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Trichodion, a new inhibitor of inflammatory signal transduction pathways from a *Trichosporiella* species

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Abstract In a search for new inhibitors of the IFN-γ mediated signal transduction in HeLa S3 cells using secreted alkaline phosphatase (SEAP) as reporter gene, the novel pyran-dione trichodion was isolated from fermentations of the imperfect fungus *Trichosporiella* sp. 20-95. The compound inhibits the IFN-γ mediated expression of the reporter gene with IC_{50} values of 21–42 μM (5–10 μg/ml). The NF-κB and AP-1 mediated expression of the reporter gene are inhibited with IC_{50} values of 42–84 μM (10–20 μg/ml) and 21 μM (5 μg/ml) respectively. Western blotting with COX-2 and NOS II antibodies showed that the expression of both proinflammatory enzymes is almost completely inhibited at 21–42 μM (5–10 μg/ml) in LPS/IFN-γ stimulated J774 mouse macrophages. Studies on the mode of action of the compound revealed that the inhibition of the NF-κB dependent pathway is due to the stabilization of the IκB protein and the inhibition of the IFN-γ dependent signaling is caused by an inhibition of the phosphorylation of the STAT1α transcription factor.

Key words: JAK/STAT pathway; Inhibitor; Inflammatory signal transduction pathway

1. Introduction

Altered gene expression is fundamental to the ethiology of many human diseases including cancer, inflammation and immune disorders. Many genes for proinflammatory enzymes (e.g. COX-2, NOS II) and cytokines (e.g. TNF-α) contain binding sites for multiple transcription factors in their regulatory elements, which are activated by a variety of inducing agents like bacterial lipopolysaccharide (LPS), tumor promoters, cytokines (e.g. IFN-γ) and growth factors. Inhibitors which specifically interfere with components of different intracellular signaling pathways or inhibit the activation of transcription factors responsible for the expression of disease related genes may have applications as novel therapeutics in inflammation [1,2]. Besides the transcription factor NF-κB which is an immediate early transcriptional activator [3,4], components of the JAK/STAT pathway play an important role in the transcriptional activation of many inflammatory genes. Exposure of cells to interferon-γ triggers the phosphorylation of latent cytoplasmatic transcription factors termed STATs [5]. The STATs become phosphorylated on tyrosine residues by one or more members of the JAK family of protein tyrosine kinases then assemble in dimeric or oligomeric form, enter the nucleus and regulate transcription of many genes by binding to specific DNA sequences (STAT binding elements, SBE) [6]. IFN-γ has been shown to induce tyrosine phosphorylation of STAT1α and a homodimeric complex of STAT1α binds to the IFN-γ activation sequence (GAS). STAT1 and STAT2 are tyrosine phosphorylated in response to IFN-α/β and form a complex with the DNA binding protein p48, which moves to the nucleus and activates the transcription of genes bearing the interferon responsive element (ISRE) [7]. SBE and NF-κB binding sites have been found in the promoters of inflammatory genes including those encoding for NOS II and ICAM and a functional synergy of IFN-γ and TNF-α has been reported [8]. In order to search for fungal metabolites inhibiting the IFN-γ dependent signaling pathway, reporter gene vectors were constructed which express the reporter gene under the control of five copies of the GAS/ISRE responsive element. A screening of 500 mycelial cultures of basidiomycetes, ascomycetes and imperfect fungi resulted in the isolation of trichodion from the deuteromycete *Trichosporiella* sp. In this paper the biological properties of trichodion are described.

2. Materials and methods

2.1. Producing organism

The deuteromycete strain 20-95 was isolated from a soil sample collected in Germany. The specimen showed the characteristics of the genus *Trichosporiella* as described by Domsch et al. [9]. The species however could not be identified. The strain was kindly provided by H. Anke and is deposited in the culture collection of the LB Biotechnologie, Universität Kaiserslautern.

2.2. Fermentation

For maintenance on agar slants the strain was kept on YMG medium [10]. For submerged cultivation, strain 20-95 was grown in malt extract medium (40 g/l malt extract). A well grown seed culture of *Trichosporiella* sp. 20-95 (200 ml YMG medium) was used to inoculate a Biolafitte C-6 fermenter containing 20 l of malt extract medium with aeration (3 l air/min) and agitation (120 rpm) at 22°C. The production of trichodion was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluid in the reporter gene assay as described below.

