

Research Article

Hop bitter acids efficiently block inflammation independent of GR α , PPAR α , or PPAR γ

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Hop (*Humulus lupulus* L.) is an essential ingredient of beer, where it provides the typical bitter taste, but is also applied in traditional folk medicine for sedative and antibacterial purposes. In this study, we demonstrate and compare the anti-inflammatory effect of various classes of hop bitter acids (HBA), including α -acids (AA), β -acids (BA), and iso- α -acids (IAA), in fibroblasts, which are important players in the inflammatory response. All three studied classes of HBA blocked the tumor necrosis factor alpha (TNF)-induced production of the cytokine IL6, and inhibited the transactivation of the pro-inflammatory transcription factors nuclear factor kappa B (NF- κ B), activator protein-1 (AP-1), and cAMP-response element-binding protein (CREB). In this respect, the six-membered ring compounds AA and BA showed equal potency, whereas the five-membered ring compounds, IAA, were effective only when used at higher concentrations. Furthermore, with regard to the mechanism of NF- κ B suppression, we excluded a possible role for glucocorticoid receptor alpha (GR α), peroxisome proliferators-activated receptor alpha/gamma (PPAR α or PPAR γ), nuclear receptors (NRs) that are also known to inhibit inflammation by directly interfering with the activity of pro-inflammatory transcription factors. Interestingly, combining hop acids and selective agonists for GR α , PPAR α , or PPAR γ resulted in additive inhibition of NF- κ B activity after TNF treatment, which may open up new avenues for combinatorial anti-inflammatory strategies with fewer side effects. Finally, systemic administration of HBA efficiently inhibited acute local inflammation *in vivo*.

Keywords: Hop bitter acids / *Humulus lupulus* L. / Inflammation / NF- κ B / Nuclear receptors

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1 Introduction

The treatment of chronic inflammatory disorders, such as arthritis, atherosclerosis, and cancer, make up a major challenge in recent medicine. Despite tremendous efforts to selectively alleviate symptoms and slow down disease pro-

gression, the commonly used drugs, such as glucocorticoids, cope with unwanted side effects upon long-term usage. In search for new anti-inflammatory drugs, several food constituents proved themselves as interesting candidates, including resveratrol (derived from red wine) [1], curcumin (derived from turmeric powder) [2], and epigallocatechin gallate (EGCG, derived from green tea) [3].

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Abbreviations: AP-1, activator protein-1; AA, hop α -acids; BA, hop β -acids; CI, confidence interval; CREB, cAMP-response element-

binding protein; COX-2, cyclo-oxygenase-2; DEX, dexamethasone; FF, fenofibrate; GR α , glucocorticoid receptor alpha; HBA, hop bitter acids; IAA, hop iso- α -acids; NF- κ B, nuclear factor kappa B; NR, nuclear receptor; PPAR α / γ , peroxisome proliferators-activated receptor alpha/gamma; ROSI, rosiglitazone; STS, staurosporine; TNF, tumor necrosis factor alpha; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VEGF, vascular endothelial growth factor

Actually, plants have been used since ages in traditional folk medicine to treat inflammation [4, 5]. However, it is often difficult to obtain sufficient amounts of bioactive ingredients from a normal human diet. Currently, food supplements containing (mixtures of) individual food compounds (nutraceuticals) or plant extracts (phytotherapeutics), are currently being commercialized claiming “to relieve pain and promote health,” and are consequently used in the self-treatment of several inflammatory disorders. Only recently, scientists have begun to focus on the identification of the bioactive ingredients and their coupled molecular targets [6].

Nuclear factor-kappa B (NF- κ B) functions as a key transcription factor in the cellular response to inflammatory signals. In response to various stimuli, including cytokines (tumor necrosis factor alpha (TNF), IL1 β), pathogens (LPS), irradiation and oxidative stress, NF- κ B is released from its inhibitor in the cytoplasm (I κ B), translocates to the nucleus and binds to the conserved DNA-binding site 5'-GGGRNNYYCC-3' (R, purine; Y, pyrimidine; N, any base) in the promoter or enhancer regions of target genes, such as cytokines, enzymes, and adhesion molecules (for detailed review, refer to [7]) [8–10]. Since the regulation of NF- κ B activity is essential to maintain cellular homeostasis, constitutive NF- κ B activation is directly linked to the pathogenesis of several diseases like atherosclerosis, rheumatoid arthritis, cystic fibrosis, and cancer [8, 11].

Members of the nuclear receptor (NR) superfamily have been intensively studied because of their physiological roles as negative regulators of inflammatory responses. Upon ligand binding, the receptors directly regulate their own gene programs by binding the DNA at typical response elements. Moreover, they can also interfere with other signaling pathways, such as those driven by NF- κ B, activator protein-1 (AP-1) or cAMP-response element-binding protein (CREB) by a broad range of non-exclusive mechanisms, remaining to be solved in detail [12, 13]. In this respect, the glucocorticoid receptor alpha (GR α) and the peroxisome-proliferator-activated receptors (PPARs) have received the most attention, as they effectively target a number of cytokines and chemokines, such as IL6 and IL8 [14–16]. Glucocorticoid hormones are secreted by the adrenal cortex and mediate their effects by activating the intracellular GR α receptor. They play a role in diverging processes ranging from protein and carbohydrate metabolism, development, and differentiation, to immune regulation. Endogenous and synthetic agonists for the PPARs, including fatty acids, eicosanoids, and hypolipidemic drugs, modulate various physiological processes, such as the regulation of lipoprotein and lipid metabolism and glucose homeostasis [17]. Both fibrates, PPAR α agonists used in the treatment of dyslipidemia, and glitazones, agonists for the PPAR γ receptor and clinically used to reduce insulin resistance in diabetes mellitus type II, decrease plasma concentrations of inflammatory cytokines by interfering with intracellular

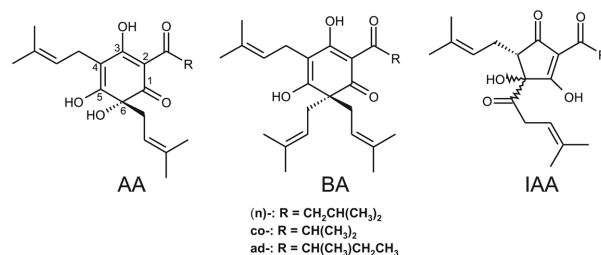


Figure 1. Structures of HBA: α -acids (AA or humulones), β -acids (BA or lupulones), and iso- α -acids (IAA or isohumulones).

inflammatory pathways [14, 18, 19]. It is generally believed that mainly non-DNA-binding mechanisms of transrepression are responsible for the anti-inflammatory activity exerted *via* PPARs and GR α [20].

Besides being an essential ingredient in beer production, hop (*Humulus lupulus* L.) is also recognized in herbal medicine, where it is applied for its antibacterial [21], sedative [22], and – more recently discovered – estrogenic [23] and anticarcinogenic properties [24]. The latter biological activities have been linked to specific polyphenols in the lupulin powder secreted by the female hop cones. 8-Prenylnaringenin, a prenylated flavanone, has been shown to be one of the strongest phyto-estrogens currently known [25], while xanthohumol, a prenylated chalcone, exerts important anticancer effects [24]. In recent years, evidence has been found for biological activities of the fraction of hop bitter acids (HBA), which was before merely known for its bittering, foam-stabilizing, and antibacterial properties delivered to beer [21, 23, 26]. HBA, which may comprise up to 30% of the dry weight of the cones, can be classified into α -acids (AA) and β -acids (BA) (also called humulones and lupulones, respectively), and are characterized as prenylated phloroglucinol derivatives. Depending on the acyl side chain, three major homologs can be identified (Fig. 1). During the brewing process, the nonbitter AA isomerize to the bitter-tasting iso- α -acids (IAA), which are present in all beers in concentrations ranging from 10 up to 100 mg/L [27]. Each IAA homolog prevails as an epimeric mixture of *cis*- and *trans*-isomers, where the stereochemical notation refers to the relative orientation of the hydroxyl at C(4) and the prenyl group at C(5) (Fig. 1).

