Inhibitory Activity of Tryptanthrin on Prostaglandin and Leukotriene Synthesis

Heinrich Mustermann 1, Christine Müller 2, Matthias Hamburger 1

Affiliation

1 Institute of Pharmaceutical Biology, University of Basel, Basel, Switzerland
2 Institute of Pharmacy, University of Nowhere, Seldwyla, Switzerland

Correspondence

Prof. Dr. Matthias Hamburger, Institute of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland. E-mail: matthias.hamburger@unibas.ch Phone: +41 61 267 14 25 Fax: +41 61 267 14 74
Abstract

The indolo[2,1-b]quinazoline alkaloid tryptanthrin has previously been identified as the cyclooxygenase-2 inhibitory principle in the extract ZE550 prepared from the medicinal plant *Isatis tinctoria*. We here investigated the potential inhibitory activity of tryptanthrin and ZE550 on cyclooxygenase-2 and cyclooxygenase-1 in cellular and cell-free systems. A certain degree of selectivity towards cyclooxygenase-2 was observed when cyclooxygenase-1 dependent formation of thromboxane B2 in HEL cells and cyclooxygenase-2 dependent formation of 6-keto prostaglandin F1α in Mono Mac 6 and RAW 264.7 cells were compared. Preferential inhibition of cyclooxygenase-2 by two orders of magnitude was found in phorbol myristate acetate activated bovine aortic coronary endothelial cells. Assays with purified cyclooxygenase isoenzymes from sheep confirmed the high selectivity towards cyclooxygenase-2. The leukotriene B4 release from calcium-ionophore-stimulated human granulocytes (neutrophils) was used as a model to determine 5-lipoxygenase activity. Tryptanthrin and the extract ZE550 inhibited leukotriene B4 release in a dose dependent manner and with a potency comparable to that of the clinically used 5-lipoxygenase inhibitor zileuton.

Key words
tryptanthrin, *Isatis tinctoria*, Brassicaceae, cyclooxygenase, 5-lipoxygenase, 6-keto-PGF1α, TxB2
Introduction

There is still an unmet need for medicinal plants and phytopharmaceuticals with scientifically proven anti-inflammatory and antirheumatic efficacy. Although herbal drugs such as devil’s claw (Harpagophytement procumbens DC., Pedaliaceae), willow bark (Salix alba L., Salicaceae) and others have been positively reviewed in the 1980’s by the subcommittee for phytotherapy (the so-called Kommission E) of the German Drug Agency [1], none of these herbs nor phytopharmaceuticals obtained from their extracts satisfies the criteria of orthodox medicine. Due to the good acceptance of herbal drugs among the population, phytopharmaceuticals with demonstrated clinical efficacy could become a suitable therapeutic alternative to current medication for specific indications such as the adjuvant treatment of mild or chronic forms of rheumatic and other inflammatory ailments [2].

In a survey of historical accounts on old folk medicines with potential antiinflammatory properties, woad (Isatis tinctoria L., Brassicaceae) was selected for further investigation. Subsequently, lipophilic woad extracts such as ZE550 were found to display a promising in vitro pharmacological profile. In cell culture assays a pronounced inhibition was found for cyclooxygenase-2 (COX-2) only, but not for cyclooxygenase-1 (COX-1). Furthermore, a distinct effect on purified 5-lipoxygenase (5-LOX), and inhibition of histamine and serotonin release from mast cells was shown. With the aid of activity directed fractionation tryptanthrin (Fig. 1) was identified as the COX-2 inhibitory principle in the extracts [3, 4]. Given the compound’s rather remarkable potency in a cell based assay for COX-2 activity with Mono Mac 6 cells, we conducted more detailed investigations to elucidate the in vitro pharmacological effects of tryptanthrin on key enzymes involved in the eicosanoid synthesis.

