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S14-95, a Novel Inhibitor of the JAK/STAT Pathway from a *Penicillium* species

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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, a novel compound, designated as S14-95 was isolated from fermentations of the imperfect fungus *Penicillium* sp. 14-95. The compound inhibits the IFN-γ mediated expression of the reporter gene with IC_{50} values of 2.5~5 μg/ml (5.4~10.8 μM). Furthermore the compound inhibited the expression of the proinflammatory enzymes COX-2 and NOS II at 5 μg/ml (10.8 μM) in LPS/IFN-γ stimulated J774 mouse macrophages. Studies on the mode of action of the compound revealed that the inhibition of the IFN-γ dependent signaling pathway is caused by an inhibition of the phosphorylation of the STAT1α transcription factor. In addition, S14-95 inhibited the activation of the p38 MAP kinase, which is involved in the inducible expression of many proinflammatory genes.

The inducible expression of proinflammatory enzymes like cyclooxygenase 2 (COX-2), NO-synthase II (NOS II) and cytokines like interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) is regulated by the activation of multiple signal transduction pathways which are induced by a variety of extracellular stimuli like bacterial lipopolysaccharide (LPS), tumor promoters, cytokines (*e.g.* interferon-γ) and growth factors. The endpoints of these different signaling pathways are the activation of transcription factors like nuclear factor-kappaB (NF-κB), nuclear factor of activated T-cells (NF-AT), activator protein-1 (AP-1) or signal transducers and activators of transcription (STATs), which bind to the corresponding binding sites in the promoter of proinflammatory genes and induce transcription[1]. Regulation of transcription of most proinflammatory genes is believed to be the most important step in their inducible expression and therefore compounds, which 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[2]. Besides the transcription factor NF-κB, which is an immediate-early transcriptional activator, components of the JAK/STAT pathway play an important role in the activation of many inflammatory genes. Exposure of cells to interferon-γ (IFN-γ) triggers the phosphorylation of latent cytoplasmatic transcription factors termed STATs. 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). 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)[3,4]. 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[5].

In order to search for fungal metabolites inhibiting the IFN-γ dependent signaling pathway, reporter gene vectors were constructed which express the reporter gene SEAP under the control of five copies of the GAS/ISRE

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responsive element. A screening of 500 mycelial cultures of basidiomycetes, ascomycetes and imperfect fungi resulted in the isolation of S14-95 from a *Penicillium* species. In this paper the structure elucidation and some biological properties of S14-95 are described.

Materials and Methods

General

Spectral data were recorded with the following instruments: ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference. The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. COSY, HMQC and HMBC experiments were recorded with gradient enhancements using sine shaped gradient pulses. For the 2D heteronuclear correlation spectroscopy the refocusing delays were optimised for $^{1}J_{CH}=145$ Hz and $^{n}J_{CH}=10$ Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker XWIN-NMR software (rev. 010101). EI mass spectra (70 eV) were recorded with a Jeol SX102 spectrometer, while the UV and the IR spectra were recorded with a Perkin Elmer λ 16 and a Bruker IFS 48 spectrometer. The optical rotation was measured with a Perkin-Elmer 141 polarimeter at 22°C.

Producing Organism

The deuteromycete strain 14-95 was isolated from a soil sample collected in Germany. The specimen showed the characteristics of the genus *Penicillium* as described by DOMSCH *et al.*[6]. 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 (Paul-Ehrlich-Str. 23, D-67663 Kaiserslautern, Germany).