Epidemiological studies suggest that a light to moderate consumption of either wine or beer reduces the risk of coronary heart disease (CHD), resulting in a lower all-cause mortality. CHD is regarded as arising from inflammation at the vascular wall, where accumulation of fat and infiltration of macrophages leads to the development of atherosclerotic lesions and eventually to blocked arteries [28]. At first, it was suggested that ethanol itself might be largely responsible for the potential anti-inflammatory effects of these beverages [29]. However, recent findings show that alcohol-free beer can also down-regulate activated peripheral blood

mononuclear cells *in vitro* [30], thus questioning the claimed exclusive anti-inflammatory role of ethanol. Interestingly, the main hop AA *n*-humulone (or simply, humulone), has been reported to possess anticarcinogenic properties, such as inhibition of angiogenesis [31] and prevention of tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin [32, 33]. Furthermore, humulone potentially reduces the production of the enzyme cyclooxygenase-2 (COX-2) [34] and the vascular endothelial growth factor (VEGF), both known to be involved in inflammation and cancer.

In the current work, we aimed to take these studies further and to investigate a broad range of hop acids, including total AA, BA, and IAA for their ability to influence pro-inflammatory gene expression induced by TNF. Furthermore, we investigated their GR α -, PPAR α -, and PPAR γ -modulating activities in inhibiting NF- κ B, a major transcription factor in inflammation. Finally, we present *in vivo* evidence for the inhibitory effect of HBA on acute local inflammation. As a cellular model, we chose L929sA mouse fibroblasts. Fibroblasts are not only important for structural purposes, but are moreover essential sentinels for reacting to early danger signals. They are equipped with numerous surface and intracellular receptors, and possess a highly developed molecular machinery to efficiently translate inflammatory triggers into a corresponding expression of immunomodulating factors, such as cytokines, lipid mediators, and growth factors. In this way, they coordinate the recruitment of cells to the affected region, and as such may be considered extensions of the “professional” immune system [35].

2 Materials and methods

2.1 Cell culture and reagents

Mouse fibrosarcoma L929sA cells and monkey kidney COS1L2A fibroblasts were maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal calf serum and 5% newborn calf serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Recombinant murine TNF was produced in our laboratory [36]. Staurosporine (STS) was purchased from Calbiochem-Novabiochem International (San Diego, CA, USA). Forskolin (FK), dexamethasone (DEX), RU486, GW9662, and GW6471 were purchased from Sigma Chemical (St. Louis, MO, USA). GW9578 was a kind gift from Dr. B. Staels (Institut Pasteur, Lille, France) and previously described [37]. Rosiglitazone (ROSI) and fenofibrate (FF) were obtained from Cayman Chemicals. The luciferase assay reagent comprised 270 μ M coenzyme A, 470 μ M luciferin (both from Sigma), and 530 μ M adenosine triphosphate (Boehringer, Mannheim, Germany) in a reaction buffer containing 20 mM tricine, 1 mM (MgCO₃)₄ · Mg(OH)₂, 2.7 mM MgSO₄, 0.1 mM EDTA, and 33.3 mM DTT (all from Sigma). Inductions

were performed in serum-free DMEM to exclude interference of serum components on the results of the assay. We verified that the highest solvent concentration (not exceeding 0.1%) of the test compounds did not interfere with any of the assays. Each experiment was performed at least in triplicate in three independent setups, unless mentioned otherwise in the figure legends.

2.2 Cytotoxicity assay

An annexin-V-FITC/propidium iodide staining of L929sA cells was performed, using a commercial kit (BenderMed Systems, USA). After incubation with HBA in serum-free medium, cells were harvested using trypsin. Following centrifugation, the cell pellet was resuspended in 200 μ L binding buffer supplemented with 1 μ L annexin-V-FITC, and incubated for 15 min in the dark. Next, 4 μ L propidium iodide and 100 μ L binding buffer was added to the sample and fluorescence was immediately measured on a Beckman Coulter Cytomics FC500 5 color flow cytometer equipped with a 488 nm argon ion laser.

2.3 Purification and analysis of HBA

AA and BA were purified from a CO₂-hop extract (NateCO₂, Wolnzach, Germany) as described previously [38]. Alternatively, AA were released from their lead(II) salts, and BA were purified from the BA-rich hop paste, Aromahop (John Haas, Yakima, Washington DC, USA). IAAs were obtained from Isohop® (J. I. Haas, Yakima, WA, USA) after acidification. Concentrations were determined by analytical HPLC (Waters Alliance 2695 coupled to a Photodiode Array Detector Waters 996) and structural identities were confirmed by UV spectra and HPLC-retention times, as compared to international calibration extracts. Stock solutions of 100 mM were prepared in ethanol, aliquoted and stored at -70°C . Under these conditions, hop acid composition and concentration have been verified to remain essentially unchanged for at least 1 year. Moreover, endotoxin levels in stock solutions of HBA (100 mM) were measured by a Limulus amoebocyte lysate gel-clot assay (E-Toxate Kit, ET0200, Sigma), and were found to be less than 1.25 EU/mL. Thus, at the concentrations used *in vitro*, endotoxin levels were less than 1.25×10^{-4} EU/mL for AA and BA (10 μ M), and less than 1.25×10^{-3} EU/mL for IAA (100 μ M).

2.4 Plasmids

The recombinant plasmids p(IL6 κ B)₃50hu · IL6P-luc⁺ and p(GRE)₂50hu · IL6P-luc were described previously [39, 40]. pE-selectin-luc⁺, containing the E-selectin promoter, was a kind gift from Dr. D. Goeddel (Tularik, San Francisco, CA, USA). p546 · IL8P-luc⁺, containing an IL8 promoter fragment of 546 bp, was donated by Dr. N. Mukaida (Can-

cer Research Institute, Kanazawa, Japan). pPGK β GeobpA, constitutively expressing a neomycin-resistant β -galactosidase fusion protein under control of the 3-phosphoglycerate kinase promoter, was provided to us by Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). pcDNA₃ was purchased from Invitrogen. The TK-promoter-containing TRE-driven reporter gene, pTRE-luc⁺, was kindly donated by Dr. M. R sche-Rigon (Marion Hoechst Roussel-Uclaf, Paris, France). The synthetic reporter gene construct pCRE-luc⁺ containing multimerized responsive elements in front of a minimal promoter, was purchased from Stratagene Cloning Systems (La Jolla, CA, USA). pSG5-hPPAR α , pSG5-hPPAR γ , and a PPRE-containing reporter plasmid J₃-tk-luc were a generous gift from Dr. B. Staels (Institut Pasteur, Lille, France) [15].