2.3. Isolation of trichodion

After 9 days the culture fluid was separated by filtration and applied onto Mitsubishi DIAION HP 21 resin. The resin was washed with water and the active compound was eluted with acetone. The eluate was concentrated and the remaining aqueous residue extracted with EtOAc. The solvent was evaporated and the crude product (1.16 g) was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (50:50) as eluent resulting in 155 mg of an enriched product. Precipitation from acetonitrile yielded 62 mg of pure

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Abbreviations: COX, cyclooxygenase; NOS, NO-synthase; IFN, interferon; LPS, lipopolysaccharide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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trichodion. The structural elucidation was done by spectroscopic methods and will be published elsewhere.

2.4. Biological assays

COS-7 (ATCC CRL 1651) and HeLa S3 (ATCC CCL 2.2) cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. J774 (DSMZ ACC 170) and Jurkat (ATCC TIB 152) cells were grown in RPMI 1640 medium with 10% FCS. The assays for antimicrobial activity, cytotoxicity as well as macromolecular syntheses in HeLa S3 cells were carried out as described previously [11].

2.5. Reporter gene assays

The reporter plasmids pGE3-NF1 and pGE2-AP1 have been described recently [12]. Both plasmids carry the reporter gene secreted alkaline phosphatase (SEAP) under the control of a thymidine kinase promoter and 5×NF-κB or an enhancerless SV40 promoter and 3×AP-1 binding sites. The reporter plasmid pGE3-GAS/ISRE was constructed essentially as described earlier by cloning five copies of an GAS/ISRE consensus oligonucleotide (5′-AAGTACTTT-CAGTTTCATATTACTCTA-3′) immediately upstream of the thymidine kinase promoter driven SEAP reporter gene [12]. The reporter plasmid pGE3-NFAT contains the reporter gene SEAP under the control of the thymidine kinase promoter and five copies of the distal NF-AT/AP-1 binding site from the human IL-2 promoter [13]. The reporter plasmid pGE2-GRE contains the reporter gene SEAP under the control of the enhancerless SV40 promoter and three copies of the glucocorticoid responsive element (GRE) [14]. In addition, the activation of the glucocorticoid receptor mediated expression of the reporter gene in COS-7 cells has been determined by cotransfection of the human glucocorticoid receptor-α (pRShGR, ATCC 67200). Transfection of HeLa S3 and COS-7 cells and determination of the activity of the expressed SEAP were performed as described previously [15]. The transfection of Jurkat cells was performed by electroporating (Bio-Rad, Gene Pulser) 6×10^7 cells/ml in 0.2 ml HEBS buffer (10 mM HEPES, pH 7.05, 68.5 mM NaCl, 2.5 mM KCl, 0.35 mM Na_2HPO_4, 3 mM dextrose) together with 30 μg of the pGE3-NFAT plasmid at 500 V/cm. After electroporation the cells were seeded at 1×10^6 cells/ml OPTI-MEM containing 10% FCS in a 24 well plate with and without test compounds and SEAP expression was induced with 32 nM TPA and 2 μM ionomycin. The activity of the SEAP in the culture medium was determined 24 h after transfection using the Phospha-Light chemoluminescent reporter gene assay (Tropix, MA) according to the manufacturer's instructions with a liquid scintillation counter.

2.6. Western blots

J774 cells were starved for 16 h in DMEM medium with 0.5% FCS, treated for the indicated times with test compounds and induced with 1 μg/ml LPS and 10 ng/ml IFN-γ in DMEM medium containing 0.5% FCS. Total cell extracts were prepared using a high salt detergent buffer (20 mM HEPES, pH 7.4, 350 mM NaCl, 20% glycerin, 1% NP-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 10 mM NaF, 1 mM Na_3VO_4), complete protease inhibitor cocktail 1:50 (Roche Diagnostics, Germany) and cell extracts (50–100 μg protein) were subjected to 10% SDS–PAGE, transferred onto a nitrocellulose membrane, probed with either an anti-IκB antibody, anti-COX-2 antibody, anti-NOS II antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), an anti-phosphotyrosine-STAT1α antibody or an antiphospho-p38 antibody (NEB Inc.) and then with the appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were visualized by the enhanced chemoluminescent detection system (ECL system, Amersham International, UK).

IL-2 production in Jurkat cells pretreated for 1 h with or without test compounds and stimulated with 32 nM TPA and 2 μM ionomycin for additional 24 h, was determined by ELISA (Quantikine human IL-2, R and D Systems, UK) according to the manufacturer's instructions.