Fermentation and Isolation of S14-95

For maintenance on agar slants the strain was kept on YMG medium (yeast extract 0.4%, malt extract 1%, glucose 1%, pH 5.5 and agar 1.5% for solid media). For submerged cultivation, the strain was grown in malt extract medium (40 g/liter malt extract). Well-grown seed cultures of the producing strain (200 ml YMG medium) were used to inoculate a Biolafitte C-6 fermenter containing 20 liters of malt extract medium with aeration (3 liters air/minute) and agitation (120 rpm) at 22°C. The production of the compounds was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluids or mycelia in the GAS/ISRE dependent reporter gene assay as described below. The compound S14-95 was isolated from the mycelium of the *Penicillium* strain 14-95 by bioactivity-guided fractionation. After seven days the mycelium was separated by filtration and extracted with 2 liters of acetone. The organic phase was evaporated and the remaining aqueous residue was extracted with ethyl acetate, dried with $NaSO_4$ and concentrated *in vacuo* (40°C). The crude mycelial extract (4.2 g) was separated by subsequent chromatography on silica gel (Merck 60, eluent: cyclohexane : EtOAc, 50 : 50 v/v). The enriched product (660 mg) was further purified by preparative HPLC (Merck LiChrosorb Diol; column 250×25 mm, flow 5 ml/minute; eluent: cyclohexane : *tert.*-butylmethylether (60 : 40 v/v) to yield 75.7 mg pure S14-95. Detection at 210 nm and 238 nm. The Retention time (Rt) of the fractions containing S14-95 was 150 minutes.

Biological Assays

HepG2 (ATCC HB 8065) 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), Jurkat (ATCC TIB 152) and U937 (ATCC CRL 1593) 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[7].

The reporter plasmid pGE3-GAS/ISRE was constructed essentially as described earlier by cloning five copies of a GAS/ISRE consensus oligonucleotide immediately upstream of the thymidine kinase promoter driven SEAP reporter gene[8]. The reporter plasmids pGE3-NF1 and pGE2-AP1 have been described recently. 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[8]. The IL-6 inducible SEAP reporter gene vector pMW-IRF7, which contains eight copies of an IL-6 responsive element cloned upstream of the thymidine kinase promoter has been described recently[9]. The 1.2 kb human TNF-α and 1.1 kb human COX-2 promoter driven reporter plasmids have been previously reported[10,11]. The plasmid pRL-CMV for normalizing transfection efficiency was obtained from PROMEGA (Dual-Luciferase-Reporter-Assay).

Transfections of HeLa S3 cells were performed by

electroporating 3×10^6 cells suspended in 1 ml phosphate buffered saline (PBS) containing 30 μg of the reporter constructs at 500 V/cm and τ=20~23 ms using a gene pulser apparatus (BioRad). After electroporation the cells were seeded at 1×10^5 cells/ml Opti-MEM (GIBCO, BRL) containing 10% FCS in a 24 well tissue culture plate and allowed to recover for 16 hours. For induction of SEAP expression, cells were treated with the indicated inducers with or without test compounds in Opti-MEM containing 0.5% FCS. The activity of the SEAP in the culture medium was determined 60 hours after transfection using the Phospha-Light chemiluminiscent reporter gene assay (TROPIX, MA) with a luminometer according to the manufacturer's instructions. The transfection of HepG2 cells was performed by electroporation as described recently[12].

J774 cells were starved for 16 hours 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 and complete protease inhibitor cocktail 1 : 50 (Roche Diagnostics, Germany). Cell extracts (50~100 μg protein) were subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with either an anti-COX 2 antibody, anti-NOS II-antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-phosphotyrosine-STAT1 α-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 chemoluminiscent detection system (ECL system, Amersham International, UK).