2.5 Transfection procedure

Stable transfection of L929sA cells was performed by the calcium phosphate precipitation procedure according to standard protocols [41] using a ten-fold excess of the plasmid of interest over the selection plasmid pPGK β GeobpA. Transfected cells were selected in 500 μ g/mL G418 for 2 wk, after which the resistant cell clones were pooled for further experiments. Thus, the individual clonal variation in expression was averaged, providing a reliable response upon induction. The cotransfected plasmid pPGK β GeobpA, conferring resistance to G418 and expressing constitutive β -galactosidase enzymatic activity, was further used as an internal control. Transient transfection of COS1L2A cells was performed using the lipofectamin reagent (Invitrogen) according to the manufacturer's instructions.

2.6 Reporter gene analysis

After the appropriate inductions, cells were washed with PBS, lysed in lysis buffer (Tropix, Bedford, MA, USA), frozen overnight and samples were assayed for their reporter gene activity according to the manufacturer's instructions (Promega, Madison, WI, USA). Light emission was measured in a luminescence microplate counter (Victor X3; Perkin Elmer, Turku, Finland). Luciferase activity, expressed in arbitrary light units, was corrected for the protein content of the sample, as indicated, by normalization to the coexpressed β -galactosidase levels. β -Galactosidase protein levels were quantified using a chemiluminescent reporter assay GalactoLight kit (Tropix) [40, 41].

2.7 Reverse transcription-PCR (RT-PCR) and quantitative PCR (qPCR)

RNA was isolated from L929sA cells by using TRIzol reagent (Invitrogen) according to the manufacturer's

instructions. RT-PCR reactions were carried out according to the instructions of Promega. Reverse transcription was performed on 1 μ g total RNA to prepare cDNA for qPCR in order to quantify the initial mRNA levels of IL6 (sense 5'-GGAGTACCATAGCTACCTGG-3', antisense 5'-GACCA-CAGTGAGGAATGTCC-3'), RANTES (sense 5'-AGCT-GCCCTCACCATCATC-3'; asense 5'-CTCTGGGTTGGC-ACACACTT-3'), and TNF- α (sense 5'-CACAAGATGCTGGGACAGTGA-3'; asense 5'-GCTCCAGTGAATTCG-GAAAGC-3').

PCR mixes contained 1 μ L cDNA, 7.5 μ L SybrGreen mastermix (Invitrogen), and 0.75 μ L of each primer in a total volume of 15 μ L *per* reaction. qPCR was performed using a BioRad iCycler[®] according to the manufacturer's instructions and melting curves were generated at the end of the protocol to check for false priming. Normalization was done using a primer set for the common household gene HPRT.

2.8 ELISA

A murine IL6 ELISA was performed, using a commercial kit, according to the manufacturer's instructions (Bio-source, USA).

2.9 Zymosan-induced inflamed paw model

Female C57BL/6 mice were obtained from Iffa-Credo (Saint Germain-sur-l'Arbresle, France) and used at the age of 8–10 wk. Mice were kept in a temperature-controlled, air-conditioned animal house with 14 h/10 h light/dark cycles; they received food and water *ad libitum*. Mice were pretreated with PBS or with water-soluble compounds *i.p.* in a volume of 250 μ L *per* mouse, or with DMSO or DMSO-soluble compounds *i.p.* in a volume of 60 μ L *per* mouse. After 30 min, mice were given 20 μ L of a zymosan solution (15 mg/mL in PBS, sterilized) in the right footpad. 6, 12, 24, and 48 h after zymosan injection, the thickness of both footpads was measured using a caliper and the difference between zymosan-injected and the control footpad was compared for all treated groups (5 mice *per* group). All the experiments were approved by the Ethics Committee for Animal Experimentation at the Ghent University.

2.10 Statistics

Nonlinear regression analysis was performed using Graph-Pad Prism 4 Software (sigmoidal dose-response model). Ninety-five percent confidence interval (CI) of the regression was calculated by the same program and depicted in graphs as dotted lines. Statistical analysis was done using the same program, performing Student's *t*-tests to compare two groups (test group *vs.* control). *p*-Values lower than 0.05 were considered as significant.

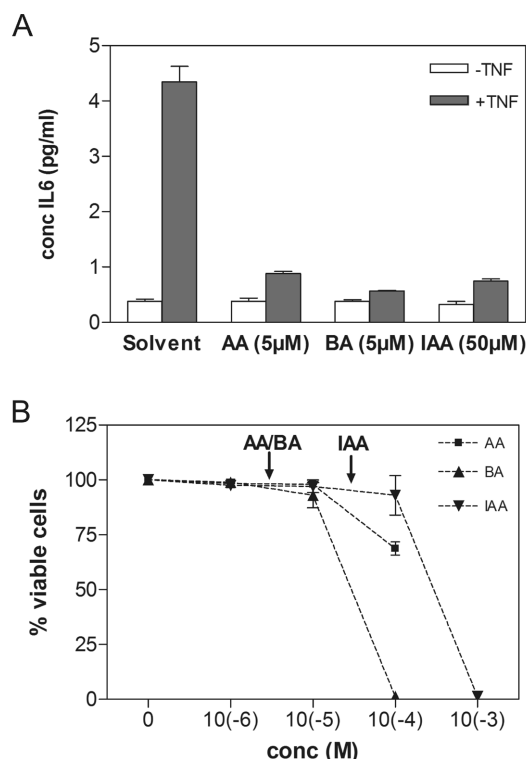


Figure 2. Effects of different classes of HBA on TNF-induced production of IL6 protein in L929sA cells. (A) Cells were exposed for 1 h to AA (5 μM), BA (5 μM), or IAA (50 μM), before adding TNF (2000 IU/mL) for a total period of 6 h. Afterwards, medium was collected to perform an IL6 ELISA. Data are means + SD of triplicate results. (B) Cells were treated with different concentrations of HBA for 24 h in serum-free DMEM. Afterwards, the number of viable cells was determined by FACS analysis, after staining early apoptotic and death cells with annexin-V-FITC and propidium iodide, respectively. Results were normalized to the number of viable cells in the control sample, which was set to 100%. Data are means + SD of triplicate results. Arrows indicate the concentration of individual HBA used in (A).

3 Results

3.1 HBA lower the TNF-stimulated IL6 protein production

The cytokine IL6 plays a central role in host defense, and elevated levels can be used as a marker for inflammation [42, 43]. As depicted in Fig. 2A, IL6 protein levels are highly elevated by the treatment of L929sA fibroblasts with TNF, a pro-inflammatory mediator. Pretreatment with AA, BA (both 5 μM), or IAA (50 μM) resulted in a significant reduction of IL6 protein expression, to almost baseline levels.

To verify that the reduced IL6 production was not caused by a direct cytotoxic effect of the compounds, we tested cell viability after 24 h incubation with HBA. Both early apoptotic, as well as late apoptotic and dead cells were excluded

from the viable cell population, by measuring fluorescence from membrane-bound annexin-V-FITC and DNA-bound propidium iodide, respectively. It is clear from Fig. 2B that at the used concentrations, HBA did not affect cell viability (see arrows in figure). Similar results were obtained with an MTT cell proliferation assay (data not shown). Based on these data, in all subsequent experiments, the concentration of 10 μM for AA and BA, or 200 μM for IAA was never exceeded.

3.2 HBA inhibit the expression of IL6 and other inflammation-related genes

We wondered whether HBA block IL6 production at the transcriptional level. Real-time quantitative PCR analysis demonstrates that HBA substantially lower TNF-induced IL6 mRNA expression, in a dose-dependent manner (Table 1). In agreement with the IL6 protein data, AA- and BA-mediated reduction of TNF-induced IL6 mRNA levels is very efficient at 1–5 μM, whereas the repressive capacity of IAA only occurs at higher concentrations (50–100 μM). Interestingly, the expression of other genes involved in the inflammatory process, such as the chemokine RANTES and the cytokine TNF-α, were also dose-dependently inhibited.

