Inhibition of interleukin-6 signaling by galiellalactone

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Abstract A search for inhibitors of the IL-6-mediated signal transduction in HepG2 cells using secreted alkaline phosphatase (SEAP) as reporter gene resulted in the isolation of galiellalactone (1) from fermentations of the ascomycete strain A111-95. Galiellalactone inhibits the IL-6-induced SEAP expression with IC_{50} values of 250–500 nM by blocking the binding of the activated Stat3 dimers to their DNA binding sites without inhibiting the tyrosine and serine phosphorylation of the Stat3 transcription factor. Due to its selective activity, galiellalactone may serve as a lead compound for the development of new therapeutic agents for diseases originating from the inappropriate expression of IL-6 and as molecular tool to dissect the JAK/STAT pathways.

Key words: Galiellalactone; Acute phase response; IL-6 signaling; Inhibitor

1. Introduction

Cytokines are intercellular signaling polypetides produced by activated cells. Inflammation-associated cytokines include interleukin-6 (IL-6), interleukin-1β, tumor necrosis factor (TNF-α), interferon-γ (IFN-γ), and transforming growth factor β [1]. IL-6 is the main inducer of the acute-phase response in liver cells and stimulates the production of most acute phase proteins (APPs) [2–5]. IL-6 initiates its action by binding to its receptor, which is composed of the 80 kDa ligand-binding subunit (IL6-R) and the signal-transducing subunit, gp130 [6]. After ligand binding the gp130 subunits aggregate into a complex consisting of the IL-6R, two gp130 molecules and one IL-6 molecule [7]. This aggregation activates the gp130-associated protein-tyrosine kinases Jak1, Jak2, and Tyk2, which tyrosine phosphorylate gp130, themselves, and signal transducer and activator of transcription (Stat) 3 and 1. Tyrosine phosphorylation of Stat3/1 occurs at a single residue (tyrosine 705, Stat3; tyrosine 701, Stat1) which is located in the conserved Src homology (SH2) domain allowing homodimerization as well as heterodimerization [8]. During translocation to the nucleus Stat3 and Stat1 are specifically phosphorylated on a serine by a yet unknown kinase which is required for full transcriptional activation [9]. In the nucleus the Stat dimers bind to specific consensus sequences (IL-6REII, class II IL-6 responsive elements) and activate the transcription of the target genes including the genes of the APPs [10,11].

In view of the central role of the IL-6-dependent JAK/STAT pathway for the induction of the acute phase response, we developed a cell-based screening system for the search of new inhibitors of the IL-6-dependent signal transduction in HepG2 cells. Screening of 3100 mycelial cultures of basidiomycetes, ascomycetes and deuteromycetes for the production of inhibitors resulted in the isolation of galiellalactone (**1**), a tetrahydro-isobenzofuranone derivative, from the ascomycete strain A111-95. Galiellalactone has been described previously as a weak inhibitor of gibberellic acid-induced de novo synthesis of α-amylase in embryoless halves of wheat seeds [12]. In this paper the biological properties and the mode of action of galiellalactone (**1**) on the IL-6 induced activation of the JAK/STAT pathway in liver cells are reported.

2. Materials and methods

2.1. Producing organism, fermentation and isolation of galiellalactone

The ascomycete strain A111-95 was isolated from wood. 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. For maintenance on agar slants the strain was kept on YMG medium composed of: yeast extract 0.4%, malt extract 1%, glucose 1%, pH 5.5 and agar 1.5% for solid media. Fermentations were carried out in a Braun Biostat A-20 fermenter containing 20 liters of YMG medium with aeration (3 l air/min) and agitation (120 rpm) at 22°C. The production of galiellalactone (**1**) was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluid in the IL-6-dependent reporter gene assays as described below. After 700 h of fermentation, the culture fluid was separated from the mycelium by filtration and extracted with EtOAc. The solvent was evaporated and the crude product (2.3 g) was separated by chromatography on silica gel (Merck 60) with cyclohexane:EtOAc (70:30) as eluent resulting in 1.2 g of an enriched product. Preparative HPLC (Macherey-Nagel Nucleosil 100-7 C-18, column 40×250 mm) with water:MeOH (46:54) as eluent yielded 635 mg galiellalactone (**1**). The structure elucidation was performed by spectroscopic methods.

