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Sporogen, S14-95, and *S*-Curvularin, Three Inhibitors of Human Inducible Nitric-Oxide Synthase Expression Isolated from Fungi

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ABSTRACT

The induction of human inducible nitric-oxide synthase (iNOS) expression depends (among other factors) on activation of the signal transducer and activator of transcription 1 (STAT1) pathway. Therefore, the STAT1 pathway may be an appropriate target for the development of inhibitors of iNOS expression. HeLa S3 cells transiently transfected with a γ-activated site (GAS)/interferon-stimulated response element-driven reporter gene construct were used as the primary screening system. Using this system, three novel inhibitors of interferon-γ-dependent gene expression, namely, sporogen, S14-95, and *S*-curvularin, were isolated from different *Penicillium* species. These three compounds also inhibited cytokine-induced, GAS-dependent reporter gene expression in stably transfected human A549/8-pGASLuc cells, confirming the data obtained with the above-mentioned screening system. Furthermore, in A549/8 cells, sporogen, S14-95, and *S*-curvularin inhibited cytokine-induced activity of the human iNOS promoter [a 16-kilobase (kb) fragment in stably transfected A549/8-pNOS2(16)Luc cells], cytokine-induced iNOS mRNA expression, and cytokine-induced nitric oxide (NO) production in a concentration-dependent manner. The proliferation of A549/8 cells, and the activity of the human eNOS promoter (a 3.5-kb fragment in stably transfected ECV-pNOS III-Hu-3500-Luc cells), were only influenced marginally by the three compounds. Sporogen, S14-95, and *S*-curvularin also inhibited cytokine-induced activation of STAT1α in A549/8 cells. In conclusion, sporogen, S14-95, and *S*-curvularin represent new transcriptionally based inhibitors of iNOS-dependent NO production, acting on the Janus tyrosine kinase-STAT pathway. These compounds may represent lead structures for the development of drugs inhibiting iNOS-dependent overproduction of NO in pathophysiological situations.

Nitric oxide (NO) has been found to play an important role as a regulatory and cytotoxic effector molecule of the immune response (Kleinert et al., 2000; Bogdan, 2001). There are three isoforms of NO synthase (NOS). The endothelial (eNOS) and the neuronal NOS are constitutively expressed enzymes that, upon stimulation, synthesize NO in a pulsatile and Ca^{2+}/calmodulin-regulated manner. In contrast, the inducible NO synthase (iNOS) is normally absent from resting cells (Kleinert et al., 2000). After activation of cells by different inducers (bacterial lipopolysaccharides, cytokines) iNOS is expressed and active for hours to days as a "high-output" enzyme (MacMicking et al., 1997). The large amounts of NO generated by this enzyme can have beneficial effects, such as antimicrobial, antiatherogenic, or antiapoptotic actions (Kleinert et al., 2000). Also, iNOS-generated NO is involved in wound healing of the skin (Stallmeyer et al., 1999) and can protect liver cells against different forms of stress (Kim et al., 1997). In contrast, aberrant iNOS induction may have detrimental consequences and seems to be involved in the pathophysiology of several human diseases such as asthma, arthritis, multiple sclerosis, colitis, psoriasis, neurodegenerative diseases, tumor development, transplant rejection, or septic shock (Kröncke et al., 1998; Kleinert et al., 2000; Bogdan, 2001). Therefore, pharmacological inhibition of iNOS-dependent NO production may be useful in the treatment of these diseases.

The regulation of iNOS expression is cell- and species-specific, with a wide variety of signal transduction pathways

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; NF-κB, nuclear factor-κB; JAK, janus kinase; STAT, signal transducer and activator of transcription; IFN, interferon; kb, kilobase(s); GAS, γ-activated site; BSA, bovine serum albumin; IL, interleukin; TNF, tumor necrosis factor; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; ISRE, interferon-γ–stimulated responsive element; CM, cytokine mixture; nt, nucleotide; SEAP, secreted alkaline phosphatase; OD, optical density; MTS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TBS, Tris-buffered saline.

involved (Kleinert et al., 2000). iNOS induction in human cells seems to be partially dependent on activation of the NF-κB signal pathway (Kleinert et al., 1996; Geller and Billiar, 1998). In addition to NF-κB activation, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway had been shown to be essential for human iNOS expression (Kleinert et al., 1998a,b; Ganster et al., 2001). Exposure of cells to interferon-γ (IFN-γ) results in IFN-γ receptor dimerization and activation of cytoplasmic-located JAKs that tyrosine phosphorylate themselves, the IFN-γ receptor, and latent cytoplasmic-located transcription factors named STATs. After tyrosine-phosphorylation the STATs dimerize, translocate to the nucleus, and activate STAT-dependent gene expression.

