlabeled PGE_2 from the bioassay binds.

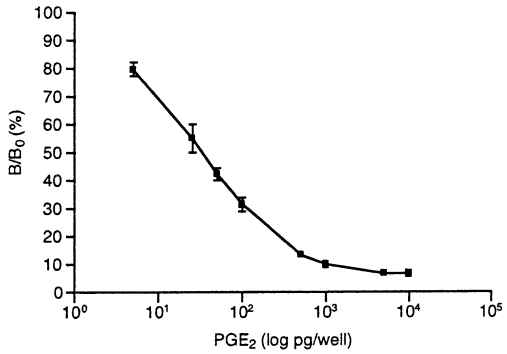

Figure 2. Representative standard curve for PGE_2 (i.e., relative binding vs log amount of PGE_2 in pg/well). The standard curves from various occasions show minor variations. Each point represents the mean ± SEM (n = 4−8).

Results and Discussion

In our current system developed for screening of inhibitors of COX-2-catalyzed PGE_2 biosynthesis, the selected SPA beads consisted of the hydrophobic polymer polyvinyl toluene (PVT) and were precoated with a secondary anti-rabbit antibody. They could thus be used to capture the primary anti-prostaglandin E_2 (rabbit) antibody. A tritium-labeled PGE_2 tracer is added, which competes with the unlabeled PGE_2 from the enzyme reaction for the binding sites of the PGE_2 antibodies (Figure 1). The SPA beads consisting of PVT were selected to enable homogeneity during dispensions, since they have considerably lower density than beads of yttrium silicate. By using a competitive method, the risk of detection of false positives caused by color quenching was reduced; however, there is a risk of underestimation of the potencies of color quenching compounds.

To achieve a detectable signal, the amounts of PGE_2 antibody, labeled PGE_2, and SPA beads were titrated, resulting in the following concentrations for each well: 100 pg of ^{3}H-labeled PGE_2, 1:5000 dilution of antibody, and 1:4 dilution of the stock solution of SPA beads (results not shown). An estimated standard curve was used to quantify the amounts of PGE_2 produced (Figure 2). The linear part (5−500 pg/well) defined the detecting range of the system, which is well suited for the described COX-2 enzymatic assay, since the amount of produced PGE_2 did not exceed 200 pg/well. The sensitivity for PGE_2 was 5 pg/well, which corresponds to the amount of unlabeled PGE_2 required to displace 20% of ^{3}H-PGE_2 from the antibody (i.e., B/B_0 = 80%). A SPA system for detecting PGE_2, using ^{125}I-labeled PGE_2 as tracer, was previously available from Amersham. Although this system was more sensitive for PGE_2 quantification, more steps were needed in the procedure, since methyl oximation of PGE_2 was required to increase the

Table 1. Natural Compounds (100 μM) Evaluated for Inhibition of COX-2-Catalyzed PGE_2 Biosynthesis[a]

compound	inhibition (%)	compound	inhibition (%)
Alkaloids		**Steroid**	
atropine	–	ouabain	–
caffeine	–	**Terpenes**	
emetine HCl	–	abietic acid	–
nicotinic acid	–	betulin	–
pipecolic acid	–	α-bisabolol	–
(–)-scopolamine HCl	–	(+)-camphor	–
stachydrine HCl	–	citronellal	–
theophylline	–	DL-citronellol	–
trigonelline HCl	–	*β*-escin	–
Anthraquinone		eucalyptol	–
sennoside B	–	(*R*)-(+)-limonene	–
Flavonoids		α-lupeol	–
chrysin	–	(–)-menthol	–
luteolin	–	thymol	–
myricetin	62 ± 7	**Other**	
naringenin	–	arbutin	–
rutin	–	hydroquinone	–
Phenylpropanes		gallic acid	–
caffeic acid	32 ± 16	pyrogallol	57 ± 7
chlorogenic acid	–	(–)-quinic acid	–
cinnamaldehyde	45 ± 7	α-terthienyl	–
coumarin	–	thioctic acid	–
p-coumaric acid	–	shikimic acid	–
3,4-dimethoxycinnamic acid	–		
esculin	–		
eugenol	40 ± 7		
isovanillic acid	–		
podophyllotoxin	–		
scopoletin	–		
umbelliferone	–		

[a] Compounds marked "–" showed less than 30% inhibition.