2.2. Biological assays

HepG2 cells (ATCC HB 8065) and HeLa S3 (ATCC CCL 2.2) were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) und 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. Jurkat cells (ATCC TIB 152) and HL-60 cells (ATCC CCL240) were grown in RPMI 1640 medium with 10% FCS. The assays for antimicrobial activity and cytotoxicity were carried out as described previously [13]. Mutagenicity was tested as described by Venitt et al. [14].

The influence of galiellalactone on the syntheses of macromolecules in HepG2 cells was analyzed as follows. HepG2 cells were seeded in 24 well plates (6×10^5 cells/ml in OPTIMEM containing 10% FCS) and allowed to grow for 16 h. Galiellalactone and 0.1 μCi of

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Abbreviations: APP, acute phase protein; AP-1, activating protein-1; IL-6, interleukin-6; IFN-γ, interferon-γ; NF-κB, nuclear factor κB; STAT, signal transducer and activator of transcription; TNF-α, tumor necrosis factor-α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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[2-^{14}C]thymidine, [2-^{14}C]uridine or [1-^{14}C]leucine, respectively, were added and the cells were incubated for an additional 24 h. The medium was removed and the cells were washed with ice-cold PBS. The cells were lysed by addition of 1 ml TCA (15%) per well, the precipitate was collected by centrifugation at 21 000×*g* for 10 min at 4°C, washed with 1 ml TCA (10%) and the radioactivity in the acid insoluble material was measured with a liquid scintillation counter.

2.3. Reporter gene assays

The reporter plasmid pMW-IRF7 was constructed by cloning eight copies of a class II IL-6 responsive element (IL-6RE II) of the IRF promoter (5′-GCTAGCGATTTCCCCGAAATGGCTAGC-3′ with overlapping *Nhe*I sites) [15] immediately upstream of a thymidine kinase promoter-driven secreted alkaline phosphatase (SEAP) reporter gene [16]. The 1.2 kb human TNF-α promoter was amplified by PCR from genomic DNA extracted from HeLa S3 cells as described recently [17]. The PCR product was cloned into the *Xho*I–*Hin*dIII site of the pGL3-Basic vector (Promega) to generate the TNF-α promoter-driven luciferase reporter plasmid pJR-TNF-pro. The plasmid pCH110 for normalizing transfection efficiency was obtained from Amersham. The reporter plasmids pGE3-NF1 [16], pTK-activating protein-1 (AP-1) [18], pGE3-GAS/ISRE and pGE3-NF-AT [19] have been described recently. Transfection of HepG2 cells was performed by electroporating (Bio-Rad, GenePulser) 1×10^7 cells/ml in 0.4 ml HEBS buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose) together with 80 μg of the pMW-IRF7 plasmid at 500 V/cm. After electroporation the cells were seeded at 2×10^5 cells/ml and allowed to recover for 16 h. For induction of SEAP expression, the cells were treated with 10 ng/ml IL-6 with or without test compounds in OPTIMEM containing 0.5% FCS. The activity of the SEAP in the culture medium was determined 24 h after transfection using the Phospha-Light chemoluminescent assay (Tropix, MA, USA) with a luminometer according to the manufacturer's instructions.

Jurkat cells were electroporated similarly (6×10^7 cells/ml in 0.2 ml 0.5× HEBS buffer, 50 μg of the pJR-TNF-pro vector or pGE3-NF-AT vector). After electroporation the cells were seeded in 96 well plates (6×10^5 cells/ml in OPTIMEM containing 10% FCS) with and without test compounds and reporter gene expression was induced with 32 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 2 μM ionomycin. The reporter gene activity was measured 24 h after transfection as described above. Transfection of HeLa S3 cells with pGE3-NF1, pGE3-GAS/ISRE or pTK-AP-1 and determination of the activity of the expressed SEAP was performed as described previously [20].

