Appendix S1

MATERIALS AND METHODS

Cell cultures

Normal human epidermal keratinocytes (NHEK) from juvenile foreskin were purchased from Promocell (Heidelberg, Germany) and cultured in serum-free medium (KGM2, Promocell). For the experiments, serum-free medium additionally lacking epidermal growth factor (EGF) and hydrocortisone were used. Only cells in passage 3–5 were used. The immortalized human keratinocyte cell line HaCaT, purchased from the German Cancer Research Center (DKFZ, SR7), was cultured in DMEM culture media (Dulbecco's Modified Eagle's Medium, Sigma Aldrich, Seelze, Germany) supplemented with 10% foetal calf serum, 2 mM glutamine and penicillin/streptomycin (GE Healthcare, Solingen, Germany). The human mast cell line LAD2 was received from Dr A. Kirshenbaum (National Institutes of Health, Bethesda, MD, USA) (SR8). Cells were cultured in Stem Pro 34 SFM (Life Technologies, Darmstadt, Germany) with nutrient supplements, 1% glutamine, 1% antibiotics and 100 ng/ml human stem cell factor (PeproTech, Hamburg, Germany). All cells were sub-cultured in 75 cm² tissue culture flasks at 37°C in a humidified atmosphere of 95% air/5% CO_2.

Chemicals

[$Sar^9Met(O_2)^{11}$]-substance P, calcium ionophore (A23187), PIPES sesquisodium salt, 4-nitrophenyl N-acetyl-beta-D glucosaminide (pNAG), 3-indoleacetonitrile (IAN) and 1-fluoro-2,4-dinitrobenzene (DNFB) were obtained from Sigma Aldrich (Seelze, Germany). Tryptanthrin (TRY, indolo(2,1-b)quinazoline-6,12-dione) was purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and *p*-cumaric acid methyl ester (pCM) from Chemos (Regenstauf, Germany).

Plant material, sample preparation and extraction

The *I. tinctoria* L. plants for this project were cultivated under controlled conditions by Dr Junghanns GmbH (Groß Schierstedt, Germany). After delivery, the leaves were dried and milled for subsequent extraction. To determine the extraction parameters for the highest extraction yield and content of effective compounds, the accelerated solvent extraction with ethanol using an ASE 200 (Dionex, Sunnyvale, CA, USA) was performed with 2 g leaves of a mixed genotype, harvested in 2012. Due to the high cytotoxicity of this ethanol extract (1), determined by XTT assay, a new manual extraction scheme was designed to separate the toxic compounds, based on a manual solid-liquid and liquid-liquid extraction of the dried and milled leaves, harvested in 2013. This scheme was used for all further extractions.

For the genotype-screening, necessary for the selection of the suitable plant material with the highest extract yield and effective compound content, 50 g of plant material were extracted 5 times with ethanol using the ultra turrax (IKA, Staufen, Germany). The crude extracts were evaporated, dissolved in 200 ml water and stored overnight at 6°C for cold precipitation. After separation of the solid residue via centrifugation (4 min, 3,500 rpm) and sanction filtration, liquid-liquid extraction with petroleum ether and ethyl acetate, 4 times each, was then performed. The degreased polar water extract (2), the lipophilic petroleum extract (4.1) and the middle polar ethyl acetate extract (3) were lyophilized or evaporated and analysed for content of effective compounds and entered into the biological test systems.

Because of the high level of effectiveness of lipophilic extract 4.1, examined in the biological test systems, there was an objective to increase the extraction yield. Therefore a scale-up-extraction method was developed only by enhancing the amount of dried leaf material from 50 to 500 g and the utilized solvents from 200 ml to 1.5 l. Following this extraction procedure, lipophilic petroleum ether extract 4.2 was obtained.

For the biological experiments, TRY and IAN were dissolved in dimethyl sulphoxide (DMSO), pCM and the woad extract 4.1 and 4.2 in pure ethanol. For the COX assay, the woad extract samples were also dissolved in DMSO. For the HPLC analyses HPLC-eluent was used for all substances and extracts.