S14-95

S14-95 (**1**, IUPAC name: (3*S**,4a*R**,6a*R**,12a*R**,12b*S**)-4,4,6a,12b-tetramethyl-11-oxo-9-phenyl-1,3,4,4a,5,6,6a,12,12a,12b-decahydro-2*H*,11*H*-benzo[f]pyrano[4,3-*b*]chromen-3-yl acetate) was obtained as a colourless oil. $[\alpha]_D^{22}$ +94° (*c* 0.9 in $CHCl_3$). UV λ_{max}^{MeOH} 238 nm (log ε 4.28) and 322 nm (log ε 4.12). IR (KBr): 3440, 2945, 1715, 1645, 1580, 1405, 1245, 1125, 1030, 1005, 985, 765 and 690 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$, atom numbering according to Figure 1) δ 7.77 (2H, m, 7'-H), 7.42 (3H, m, 8'-H and 9'-H), 6.36 (1H, s, 4'-H), 4.50 (1H, dd, $J_{2\alpha\text{-}3}$=4.7, $J_{2\beta\text{-}3}$=11.7, 3-H), 2.51 (1H, dd, $J_{9\text{-}11\alpha}$=4.7, $J_{11\alpha\text{-}11\beta}$=17.0, 11-H$\alpha$), 2.23 (1H, dd, $J_{9\text{-}11\beta}$=12.9, $J_{11\alpha\text{-}11\beta}$=17.0, 11-H$\alpha$), 2.13 (1H, ddd, $J_{6\alpha\text{-}7\beta}$=3.2, $J_{6\beta\text{-}7\beta}$=3.1, $J_{7\alpha\text{-}7\beta}$=12.6, 7-H$\beta$), 2.05 (3H, s, 3-OAc), 1.81 (1H, m, 1-Hβ), 1.79 (1H, m, 6-Hα), 1.76 (1H, m, 2-Hβ), 1.70 (1H, m, 7-Hα), 1.66 (1H, m, 2-Hα), 1.51 (1H, dd, $J_{9\text{-}11\alpha}$=4.7, $J_{9\text{-}11\beta}$=12.9, 9-H), 1.44 (1H, dddd, $J_{5\text{-}6\beta}$=12.3, $J_{6\alpha\text{-}6\beta}$=13.6, $J_{6\beta\text{-}7\alpha}$=3.1, $J_{6\beta\text{-}7\beta}$=13.6. 6-Hβ), 1.26 (3H, s, 12-H_3), 1.18 (1H, ddd, $J_{1\alpha\text{-}1\beta}$=13.4, $J_{1\alpha\text{-}2\alpha}$=3.8, $J_{1\alpha\text{-}2\beta}$=13.0, 1-H$\alpha$), 1.08 (1H, dd, $J_{5\text{-}6\alpha}$=2.1, $J_{5\text{-}6\beta}$=12.3, 5-H), 0.93 (3H, s, 15-H_3), 0.91 (3H, s, 13-H_3), 0.89 (3H, s, 14-H_3). ^{13}C NMR (125 MHz, $CDCl_3$, atom numbering according to Figure 1) δ 170.8 (C=O, 3-OAc), 164.4 (C-1'), 163.2 (C-3'), 158.2 (C-5'), 131.5 (C-6'), 130.4 (C-9'), 128.8 (C-8', double intensity), 125.4 (C-7', double intensity), 99.4 (C-2'), 98.3 (C-4'), 80.5 (C-8), 80.2 (C-3), 55.1 (C-5), 51.5 (C-9), 40.3 (C-7), 37.7 (C-4), 37.2 (C-1), 36.8 (C-10), 28.1 (C-13), 23.5 (C-2), 21.2 (CH3, 3-OAc), 20.7 (C-12), 19.3 (C-6), 17.3 (C-11), 16.6 (C-14), 15.1 (C-15). MS *m/z* 450.2406 (100 %, M^+, $C_{28}H_{34}O_5$ requires 450.2406), 375 (6%), 369 (8%), 317 (6%), 267 (8%), 254 (13%), 201 (55%), 189 (17%), 162 (11%), 151 (9%), 105 (18%).