2.4. Western blot analysis

HepG2 cells were seeded at 5×10^5 cells/ml in 100 mm diameter dishes and allowed to grow for 24 h. The cells were then washed with OPTIMEM containing 0.5% FCS, fed with 10 ml of OPTIMEM containing 0.5% FCS, incubated for an additional 48 h and pretreated for 60 min with or without different concentrations of galiellalactone. Thereafter the cells were treated with 50 ng/ml IL-6 for 20 min. Total cell extracts were prepared by lysing the cells in 1.5 ml lysis buffer (1% Triton X-100, 10 mM EDTA, 2 mM EGTA, 500 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 1:50 complete protease-inhibitor cocktail (Roche Diagnostics), 50 mM Tris–HCl, pH 7.5). The cellular debris was removed by centrifugation (3000×*g*, 30 min, 4°C) and unspecific binding was blocked by incubation with 90 μl protein-A-Sepharose (50:50 slurry in PBS, Amersham Pharmacia Biotech, UK) for 2 h at 4°C. The suspensions were then centrifuged (3000×*g*, 5 min, 4°C) and the supernatants were incubated with 5 μl anti-Stat3 antibodies (New England Biolabs, MA, USA) and rotated end-over-end overnight at 4°C. 90 μl protein-A-Sepharose (50:50 slurry in PBS, Amersham Pharmacia Biotech, UK) was added to the samples and incubation was continued for 2 h at 4°C. Immunocomplexes were collected by centrifugation, washed twice with buffer A (100 mM NaCl, 2 mM EDTA, 0.2% NP-40, 10 mM Tris–HCl, pH 7.5), once with buffer B (500 mM NaCl, 2 mM EDTA, 0.2% NP-40, 10 mM Tris–HCl, pH 7.5) and with buffer C (10 mM Tris–HCl, pH 7.5) and dissolved in SDS-sample buffer. The samples were analyzed by SDS–polyacrylamide gel (6.5%) electrophoresis, transferred to nitrocellulose (Optitran, Schleicher and Schüll, Germany) and probed with an anti-phospho-Stat3 antibody (New England Biolabs, MA, USA) and then with a goat anti-rabbit antibody conjugated to horseradish peroxidase. The blots were detected by enhanced chemoluminescence (New England Biolabs, MA, USA). Thereafter the blots were stripped and reprobed with an anti-Stat3 antibody (New England Biolabs, MA, USA).

HepG2 cells were starved for 24 h in DMEM with 0.5% FCS, pretreated with galiellalactone for 1 h and induced with 50 ng/ml IL-6. Total cell extracts were prepared as described above and 50 μg of protein were loaded in each lane of the 10% SDS–polyacrylamide gel. Following electrophoretic transfer to nitrocellulose, the membrane was probed with an anti-phospho-p38-MAPK-antibody (New England Biolabs, MA, USA) and then with a goat anti-rabbit antibody conjugated to horseradish peroxidase. The blots were detected by enhanced chemoluminescence (New England Biolabs, MA, USA). Thereafter the blots were stripped and reprobed with an anti-p38-MAPK-antibody (New England Biolabs, MA, USA).

2.5. Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out essentially as described by Cantwell et al. [21]. Nuclear extracts for Stat binding assays were prepared as follows. HepG2 cells were seeded at 5×10^5 cells/ml in 100 mm diameter dishes and allowed to grow for 72 h. The cells were then starved for 24 h in OPTIMEM with 0.5% FCS, pretreated for additional 60 min with different concentrations of galiellalactone and induced with 100 ng/ml IL-6 for 20 min. The medium was removed and the cells were washed twice with ice-cold PBS and once with ice-cold PBS containing 1 mM Na_3VO_4 and 5 mM NaF. Cells were then washed with hypotonic buffer (20 mM HEPES pH 7.9, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1:50 complete protease-inhibitor cocktail (Roche Diagnostics), and 150 μl of hypotonic buffer containing 0.2% Nonidet P-40 was added. Lysates were scraped into microcentrifuge tubes, and the nuclei were pelleted by centrifugation at 16 000×*g* for 20 s at 4°C. The supernatant was removed, and the pellet was resuspended in 30 μl of high-salt buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 30% glycerol, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1:50 complete protease-inhibitor cocktail and gently rocked at 4°C for 30 min. Nuclear debris was removed by centrifugation at 16 000×*g* for 20 min at 4°C. The extracts were aliquoted and stored at −70°C.

