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Prostaglandin-H-synthase (PGHS)-1 and -2 microtiter assays for the testing of herbal drugs and in vitro inhibition of PGHS-isoenzyms by polyunsaturated fatty acids from Platycodi radix

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Abstract

In order to test inhibition of prostaglandin-H-synthase-1 and -2 (PGHS-1 and -2) by plant extracts, we have established two enzyme based in vitro assays with enzyme immunoassay (EIA) evaluation. The assays have been evaluated with known synthetic inhibitors and with plant extracts.

In a screening of traditionally used Chinese herbs for anti-inflammatory activity, a series of *n*-hexane and dichloromethane extracts showed significant inhibitory effect in comparison with the known specific PGHS-2 inhibitors NS-398 ($IC_{50} = 2.6\,\mu M$) and nimesulide ($IC_{50} = 36\,\mu M$).

The lipophilic extracts of the Chinese drug Jiengeng, the dried roots of *Platycodon grandiflorum* (Jacq.) A. DC. (Campanulaceae), showed good inhibitory activity against both PGHS isoenzymes. The directly prepared DCM-extract exhibited better activity against PGHS-2 ($IC_{50} = 4.0\,\mu g/ml$) than against PGHS-1 ($IC_{50} = 17.6\,\mu g/ml$). We identified fatty acids as main active constituents and quantified them. Linoleic acid showed the highest content (ca. 20% of the dried extract) and a high and preferential PGHS-2 inhibitory activity (IC_{50} (PGHS-1) = 20 μM; IC_{50} (PGHS-2) = 2 μM). The comparison of the concentration of linoleic acid and the inhibitory activity of the direct DCM-extract showed, that linoleic acid is mainly responsible for the in vitro activity of the extract on PGHS-2.

Keywords: COX-1; COX-2; In vitro assays; Chinese herbs; *Platycodon grandiflorum*; Fatty acids; PGHS-isoenzymes

Introduction

Prostaglandin-H synthase (PGHS) catalyses the first two steps of the biosynthesis of prostaglandins (PGs) from arachidonic acid (AA). It forms in the first step PGG_2, a hydroperoxyendoperoxide, and by reduction in the second step PGH_2, a hydroendoperoxide. This reacts subsequently enzymatically or non-enzymatically to various PGs (Vane et al., 1998). PGs, especially PGE_2, support the release of further mediators of inflammation and cause the typical symptoms at sites of inflammation. Therefore, PGHS has been regarded for a long time as an important target of most non-steroidal-antiinflammatory drugs (NSAIDs) like indomethacin or diclofenac (Herschman, 1996).

PGHS exists in several isoforms. Constitutive PGHS-1 is important in generating PGs for physiological purposes and acts as a housekeeping enzyme for

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homeostasis of various physiologic functions like protection of the stomach or bronchodilatation (Lands et al., 1973). Therefore its inhibition results in a number of unwanted side effects especially on the gastrointestinal tract and on the kidneys. The inducible isoenzyme PGHS-2 is stimulated by many pro-inflammatory agents and increases PGs biosynthesis rapidly at sites of inflammation (Seibert et al., 1994). The results of long-term treatment with COX-2-selective drugs suggested that they possess lower gastrotoxicity than non-selective NSAIDs. In recent years there has been accumulated evidence of positive effects of COX-2 inhibitors in cancer and Alzheimer's disease. However, recent findings of an elevated incidence of myocardial infarction after long term use of rofecoxib questioned the safety of these drugs (Simmons et al., 2004). Therefore, dual inhibition of PGHS-2 and 5-lipoxygenase (5-LOX) has been considered now as a new strategy to provide safer non-steroidal anti-inflammatory drugs (Charlier and Michaux, 2003).