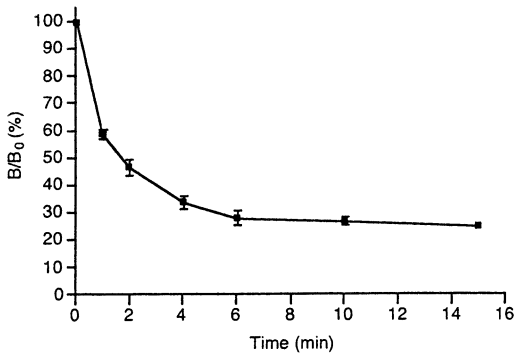

Figure 3. Time-course of COX-2-catalyzed PGE_2 biosynthesis conducted after 1.0, 2.0, 4.0, 6.0, and 15.0 min of reaction. To end the reaction, a stop-time of 6 min was selected. Each point represents the mean ± SEM (n = 3).

stability of PGE_2.[20] However, the present assay is rapid, with a handling time of about 30 min. The method may easily be adapted for robotics, since only pipeting steps are required.

The time-course of the COX-2 system was examined to estimate a stop-time for the reaction (Figure 3). The reaction velocity of the enzyme leveled off after about 6 min. Ideally, the chosen stop-time would lie within the period before reaching the steady-state level.[21] However, to obtain a reproducible and robust assay for analysis of a large number of samples, a stop-time of 6.0 min was selected. This time marks the beginning of the steady state and might lead to an underestimation of the potency of the inhibitors. The K_m value of COX-2 for arachidonic acid was estimated to be 1.0 μM, which accords with the 2.1 μM estimate reported by Johnson et al.[22]

The SPA assay was validated with two natural compounds that we had previously identified as COX-2 inhibitors.[4] In the developed assay, the triterpenoids ursolic and oleanolic acid inhibited COX-2-catalyzed PGE_2 biosynthesis, with IC_{50} values of 86 and 87 μM, respectively. Further, the inhibition of COX-2-catalyzed PGE_2 biosynthesis of four known synthetic COX inhibitors, NS-398, rofecoxib, indomethacin, and aspirin, was investigated.

The compound NS-398 was the most potent inhibitor of COX-2-catalyzed PG biosynthesis, followed by rofecoxib, with IC_{50} values of 0.16 and 0.76 μM, respectively. Both compounds are known to be COX-2 selective compounds.[2] As expected, indomethacin and aspirin, inhibitors that are known to be more COX-1 selective, were found to be less potent, with IC_{50} values of 3.9 and 780 μM, respectively. The COX-2 inhibitory rank-order of the three synthetic reference compounds (NS-398, indomethacin, and aspirin) accords with that of other assays using the same enzyme source.[13,22] Among the 49 compounds screened for COX-2 inhibition, five compounds, namely, pyrogallol, eugenol, cinnamaldehyde (cinnamic aldehyde), myricetin, and caffeic acid, showed more than 30% inhibition at a 100 μM concentration (Table 1). Dose–response experiments of these compounds were therefore run. At the lower concentration (10 μM) that was tested, none of the compounds inhibited COX-2-catalyzed PGE_2 biosynthesis. Eugenol and pyrogallol were found to be equipotent at 100 μM, while cinnamaldehyde was less potent (Figure 4). In dose–response experiments, eugenol and pyrogallol were found to be equipotent, and cinnamaldehyde was less potent (Figure 4), while myricetin and caffeic acids showed no dose-dependent inhibition.

The most potent COX-2 inhibitor, eugenol, is a phenylpropane that naturally exists in *Syzygium aromaticum* L., for example. Eugenol inhibited the COX-2-catalyzed production of PGE_2 with an IC_{50} value of 129 μM. After monitoring the initial rate of oxygen consumption, Kelm et al. reported that eugenol inhibited COX-2 activity.[23] Eugenol has also been described to inhibit COX-1-catalyzed

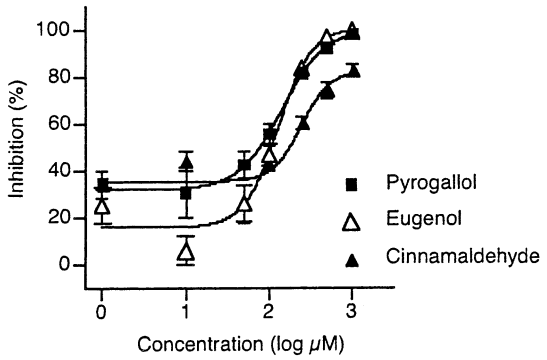

Figure 4. Inhibitory effects of pyrogallol, eugenol, and cinnamaldehyde on COX-2-catalyzed PGE_2 biosynthesis. Each point represents the mean ±SEM (n = 4−8).