3.3 HBA inhibit NF-κB-dependent gene expression

Gene expression of IL6, RANTES, and TNF-α is regulated at the promoter level by the transcription factor NF-κB, which plays a central role in controlling inflammatory processes and can be activated by pro-inflammatory signals, such as TNF. Therefore, we explored the possibility that the investigated HBA could differentially influence gene expression of several NF-κB-dependent target genes. We studied the repressive capacity of HBA on TNF-induced IL8 and E-Selectin (ELAM) promoter regulation, both containing NF-κB-binding sequences, in comparison to a synthetic construct containing a triple κB repeat (κB3), all cloned in luciferase-coupled reporter gene systems [39]. From Fig. 3, it is clear that TNF treatment highly increases NF-κB reporter gene activity. AA (Fig. 3A), BA (Fig. 3B), and IAA (Fig. 3C) dose-dependently and efficiently repress the TNF-induced promoter activities of all three NF-κB-dependent reporter genes. HBA alone also slightly reduce background levels, which can be explained by the effect of these compounds on basal NF-κB activity. Importantly, they do not affect the expression of β-galactosidase protein levels, used to correct for protein content (PGK), thus strongly indicating that the observed effect is promoter-specific. In the next step, we created sigmoidal dose-response models of the κB3-luc induction levels (Fig. 3D) and analyzed the data in terms of the concentration able to result in 50% repression of the TNF response (IC₅₀, Table 2). As a positive control, we

Table 1. Effects of different classes of HBA on TNF-induced production of inflammatory gene transcripts in L929sA cells

	Solvent	AA	BA	IAA
IL6	29.47 ± 6.13	10.50 ± 2.30	4.37 ± 1.01	6.01 ± 2.24
RANTES	11.76 ± 2.17	6.11 ± 1.39	3.09 ± 1.23	5.25 ± 1.28
TNF- α	11.01 ± 2.38	2.40 ± 0.45	1.49 ± 0.45	5.11 ± 0.19
				3.83 ± 0.54
				2.76 ± 1.95
				1.97 ± 0.29

After treatment of the cells for 1 h with AA (1 and 5 μ M), BA (1 and 5 μ M), or IAA (50 and 100 μ M), followed by TNF (2000 IU/mL) for 5 h, cells were washed with PBS, lysed, and total RNA was recovered using Trizol® reagent. Reverse transcription was performed to prepare cDNA for quantitative mRNA analysis *via* real time PCR with Sybr Green detection. mRNA expression levels are normalized to HPRT expression, and data presented are fold expression + SD to noninduced solvent control.

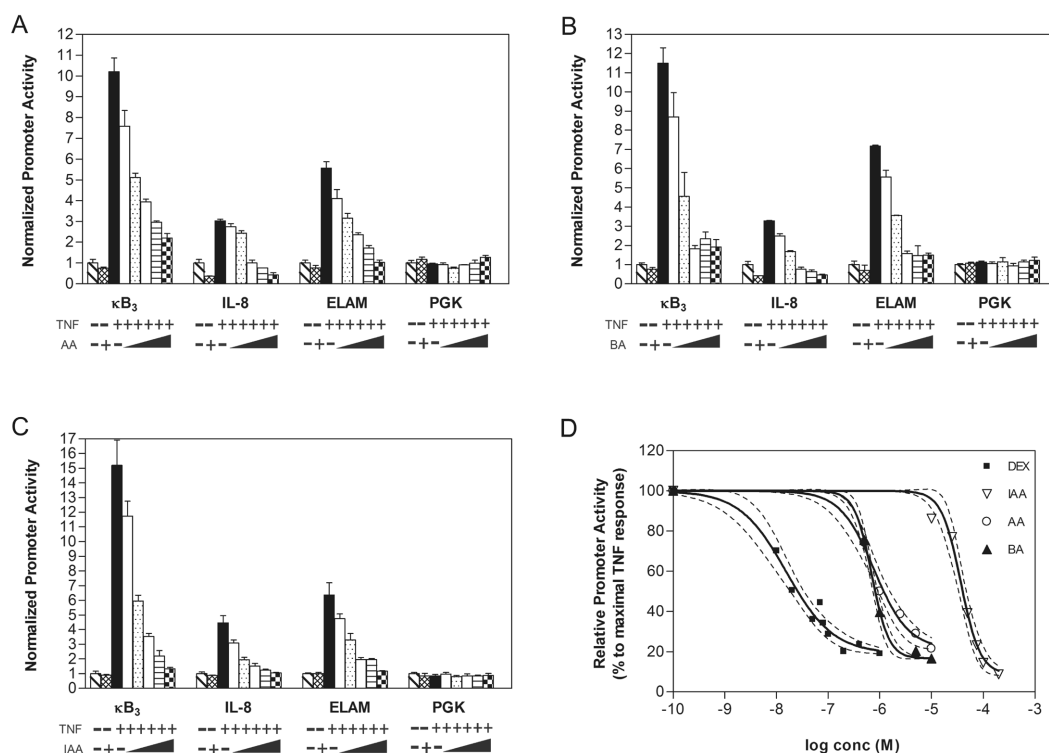


Figure 3. HBA block TNF-induced transactivation of NF- κ B-dependent promoters in luciferase reporter gene assays. L929sA mouse fibroblasts were stably transfected with either p546 · IL8P-luc⁺ (IL8), p(IL6 κ B)₃50hu · IL6P-luc⁺ (κ B₃), or pE-selectin-luc⁺ (ELAM) as described in Section 2. Cells were pretreated for 1 h with increasing concentrations of AA (A) or BA (B) (both 0.5–1–2.5–5–10 μ M), or IAA (C) (10–25–50–75–100–200 μ M), before adding TNF (2000 IU/mL) for another 5 h. Afterwards, cells were washed in PBS, lysed, frozen overnight, and enzyme activity was measured. The β -galactosidase protein expression of the cotransfected pPGK β GeobpA (PGK) is also depicted, under the same induction conditions. Results are means + SD of triplicate measurements (luciferase levels corrected for β -galactosidase activity) and data are normalized to the solvent control, which was arbitrarily set to 1. Out of three independent experiments with similar results, one representative is shown. (D) Presents the results of the NF- κ B-dependent κ B₃-reporter gene assays, now used to calculate IC₅₀ values for the different hop compounds in comparison to DEX, serving as a positive control for transrepression. Therefore, values of % induction to maximal TNF response were used to perform a nonlinear regression analysis as described in Section 2. The calculated 95% CI of the regression is indicated by the dotted lines.

included DEX, an extremely potent glucocorticoid commonly used to treat inflammatory diseases, which represses NF- κ B-driven inflammatory gene expression *via* binding to the glucocorticoid receptor. From Table 2, we can conclude that AA and BA show a similar potency in reducing inflammation, with an IC₅₀ of 0.78 and 0.70 μ M, respectively. In

clear contrast, higher concentrations of IAA are needed to be equally effective (IC₅₀ of 37.04 μ M). As expected, DEX is most effective and able to reduce inflammation for 50% at a concentration of 0.016 μ M.