Etc.
Results

The inhibition of eicosanoid formation in LPS-stimulated Mono Mac 6 cells was determined by the measurement of 6-keto-PGF$_{1\alpha}$ synthesis. Preliminary experiments had established the comparability of data with those obtained in a PGE$_2$ assay. First, dose-inhibition curves for tryptanthrin and a number of known COX-inhibitors were obtained (Fig. 2A). The IC$_{50}$ value of tryptanthrin (0.037 µM) was comparable to that of nimesulide (IC$_{50}$ 0.027 µM), a clinically used preferential COX-2 inhibitor. The potency of acetyl salicylic acid was lower by two orders of magnitude (IC$_{50}$ 3.8 µM). This finding is in line with the fact that the latter compound is a selective COX-1 inhibitor [5]. Diclofenac, a clinically used non-selective COX inhibitor, and NS 398, a selective COX-2 inhibitor, showed IC$_{50}$ values of 0.002 and 0.003 µM, respectively. The relative inhibitory potencies of tryptanthrin, nimesulide and NS 398 on 6-keto-PGF$_{1\alpha}$ synthesis in RAW 264.7 cells (Fig. 2B) was comparable to those in Mono Mac 6 cells, although all IC$_{50}$ values were shifted by about one order of magnitude (IC$_{50}$ of 0.25 µM, 0.21 µM and 0.013 µM, respectively).

Discussion

With respect to the mode of action of tryptanthrin there is evidence of its inhibitory activity on key enzymes of the arachidonic acid cascade in various cell types. Tryptanthrin strongly inhibited prostaglandin synthesis in stimulated Mono Mac 6, RAW 264.7 cells and BAECs expressing COX-2, whereas significantly weaker inhibition was observed in HEL cells and thrombocytes which both constitutively express COX-1. The difficulties arising with assessment of isoenzyme selectivity due to a lack of standard in vitro assays has been pointed out earlier [14]. The consistency of our findings in a range of assay systems using known reference compounds and different endpoint measures,
however, strongly corroborate the selectivity of the compound.

Etc.
Materials and Methods

Cell lines, chemicals and biochemicals
HEL (human erythroleukemia) and Mono Mac 6 (human acute monocytic leukemia) cell lines were purchased from the DMSZ. Tryptanthrin was synthetized according to Friedlaender and Roschdestwensky [20]. Purity was assessed by HPLC, NMR and ESI-MS as > 99%. Extract ZE550 was prepared according to Danz et al. [4]. The tryptanthrin content of ZE550 was 0.10%. Nimesulide (purity >98% by HPLC), indometacin, diclofenac (purity >99% by HPLC), acetylsalicylic acid, arachidonic acid, OPI media supplement Hybri-Max, glutathione, hematin, epinephrine and lipopolysaccharide (LPS, from E. coli) were purchased from Sigma Aldrich. ELISA kits for thromboxane B2 (TxB2), 6-keto prostaglandin F1α (6-keto PGF1α) and leukotriene B4 (LTB4) were purchased from R&D Systems. The assays were performed according to manufacturer’s instructions. Unless stated otherwise, all other reagents were from Sigma.

Plant material
Leaves of Isatis tinctoria L. were collected in Martigny, Switzerland, in June 2004, and identified by Dr. C. Mustermann, University of Basel. A voucher specimen (U 36251) is deposited at the Herbarium of the Department of Botany, University of Basel, Switzerland.

Extraction and isolation
Air-dried leaves were ground in a cross beater mill equipped with a 1 mm sieve. An aliquot (100 g) was extracted at r.t. with CH₂Cl₂ or EtOH (3 × 1000 mL, 24 h each). Frozen fresh material was thawed, and an aliquot (100 g) extracted in a Waring blender model 38BL45 with EtOH (3 × 750 mL, 20 min each).

The EtOH extract (25 g) was submitted to vacuum liquid chromatography on silica gel (40-63 μm, 5 x 50 cm; Macherey & Nagel) using a step gradient of CHCl₃-MeOH 100:0 (3 L) and 97:3 (2 L). Fractions of 250 mL were collected and monitored by TLC (system 1) with respect to tryptanthrin
content. The fractions 4-9 (750-2250 mL, 3.5g) were combined and separated on a silica gel column (25-40 µm, 3 × 50 cm, eluent CHCl₃, flow rate 3 mL/min). 14 fractions (I-XIV) were collected. Fraction IV (600-800 mL, 101 mg) was subjected to solid phase extraction on a Bakerbond C18 cartridge (1 × 3cm) eluted with MeOH-H₂O 80:20, approx. flow 1 mL/min. Fractions 2-5 (5-20 mL, 16 mg) were combined. Pure tryptanthrin (1) (5 mg) was obtained by preparative HPLC on a LiChrosorb RP-18 column (7 µm, 2 × 30 cm i.d.; Merck) eluted with MeOH-H₂O 80:20 at 10 mL/min. (tᵣ = 7.5 min).