Binding reactions for EMSA experiments were as follows. 10 μg of nuclear protein was mixed with 4 μl of 5×binding buffer (65 mM HEPES pH 7.9, 0.75 mM EDTA, 40% glycerol, 0.1% Nonidet P-40), 1 μg of poly(dI-dC) (Boehringer Mannheim), 1 mM DTT and approximately 50 000 cpm (∼0.3 ng) of ^{32}P-labeled Stat binding probe in a total volume of 20 μl. The reaction mixtures were incubated at room temperature for 20 min and 10 μl were separated on a 4% polyacrylamide gel, transferred to 3MM paper (Whatman), dried under vacuum, and exposed to X-ray film. For competition binding assays, unlabeled oligonucleotides were added to the reaction in 100× molar excess and incubated at room temperature for 5 min prior to addition of the probe. For supershift experiments, phospho-Stat3(Tyr-705) or Stat3 antibodies (1–3 μg) were added to the reaction mixtures, which were then incubated at 4°C for 1 h prior to addition of the labeled probe.

The oligonucleotides used as EMSA probes were annealed and gel-purified prior to labeling. The sequences of the upper strands of the oligonucleotides used were as follows: IRF: 5′-CTAGCGATTTCCCCGAAATGGCTAG-3′ [15]; m67SIE: 5′-GATCCATTTCCCGTAAATC-3′ [15,22]; m67SIEmut: 5′-GATCCGGGAGGGATTTACGGGGAAATGCTG-3′ [23]. Double-stranded oligonucleotides were labeled with polynucleotide kinase and [γ-^{32}P]ATP.

3. Results

Galiellalactone (**1**) has been detected during fermentation and isolation using SEAP reporter plasmids containing multiple IL-6RE II in their promoter (pMW-IRF7). Transfection of HepG2 cells and stimulation with 10 ng/ml IL-6 resulted in a five-fold activation over the basal level of SEAP expression. Galiellalactone (**1**) inhibited the IL-6 induced SEAP expression in a dose-dependent manner with IC_{50} values of 250–500 nM (50–100 ng/ml; Table 1). Higher concentrations (>2 μg/ml) inhibited the IL-6-induced SEAP expression below the basal level. Besides galiellalactone (**1**), smaller

Table 1
Effect of galiellalactone on SEAP or luciferase reporter gene expression in HepG2, HeLa S3 and Jurkat cells

Reporter gene assay; cell line	Stimulus	Galiellalactone IC_{50} (μM)
8×IL-6RE II-SEAP; HepG2	IL-6 (10 ng/ml)	0.25–0.5
5×GAS/ISRE-SEAP; HeLa S3	IFN-γ (10 ng/ml)	10–15
3×AP-1-SEAP; HeLa S3	TPA (50 ng/ml)	10–15
5×NF-κB-SEAP; HeLa S3	TNF-α (10 ng/ml)	10–15
5×NF-AT/AP-1-SEAP; Jurkat	TPA (32 nM)+ionomycin (2 μM)	10–15
hTNF-α-pro-Luc; Jurkat	TPA (32 nM)+ionomycin (2 μM)	1.3–2.5

The cells lines were transiently transfected with the indicated reporter gene constructs and the expression of the reporter genes was induced as described in Section 2.

amounts of the biosynthetic precursors pregaliellalactone (**2**) and deoxygaliellalactone (**3**) were isolated from fermentations of the same fungus [24] (for structures see Fig. 1). Pregaliellalactone (**2**) inhibited the IL-6 induced SEAP expression in transiently transfected HepG2 cells with IC_{50} values of 5–10 μg/ml (Fig. 2), whereas deoxygaliellalactone (**3**) showed only a weak inhibition of the IL-6-induced reporter gene expression at comparatively high concentrations (10–50 μg/ml).

In order to test the specificity, we determined the effect of galiellalactone on the GAS/ISRE, AP-1, nuclear factor κB (NF-κB), NF-AT and human TNF-α promoter-driven expression of the reporter genes SEAP or luciferase in HeLa S3 and Jurkat cells.