Results and Discussion

S14-95 (**1**) was isolated from the crude mycelial extract of *Penicillium* spec., strain 14-95 by bioactivity-guided fractionation as described in the experimental section. The high resolution MS data suggested that the composition of **1** is $C_{28}H_{34}O_5$, and this was further supported by the appearance of 26 signals in the ^{13}C NMR spectrum together with the apparent presence of a phenyl group in the molecule (indicated by the three ^1H NMR signals with characteristic chemical shifts integrating for five protons attached to three carbons with characteristic chemical shifts of which the signals of two have double intensity). The structure of **1** (for structure and atom numbering, see Figure 1) was determined by COSY and HMBC 2D NMR experiments, while the relative configuration suggested in Figure 1 was determined by NOESY experiments (the absolute configuration was not determined). The phenyl group is attached to the C-4'/C-5' double bond, as shown by the HMBC correlations between 7'-H and C-5', and between 4'-H and C-5' as well as C-6'. 4'-H also correlates with C-2' and C-3', suggesting that the C-4'/C-5' double bond is conjugated with a C-2'/C-3' double bond, and HMBC correlations from 11-H_2 to C-1', C-2' and C-3' indicate that the diene is followed by a carbonyl group in the form of an ester or a lactone. By closing this part of the molecule as a pyranone ring substituted with a methylene group in position 2, an oxygen in position 3, and a phenyl group in position 5, the ^{13}C NMR shifts of the unsaturated carbons become reasonable. 11-H_2 also give HMBC

Fig. 1. Structure of S14-95.

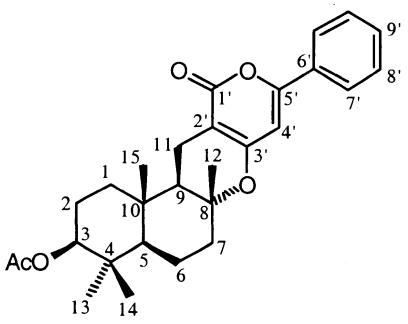
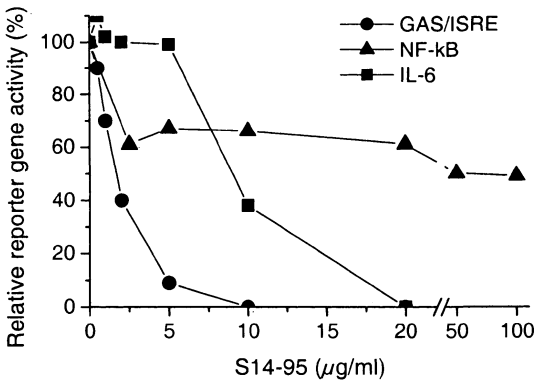


Fig. 2. Effect of S14-95 on IFN-γ, IL-6 and TNF-α induced SEAP reporter gene expression in HeLa S3 and HepG2 cells.



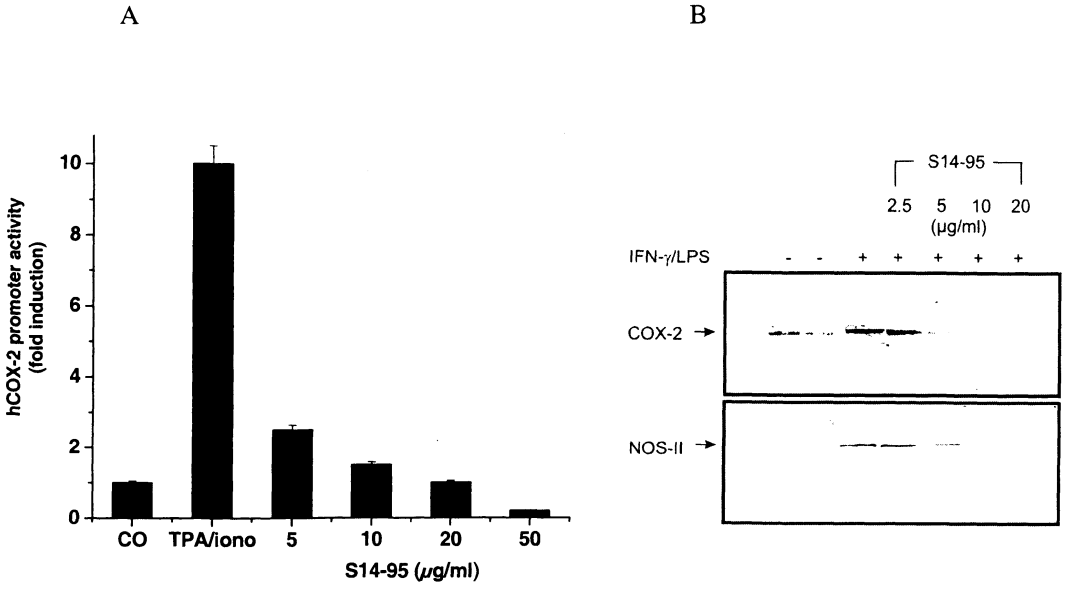
Hela S3 cells were transfected with a GAS/ISRE (5×GAS/ISRE-SEAP) or a NF-κB (5×NF-κB-SEAP) dependent SEAP reporter gene and stimulated with 10 ng/ml IFN-γ (GAS/ISRE) or 5 ng/ml TNF-α (NF-κB) for 48 hours with or without S14-95. HepG2 cells were transfected with an IL-6 RE dependent SEAP reporter gene (8×IL-6RE-SEAP) and stimulated with 10 ng/ml IL-6 for 48 hours with or without S14-95. Control (100%): stimulation only. The expression of the reporter gene SEAP was determined as described in Material and Methods.