Analytical methods

The HPLC analysis was performed by using an Agilent Technologies 1100 series HPLC system (Agilent, Waldbronn, Germany) with degasser, binary pump, autosampler, column oven, diode array detector and Chemstation for LC 3D. A Reprosil-pur C18AQ (5 µm, 125×4 mm) column equipped with a Reprosil-pur C18AQ (5 µm, 5×4 mm) guard column (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) was used as stationary phase. Eluent A was composed of ultrapure water with 0.1% acetic acid, 0.1% methanol and 0.03% ammonium acetate. Eluent B contained acetonitrile with 0.1% ultrapure water. The parameters for gradient development were as follows: gradient B [%] / time [min] 30/0, 30/3, 72/15, 100/20, 100/23, 30/27, 30/35. Flow rate was 0.5 ml/min. Detection of the 3 key components was carried out at 254 nm for TRY, IAN at 280 nm and pCM at 310 nm. A calibration was established with a mixed standard solution with 11 levels, from 50 ng/ml to 3 µg/ml (1 mg/ml of standard compounds solved in DMSO and further diluted with HPLC eluent 25 to 75). Sample preparation of the petroleum ether extract samples was performed with solid-phase extraction with Chromabond 500 mg C18 6 ml cartridges (Macherey-Nagel, Düren, Germany). The conditioning of the cartridge was executed with 4 ml of a mixture of eluent A and B 25:75 (v/v). Approximately 15 mg of PE extract samples were solved with 1.5 ml eluent B then mixed with 0.5 ml eluent A. This solution was applied on the conditioned cartridge. The elution was performed 4 times with 0.5 ml of eluent A and B 25:75 (v/v) with interjectional drying steps. The collected solution was filled up to 5 ml. Then 0.5 ml of this solution was mixed with 1.5 ml eluent A and cleaned up for injection with a 0.45 µm PTFE-syringe filter (Macherey-Nagel, Düren, Germany). The standard and sample injection volume was 20 µl. Furthermore, the proportions of various fatty acids in the PE extract 4.2 and in an extract from fresh woad leaves were determined using gas chromatography with a flame-ionization detection method.

Cell viability assay

Cell proliferation of keratinocytes was determined using an XTT assay (2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; Applichem, Darmstadt, Germany). HaCaT cells (10,000/well) were seeded in 96-well plates and allowed to adhere overnight before they were treated with the 3 main compounds (from 10 nM to 10 µM), the woad extracts (from 10 µg/ml to 1 mg/ml) or the vehicle controls (DMSO or ethanol) for 24 h at 37°C. Hereafter, 50 µl XTT reagent was added to each well and incubated for 2 h. The absorption was determined using an EL808 ELISA plate reader (Biotek, Bad Friedrichshall, Germany) at 490 nm (reference 630 nm). Values were corrected against the blank control and viability of treated cells was calculated as the percentage ratio of untreated cells.

COX-2 activity assay

The screening to determine promising *Isatis tinctoria* L. extract samples was performed with the cyclooxygenase (COX) Inhibitor Screening Assay Kit (Item No. 560131, Cayman Chemical, MI, USA). The assay was executed according to the manufacturer's

procedure. The assay was conducted with COX-2 enzyme. The samples and standard substances were first solved in pure ethanol, subsequently in DMSO. Various woad extracts (water, ethyl acetate, ethanolic, PE) and the sap were tested for their potential COX-2 inhibition. The ethanol extract (1) and the fermented sap were used for determination of the application concentration, which should result in a maximum inhibition rate of 50% of the enzyme. Thereupon the application concentration of the tested extracts 2, 3, and 4.1 was set to 20 µg/ml.

The 4.2 PE samples were applied in 3 levels up to an assay concentration of 28 µg/ml in order to examine their concentration-dependent effect. The high level sample was used to determine possible interferences with the enzyme immunoassay by incubating with inactivated enzyme. The plate reading was performed at 405 nm every 5 min of development by a Victor2 1420 multi label counter (PerkinElmer, Hamburg, Germany). The percentage of inhibition was calculated by using a logistic fit curve. The fit curve was estimated by relative binding values of standard calibration from 15.6 to 2,000 pg prostaglandin per ml EIA buffer.

Animals

For the experiments female C57BL/6J (WT, 8–10 week-old) mice were purchased from Harlan Laboratories, Inc. All mice were housed under specific pathogen-free conditions in the animal facility of the University of Münster and the study was approved by the local authorities (District Government and District Veterinary Office, Münster, Germany; 87-51.04.2011.A044).

DNFB-induced contact hypersensitivity

The in vivo hapten-induced model of contact hypersensitivity (CHS) was used as described previously (SR9; SR10). Briefly, mice were sensitized with 80 µl 0.5% DNFB (dissolved in acetone/olive oil 4:1) on shaved abdominal skin. After 5 days both sides of the right ears were challenged with 15 µl 0.3% DNFB (in A/O) and the combination TRY+pCM+IAN or 4.2 (100 µg/ml). The left ears served as internal control. Control mice were only challenged with acetone/olive oil without DNFB. Ear swelling was measured before challenge and 24 h and 48 h after challenge with a dial thickness gage (Mitutoyo, Neuss, Germany). For net ear swelling, values of untreated controls were subtracted from the treated ears. Animal ears were harvested 48 h after challenge.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from harvested animal material or cultured cells using the innuPrep RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer's protocol. An amount of 0.5 µg RNA was reverse transcribed with the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Schwerte, Germany). Expression of interleukin (IL)-1 beta, IL-6, IL-33, ST2 and interferon (IFN)-γ in the mouse study, respectively, IL-6 and IL-33 in the cell stimulation experiments were analysed using the qTower 2.2 real-time PCR system (Analytik Jena, Jena, Germany) using the Kappa SYBR Fast Universal Kit (Peqlab, Erlangen, Germany) and sequence-specific oligonucleotides (see **STable I**). Beta-actin or peptidylprolyl isomerase A (PPIA) served as reference gene for normalization. The PCR protocol consisted of 40 cycles of denaturation at 95°C for 3 s; annealing at 60°C for 25 s; and elongation at 72°C for 3 s. All reaction experiments were performed in duplicate. Melt curves were analysed to ensure amplification specificity of the PCR products and transcript levels were quantified with the ΔΔCt method (SR11). Unless otherwise stated, the oligonucleotides were designed using Primer-BLAST (NCBI, Bethesda, USA) and purchased from VBC Biotech (Vienna, Austria; 50 nmol, HPLC purified).