PGHS activity has long been studied in preparations from ram and bull seminal vesicles (Hemler et al., 1976). Investigations showed, that in these tissues PGHS-1 is the exclusively active isoform. The second isoform is found in sheep placental cotyledones and its microsomal preparations have become a target for the discovery of selective PGHS-2 inhibitors (Johnson et al., 1995; Pairet and van'Ryn, 1998).

In order to test for active compounds in plants, we have developed two enzyme based in vitro assays with enzyme immunoassay (EIA) evaluation useful for high throughput screening. The assays should test both enzymes and each assay should be comparable to the other isoenzyme. The assays were evaluated with known synthetic inhibitors like indometacin, NS-398 and nimesulide (Lands et al., 1973; Huff et al., 1995) and with plant extracts.

Materials and methods

Pharmacological assays

PGHS-2 assay

The assay was performed in a microtiter scale with purified PGHS-2 from sheep placental cotyledones (Cayman Chemical) (Johnson et al., 1995). The incubation mixture contained 180 µl 0.1 M TRIS (USB)/HCl buffer (pH 8.0), 50 µM Na_2EDTA (Merck), 18 mM epinephrine-hydrogentartrate (Merck) and 5 µM hematin (Sigma). Each plant extract (10 µl), compound or inhibitor dissolved in ethanol (EtOH) p.a. and 0.2 units PGHS-2 enzyme in 1 µl 80 mM TRIS/HCl buffer (pH 8.0) with 0.1% TWEEN 20 (Cayman Chemical) and 300 µM DDC (Fluka) were added and preincubated for 5 min at room-temperature. The reaction was started by adding 10 µl of 5 µM AA (Cayman Chemical) dissolved in EtOH p.a. and incubated for 20 min at 37 °C. The reaction was terminated by addition of 10 µl formic acid 10%.

PGHS-1 assay

The determination of PGHS-1 inhibition was performed according to the PGHS-2 assay with 0.2 units purified PGHS-1 enzyme from ram seminal vesicles (Cayman Chemical), but without Na_2EDTA and instead additional 10 µl of 80 mM TRIS/HCl buffer (pH 8.0).

Determination of PGE_2

Assay of the successively prepared extracts

The concentration of PGE_2 was determined by a PGE_2 EIA kit, which was used as described by Cayman Chemical company, Ann Arbor (Cayman Chemical Company, 1998). All samples were diluted 1:100 in EIA-buffer. The determination was achieved by a competitive enzyme immunoassay with PGE_2-acetylcholinesterase conjugate as tracer, specific rabbit antiserum and Ellman's Reagent (Pradelles et al., 1985). The absorbance is reversed proportional to the concentration of PGE_2.

Assay of DCM extracts and pure compounds

Later on PGE_2 was determined by a competitive PGE_2 EIA kit from R&D Systems (Cat. No. DEO100), which has been used like the kit from Cayman Chemical Company. Because of the use of alkaline phosphatase, the procedure was shortened to 2 h incubation time and 45 min for the development process, which involves *p*-nitrophenyl phosphate (pNPP) acting as a substrate. The development is stopped by adding 10 µl of 2N-NaOH. All samples were diluted 1:15 in EIA-buffer.

Calculation of the inhibitory effects

The EIAs were evaluated with the ELISA reader "rainbow" (TECAN). Inhibition refers to reduction of PGE_2 formation in comparison to a blank without inhibitor. The inhibition is calculated according to the following equation:

$$\text{Inhibition}\,[\%] : 100 - \frac{\text{conc. PGE}_2\ \text{(sample)} \times 100}{\text{conc. PGE}_2\ \text{(control)}}.$$

Extraction

Plant material was provided by the TCM-Hospital, Kötzting, Germany. Voucher specimens are deposited at the Karl-Franzens-Universität, Graz, Austria.

For successive extraction 5 g powdered plant material was extracted for 2 h with 60 ml of *n*-hexane under reflux. The same plant material was further extracted with 60 ml DCM, followed by methanol and pure water. The extracts were evaporated to dryness and dissolved in EtOH p.a. at a concentration of 1 mg/ml. For the directly prepared DCM extract 5 g powdered plant material was extracted for 2 h with 60 ml DCM under reflux. After evaporation to dryness it was dissolved in EtOH p.a. at necessary concentrations.