PG biosynthesis.[24−27] Another phenylpropane, cinnamaldehyde, a main constituent of the volatile oil from the bark of *Cinnamomum zeylanicum* Blume, inhibited the production of PGE_2 (with an IC_{50} value of 245 μM). Takenaga et al. reported that cinnamic aldehyde has no effect on COX-catalyzed biosynthesis of TXB_2 and HHT in human platelets, with arachidonic acid added exogenously.[28] This enzymatic effect was later ascribed to COX-1 activity.[29]

The tannin pyrogallol was found to dose-dependently inhibit COX-2-catalyzed production of PGE_2, with an IC_{50} value of 144 μM. This tannin, depending on the concentrations used, is reported to both stimulate and inhibit the production of PGE_2 in Ca ionophore A23187-stimulated human polymorphonuclear leukocytes.[30] For the other plant metabolites screened for COX-2-catalyzed PGE_2 production, an inhibition of less than 30% was observed.

Other research groups have also investigated some of these compounds for COX-2 enzymatic activity. However, assay conditions vary substantially between laboratories. Hence, the IC_{50} values are not directly comparable, especially when the enzymatic activity is measured in whole cells stimulated to express COX-2. Consistent with our results, Lee et al. did not observe inhibition of COX-2-catalyzed PGE_2 production by luteolin (IC_{50} < 100 μg/mL).[31] Variation between different assays for COX-2 measurements is evident, however, since Moon et al. observed 80% inhibition in COX-2 enzyme activity by luteolin (2.5 μg/mL) in a study of PGD_2 production in bone marrow-derived mast cells stimulated with a solution of ckit-ligand, Il-10, and LPS.[32] Chen et al. report a small inhibition with rutin (20 and 40 μM) of LPS-induced PGE_2 production in RAW 264.7 macrophages.[33] Yet the same effect was not observed when arachidonic acid was added to the cells (direct enzymatic activity).[33] Naringenin, which was also tested for enzyme activity in a LPS-stimulated mouse macrophage J774.A1 cell line, inhibited PGE_2 production at 50 μM.[34] Murakami et al. observed no suppression by the phenylpropane umbelliferone of PGE_2 production in LPS-stimulated RAW 264.7 cells,[35] which is consistent with our results.

Of the 49 plant metabolites investigated and detected as COX-2 inhibitors, most have phenolic moieties. None of the alkaloids, anthraquinones, steroids, and terpenes tested inhibited COX-2. However, one cannot exclude that, in a larger survey, compounds from these substance classes will be identified as COX-2 inhibitors. Furthermore, it is likely that many other phenolic compounds will inhibit COX-2. We suggest therefore that these compounds should be dereplicated when plant extracts are subjected to bioassay-guided separation. Phenolic compounds are known to be inhibitors of COX-1[24,36,37] and are therefore likely to also inhibit COX-2, since the active sites of COX-2 and COX-1 are almost identical.[1] The compounds are known to modulate arachidonic acid metabolism through different mechanisms, such as by direct competitive reversible inhibition, acting as electron donors used in the hydroperoxidase reaction, and protecting the enzyme from self-inactivation.[24]

In summary, we developed a rational assay that can provide a rapid alternative to other COX-2 enzymatic assays for studying the inhibition of COX-2-catalyzed PGE_2 biosynthesis in both natural and synthetic compounds. The use of SPA technology reduces handling time by omitting time-consuming separation steps, such as separation of unmetabolized arachidonic acid from PGE_2 that is necessary in a radiochemical assay or separation of free from bound ligands that is required in RIA and EIA. This study shows that the SPA-based assay is suitable for screening natural compounds for inhibitory effects on COX-2. Further, 3 out of 49 screened compounds (eugenol, cinnamaldehyde, and pyrogallol) were found to inhibit COX-2-catalyzed PGE_2 biosynthesis.