**LC-DAD-MS analysis**

LC-DAD-MS data were obtained with an Agilent 1100 series HPLC system consisting of an autosampler, high-pressure mixing pump, column oven and DAD detector connected to a Perkin Elmer API 165 single quadrupole instrument equipped with a PE Sciex Turbo ion probe.

HPLC conditions: LiChrospher 100 RP18 cartridge, (5 µm, 4 × 125 mm; Merck), solvent system: A - H₂O, B - MeCN; isocratic 10% B for 5 min, 10-90% B over 45 min, 90% B for 15 min; flow rate: 1 mL/min; injection volume: 20 µL; Sample concentration: 10 mg/mL in CH₂Cl₂. DAD conditions: 220, 254, 328, 387, 540 nm, or spectra 190-700 nm (step 2 nm).

ESI-MS conditions: positive ion mode; split ratio: 1 : 4; scan range: 140-1000 amu; source temperature: 350°C; ion spray voltage 5 kV; focussing potential: 230 V; declustering potential: 20 V.

**COX-1 and COX-2 assays with HEL, RAW 264.7 and Mono Mac 6 cells**

The protocol by Berg et al. [21] was used for the assay with Mono Mac 6 cells, with some modifications. Cells were cultured in VLE RPMI 1640 medium with 10% fetal calf serum, 1% of penicillin/streptomycin stock solution, 2 mM L-glutamine, non-essential amino acid stock solution, supplemented with OPI media supplement Hybri-Max (0.5 x). Cells were cultured at a density of approx. 2 x 10⁵ cells/mL, harvested and resuspended in fresh medium at 1 x 10⁶ cells/mL. After addition of test solutions, cells were incubated for 30 min at 37°C. An LPS solution (10 µL, final LPS concentration in assay 100 ng/mL) was added and the cells were incubated for additional 6 h. The
further steps were carried out as for HEL cells, with the exception that formation of 6-keto-PGF$_{1\alpha}$ was
determined by ELISA. Preliminary comparative experiments using a PGE$_2$ ELISA kit (R&D Systems)
instead were carried out. The IC$_{50}$ values found for tryptanthrin and reference compounds in both
assays were comparable (data not shown). Diclofenac and nimesulid were used as positive controls.

**COX-1 and COX-2 assays with purified enzymes**

The procedure of Mitchell et al. [22] was employed with some modifications. Briefly, 40 µL of
enzyme solution (100 U/mL) were diluted with 140 µL Tris buffer, and 80 µL of test compound
solution in Tris buffer (final DMSO concentration 1.25%) were added and incubated for 10 min at
37°C. 140 µL of a substrate solution consisting of an ethanolic stock solution of arachidonic acid and
a cofactor solution containing epinephrin, glutathione and hematin in Tris buffer were added. The
final volume of the reaction mixture was 400 µL, the concentrations in the assay were 6.6 µM for
arachidonic acid, 5 mM for epinephrin and glutathione, 1 µM for hematin, 0.25% for DMSO and
0.16% for ethanol. After incubation for 10 min at 37°C, the reaction was stopped by addition of 20 µL
1 N HCl and immediately centrifuged (13’000 rpm for 3 min). The supernatant was removed and
neutralized with 1 N NaOH prior to determination of the 6-keto-PGF$_{1\alpha}$ concentration by ELISA.

**Statistical analysis**

Statistical calculations were carried out with the SPSS 10.0 for Windows software package
(Statistica). Results are expressed as the mean ± S.E.M. of 5 independent experiments. Student’s $t$-test
was used for statistical analyses; P values > 0.05 were considered to be significant.