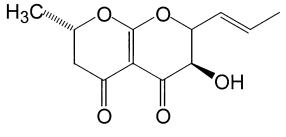

Fig. 1. Structure of trichodion.

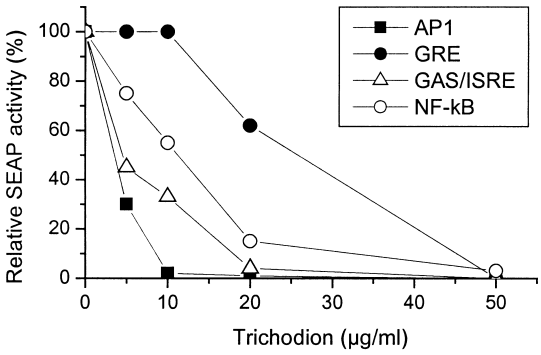

Fig. 2. Effect of trichodion on SEAP reporter gene expression in HeLa S3 and COS-7 cells. HeLa S3 cells were transfected with a GAS/ISRE (5×GAS/ISRE-SEAP) or an NF-κB (5×NF-κB-SEAP) dependent reporter gene and stimulated with 10 ng/ml IFN-γ (GAS/ISRE) or 5 ng/ml TNF-α (NF-κB) for 48 h with or without trichodion. COS-7 cells were transfected with an AP-1 dependent reporter gene (3×AP-1-SEAP) or a glucocorticoid responsive reporter gene (3×GRE-SEAP) together with an expression vector for the human glucocorticoid receptor-α and stimulated with 50 ng/ml TPA (AP-1) or 1 μM dexamethasone (3×GRE) for 48 h with or without trichodion. Control (100%): stimulation only.

3. Results

Trichodion (Fig. 1) has been detected during fermentation and isolation using SEAP reporter plasmids containing multiple GAS/ISRE sites in their promoter (pGE3-GAS/ISRE). Transfection of HeLa S3 cells and stimulation with 10 ng/ml IFN-γ resulted in a 8–10-fold increase in SEAP expression compared to the uninduced control. Trichodion inhibited the IFN-γ induced SEAP expression with IC_{50} values of 21–42 μM (5–10 μg/ml; Fig. 2). The TNF-α induced expression of the NF-κB dependent reporter plasmid (pGE3-NF1) in transiently transfected HeLa S3 cells was inhibited with IC_{50} values of 42–84 μM (10–20 μg/ml). In addition trichodion suppressed the AP-1 mediated gene activation in transiently transfected, TPA stimulated COS-7 cells with an IC_{50} value of 21 μM (5 μg/ml). In order to determine the effect of trichodion on the glucocorticoid receptor mediated gene expression, COS-7 cells were transiently transfected with a GRE driven reporter vector together with an expression vector for the human glucocorticoid receptor-α. The inhibition of the glucocorticoid receptor dependent SEAP expression was about 5-fold lower than the inhibition of the IFN-γ or TPA induced expression of the reporter gene (IC_{50}: 25 μg/ml, 105 μM).

The inhibition of the reporter gene expression did not result from a non-specific interference with cellular DNA, RNA, and protein syntheses or a decrease in cell viability since a pronounced influence on the macromolecular syntheses in HeLa S3 cells or cytotoxic effects could only be observed at concentrations higher than 50 μg/ml of trichodion (Fig. 3).

It has been shown that the inducible expression of the proinflammatory enzymes COX-2 and iNOS in murine and human cell lines by LPS, TNF-α, IL-1β, TPA or IFN-γ requires consensus sequences for the transcription factors NF-κB, AP-1 and STAT1α in their promoters, which are activated by different signal transduction pathways [16–19]. In order to determine the effect of trichodion on COX-2 and

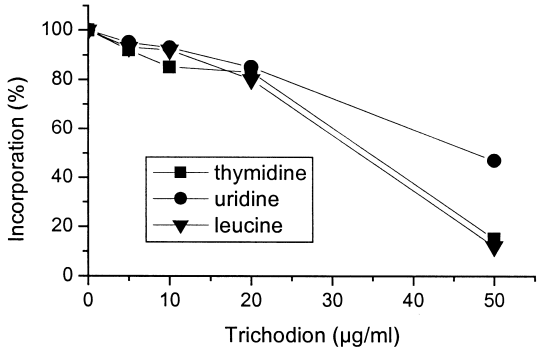

Fig. 3. Effect of trichodion on the incorporation of radiolabelled precursors into macromolecules of HeLa S3 cells. Control without test compound (100%): [^{14}C]thymidine, 7332 cpm, [^{14}C]uridine, 42737 cpm, [^{14}C]leucine, 39565 cpm.