Reference substances

NS-398, nimesulide and indometacin were purchased from Cayman Chemical Company. They were dissolved in EtOH p.a. at a concentration of 1 mM and further diluted with EtOH p.a.

Palmitic acid, stearic acid, oleic acid, linoleic acid and α-linolenic acid were purchased from Sigma. They were dissolved in EtOH p.a. at a concentration of 10 mM and further diluted with EtOH p.a.

Statistics

All IC_{50} values were determined for both enzymes by measuring at least three concentrations, all inhibition values are means of at least three experiments. The calculation of IC_{50} values was performed by a semi-logarithmical presentation and logarithmical regression analysis.

Quantification of fatty acids

Instrumentation: Gas chromatograph HP 5890 with FID detector; column: MN Optima-1 LT = 0.25 μm; 25 m × 0.25 mm; Gradient: 165 °C isotherm for 6 min, 165–180 °C with 19 °C/min, 180 °C isotherm for 20 min.

Preparation of the methyl esters was performed by boiling the pure fatty acid or extracts with 2 ml 0.5 N methanolic NaOH under reflux for 5 min, another 2 min after adding 2 ml 14% methanolic BF_3, again 2 min after adding 4 ml *n*-heptane. The methylesters were then floated using a saturated sodium chloride solution. The *n*-heptane layer was dried over Na_2SO_4 and directly injected after filtration (modified procedure according to van Wijngaarden, 1967).

Quantification results were calculated with myristic acid as internal standard and correction factors for each fatty acid were established with the pure compounds.

Results and discussion

The design of the in vitro assays was adopted from an established PGHS-1 in vitro assay, which was based on microsomes from ram seminal vesicles and performed with radioactive AA (Redl et al., 1994). The presented assays are performed with purified PGHS-2 from sheep placental cotyledones (Johnson et al., 1995) and purified PGHS-1 from ram seminal vesicles, respectively, to achieve high specificity and comparability. For optimal effectivity of the enzyme and high reproducibility 18 mM epinephrine-hydrogentartrate are necessary as cofactor to scavenge radicals, which are produced during peroxidase-reaction of PGHS. Epinephrine-hydrogen tartrate (18 mM) turned out to be the optimum, since the highest production of PGE_2 and less production of side-products was achieved at this concentration (Fig. 1A and B).

Hematin is a second necessary cofactor. This prosthetic group is lost during purification process of PGHS. During preincubation the apoenzyme and hematin form the active holoenzyme. At the concentration of 5 μM hematin, the formation of PGE_2 was reproducible high, but it showed no affect on the inhibition of substances (data not shown).

Na_2EDTA protects PGHS-2 from inactivation by metalloproteases, but does not interfere with the enzyme activity. It is not necessary for PGHS-1, because this enzyme shows higher purity than PGHS-2 (Johnson et al., 1995).

These conditions lead to a transformation of AA to PGE_2. Only few other products like PGD_2, 12-HHT and 5-HETE could be detected (Fig. 1A).

In order to achieve a high throughput, the determination of the PGE_2 concentration was performed by a specific EIA (Pradelles et al., 1985). This also allows the use of non-radioactive AA, compared to previous assays (Redl et al., 1994). Because of the high sensitivity of the EIA, the amount of enzyme could be reduced. The necessary amount was 0.2 U calculated according to the specific activity labelled by the manufacturer. The concentration of 5 μM AA guarantees to work with a