The IFN-γ-induced expression of the GAS/ISRE-driven reporter plasmid (pGE3-GAS/ISRE) in transiently transfected HeLa S3 cells was inhibited by galiellalactone far less efficient than the IL-6-dependent SEAP expression (IC_{50}: 10–15 μM, Table 1). In addition galiellalactone inhibited the AP-1 and NF-κB-mediated SEAP expression in transiently transfected HeLa S3 cells with IC_{50} values of 10–15 μM (2–3 μg/ml, Table 1). These effects were comparable to the inhibition of the IFN-γ-induced reporter gene expression. The same result was obtained for the TPA and ionomycin induced NF-AT-mediated SEAP expression in Jurkat T-cells (IC_{50}: 10–15 μM).

TNF-α is an important mediator of inflammation. The regulation of TNF-α gene expression in human cells is complex with differences across species and cell types [25–27]. The transcriptional activation of the human TNF-α promoter is dependent on binding sites for NF-κB, NF-AT and AP-1 families of transcription factors [28,29]. We therefore investigated the influence of galiellalactone on the expression of a human TNF-α transcriptional reporter in Jurkat cells. Galiellalactone inhibited the TPA/ionomycin-stimulated expression of the hTNF-α promoter-mediated luciferase expression in Jurkat cells with IC_{50} values of 1.3–2.5 μM (0.25–0.5 μg/ml, Table 1).

Cellular DNA-, RNA-, and protein syntheses were examined in HepG2 cells by determining the incorporation of [2-^{14}C]thymidine, [2-^{14}C]uridine and [1-^{14}C]leucine in TCA-insoluble fractions. Up to a concentration of 25 μM (5 μg/ml) galiellalactone, only weak inhibitory effects on all three macromolecular syntheses could be observed during 24 h incubation suggesting that galiellalactone does not interfere with replication, transcription and translation in a general manner (data not shown).

Galiellalactone exhibited up to 128 μg/ml only moderate antibacterial, antifungal or cytotoxic activities. In the test for mutagenicity no induction of revertants of *S. typhimurium* TA 98 and TA 100 could be observed at 32 μg/ml of any of the isolated compounds (pour plate assay with and without addition of rat liver microsomes) [14].

IL-6 induces the activation of Stat1 and Stat3 transcription factors by phosphorylation of tyrosine 701 and serine 727 on Stat1 and tyrosine 705 and serine 727 on Stat3 [8,9]. To elucidate the mechanism responsible for the inhibition of the IL-6-induced expression of the reporter gene, we examined the effect of galiellalactone on the phosphorylation of these residues upon induction of HepG2 cells with IL-6 by Western blot analysis using anti-Stat3 antibodies specific for the phosphorylation on tyrosine 705 or serine 727 and an anti-Stat1 antibody specific for the phosphorylation on tyrosine 701.

As shown in Fig. 3A–C treatment of HepG2 cells with 50 ng/ml IL-6 resulted in a strong induction of tyrosine phosphorylation of the Stat3 and Stat1 proteins as well as serine phosphorylation of Stat3. Pretreatment of HepG2 cells with galiellalactone and stimulation with 50 ng/ml IL-6 showed no inhibition of the tyrosine and serine phosphorylation of both transcription factors up to a concentration of 16 μg/ml (80 μM). These data suggest that galiellalactone does not act as an inhibitor of one or several of the Janus-kinases (Jak1, Jak2 and Tyk2) which phosphorylate the Stat3/Stat1 transcription factors [6,30].

An involvement of p38 mitogen-activated protein kinase (MAPK) in the IL-6-induced phosphorylation on serine 727

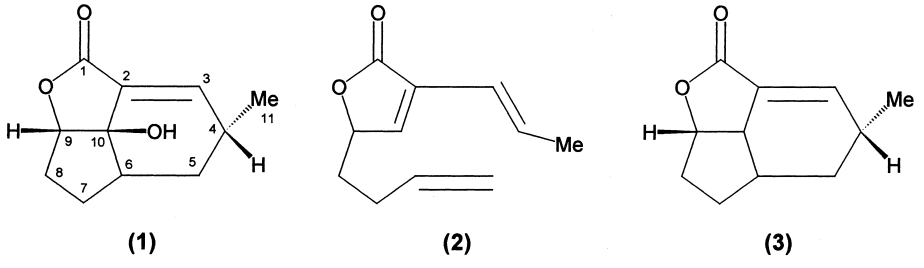

Fig. 1. Structure of galiellalactone (**1**) and its biosynthetic precursors deoxygaliellalactone (**2**) and pregaliellalactone (**3**).