From the combined aforementioned results, we can conclude that AA, BA, and also, albeit to a lesser extent, IAA,

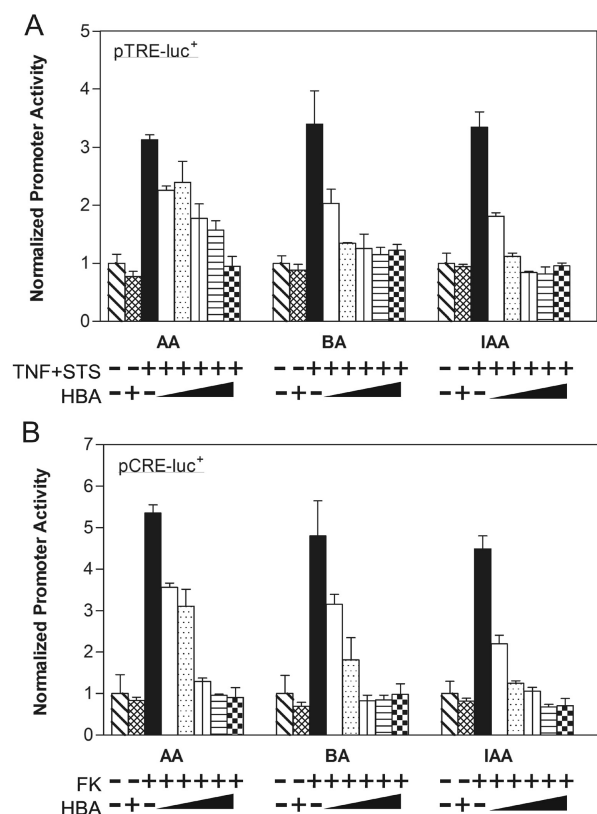


Figure 4. HBA affect AP-1- and CREB-driven gene expression. L929sA cells were stably transfected with luciferase reporter gene vectors for either AP-1- (A) or CREB-driven (B) gene expression. Cells were pretreated with increasing concentrations of AA or BA (both 0.5–1–2.5–5–10 μ M), or IAA (10–25–50–75–100–200 μ M) for 1 h, after which a combination of STS (60 nM) and TNF (2000 IU/mL) was added to activate TRE-luc, or forskolin (FK, 10 μ M) to induce CREB activation. After 5 h, cells were washed with PBS and lysed. Luciferase and β -galactosidase activities were measured. Data are means \pm SD of luciferase levels, corrected for β -galactosidase activity, and normalized to solvent control. Out of three independent experiments of which each sample was measured in triplicate, one representative is shown.

effectively inhibit inflammatory protein production, most probably through the inhibition of NF- κ B-dependent gene expression.

3.4 HBA also affect AP-1- and CREB-driven gene expression

To explore the possibility that HBA exert a more general inhibitory effect on pro-inflammatory signaling, we investigated their effect on AP-1- and CREB-driven gene expression. Besides NF- κ B, AP-1 and CREB are also transcription factors involved in cellular homeostasis and inflammatory responses, for which binding sites are present in the promoter region of many cytokines, including IL6 [42]. Once activated by phosphorylation through upstream pro-

Table 2. IC₅₀ values, as calculated from the NF- κ B-dependent κ B₃-reporter gene assays

κ B ₃ -luc ⁺	IC ₅₀	95% CI	
		Lower limit	Upper limit
DEX (μ M)	0.016	0.011	0.022
AA (μ M)	0.78	0.61	0.99
BA (μ M)	0.70	0.63	0.77
IAA (μ M)	37.04	31.15	44.04

tein kinases, CREB recognizes cAMP response elements (CRE), and activated AP-1 binds to TPA response elements [44–46]. We performed reporter gene assays on a TRE-driven luciferase reporter, induced with a combination of STS and TNF (as described in ref. [47]). It is clear from Fig. 4A that AA, BA, and IAA inhibit AP-1-driven gene expression in a dose-responsive manner. Similar results were obtained with a CRE-driven luciferase reporter in L929sA fibroblasts, induced with forskolin (Fig. 4B). Again, AA and BA show similar repression levels, while IAA is effective at higher concentrations.

Taken together, these results suggest that HBA affect upstream targets, common in the pathway leading to activation of NF- κ B, AP-1 as well as CREB, and independent of the stimulus for activation.

3.5 HBA do not mediate GR α -, PPAR α -, or PPAR γ -dependent gene transcription

Ligands for the NRs GR α , PPAR α , and PPAR γ have been shown to reduce inflammation [48, 49], either through the transactivation of anti-inflammatory proteins, or through the downregulation of pro-inflammatory cytokines. To investigate whether AA, BA, or IAA could mediate their anti-inflammatory actions through any of these NRs, we first checked whether these compounds can transactivate the respective NRs by using specific luciferase-coupled reporter genes. As expected, DEX increases the luciferase activity of a GRE-containing reporter 12-fold in L929sA cells (Fig. 5A). In contrast, incubating the transfected cells with either AA, BA, or IAA at concentrations which give significant repression of NF- κ B activation (10 μ M for AA and BA, 50 μ M for IAA), does not increase the GRE-driven promoter activity. Treatment with the PPAR α -specific agonist FF stimulates the promoter activity of a PPRE-containing reporter three-fold in COS1L2A cells cotransfected with PPAR α . Again, treatment with AA, BA, or IAA does not stimulate PPRE-driven gene expression (Fig. 5B). When exchanging PPAR α for PPAR γ (Fig. 5C), PPRE-driven promoter activity is four-fold enhanced after treating the cells with the PPAR γ -specific agonist ROSI, whereas the HBA fail to do so. Overall, these data show that AA, BA, and IAA do not mediate typical DNA-dependent transactivation of GR α , PPAR α , or PPAR γ reporter gene sys-

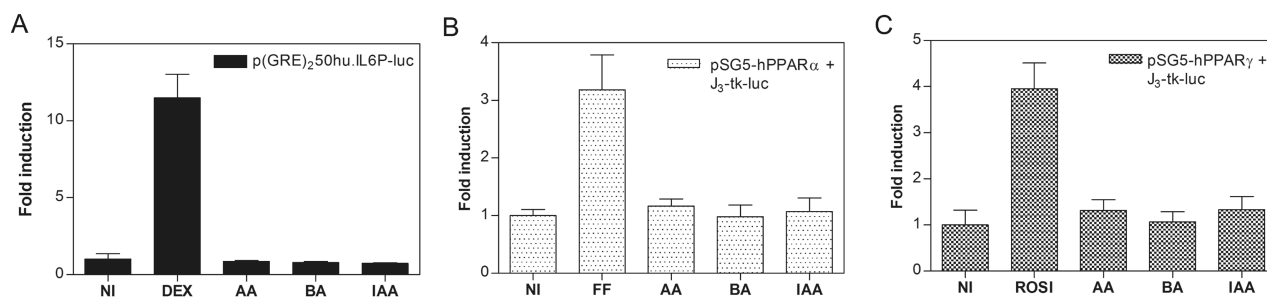


Figure 5. HBA do not mediate GR α -, PPAR α -, or PPAR γ -dependent transactivation. L929sA cells were stably transfected with p(GRE)₂₅₀hu · IL6P-luc and treated with compounds indicated on the graph for 24 h, after which protein lysates were made and luciferase and β -galactosidase protein levels were measured (A). COS1L2A cells were transiently transfected with the reporter J₃-tk-luc, and an expression vector for PPAR α (B) or PPAR γ (C). Twenty-four hours after transfection, inductions were performed for another 24 h. Data are expressed as luciferase levels, corrected for β -galactosidase activity, relative to the solvent-treated control (noninduced, NI). The concentrations used are 1 μ M DEX, 50 μ M FF, 1 μ M ROSI, 10 μ M AA or BA, and 50 μ M IAA. Each experiment was repeated at least two-fold with similar results.

tems, and may thus not be surrogate ligands for the NRs involved.