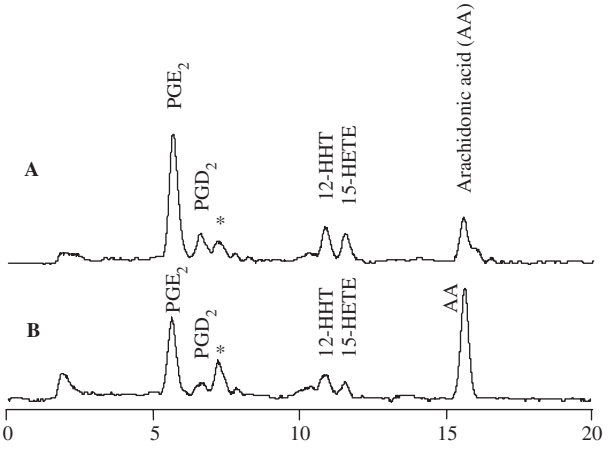

Fig. 1. HPLC-chromatogram of the metabolites of arachidonic acid after incubation measured according to Redl et al. (1994). (A) with 18 mM epinephrine-hydrogentartrate and 2.5 U/sample PGHS-2. (B) with 3 mM epinephrine-hydrogentartrate and 2.5 U/sample PGHS-2. *Unidentified metabolite.

Table 1. Specificity of the EIA from Cayman company (Cayman Chemical Company, 1998)

Metabolite	Specifity (%)
Prostaglandin E_2	100
Prostaglandin E_3	43
Prostaglandin E_1	18.7
Prostaglandin B_2	<0.01
Prostaglandin D_2	<0.01
15-keto prostaglandin E_2	<0.01
Prostaglandin $F_{2\alpha}$	<0.01
Thromboxane B_2	<0.01

surplus of substrate. This is important to prevent an inhibition simulated because of the missing of substrate.

The EIA from Cayman Chemical Company was about 1000 times more sensitive than the HPLC method with radioactive AA. The EIA purchased from R&D-Systems showed 150 times higher sensitivity than the radioactive method (data not shown). Therefore, they are more efficient than assays using chromatographic determination of AA metabolites (Noreen et al., 1998). The incubation mixture can be used for the determination of PGE_2 without any sample cleanup. There is almost no cross reactivity to other metabolites (Table 1).

The PGHS-1 and PGHS-2 assays are directly comparable, because both enzymes are purified and from the same species. They are used with the same amount of specific activity. Although the conditions are not fully physiological (Otto and Smith, 1995), the in vitro assays are suitable to compare the selectivity of substances and to guarantee fast and high throughput. The time for testing 30 samples with the new assays could be shortened to one-eighth compared to the previously used assay with HPLC-evaluation (Redl et al., 1994).

Common NSAIDs are more potent against PGHS-1 or equipotent against both isoenzymes (Seibert et al., 1994). Our test system showed equipotent inhibitory effect for indometacin ($IC_{50} = 0.8\,\mu M$). Known specific PGHS-2 inhibitors showed better inhibitory effects for PGHS-2 than PGHS-1 e.g. NS-398 (IC_{50} PGHS-2 = 2.6 μM; IC_{50} PGHS-1 = 50.7 μM), nimesulide (IC_{50} PGHS-2 = 36.4 μM; IC_{50} PGHS-1 = 246.3 μM) (Fig. 2) and rofecoxib (IC_{50} PGHS-2 = 44 nM; IC_{50} PGHS-1 > 1.6 mM) (not shown in Fig. 2).

A series of *n*-hexane extracts and successively prepared DCM-extracts from plants, which have been used for anti-inflammatory purposes in traditional Chinese medicine, were screened with these PGHS-1 and -2 in vitro assays.