correlations to C-8, C-9 and C-10, and COSY correlations to 9-H, and the HMBC correlations from 12-H_3 (to C-7, C-8 and C-9) and 15-H_3 (to C-1, C-5, C-9 and C-10) show that C-9 is positioned between C-8 and C-10 which both have a methyl group attached to them. The two geminal methyl groups are positioned on C-4, with C-3 (substituted with an acetoxy group) and C-5 (already shown to be close to C-15) on each side as judged by the HMBC correlations between 13-H_3 as well as 14-H_3 and C-3, C-4 and C-5. The final links in the decalin system, between C-1 and C-3 *via* C-2, and between C-5 and C-7 *via* C-6, were demonstrated by numerous COSY and HMBC correlations. As C-8 as well as C-3′ are oxygenated, and all oxygen atoms have been accounted for, the only possibility is that the two carbons are joined by an ether link. From the correlations observed in the NOESY spectrum, it is obvious that C-12, C-14 and C-15 all are on the same side of the molecule and that they are axial, as are 2-Hβ and 6-Hβ. On the other side, 1-Hα, 3-H, 5-H and 7-Hα are axial, as demonstrated by the NOESY correlations between 5-H and all three other protons. The relative configuration of **1** is therefore the same as in most terpenoids (the absolute configuration was not determined) containing the carbon skeleton of the decaline system, and the possibility that **1** originates from a drimane sesquiterpene and a modified polyketide is reflected in the numbering of the carbon skeleton of **1** shown in Figure 1.

Biological Properties

S14-95 (**1**) has been detected during fermentation and isolation using an IFN-γ dependent transcriptional reporter plasmid containing multiple GAS/ISRE sites in the 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 the expression of the reporter gene SEAP compared to the uninduced control. As shown in Figure 2, S14-95 inhibited the IFN-γ induced SEAP expression in HeLa S3 cells in a dose dependent manner with IC_{50}-values of 2.5~5 μg/ml (5.4~10.8 μM). The reporter gene activity was reduced to around the basal level at 10 μg/ml (21.6 μM). In order to test the specificity of the isolated compound we tested the inhibitory activity of S14-95 on the related IL-6 signalling pathway, which results in the activation of a homo- as well heterodimeric complex of STAT1/STAT3 transcription factors by using an IL-6 dependent transcriptional reporter. The compound S14-95 inhibited the IL-6 induced SEAP expression in HepG2 cells with an IC_{50} value of 10 μg/ml (21.6 μM). S14-95 only partially inhibited the TNF-α induced NF-κB dependent transcriptional reporter in HeLa S3 cells with a remaining activity of 50% of the induced control even at the highest concentration tested (216 μM, 100 μg/ml). S14-95 showed no inhibition of a TPA inducible AP-1 transcriptional reporter in HeLa S3 cells. These results indicate a more preferential target within the JAK/STAT pathway.