Regulation of the transcription of the iNOS gene is believed to be the most important control mechanism for iNOS expression. Transient transfection experiments with human A549/8, AKN, or DLD-1 cells have demonstrated that maximal induction of the human iNOS promoter depends on transcription factor binding sites upstream of position −5 kb (de Vera et al., 1996; Ganster et al., 2001; Kristof et al., 2001; H. Kleinert and U. Forstermann, unpublished data). A549/8 or DLD-1 cells stably transfected with a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene showed basal iNOS promoter activity that was enhanced 5- to 8-fold by cytokines (Hausding et al., 2000; Rodriguez-Pascual et al., 2000). The published sequence of the human iNOS promoter exhibits homologies to numerous binding sites for transcription factors such as AP-1, C/EBP, c-ETS-1, CREB, GATA, IRF-1, NF-1, NFAT, NF-κB, NF-IL6, Oct-1, PEA3, p53, Sp1, SRF, and STAT1 (GenBank AC005697). Functionality has only been proven for a small number of these putative binding sites. Studies using the human iNOS promoter showed important regulation of the iNOS promoter activity by the transcription factors NF-κB, AP-1, and STAT1 (de Vera et al., 1996; Kleinert et al., 1998b, 2000; Taylor et al., 1998; Ganster et al., 2001; Kristof et al., 2001). Mutation or deletion of the NF-κB sites located between positions −5.2 to −6.1 markedly reduced human iNOS promoter activity in transient transfection experiments (Taylor et al., 1998). In a recent publication, Ganster et al. (2001) described the dependence of human iNOS promoter activity on a composite GAS/NF-κB element located at position −5.8 kb in the human iNOS promoter. Therefore, the IFN-γ-JAK-STAT pathway seems to be a good target for inhibition of human iNOS promoter activation and hence iNOS expression in pathophysiological situations. In the current study, we identified three novel compounds that inhibit iNOS induction by acting on this pathway.

Materials and Methods

Reagents. Trypsin, glutamine, and pyruvate solutions; agarose; tRNA; and bovine serum albumin (BSA) were purchased from Sigma Chemie (Deisenhofen, Germany). Isotopes were obtained from ICN Biomedicals (Eschwege, Germany). T3 and T7 RNA polymerase, RNase A, RNase T1, DNase I, and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate were obtained from Roche Diagnostics (Mannheim, Germany). Human IFN-γ, IL-1β, and TNF-α were obtained from Strathmann (Hannover, Germany). Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from PAN-Systems (Nürnberg, Germany). The dual-luciferase reporter assay system, CellTiter 96 AQ_{ueous} one solution cell proliferation assay, passive lysis buffer, and pGL3-Basic were purchased from Promega (Heidelberg, Germany). pCR-Script and pGAS-Luc were from Stratagene (Heidelberg, Germany). Restriction enzymes, Klenow DNA polymerase, dNTPs, NTPs, and the PhosphoPlus Stat1 (Tyr701) antibody kit were from New England Biolabs (Frankfurt a.M., Germany).

Fermentation of Fungi, Isolation of Compounds, and Determination of Structures of Compounds. The deuteromycete strains 14-95, 48-93, and 9-93 were isolated from soil samples collected in Germany. The specimens showed the characteristics of the genus *Penicillium* as described by Domsch et al. (1993). The species, however, could not be identified.

For maintenance on agar slants, the strains were kept on YMG medium. For submerged cultivation, all strains were grown in malt extract medium (40 g/l malt extract). Well grown seed cultures of the producing strains (200 ml of YMG medium) were used to inoculate a Biolafitte C-6 fermenter containing 20 liters of malt extract medium with aeration (3 liters of air/min) 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 the mycelium in the GAS/ISRE-dependent reporter gene assay as described below.

Compound S14-95 was isolated from the mycelium of the *Penicillium* strain 14-95 by bioactivity-guided fractionation using standard chromatographic methods. The structure elucidation of the new fungal secondary metabolite S14-95 was performed by spectroscopic methods and will be published elsewhere. *S*-Curvularin and sporogen were isolated from the culture fluids of *Penicillium* strains 9-93 and 48-93, respectively, by bioactivity-guided fractionation using standard chromatographic methods. The structure elucidation was done by spectroscopic methods in comparison with an authentic sample.