Extracts of Angelicae sinensis radix, Angelicae dahuricae radix, Atractylodis lanceae rhizoma, Notopterygii rhizoma seu radix, Piperi sarmentosi fructus, Platycodi radix and Zingiberis rhizoma showed high inhibitory effects at a concentration of 50 μg/ml. They

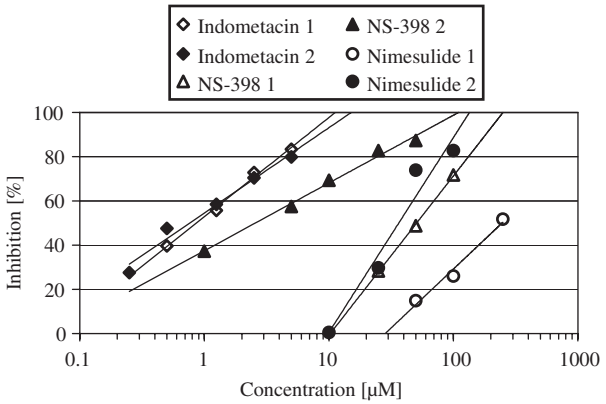

Fig. 2. Determination of the IC_{50}-values of known PGHS-inhibitors as positive controls (1) PGHS-1; and (2) PGHS-2.

all exhibited inhibitory activity towards PGHS-1 and PGHS-2 in a similar range (Table 2).

Platycodi radix, Jiegeng, used as an antiinflammatory and antitussive agent in traditional Chinese medicine, turned out to be an interesting drug for further investigations. The *n*-hexane and DCM extracts exhibited in comparison with the other tested extracts a very high inhibition of both enzymes.

For a systematic evaluation of this drug the dried roots of *Platycodon grandiflorum* (Jacq.) A. DC. (Campanulaceae) were successively extracted with *n*-hexane, DCM, methanol and water obtaining extracts of different polarity with yields of 0.5% (*n*-hexane), 0.1% (DCM), 15.6% (methanol) and 10.3% (water). At a concentration of 50 μg/ml the lipophilic extracts, *n*-hexane (50 ± 6%) and DCM (70 ± 8%) exhibited good inhibitory activity on PGHS-1 and PGHS-2, however, without a statistical significant selective inhibitory effect on one of the two PGHS isoforms. Hydrophilic extracts were not active.

Only when the raw material was extracted directly with DCM, the extract showed better PGHS-2 than PGHS-1 inhibitory activity (IC_{50} (PGHS-1) = 17.6 μg/ml; IC_{50} (PGHS-2) = 4.0 μg/ml). We therefore expected constituents with preferential PGHS-2 inhibitory activity. By chromatographic methods (TLC, GC-MS) we identified fatty acids as main compounds in this extract. Fatty acids are known to act as competitive inhibitors of both enzymes (Johnson et al., 1995; Huff et al., 1995). Therefore, we expected a contribution to the inhibitory effect of the extract.

The quantification of palmitic acid, linoleic acid, oleic acid and stearic acid showed that the content of linoleic acid was remarkably high (ca. 20% of the dried DCM extract). Palmitic acid was present in a concentration of about 7% of the dried extract. The amount of other fatty acids was less than 1% (Table 3). The IC_{50} values of linoleic acid and the other fatty acids were determined for both enzymes (Table 3). The polyunsaturated fatty

Table 2. Inhibition of PGHS-1 and PGHS-2 by several successively prepared extracts from TCM drugs (screening concentration 50 µg/ml)

No.			*n*-hexane extract		Successively prepared DCM-extract	
	Inhibition (%) of		PGHS-2	PGHS-1	PGHS-2	PGHS-1
1	Angelicae dahuricae radix	Baizhi	42.4	52.5	38.8	0.1
2	Angelicae sinsesis radix	Danggui	61.5	73.0	55.8	75.0
3	Atractylodis lanceae rhizoma	Cangzhu	68.3	67.4	50.3	46.9
4	Atractylodis macrocephalae rhizoma	Baizhu	48.9	46.1	47.0	58.6
5	Cinnamomi ramulus	Guizhi	23.6	46.6	48.4	73.5
6	Houttuyniae herba	Yuxingcao	43.4	50.3	40.9	46.8
7	Notopterygii rhizoma seu radix	Qianghao	64.9	69.6	−2.1	42.6
8	Piperis sarmentosi herba	Jiaju	65.0	52.4	10.1	47.2
9	Platycodi radix	Jiegeng	55.1	48.7	70.1	77.8
10	Zanthoxyli pericarpium	Huajiao	24.9	48.5	31.3	18.3
11	Zingiberis rhizoma	Gangjiang	77.5	83.4	41.3	52.9