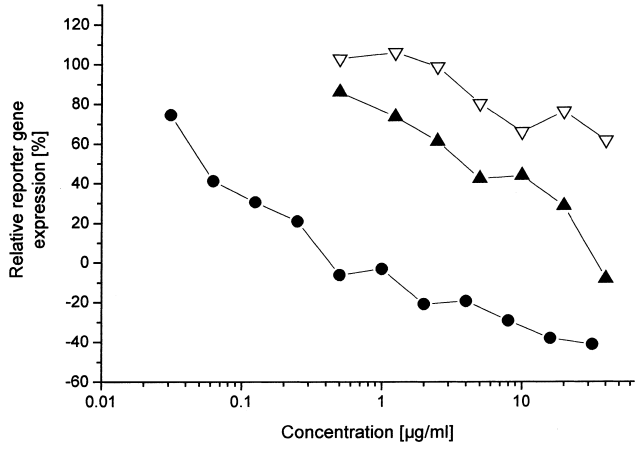

Fig. 2. Effect of galiellalactone (●), deoxygaliellalactone (▽) and pregaliellalactone (▲) on IL-6-dependent SEAP reporter gene expression in HepG2 cells. HepG2 cells were transfected with a 8×IL-6 RE II-dependent SEAP reporter gene construct and stimulated with 10 ng/ml IL-6 for 24 h with or without test compounds as described in Section 2. Control (100%): stimulation only.

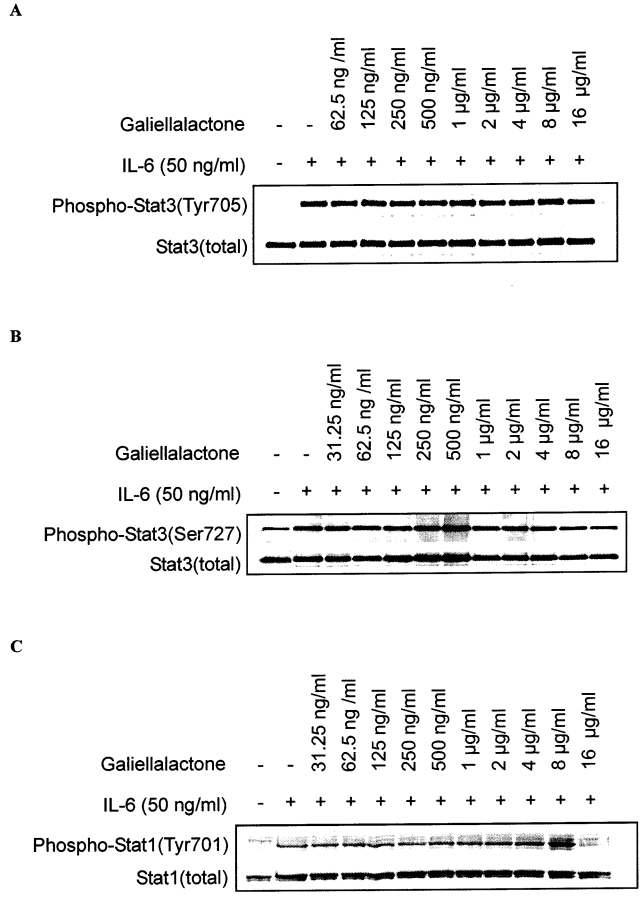

Fig. 3. Effect of galiellalactone on Stat3 and Stat1 phosphorylation. HepG2 cells were pretreated for 1 h with or without galiellalactone and stimulated with 50 ng/ml IL-6 for 30 min. Subsequently total cell extracts were prepared and in case of (A) and (B) immunoprecipitated with an anti-Stat3 antibody prior to Western blot analysis (experimental details are described in Section 2). A: Phosphorylation of Stat3 on tyrosine 705. B: Phosphorylation of Stat3 on serine 727. C: Phosphorylation of Stat1 on tyrosine 701.