iNOS expression J774 cells were stimulated for 12 h with LPS and IFN-γ with or without test compound and immunoblotted for COX-2 and iNOS proteins. As shown in Fig. 4 treatment of J774 cells with 1 μg/ml LPS and 10 ng/ml IFN-γ resulted in a strong induction of iNOS and COX-2 expression. Cotreatment with trichodion caused a dose dependent decrease in LPS/IFN-γ mediated induction of both enzymes. At 5 μg/ml (21 μM) the expression of the iNOS and COX-2 protein is almost completely inhibited. To elucidate the mechanism responsible for the inhibition of COX-2 and iNOS expression we determined the influence of trichodion on the LPS/IFN-γ mediated degradation of the inhibitory protein IκB-α in J774 cells. As shown in Fig. 5A stimulation of J774 cells with IFN-γ and LPS for 30 min resulted in the disappearance of the IκB protein which was completely inhibited by 10 μg/ml (42 μM) of trichodion. These data fit well to the ones obtained with the NF-κB dependent reporter gene assay and suggest that trichodion inhibits the LPS/IFN-γ induced activation of NF-κB in J774 macrophages.

To determine whether the inhibition of the IFN-γ dependent reporter gene in HeLa S3 cells and the LPS/IFN-γ induced expression of the proinflammatory enzymes COX-2 and iNOS in J774 cells is due to an inhibition of tyrosine phosphorylation of the STAT1α transcription factor, Western

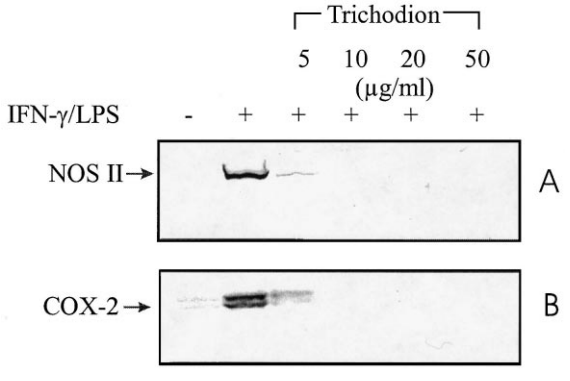

Fig. 4. Effect of trichodion on NOS II and COX-2 expression in J774 cells. J774 cells were pretreated for 1 h with or without trichodion and stimulated with 1 μg/ml LPS and 10 ng/ml IFN-γ for 12 h. Subsequently total cell extracts were prepared and equal amounts of protein (80 μg) analyzed by Western blotting for A: NOS II expression with an NOS II antibody and B: COX-2 expression with a COX-2 antibody.

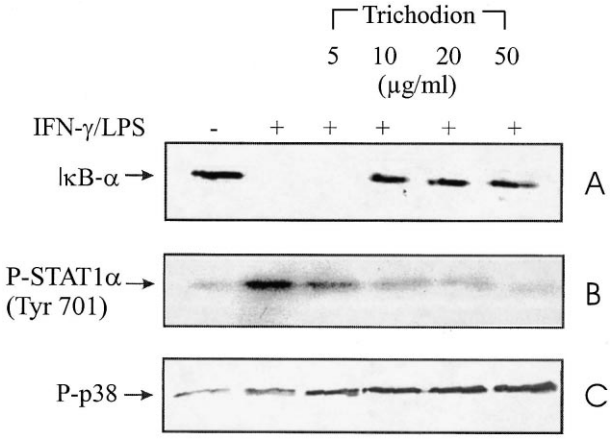

Fig. 5. Effect of trichodion on IκB degradation, STAT1α and p38 phosphorylation in J774 cells. J774 cells were pretreated for 1 h with or without trichodion and stimulated with 1 μg/ml LPS and 10 ng/ml IFN-γ for 30 min. Subsequently total cell extracts were prepared and equal amounts of protein (80 μg) analyzed by Western blotting for A: IκB degradation using a IκB-α antibody, B: STAT1α phosphorylation with a phospho-STAT1α antibody and C: p38 phosphorylation with a phospho-p38 antibody.