Stimulation of keratinocytes

NHEK cells were seeded in 12-well-plates and grown to a confluence of 80%. Cells were washed twice with PBS and stimulated with 20 ng/ml IFN-γ (Peprotech, Hamburg, Germany) or 10 µg/ml polyinosinic:polycytidylic acid (Poly I:C, Sigma Aldrich, Munich, Germany) for 16 h for mRNA isolation in nutrient-deficient medium. In addition, different doses of 4.2 (1, 5 and 10 µg/ml) or a combination of the main compounds (each 1 µM) was added directly after the stimulation to the cells.

Mast cell degranulation and β-hexosaminidase release assay in LAD2 cells

Release of histamine containing granules was quantified via the measurement of β-hexosaminidase in the cell supernatants. For that, cells of the humane mast cell line LAD2 (1×10^5) were incubated with different doses of PE extract (10, 25, 50 and 75 µg/ml) or a combination of the main compounds (each 1 µM) for 30 min at 37°C under shaking conditions. Degranulation of the cells was induced by stimulation with calcium ionophore (CI, 2 µM) or substance P (30 µM) in Pipes-CM buffer (25 mM Pipes, 119 mM NaCl, 5 mM KCl, 2.8 mM $CaCl_2$, 1.4 mM $MgCl_2$; pH 7.4) supplemented with 0.1% bovine serum albumin (BSA) for an additional 30 min at 37°C. Unstimulated cells served as control for spontaneous release and lysed cells (Triton-X 100, 1%) as control for total content. Supernatants were harvested and incubated with an equal volume of 4-nitrophenyl N-acetyl-beta-D glucosaminide (pNAG, in 0.05 M citrate buffer, pH 4.5) substrate for 1 h at 37°C. To determine enzyme activity, absorbance at 405 nm (ref 630 nm) was measured using an ELISA reader. The specific percentual release of β-hexosaminidase was calculated in relation to the total content.

Histology and immunohistochemically staining

Mouse ears were either fixed in 4% formalin for paraffin embedding or frozen in section medium (NEG50, Thermo Fisher Scienti-

STable I. Sequences of oligonucleotides used to amplify mouse and human genes

	Forward	Reverse
Oligonucleotides used in the mouse study		
Interleukin-1b	cttcaggcaggcagtatcactcat	tctaatgggaacgtcacacaccag
Interleukin-6	gccttcttgggactgatgct	gaattgccattgcacaactct
Interleukin-33	aagaccaagagcaagaccagg	gagtagtccttgtcgttggca
Interleukin-1 Receptor Like 1	cgcggagaatggaaccaact	ccacaggacatcagccaagaa
Interferon gamma	cggcacagtcattgaaagcc	tgtcaccatccttttgccagt
beta-Actin	gatcaagatcattgctcctcctg	agggtgtaaaacgcagctca
Oligonucleotides used in the experiments with humane keratinocytes		
Interleukin-6	agccctgagaaaggagacatgtaac	accaggcaagtctcctcattg
Interleukin-33	acggtgttgatggtaagatgtt	tatgaaggacaaagaaggcctgg
Peptidylprolyl Isomerase A	atggtcaaccccaccgtgt	tctgctgtctttgggaccttgtc

fic) for cryosections. Paraffin-embedded samples were stained with haematoxylin and eosin (H&E) or Giemsa. Frozen samples were pre-incubated with 2% BSA (Fraction V in phosphate-buffered saline (PBS)), and then incubated with anti-CD4 (mouse, 1:100, BioLegend, San Diego, CA, USA) antibody at 4°C. After washing (PBS + Tween 0.05%) specimen were stained with secondary antibody (Alexa Fluor 594, donkey, Life Technologies, Darmstadt, Germany) for 3 h at room temperature and counterstained with DAPI. All stained sections were evaluated using a BX43 fluorescence microscope (Olympus, Hamburg, Germany) and the CellSense Dimension software (version 1.7.1; Olympus) and Photoshop (version CS5, Adobe, Munich, Germany).

Data analysis and statistics

Values are shown as means ± SEM. All reported *p*-values were calculated using Sigma Plot 12.5 (Systat Software, Erkrath, Germany). Student's unpaired 2-tailed *t*-test or Mann-Whitney rank sum test and were significant at levels of less than 0.05.

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