Table 3. Content of fatty acids in the directly prepared DCM-extract of Platycodi radix and inhibition of PGHS-1 and PGHS-2 by fatty acids (IC_{50}-values)

	Content of fatty acids of the DCM-extract (%)	IC_{50} PGHS-1 (µM)	IC_{50} PGHS-2 (µM)	Ratio PGHS-1/PGHS-2
Linoleic acid	20	19.8	2.6	7.6
Palmitic acid	7	—	—	—
Stearic acid	<1	—	—	—
Oleic acid	<1	124.5	122.6	1.0
α-linolenic acid	<1	21.2	3.0	7.1
Indometacin	—	0.9	0.8	1.1
NS-398	—	50.7	2.6	19.5

acids, linoleic acid and α-linolenic acid showed better inhibitory activity on PGHS-2 than on PGHS-1. The exchange of the voluminous amino acid isoleucine in PGHS-1 by the smaller valin in PGHS-2 causes the bigger and more flexible channel to the active site of PGHS-2. This fact suggests, that PGHS-2 accepts a wider range of fatty acids as a substrate (Otto and Smith, 1995; Gierse et al., 1996).

Palmitic acid and stearic acid showed no significant inhibitory effect on both enzymes. Oleic acid has weak inhibitory activity, but on both enzymes to the same extent.

Fig. 3 show the comparison of the amount of linoleic acid in the directly prepared DCM-extract and the inhibitory activity of this extract. The inhibitory effect of linoleic acid is shown as a reference at comparable concentrations in the same figure. According to these results, linoleic acid is mainly responsible for the in vitro PGHS-2 inhibitory activity of this extract. Other compounds of the extract seem to be not relevant for PGHS-2 inhibition.

On the other hand, the comparison of PGHS-1 inhibitory activity of the extract and the content of

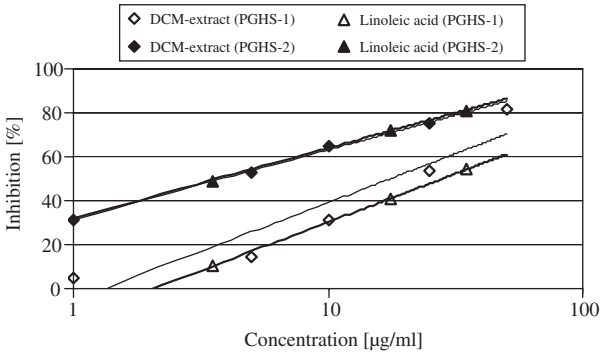

Fig. 3. Comparison of the inhibitory activity of directly prepared DCM- extract of Platycodi radix and linoleic acid (concentration of linoleic acid is calculated in relation of its concentration in the extract).

linoleic acid demonstrated, that there is a significant discrepancy between the activity of linoleic acid and the DCM extract. In this case there must be other compounds contributing to the inhibitory activity of the extract, e.g. phytosterols which we identified as another major group of compounds in Jiegeng with weak PGHS-1 inhibitory activity. A mixture of

α-spinasterole, campsterol and Δ7 stigmastenole as a precipitate of the DCM-extract exhibited in a concentration of 50 μg/ml 30% inhibition of PGHS-1, but no effect on PGHS-2.

In conclusion, high concentration of polyunsaturated fatty acids, especially linoleic acid, can be the reason for selective in vitro PGHS-2 inhibitory effects of plant extracts. Thus the testing for fatty acids and their quantification is compulsory before testing extracts for PGHS-1 and PGHS-2 inhibition.

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