Cell Culture, Cytokine Treatment, RNA Isolation, and Nitrite Measurement. HeLa S3 (American Type Culture Collection, Manassas, VA) cells were maintained in DMEM medium supplemented with 10% FCS and 65 μg/ml penicillin G and 100 μg/ml streptomycin sulfate. The human alveolar epithelial A549/8 cells (Edgell et al., 1983) and human ECV304 cells (Takahashi et al., 1990) were grown in DMEM with 5 to 10% fetal bovine serum, 2 mM L-glutamine, penicillin, and streptomycin. For RNA isolation, they were plated onto 10-cm-diameter (58 cm^2/well) dishes, whereas those experiments involving luciferase activity determinations or NO production studies were performed with cells plated onto six-well plates (9.6 cm^2/well) or 24-well plates (1.75 cm^2/well). Eighteen hours before cytokine induction, cells were washed with phosphate-buffered saline solution and incubated with DMEM containing 2 mM L-glutamine in the absence of serum and phenol red. During this incubation time as well as the following induction period, the cells were treated with or without different concentrations of sporogen, S14-95, or *S*-curvularin. A549/8 cells were induced with a cytokine mixture (CM) composed of IFN-γ (100 U/ml), IL1-β (50 U/ml), and TNF-α (10 ng/ml) for the time periods indicated. Afterward, the supernatant of the cells (300 μl) was used to measure NO_2^- by the Griess reaction, and cells were processed for RNA isolation by guanidinium thiocyanate/phenol/chloroform extraction as described previously (Chomczynski and Sacchi, 1987; Kleinert et al., 1998b).

RNase Protection Analysis. To generate a plasmid containing a cDNA fragment of the human GAPDH mRNA for in vitro transcription, a 105-base pair reverse transcription-polymerase chain reaction fragment of the human GAPDH mRNA (positions 486–590) was cloned into the *Xcm*I site of pXcmI (Borovkov and Rivkin, 1997), generating pXcm_GAPDH_human. DNA sequences of the clones were determined using the dideoxy chain termination method with a sequencing kit from Amersham Biosciences (Freiburg, Germany). For the generation of radiolabeled human iNOS- and human GAPDH-antisense probes for RNase protection assays, 0.5 μg of the linearized plasmids pCR_iNOS_human (Kleinert et al., 1996) or pXcm_GAPDH_human were in vitro transcribed using T3 or T7 RNA

polymerase and [α-^{32}P]UTP. To quantify human iNOS mRNA levels, RNase protection experiments were performed as described previously (Kleinert et al., 1998b). In all experiments, GAPDH mRNA expression was determined for normalization purposes. Densitometric analyses were performed using a PhosphorImager (Bio-Rad, Munich, Germany). The protected fragments of human iNOS- and human GAPDH-mRNA were 386 nt or 195 and 105 nt, respectively.

Transient Transfection of Reporter Gene Constructs and Determination of Secreted Alkaline Phosphatase (SEAP) Activity. The reporter plasmid pGE3-GAS/ISRE was constructed essentially as described previously by cloning five copies of a GAS/ISRE consensus oligonucleotide (5′-AAGTACTTTCAGTTTCATATTACTCTA-3′) immediately upstream of the thymidine kinase promoter-driven SEAP reporter gene (Erkel et al., 1996). 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 of phosphate-buffered saline containing 30 μg of the reporter constructs at 500 V/cm and $\tau = 20$ to 23 ms using a gene pulser apparatus (Bio-Rad). After electroporation, the cells were seeded at 1×10^5 cells/ml Opti-MEM (Invitrogen, Carlsbad, CA) containing 10% FCS in a 24-well tissue culture plate and allowed to recover for 16 h. 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 h after transfection using the Phospha-Light chemiluminescent reporter gene assay (Tropix, Bedford, MA) according to the manufacturer's instructions with a luminometer.

Stable Transfection of A549/8 and ECV304 Cells, Analysis of GAS-Dependent Reporter Gene Expression, and Analysis of Human iNOS Promoter Activity and Human eNOS Promoter Activity. To generate A549/8 cells stably transfected with a luciferase reporter gene under the control of multimerized GAS-responsive elements, cells were transfected using 4.5 μg of pGAS-Luc (Stratagene, La Jolla, CA) and 0.5 μg of pRc-CMV. To generate ECV304 cells stably transfected with a construct containing a 3.5-kb fragment of the human eNOS promoter cloned in front of a luciferase reporter gene, cells were transfected using 5 μg of pNOS III-Hu-3500-Luc-neo (Li et al., 1998). The transfected cells were selected by G418 treatment (1 mg/ml). Pools of cell clones were analyzed for luciferase activity and checked for integration of the transfected DNA by polymerase chain reaction.