**Helveticosine A (1):** yellow needles (CHCl$_3$); mp 132-138°C; $R_f$ 0.70, silica gel 60 F$_{254}$, 
CHCl$_3$/MeOH/H$_2$O (80:20:2); $R_f$ 0.15, RP-18 F$_{254s}$, MeOH/0.02M CH$_3$COONH$_4$ (pH 5) (9:1); $[\alpha]_{D}^{25} = 74.4$ (c $0.24$, MeOH); UV (MeOH) $\lambda_{\text{max}}$ (log e) 253 (4.43), 384 (4.55) nm; CD (CH$_3$CN) $\Delta\varepsilon_{211} = +17.1$, 
$\Delta\varepsilon_{231} = -10.6$; IR (KBr) $\nu_{\text{max}}$ 1680, 1540, 1035, 940 cm$^{-1}$; $^1$H NMR (CDCl$_3$, 500 MHz) $\delta$ 7.26 (1H, d, $J$ = 8.1 Hz, H-4), 6.42 - 6.05 (2H, m, H-8, H-12), 3.77 (3H, s, NCOOCH$_3$), 2.28 (1H, dd, $J$ = 7.3, 2.1
Hz, H-15); $^{13}$C NMR (CDCl$_3$, 125 MHz) $\delta$ 176.4 (C, C-4), 127.8 (CH, C-9), 100.9 (CH$_2$, OCH$_2$O), 52.5 (CH$_3$, NCOOCH$_3$), 39.2 (CH$_2$, C-5; ESI MS (pos. ion mode) $m/z$ 444.2 [M+Na]$^+$, 422.3 [M+H]$^+$; ESI MS (neg. ion mode) $m/z$ 420.3 [M-H]$^-$; HR ESIMS $m/z$ 444.21526 [M+Na]$^+$ (calcd for C$_{26}$H$_{31}$NaNO$_4$ 444.21508); anal. C 71.89%, H 12.13%, calc. for C$_{30}$H$_{64}$O$_5$, C 72.12%, H 12.65%.

Supporting information

Microscopic sections of seeds of Unknownia helvetica, $^1$H NMR spectra of compound 1, and COSY correlations for compound 1 are available as Supporting Information.

Acknowledgements

Thanks are due to Prof. C. Unknown, University of Appenzell, for the gift of RAW 264.7 cells and information on culturing. Financial support by Herbal Bonanza AG, Irgendwo, Switzerland, for part of this work is acknowledged.

Conflict of Interest

The authors declare no conflict of interest.
References


Legends for Figures

Fig. 1 Chemical structures of tryptanthrin (1), rutaecarpine (2), indigo (3) and indirubine (4).

Fig. 2 Effect of tryptanthrin, acetyl salicylic acid, diclofenac, nimesulide and NS 398 on 6-keto-PGF$_{1\alpha}$ synthesis in (A) LPS-stimulated Mono Mac 6, and (B) LPS-stimulated RAW 264.7 cells. Each symbol and bar indicates the mean ± S.E.M. of nine experiments.
Fig. 1
Fig. 2

A

B
Supporting Information

Inhibitory Activity of Tryptanthrin on Prostaglandin and Leukotriene Synthesis

Heinrich Muster 1, Christine Müller 2, Matthias Hamburger 1

Affiliation

1 Institute of Pharmaceutical Biology, University of Basel, Basel, Switzerland

2 Institute of Pharmacy, University of Nowhere, Seldwyla, Switzerland

Correspondence

Prof. Dr. Matthias Hamburger, Institute of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel, Klingelbergstrasse 50, CH-4056 Basel, Switzerland. E-mail: matthias.hamburger@unibas.ch Phone: +41 61 267 14 25 Fax: +41 61 267 14 74
Fig. 1S Cross section of *Unkownia helvetica* (left, top and bottom) and *U. pannonica* (right, top and bottom) seeds. Fluorescence microscopy with DAPI filter 365 nm. The width of the photographic image corresponds to approx. 0.7 mm. Embryos display intense blue fluorescence. The testa of *U. helvetica* is irregular in thickness, and the large cells of the mesotesta are clearly discernible. The testa of *U. pannonica* shows uniform thickness.
Fig. 2S $^1$H NMR spectrum of compound 1 (CD$_3$OD, 400.13 MHz)
**Fig. 3S** Partial structures of 1 assembled with the aid of COSY correlations.