3.6 Antagonists for GR α , PPAR α , or PPAR γ cannot revert HBA-mediated NF- κ B repression

Recent data indicate that also nontransactivating ligands or modulators of the aforementioned NRs can inhibit inflammatory signaling through a mechanism called “ligand-dependent transrepression” [20]. To study whether or not the inhibitory activity of HBA on NF- κ B is attributed to ligand-dependent transrepression *via* GR α , PPAR α , or PPAR γ , we used specific antagonists for each receptor. As expected, DEX-mediated repression of TNF-induced NF- κ B-dependent promoter activity is reversed by the addition of RU486, a GR antagonist (Fig. 6A). In sharp contrast, the repression levels of AA, BA, and IAA remain unaffected. Similar results are obtained in comparable experiments, using GW6471 as an antagonist for PPAR α and GW9662 as an antagonist for PPAR γ (Figs. 6B and C). Analogously, the repression of NF- κ B activity by PPAR-specific agonists (GW9578 for PPAR α and ROSI for PPAR γ) is reversed by pretreating the cells with their respective specific antagonists, whereas the repression levels of the HBA remain unaffected. Notably, the use of higher doses of antagonists did not influence the outcome of the assay (data not shown).

3.7 HBA cooperate with agonists for GR α , PPAR α , or PPAR γ in inhibiting the activity of NF- κ B

In a different approach, we investigated the repression of NF- κ B activity by a combination of HBA and NR-specific agonists. AA, BA as well as IAA are able to cooperate with glucocorticoids for the repression of TNF-induced NF- κ B-driven gene expression, in a dose-responsive manner (Fig. 7A). Similarly, the three classes of HBA also cooperate with the PPAR α ligand GW9578 (Fig. 7B) and with the PPAR γ ligand ROSI (Fig. 7C). These results further support

our hypothesis that the studied HBA inhibit NF- κ B without directly modulating GR α -, PPAR α -, and PPAR γ activity, but possibly affect other upstream signaling components in the activation pathway toward NF- κ B transcriptional activity.

3.8 HBA inhibit acute local inflammation *in vivo*

To test the *in vivo* relevance of our *in vitro* findings, we investigated the ability of HBA to reduce acute inflammation in mice. As BA and AA gave very similar results with regard to gene inhibition *in vitro*, we decided to focus on AA and IAA. In a preliminary dose-finding study, amounts up to 1 mg IAA could be administered *i.p.* to mice without notable toxicity, while for AA, 500 μ g was lethal at 24 h (data not shown). Therefore, we chose to treat the mice with 250 μ g AA or IAA, or 1 mg IAA. Subcutaneous injection of zymosan, an insoluble antigenic carbohydrate from the cell wall of yeast, in the paw of mice causes a local inflammatory reaction characterized by edema. When injecting 250 μ g HBA, the zymosan-induced swelling of the footpad is markedly reduced (Figs. 8A and B). Hereby, AA is slightly more effective than equal amounts of IAA. When elevating the dose of IAA to 1 mg, a small trend to further reduce the swelling is reproducibly observed. For both classes of compounds, the anti-inflammatory effects, which were already apparent at 6 h, persisted up to 24 h after zymosan injection (data not shown). Furthermore, administration of the compounds either as potassium salts in PBS (Fig. 8A), or neutral HBA in DMSO (Fig. 8B), did not influence their efficacy.

4 Discussion

The use of plant-derived compounds in the treatment or prevention of inflammatory disorders has been ongoing for centuries on an empirical basis. Only in recent years, research has been directed toward elucidating the underlying molecular mechanisms of how herbals interact with inflammatory signaling cascades and influence the activity

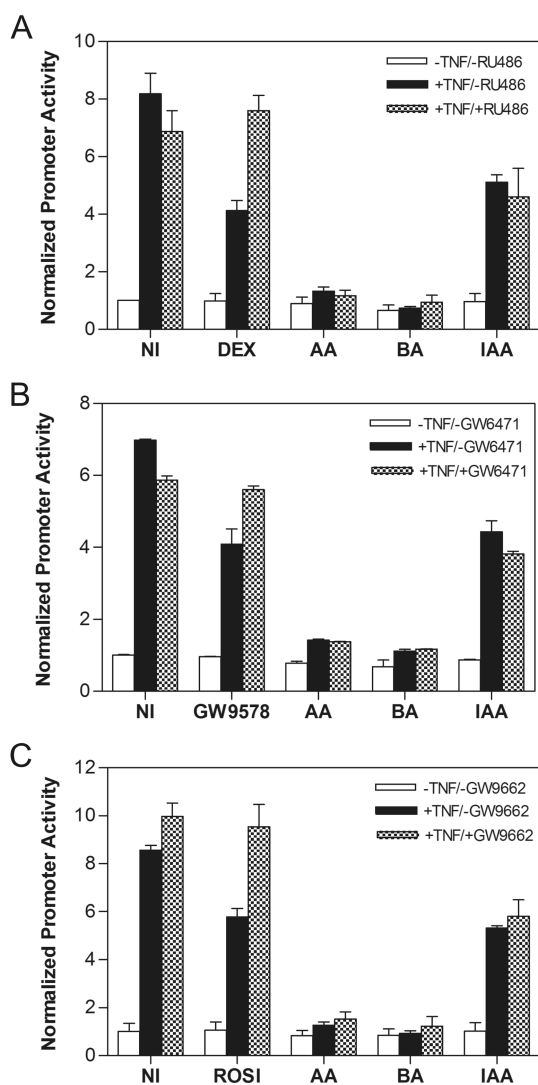


Figure 6. Antagonists for GR α , PPAR α , or PPAR γ do not affect HBA-mediated repression of TNF-induced NF- κ B activation. L929sA cells, stably transfected with p(IL6 κ B)₃50hu · IL6P-luc⁺ (κ B₃), were used to perform experiments with antagonists for GR α (A), PPAR α (B), or PPAR γ (C). The cells were exposed to the antagonists RU486 (for GR α , 1 μ M), GW6471 (for PPAR α , 2.5 μ M), or GW9662 (for PPAR γ , 5 μ M) for 1 h. Afterwards, various test compounds, as depicted on the graph, were added: DEX (0.1 μ M), GW9578 (500 nM), ROSI (500 nM), AA (10 μ M), BA (10 μ M), or IAA (50 μ M). One hour later, TNF was added to induce NF- κ B activation (2000 IU/mL) for a period of 5 h. Out of at least three independent experiments, each performed in triplicate, one representative is shown.

of nuclear transcription factors, such as NF- κ B, AP-1, and CREB. In this respect, natural constituents, including isoprenoids, phenolics (e.g., curcumin) and flavonoids (e.g., resveratrol and EGCG) have already been identified as potent inhibitors of pro-inflammatory signaling [4–6] and their molecular targets are the subject of intensive research.