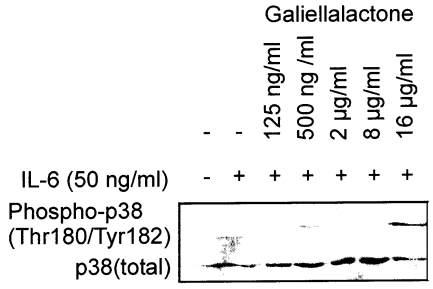

Fig. 4. Effect of IL-6 and galiellalactone on p38 phosphorylation. HepG2 cells were pretreated for 1 h with or without galiellalactone and stimulated with 50 ng/ml IL-6 for 30 min. Subsequently total cell extracts were prepared and analyzed by Western blotting.

of Stat3 in hepatocytes was recently proposed [31]. In order to determine whether galiellalactone affects the activation of the p38 MAPK, Western blot analysis was performed with an antibody specific for the phosphorylated form of p38 (threonine 180/tyrosine 182). The results are shown in Fig. 4. In contrast to the results obtained by Zauberman et al. [31] no induction of p38 phosphorylation was observed in our experiments after treatment of HepG2 cells with 50 ng/ml IL-6. However, cotreatment of the cells with 16 µg/ml galiellalactone markedly induced the phosphorylation of p38 (Fig. 4). These results are in correspondence to Schuringa et al. [32] who showed that p38 MAPK is not directly involved in Stat3 serine 727 phosphorylation, and that blocking the p38 kinase activity by the synthetic inhibitor SB203580 significantly increased the IL-6-induced Stat3 transactivation by increasing the level of Stat3 serine 727 phosphorylation. Therefore, p38 rather act as a negative regulator of IL-6-induced Stat3 transactivation by a yet unknown mechanism.

Since galiellalactone did not inhibit the phosphorylation of the Stat 3 transcription factor we further characterized the mechanism involved in the inhibition of IL-6 signaling by analyzing the influence of galiellalactone on the binding of activated (phosphorylated) Stat3 dimers to the high affinity m67SIE sequence [15,22]. EMSA's were performed with nuclear extracts from HepG2 cells pretreated with various concentrations of galiellalactone and stimulated with 100 ng/ml

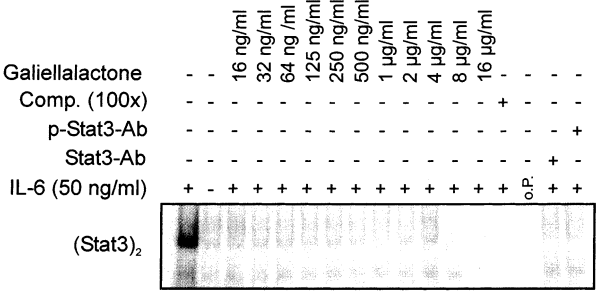

Fig. 5. Effect of galiellalactone on DNA binding of activated Stat3 dimers. HepG2 cells were pretreated with or without galiellalactone for 60 min and stimulated with 100 ng/ml IL-6 for 20 min. Subsequently nuclear extracts were prepared and tested for DNA binding activity by EMSA with a m67SIE probe. Comp. (100×): 100-fold molar excess of unlabeled probe; o.P.: sample without nuclear extract; p-Stat3-Ab: 1 µg of phospho-Stat3(Tyr-705) antibody was added to the reaction mixture; Stat3-Ab: 1 µg of Stat2 antibody was added to the reaction mixture.