Experimental Section

Materials. Arachidonic acid, aspirin, reduced glutathione, hematin, PGE_2, and indomethacin were obtained from Sigma (Sweden). Radiolabeled [5,6,8,11,12,14,15(n)-^{3}H] PGE_2 and SPA polyvinyl toluene beads (≈5 μm diameter) precoated with secondary anti-rabbit antibody were purchased from Amersham (Sweden); l-epinephrine, from Apoteksbolaget (Sweden); anti-PGE_2 (rabbit, polyclonal antiserum), from Upstate Biotechnology (USA); NS-398 ([*N*-[2-(cyclohexyloxy)-4-nitrophenyl]methane-sulfonamide]) and purified COX-2 (PGH synthase-2, obtained from sheep placental cotyledons), from Cayman Chemical Co. (USA); and sodium bisulfite (granular), from Kebo (Sweden). Rofecoxib was a gift from Merck Research Laboratories (USA). Compounds tested in the screening were obtained from Sigma (Sweden), except for betulin, α-lupeol, scopoletin, sennoside B, and stachydrin HCl (Roth, Labkemi, Sweden); caffeine, esculin, and eugenol (Merck, Sweden); nicotinic acid (Serva Labkemi, Sweden); 3,4-dimethoxycinnamic acid and limonene (Aldrich-Chemie, Labkemi, Sweden); α-bisabolol, caffeic acid, and cinnamaldehyde (Fluka AG, Sweden); and emetin HCl (WHO Centre for Chemical Reference Substances, Sweden).

Reference compounds, dissolved in 20% DMSO, were tested at seven concentrations. For screening purposes, the plant secondary metabolites were tested at two concentrations (10 and 100 μM) in no less than two individual experiments (n = 2−5). Indomethacin was used as a positive control (6 μM). Screened substances that inhibited COX-2-catalyzed PGE_2 biosynthesis by more than 30% were selected for further dose−response experiments.

Cyclooxygenase-2-Catalyzed Prostaglandin E_2 Biosynthesis Assay. Experiments were carried out (with modifications) according to Noreen et al.[13] Reactions were initiated in a 96-well plate (Optiplate No. 6005190, Packard, Sweden), with a final volume of 200 μL. COX-2 (3.0 units, 20 μL) was preincubated for 5 min at room temperature in 105 μL of cofactor solution (L-epinephrine (3.7 mM), reduced glutathione (0.9 mM), and hematin (2 μM) in 0.1 M Tris buffer (pH 8.0), resulting in final concentrations of 2.0 mM, 0.5 mM, and 1 μM, respectively). Addition of 3.0 units (125 μL) enzyme-cofactor solution to the test compounds or to the vehicle (50 μL) was followed by 10 min of incubation at room temperature. The reaction was initiated by adding arachidonic acid (25 μL, final concentration 6 μM). After 6 min reaction time, sodium bisulfite solution (50 μL, 25% w/v in 0.1 M Tris buffer, pH 8.0) was added to terminate the reaction. To equilibrate the solution, the 96-well plate was placed on a shaking table for 10 min, whereupon an aliquot of 25 μL was transferred to a detection well containing the same amount of Tris buffer (0.1

M, pH 8.0). The addition of 50 μL (100 pg) of ^{3}H-labeled PGE_2, 50 μL of anti-PGE_2 solution (final dilution 1:5000), and 50 μL of SPA beads (stock solution reconstituted in 25.0 mL of sodium azide 0.01% in 0.1 M Tris buffer, pH 8.0) was followed by incubation of the plate (90 min) before scintillation counting in a Packard Microplate Top Count scintillation counter. The manufacturer reports that the antibody for PGE_2 cross-reacts with PGE_1 (100%), $PGF_{2\alpha}$ (3%), and $PGF_{1\alpha}$ (2%).

To quantify the amounts of PGE_2 produced in the assay, a standard curve was created by increasing the concentration of unlabeled PGE_2 in the wells containing SPA beads and labeled PGE_2. The amount of produced PGE_2 in the assay was estimated from the standard curve, and the DPM values were transformed into relative values given in percent tracer binding ($B/B_0 \times 100$), where B is the signal of the test sample, and B_0, the maximal signal measured after addition of 100 pg of labeled PGE_2. The inhibition of COX-2-catalyzed PG biosynthesis was calculated as the relative decrease in produced PGE_2 of the samples containing test substance as compared with the solvent vehicle. The IC_{50} values were obtained by nonlinear regression analysis.[38]

The enzyme kinetic parameter (K_m) was estimated by measuring the enzyme velocity at six different concentrations of arachidonic acid (1.0−20.0 μM) at seven different time points (0−10 min). Subsequently, K_m was estimated by plotting enzyme velocity against the substrate concentration, and the curve was analyzed by nonlinear regression analysis using Excel Fit.

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