Fig. 3. Effect of S14-95 on human COX-2 promoter activity and COX-2 and NOS-II expression.



(A) Jurkat cells were transfected with a hCOX-2 promoter dependent luciferase reporter plasmid and stimulated with 10 ng/ml TPA and 2.5 μM ionomycin for 24 hours with or without S14-95. Control (100%): stimulation only. The expression of the luciferase reporter gene was determined as described in Material and Methods.

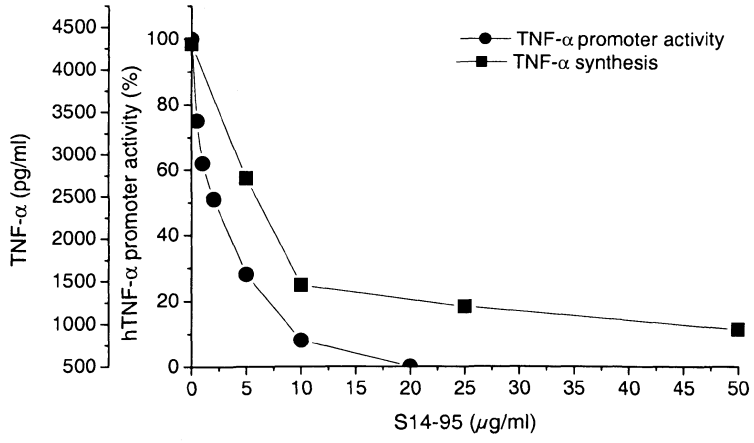
(B) J774 cells were pretreated for 1 hour with or without S14-95 and stimulated with 1 μg/ml LPS and 10 ng/ml IFN-γ for 12 hours. Subsequently total cell extracts were prepared and equal amounts of protein (80 μg) analyzed by western blotting for COX-2 expression with a COX-2 antibody and NOS II expression with a NOS II antibody.

The inhibition of the reporter gene expression did not result from a nonspecific interference with cellular DNA-, RNA-, and protein syntheses or a decrease in cell viability since weak effects on the macromolecular syntheses in Hela S3 cells or cytotoxic effects could only observed at concentrations higher than 50 μg/ml of S14-95. The compound exhibited no antibacterial or antifungal activities.

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[13,14]. We therefore investigated the influence of S14-95 on a human COX-2 transcriptional reporter in Jurkat cells (Figure 3A). S14-95 inhibited the TPA/ionomycin stimulated human COX-2 promoter mediated luciferase expression with an IC_{50}-value of 5 μg/ml (10.8 μM).

In order to determine the effect of S14-95 on COX-2 and iNOS expression, J774 cells were stimulated for 12 hours with LPS and IFN-γ with or without test compound and immunoblotted for COX-2 and iNOS proteins. As shown in Figure 3B, 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 S14-95 caused a dose-dependent decrease in LPS/IFN-γ mediated induction of both enzymes. At 5 μg/ml (10.8 μM) the expression of the iNOS and COX-2 protein is almost completely inhibited. Beside the inducible isoform of the cyclooxygenase COX-2, TNF-α is upregulated in many cell types by pro-inflammatory stimuli and it has been shown that the signaling mechanisms governing COX-2 expression also contribute to the inducible TNF-α expression[15,16]. We therefore investigated the influence of S14-95 on the expression of a human TNF-α transcriptional reporter in Jurkat T-cells. Transfection of Jurkat cells with a hTNF-α promoter driven luciferase reporter gene plasmid and stimulation with 32 nM TPA and 2 μM ionomycin resulted in a 17~20 fold activation over the basal level of luciferase expression. S14-95 inhibited the TPA/ionomycin stimulated expression of the TNF-α promoter mediated luciferase expression in Jurkat cells with IC_{50}-values of 2.5~5 μg/ml (5.4~10.8 μM, Figure 4). The influence of the compound on TNF-α production was investigated in myelomonocytic

Fig. 4. Effect of S14-95 on human TNF-α promoter activity and TNF-α production.