To investigate the effect of the different compounds on cytokine-induced GAS-dependent promoter activity or iNOS 16-kb promoter activity, the stably transfected cells [A549/8-GASLuc-cells or A549/8-pNOS2(16)Luc-cells (Hausding et al., 2000), respectively] were incubated for 18 h with DMEM without FCS and without phenol red in the presence or absence of different concentrations of sporogen, S14-95, or *S*-curvularin. After CM incubation for 4.5 h in the presence or absence of these compounds cells were lyzed in 1× passive lysis buffer.

To investigate the effect of the different compounds on the constitutive human eNOS promoter activity, stably transfected cells (ECV-pNOS III-Hu-3500-Luc-neo) were incubated for 24 h with DMEM without FCS and without phenol red in the presence or absence of different concentrations of sporogen, S14-95, or *S*-curvularin. Then the cells were lysed in 1× passive lysis buffer.

Firefly luciferase activity was determined using the dual luciferase assay kit. Protein concentrations of the extracts were determined by Bradford reagent using BSA as the standard. Protein content of the extracts was used for normalization of the luciferase activity.

Measurement of Cell Proliferation Using CellTiter 96 Nonradioactive Cell Proliferation Assay. Cytotoxicity of the compounds was measured using the CellTiter 96 nonradioactive cell proliferation assay (Cory et al., 1991) as described by the manufacturer. In brief, cells were plated at 10^4 cells/well of a 96-well plate. Next, cells were incubated for 18 h in medium without FCS and phenol red. Then cells were incubated for additional 8 h with 100 μl of medium containing 5% FCS and CM in the presence or absence of sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (3–100 μg/ml, 10–340 μM). Twenty microliters of the CellTiter 96 AQ_{ueous} one solution cell proliferation agents were added and the cells further incubated for 1 h. Then the OD at 490 nm was determined using an enzyme-linked immunosor-