IL-6 for 20 min. As shown in Fig. 5 no detectable level of Stat3 DNA binding was observed in nuclear extracts of unstimulated cells. Stimulation of HepG2 cells with IL-6 resulted in a strong increase in STAT3 DNA binding. To show that the retarded band in IL-6 treated cells was indeed Stat3, we incubated the nuclear extracts either with Stat3 or phospho-Stat3(Tyr-705) antibodies. Both antibodies completely abrogated the shifted band suggesting that the IL-6-activated $(Stat3)_2$•m67SIE complex contains Stat3 dimers. In addition the IL-6-inducible DNA complex disappeared in the presence of a 100-fold excess of the unlabeled probe (Fig. 5), and the mutant m67SIE probe (m67SIEmut) failed to bind the Stat3 proteins (data not shown). These results indicate the composition and the specificity of the Stat3 binding to the DNA. Nuclear extracts of galiellalactone treated cells showed that even at the lowest tested galiellalactone concentration (16 ng/ml) the binding of the activated Stat3 dimers was completely inhibited (Fig. 5). These results demonstrate that galiellalactone inhibited the IL-6 signaling by preventing of the DNA binding of the Stat3 proteins. Similar results were obtained when the IRF EMSA probe was used (data not shown).

4. Discussion

IL-6 is a multifunctional cytokine which is produced by a large variety of cells and functions as a regulator of immune response, acute-phase reactions and hematopoiesis [30]. Deregulated expression of IL-6 contributes to a variety of pathophysiological processes like autoimmune diseases, plasmacytoma-myeloma, inflammation and coronary heart disease [33,34]. In the hepatoma cell line HepG2 IL-6 can induce the expression of a variety of APSs, such as fibrinogen, α_1-antichymotrypsin, α_1-acidglycoprotein and haptoglobulin by activating the gp130/JAK/STAT pathway. In addition to these proteins it induces serum amyloid A, C-reactive protein (CRP) and α_1-antitrypsin in human primary hepatocytes. Small-molecule inhibitors that interfere with the IL-6 signaling cascade leading to the expression of disease-related genes would be expected to serve as novel therapeutic approaches [35,36].

We identified galiellalactone as an inhibitor of the IL-6-dependent JAK/STAT signaling cascade in a screening of fungal extracts with a cell-based reporter assay. The compound inhibited the expression of an IL-6-responsive element-driven reporter gene with IC_{50} values of 250–500 nM. The mechanism by which galiellalactone blocks the IL-6-induced JAK/STAT pathway seems to involve the direct inhibition of the binding of Stat3 dimers to their regulatory elements as shown by EMSA (Fig. 5). Furthermore we have demonstrated that galiellalactone did not have any effect on activation of the Stat3 protein since the IL-6-induced tyrosine 701 and serine 727 phosphorylation was not affected.

The distinct but related IFN-γ signaling pathway results in tyrosine 701 phosphorylation of Stat1 and a homodimeric complex of Stat1 binds to the IFN-γ activation sequence in the promoter of responsive genes [37]. The expression of an IFN-γ-induced GAS/ISRE-driven reporter gene was inhibited by galiellalactone at 20–30 times higher concentrations (10–15 μM) than the IL-6-induced reporter gene expression. As in the case for Stat3, the tyrosine 701 phosphorylation of the Stat1 protein was not affected. At higher concentrations, galiellalactone interfered with other analyzed signal transduction pathways. It only moderately blocked the induced expression of NF-κB-, AP-1- and NF-AT-dependent reporter genes (IC_{50}: 10–15 μM), whereas the expression of a human TNF-α promoter-driven reporter gene, which depends on the binding sites for NF-κB, AP-1 and NF-AT transcription factors was influenced at lower concentrations (IC_{50}: 1.3–2.5 μM).

Galiellalactone lost completely its biological activity after 24 h of incubation with a 10× molar excess of L-cysteine in PBS. The crystal structures of tyrosine-phosphorylated Stat1 and Stat3 bound to DNA have been described recently [38,39]. The Stat3 protein has a cysteine residue (Cys-468) in its DNA-binding domain which is missing in Stat1. It seems conceivable that the formation of a covalent bond between the sulfur atom of Cys-468 in Stat3 and the carbon at position 3 of galiellalactone inhibits the binding of Stat3 to the DNA. Moreover, the hydroxyl group of galiellalactone might be important for binding of galiellalactone to the DNA binding pocket of Stat3 since the biosynthetic precursor deoxygaliellalactone showed a strongly reduced inhibitory activity on the IL-6-dependent gene expression. The inhibition of the NF-κB-, AP-1- and NF-AT-dependent reporter genes might be due to reactions of galiellalactone with other cellular target proteins.

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