blots have been performed with a phospho-STAT1 (Tyr701) antibody. As shown in Fig. 5B treatment of J774 cells with LPS/IFN-γ resulted in a strong induction of tyrosine phosphorylation of the STAT1α protein. Pretreatment of the cells with trichodion inhibited the phosphorylation in a dose dependent manner. Trichodion markedly reduced the phosphorylation of the STAT1α protein at 5 μg/ml (21 μM). At 10 μg/ml the phosphorylation was reduced to the background comparable to the uninduced control. It has been shown that the expression of the inducible COX-2 and NOS II in LPS stimulated macrophages depends on the activation of different members of the mitogen activated protein kinases (MAPKs) which directly or indirectly activate a number of transcription factors including AP-1 and NF-κB [18,20,21]. Among the MAPKs, the JNK/SAPK and p38 MAPK pathways share activation by inflammatory cytokines, bacterial endotoxins and environmental stress. To determine whether trichodion inhibits the activation of the p38 MAPK Western blots were performed with a antibody specific for the phosphorylated form of p38 (Thr180/Tyr182). The results are shown in Fig. 5C. Treatment of J774 macrophages with LPS and IFN-γ alone increased the p38 phosphorylation. The level of p38 phosphorylation was markedly increased by cotreatment of the cells with various concentrations of trichodion. In addition treatment of J774 cells with trichodion alone resulted in an increase of p38 phosphorylation at concentrations starting from 20 μg/ml (data not shown).

The transcription factors AP-1, NF-κB and NF-AT have been shown to be essential for the inducible interleukin-2 (IL-2) expression in activated T cells [22]. Treatment of Jurkat cells with ionomycin which elevates intracellular levels of calcium and the phorbol ester TPA which activates protein kinase C can mimic the activation of T cells by the T cell receptor [23]. In order to determine the effect of trichodion on T cell activation Jurkat cells were transiently transfected with a reporter plasmid expressing the reporter gene under the control of five copies of the distal NF-AT/AP-1 binding site from the human IL-2 promoter immediately cloned upstream of the thymidine kinase promoter as described in Section 2.

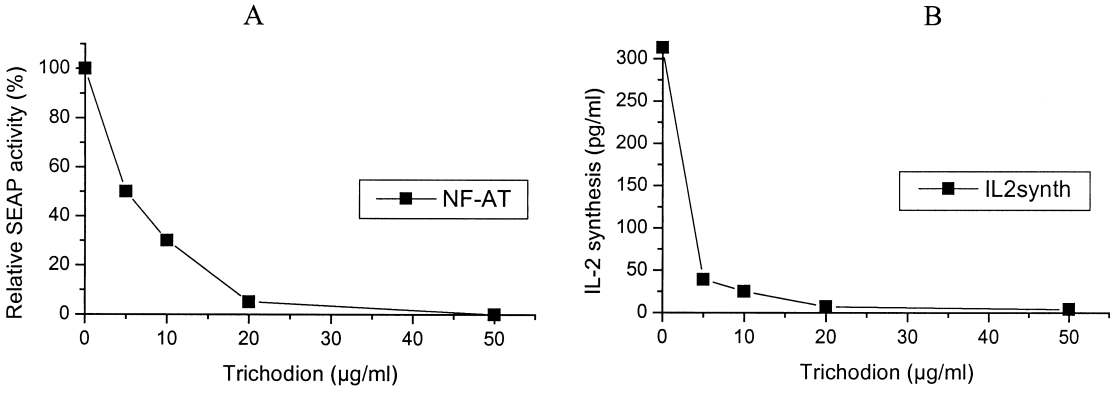

Fig. 6. Effect of trichodion on NF-AT/AP-1 dependent SEAP reporter gene expression and IL-2 synthesis in Jurkat cells. A: Jurkat cells were transfected with an NF-AT/AP-1 dependent reporter gene (5×NF-AT/AP-1) and stimulated with 32 nM TPA and 2 μM ionomycin for 24 h with or without trichodion. Control (100%): stimulation only. B: Jurkat cells were pretreated for 1 h with or without trichodion and stimulated with 32 nM TPA and 2 μM ionomycin for additional 24 h. IL-2 concentrations from cell supernatants were determined by ELISA.

Trichodion inhibited the TPA/ionomycin induced NF-AT mediated SEAP expression with an IC_{50} of 5 μg/ml (21 μM). At the same concentration the inducible IL-2 expression was reduced to 10% of the untreated control (Fig. 6A,B). Higher concentrations (>10 μg/ml) completely blocked the IL-2 synthesis in Jurkat cells.