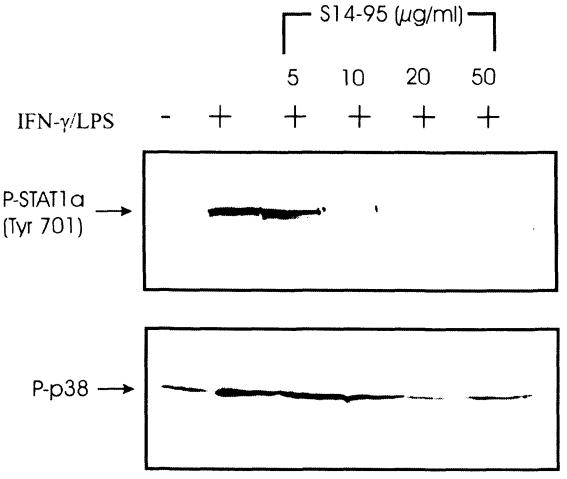
Jurkat cells were transfected with a hTNF-α promoter-dependent luciferase reporter gene plasmid and stimulated with 32 nM TPA and 2 μM ionomycin for 24 hours with or without test compounds. Control (100%): stimulation only. The expression of the reporter gene luciferase was determined as described in Material and Methods.

U937 cells were pretreated for 1 hour with or without S14-95 and stimulated with 50 ng/ml TPA for additional 16 hours. TNF-α concentrations from cell supernatants were determined by ELISA.

U937 leukemia cells, which have been shown to release significant amounts of TNF-α following TPA treatment[17]. Pretreatment of U937 cells with S14-95 and stimulation with 50 ng/ml TPA resulted in a dose dependent inhibition of TNF-α synthesis, which was comparable to the results obtained in the hTNF-α promoter reporter gene assay. As shown in Figure 4, S14-95 blocked the TNF-α synthesis with an IC_{50}-value of 5 μg/ml (10.8 μM).

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 blots were performed with a phospho-STAT1 (Tyr701) antibody. As shown in Figure 5. 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 S14-95 inhibited the phosphorylation in a dose-dependent manner. S14-95 markedly reduced the phosphorylation of the STAT1α protein at 10 μg/ml (21.6 μM). At 20 μg/ml the phosphorylation was reduced to the background comparable to the uninduced control (Figure 5). 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

Fig. 5. Effect of S14-95 on STAT1α and p38 phosphorylation in J774 cells.



J774 cells were pretreated for 1 hour with or without S14-95 and stimulated with 1 μg/ml LPS and 10 ng/ml IFN-γ for 30 minutes. Subsequently total cell extracts were prepared and equal amounts of protein (80 μg) analyzed by western blotting for STAT1α phosphorylation with a phospho-STAT1α antibody and p38 phosphorylation with a phospho-p38 antibody.

(MAPKs) which directly or indirectly activate a number of transcription factors including AP-1 and NF-κB[18~20]. Among the MAPKs, the JNK/SAPK and p38 MAPK pathways share activation by inflammatory cytokines, bacterial endotoxins and enviromental stress[21]. To determine whether S14-95 inhibits the activation of the p38 MAPK western blots were performed with an antibody specific for the phosphorylated form of p38 (Thr180/Tyr182). The results are shown in Figure 5. Treatment of J774 macrophages with LPS and IFN-γ alone increased the p38 phosphorylation. The level of p38 phosphorylation was markedly decreased by cotreatment of the cells with various concentrations of S14-95.

In conclusion we have isolated a novel compound S14-95 from fermentations of a *Penicillium* species, which inhibits the inducible expression of proinflammatory enzymes (COX-2, iNOS) and cytokines (TNF-α) in various cell lines. The mechanism by which S14-95 inhibits the expression of proinflammatory genes seems to be the result of the interference with the signal transduction pathways leading to the activation of the STAT transcription factors and an inhibition of the activation of the p38 MAP kinase. Further investigations of the inhibitory activity of S14-95 on iNOS promoter activity and expression in human cells have been published elsewhere[22].

Acknowledgements

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