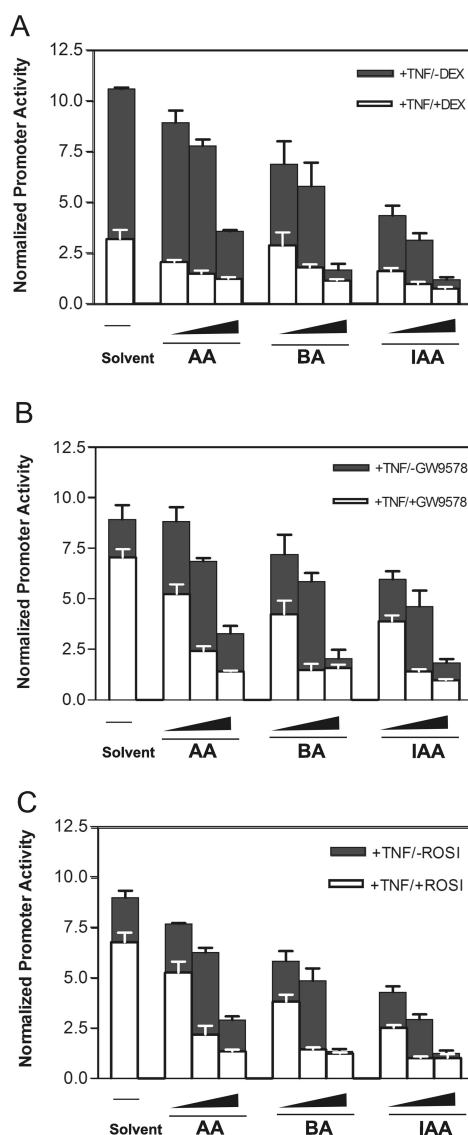


Figure 7. HBA cooperate with agonists for GR α , PPAR α , or PPAR γ to efficiently block NF- κ B. L929sA cells, stably transfected with p(IL6 κ B)₃50hu · IL6P-luc⁺ (κ B₃), were pretreated for 1 h with saturating amounts of agonists for GR α (DEX, 0.1 μ M), PPAR α (GW9578, 500 nM), or PPAR γ (ROSI, 500 nM) and different concentrations of HBA: 0.5–1–5 μ M for AA and BA, and 25–50–100 μ M for IAA, either alone or in combination. Afterwards, TNF was added for 5 h to activate NF- κ B, after which cells were washed, lysed, and enzyme activity was measured. Results from samples treated with single compounds were verified to be not significantly different from 1 (solvent control) (data not shown).

Traditionally, hop (*Humulus lupulus* L.) has been used worldwide, not only in the brewing process, but also as a herbal medicine for treating insomnia, nervousity, and post-menopausal symptoms. In previous research, the attention has mainly been focused on their high content of polyphenols, for which a broad range of bioactivities has been

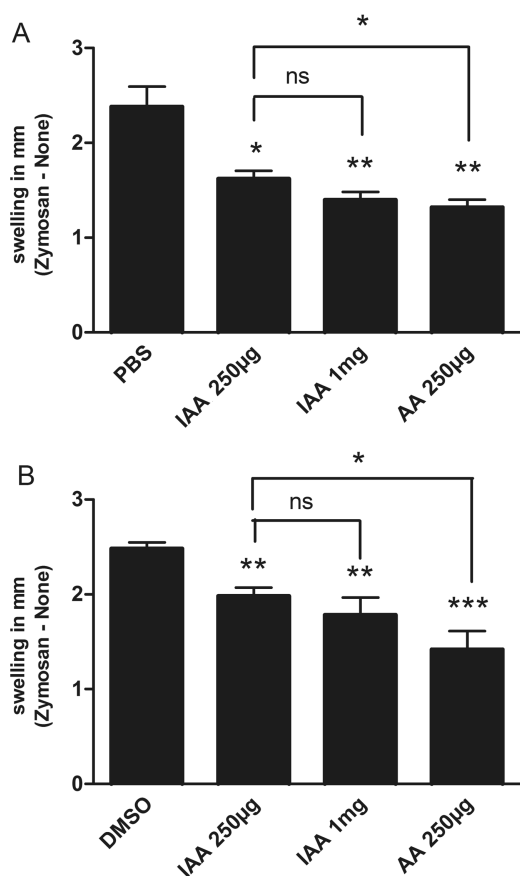


Figure 8. *In vivo* anti-inflammatory effect of AA and IAA in a mouse model of zymosan-induced paw inflammation. Bar charts represent the data on paw swelling obtained at 6 h after zymosan injection. IAA or AA were injected i.p. either as potassium salts in PBS (A) or as neutral acids in DMSO (B), 30 min prior to inducing local inflammation by subcutaneous injection of zymosan in the right paw ("Zymosan"). Thickness of the foot was measured using a caliper and values were corrected for the unaffected paw ("None"). *p*-Values were obtained comparing test groups with the solvent-treated group, unless depicted otherwise in the figure. (A) IAA 250 µg versus AA 250 µg (*): $p = 0.0340$; (all vs. PBS): IAA 250 µg (*): $p = 0.0107$; IAA 1 mg (**): $p = 0.0027$; AA 250 µg (**): $p = 0.0016$. (B) IAA 250 µg versus AA 250 µg (*): $p = 0.0294$; (all vs. DMSO): IAA 250 µg (**): $p = 0.0022$; IAA 1 mg (**): $p = 0.0072$; AA 250 µg (***): $p = 0.0008$.

described [23, 24, 50]. In contrast, the fraction of HBA remains merely known for its bittering, foam-stabilizing, and antibacterial properties delivered to beer [21, 26]. The recently increased interest in their effects on human health is justified, since they are – in contrast to the polyphenols – present in beer in relatively large amounts, even up to 100 mg/L. Consequently, several authors have reported on the possible anticarcinogenic and anti-inflammatory properties of HBA, exemplified by the reports on the inhibition of inflammatory enzyme activity of COX-2 by humulone [33, 34, 51].

The present study aimed to compare the effect of different classes of HBA, including AA, BA, and IAA, on their potential anti-inflammatory activity, to identify the specific transcription factors involved, and to investigate NRs as possible molecular targets. Pure humulone has been described to inhibit TPA- and arachidonic acid-induced ear edema in mice and TPA-induced tumor formation in mice skin [32, 52]. Furthermore, the inhibitory effect on the transcriptional activation of COX-2 has been demonstrated in cultured cells [34] and mouse skin [33]. Recently, Lee *et al.* [33] defined the transcription factors NF-κB and AP-1 as targets for the humulone-inhibited COX-2 expression in TPA-induced skin cancer. In the current study, we have focused on the anti-inflammatory effects of total AA, in comparison to those of two other classes of HBA, namely BA and IAA, using fibroblasts, which are important sentinel cells in the immune system [35]. We identified the transcription factors NF-κB, AP-1, and CREB as targets for HBA-inhibited inflammation. Furthermore, we presented important evidence excluding a role for GRα, PPARα, or PPARγ in the immune-modulating pharmacological activity of the presently studied HBA. Moreover, we show that HBA are effective in a mouse model addressing acute local inflammation.