4. Discussion

The inducible isoforms of cyclooxygenase (COX-2) and nitric oxide synthase (iNOS) are expressed by many cell types including macrophages and endothelial cells upon stimulation with proinflammatory cytokines and bacterial cell wall components. The expression of COX-2 and iNOS promotes the synthesis and release of large amounts of bioactive lipids such as prostaglandins, leukotrienes and nitric oxide which are mediators involved in the inflammatory processes [24,25]. The inhibition of the diverse signal transduction pathways leading to the expression of COX-2 and iNOS may be of therapeutic use for the treatment of chronic diseases such as rheumatoid arthritis or for the acute situations such as septic shock [26].

We have isolated a novel compound from fermentation of the deuteromycete *Trichosporiella* sp. that inhibits the LPS and IFN-γ mediated inducible expression of the proinflammatory enzymes COX-2 and iNOS in J774 macrophages. The mechanisms by which trichodion inhibits COX-2 and iNOS expression appear to involve the direct interference with several signal transduction pathways responsible for the inducible transcriptional activation of the corresponding promoters. Trichodion blocked the TNF-α induced expression of an NF-κB dependent reporter gene. In addition we have shown that the compound inhibited the LPS/IFN-γ mediated degradation of the IκB-α protein and subsequent activation of the NF-κB transcription factor. Besides NF-κB, the activation of the transcription factor AP-1 by different members of the mitogen activated protein kinases (MAPKs) has been described to function as important regulators of COX-2 and iNOS expression in various cell lines [19,21,27,28]. It has been shown that inhibition of the p38 MAPK by SB203580 and/or the MEK1,2/ERK pathway by PD98059 resulted in a reduction of COX-2 and iNOS expression in LPS or LPS/IFN-γ stimulated macrophages [20,29,30]. The compound trichodion inhibited the expression of an AP-1 dependent reporter gene in COS-7 cells after stimulation with the phorbol ester TPA (which activates the ERK1,2 pathway) and it seems conceivable that trichodion interferes with one or several kinases involved in that pathway or inhibits the activation of a common kinase upstream of IκB. In addition trichodion activated the phosphorylation of p38 as determined by Western blots with a phosphospecific antibody. These results indicate that the compound does not act as a global kinase inhibitor.

The inhibition of the AP-1 dependent signaling pathway may also contribute to the observed immunosuppressive properties of trichodion since it has been shown that the activation of the ERK cascade is required for IL-2 production and transcriptional activation of the distal NF-AT/AP-1 SEAP reporter gene in TPA/ionomycin or anti-CD3/anti-CD28 stimulated Jurkat cells [31,32]. In addition we have shown by Western blots with a phosphospecific antibody that trichodion inhibits the tyrosine phosphorylation of the STAT1α protein in LPS/IFN-γ stimulated J774 cells and suppresses the GAS/ISRE dependent expression of the SEAP reporter gene in IFN-γ stimulated HeLa S3 cells.

Several natural compounds including cyclointeinone, a terpene from the sponge *Cacospongia linteiformis*, the diterpene andalusol, curcumin, various flavonoids, salicylate and the polyphenol oroxylin have been described to inhibit the inducible expression of either COX-2 or iNOS [33–38]. The antiinflammatory properties of these chemically diverse compounds have been mainly attributed to an inhibition of the activation of the transcription factor NF-κB which participates in the regulation of expression of immediate early genes involved in immune, acute phase and inflammatory responses. Furthermore an inhibition of COX-2 gene expression in human mammary epithelial cells after stimulation with TPA has recently been described for resveratrol, a phenolic antioxidant from grapes [39]. The mechanism by which resveratrol modulates gene expression involves the inhibition of PKC translocation to the membrane and the blocking of the induction of the COX-2 promoter mediated by ERK1 and c-jun. Thus resveratrol interferes with the PKC signaling leading to the activation of the transcription factor AP-1.

The novel pyran-dione trichodion is chemically not related to the compounds mentioned above and our findings indicate that the inhibition of COX-2 and iNOS expression in LPS/IFN-γ stimulated macrophages by trichodion is the result of

the interference with different signaling pathways. Trichodion inhibits the NF-κB, AP-1 and STAT1 mediated gene expression but the exact cellular target remains to be determined. Another interesting question is which structural properties of trichodion account for the inhibition of COX-2 and iNOS expression and therefore analogues of trichodion are needed in order to determine the structure–activity relationship.

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