The transcription of the cytokine IL6, which plays a pivotal role in immune regulation, is regulated by NF-κB, AP-1, and CREB, transcription factors for which binding sites are present in the IL6 promoter [42, 53]. The present study revealed that AA, BA, and IAA effectively inhibit TNF-induced IL6 protein production (Fig. 2A), without affecting cell viability (Fig. 2B). Furthermore, IL6 mRNA levels were decreased in a dose-responsive manner, and similar effects were observed for other genes involved in the inflammatory response, such as TNFα and RANTES (Table 1). Interestingly, AA and BA were effective at lower concentrations than IAA. We identified the pro-inflammatory transcription factor NF-κB as a target by using several NF-κB-dependent reporter gene assays (Fig. 3). AA and BA showed equal potency in reducing inflammation after TNF treatment, suggesting that an extra prenylgroup at C(4) does not influence the activity of the modified AA (IC₅₀ around 0.7 µM, Table 2). On the other hand, the AA isomers (IAA) were also effective, but only when used at higher concentrations. Interestingly, IAAs are abundant in beer at ten-fold higher concentrations. Apart from the TNF-induced NF-κB activity, also AP-1 and CREB activities, induced by a combination of STS and TNF, or forskolin, respectively, were affected by all three classes of hop acids. In line with the above-mentioned findings, BA and AA showed similar repression levels at equal concentrations. Taken together, we can conclude that AA, BA and, to a lesser extent, IAA are potent inhibitors of NF-κB, AP-1, and CREB activities, independent of the activation stimulus. Our data suggest that HBA may affect (a) common upstream target(s) for these major transcription factors in inflammatory responses. Of particular interest are the

novel results of anti-inflammatory properties of BA and IAA, while our results obtained for AA are in line with those reported for humulone in mouse skin [33] and osteoblasts [34].

It is known that NRs such as GR α , PPAR α , and PPAR γ can influence inflammatory signaling through a combination of mechanisms, such as direct inhibitory interactions with NF- κ B and upregulation of inhibitory proteins, *e.g.*, I κ B- α [13, 20]. Therefore, we speculated that HBA could mediate their effects through the activation of these NRs. After all, activated GR and PPARs also target NF- κ B and AP-1. However, we could definitively rule out a possible role for these receptors in the suppression of NF- κ B by all three studied classes of HBA, at least in the used fibroblast cell system.

First of all, none of the HBA caused gene transcription through GR α , PPAR α , or PPAR γ , whereas major selective agonists did stimulate transactivation of the respective response element-driven reporter genes (Fig. 5). However, this evidence was not conclusive, and we needed to exclude the possibility that HBA could act as nontransactivating ligands for the investigated NRs. By a mechanism called “ligand-dependent transrepression,” such ligands bind to a receptor and inhibit activated transcription factors without the need for DNA-specific binding and without supporting subsequent transactivation [12]. The responsive mechanism is not yet fully understood, and a series of possible models have been suggested, including inhibitory protein–protein interactions between the receptor and the transcription factors (*e.g.*, GR α /NF- κ B, PPAR/NF- κ B, GR α /AP-1, PPAR/AP-1, GR α /CREB), competition for coactivators, disruption of the coactivator complex, inhibition of corepressor clearance and modification of histones [54, 55]. We showed that the repression of TNF-induced NF- κ B gene activation by AA, BA, and IAA did not change when blocking GR α , PPAR α , or PPAR γ by selective NR antagonists, whereas gene repression by the specific ligands DEX (for GR α), GW9578 (for PPAR α), or ROSI (for PPAR γ) was reversed (Fig. 6).

Furthermore, we showed additive inhibition of TNF-induced NF- κ B-driven reporter gene expression by combining HBA with DEX, GW9578, or ROSI (Fig. 7). Thus, it seems that HBA and agonists for GR α , PPAR α , or PPAR γ work in an independent way in order to suppress NF- κ B, which makes this observation exciting for therapeutic purposes. For example, in nasal polyposis, the use of topical corticoids is often not sufficient to combat chronic inflammation, and long-term systemical administration is avoided because of the development of side effects [56]. A combination of GC and HBA might be beneficial in clinical settings because they clearly impinge at a different level to target NF- κ B-dependent inflammatory gene expression. In another view, cotreating inflammation with both GR α , PPAR α , or PPAR γ agonists and HBA might allow to use lower doses of NR agonists, thereby possibly reducing the amount of accompanying side effects. Moreover, treatment

with HBA is expected to be well tolerated, given their long-term historical use in folk medicine and in beers without notable toxicity. However, a detailed clinical study directly addressing patient responses has yet to be carried out. In conclusion, we demonstrated that AA, BA as well as IAA inhibit the transcriptional activity of NF- κ B without activating the NRs GR α , PPAR α , or PPAR γ , and independent of the corresponding specific agonists. This leaves the possibility open that HBA affect (an)other target(s) in the pathway toward NF- κ B, such as the broad range of upstream modulating protein kinases, or, alternatively, *via* influencing nuclear chromatin-dependent mechanisms and/or cofactor assembly.

Previous studies have addressed the efficiency of topically applied HBA, more specifically of humulone, for the treatment of locally inflamed rodent skin [32, 33]. In contrast, with respect to the anti-inflammatory efficacy of HBA upon oral ingestion, Hougee *et al.* [51] have reported that a whole hop CO₂ extract, consisting of approximately 18% AA, failed to inhibit zymosan-induced joint swelling. In this report, we looked at the potential of HBA to inhibit acute local inflammation *in vivo* in mice, and administered AA or IAA systemically *via* intraperitoneal injection. Because of the very similar gene regulation profile *in vitro*, we chose to focus on AA, but we have no reason to assume that BA would behave otherwise. Administration of 250 μ g AA or IAA did effectively lower zymosan-induced paw swelling, a typical symptom of inflammation (Fig. 8). The fact that AA were found to be more effective than IAA nicely correlates with the *in vitro* obtained results on inflammatory gene regulation. Injecting a higher dose of IAA (1 mg) resulted in a further reduction, albeit slightly, of the edema, which is in agreement with the earlier discussed dose-responsive effect of IAA *in vitro*. Additionally, injecting HBA as either potassium salts in PBS or neutral acids in DMSO, did not influence their *in vivo* activity, which suggests that both formulas are equally well absorbed from the peritoneal cavity. To the best of our knowledge, this is the first report where systemic administration of AA and IAA proves to be effective in decreasing an acute local inflammation. The discrepancy with the negative results for a hop CO₂ extract, earlier reported by Hougee *et al.* [51], can most probably be explained by different routes of compound administration. Indeed, their orally administered dose of 1.25 mg crude extract, consisting of approximately 18% AA, fairly corresponds to our *i.p.* injected dose of 250 μ g AA. Thus, after oral intake, a poor gastrointestinal absorption of AA and/or of precocious breakdown in the tract most likely results in low bioactive blood levels, which – in their setting – have proven to be insufficient in causing a detectable reduction of joint swelling. However, as Hougee *et al.* also suggested, elevating the dose could overcome this drawback, but this still remains to be studied. The finding that HBA are indeed capable of systemically reducing inflammatory disorders

confirms that the observations we have made for fibroblast cells are also transferrable to the level of the whole organism. This opens up therapeutic possibilities for HBA in a whole range of chronic inflammatory disorders, including, *e.g.*, rheumatoid arthritis, nasal polyposis, severe asthma, and inflammatory bowel disease.

In conclusion, AA, BA, and IAA, although the latter to a lesser extent, inhibit TNF-induced inflammation by modulation of NF- κ B-, AP-1-, and CREB-dependent gene transcription, *via* a mechanism that runs independently of GR α , PPAR α , or PPAR γ NR activation. Further studies are needed to define which upstream signalization pathways are affected by HBA. Additionally, systemic administration of HBA proves to be effective in decreasing an acute local inflammation. Our findings imply that in search for novel anti-inflammatory strategies, devoid of side effects, a therapeutic role can be foreseen for HBA, either alone or in combination with existing hormone-based anti-inflammatory therapies.

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The authors have declared no conflict of interest.

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