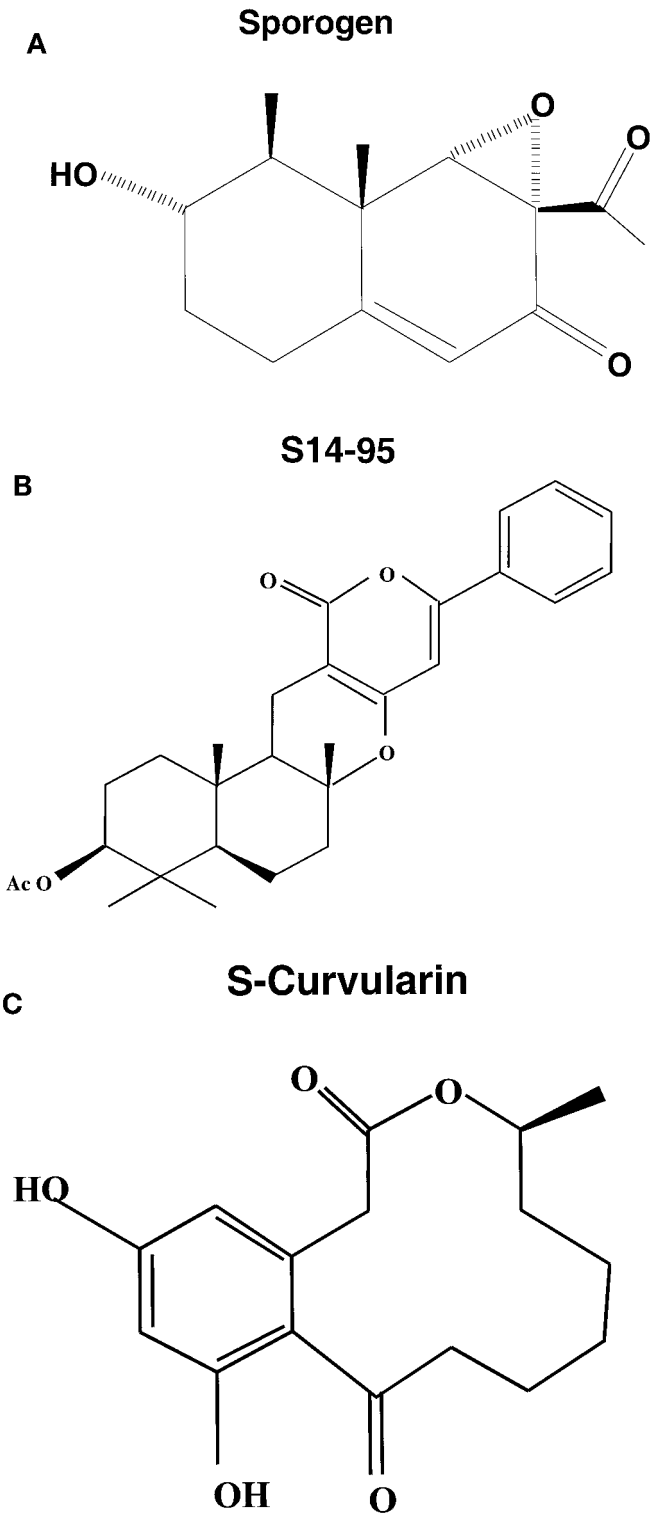

Fig. 1. Structures of sporogen (A), S14-95 (B), and *S*-curvularin (C).

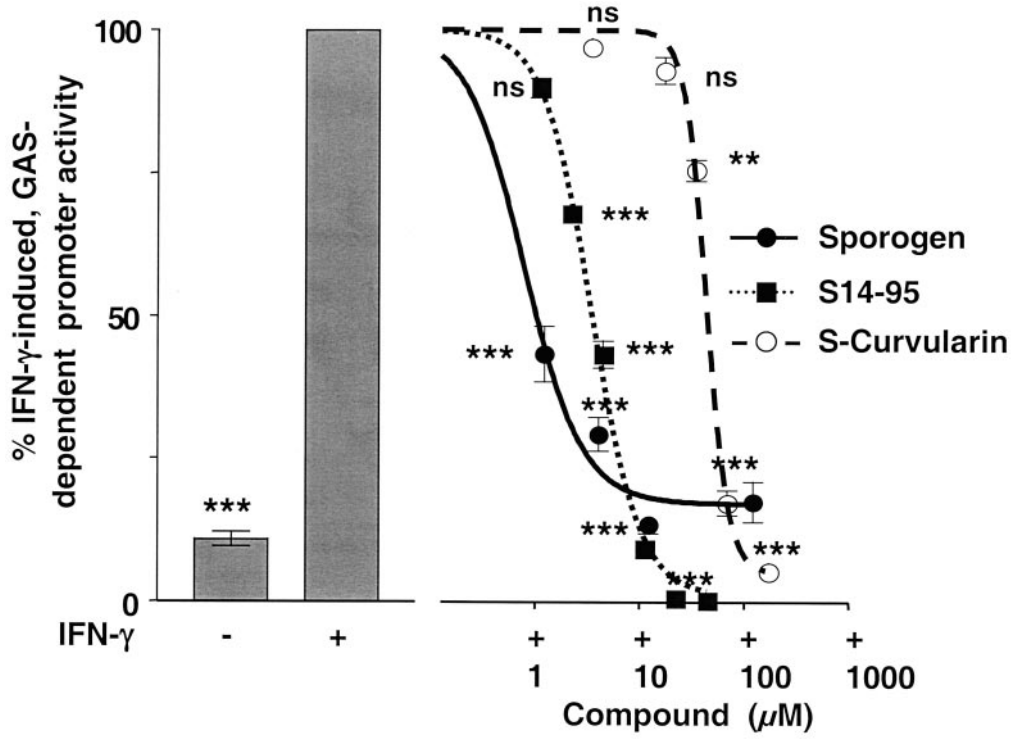

Fig. 2. Effect of sporogen, S14-95, and *S*-curvularin on SEAP reporter gene expression in HeLa S3 cells. HeLa S3 cells transiently transfected with pGE3-GAS/ISRE, containing five copies of a GAS/ISRE consensus oligonucleotide immediately upstream of the thymidine kinase promoter-driven SEAP reporter gene (Erkel et al., 1996), were left untreated or treated with 10 ng/μl IFN-γ for 60 h in the presence or absence of sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM). The activity of SEAP in the culture medium was determined 48 h later. Columns and symbols (means ± S.E.M.; $n = 10$) represent the relative SEAP activities (percentage of the IFN-γ effect) in the supernatant of transiently transfected HeLa cells, incubated with the different compounds (**, $p < 0.01$; ***, $p < 0.001$; ns, not significant versus IFN-γ). Half-maximal inhibition was achieved with 0.2 μg/ml (0.79 μM) sporogen, 1.7 μg/ml (3.5 μM) S14-95, and 13.1 μg/ml (43.8 μM) *S*-curvularin.

bent assay reader. The OD measured at 490 nm represents the amount of tetrazolium dye (MTS)-to-formazan conversion, which represents the number of viable cells.

Western Blot Experiments. Nuclear proteins from untreated A549/8 cells, or A549/8 cells treated for 5 min with CM in the presence or absence of sporogen (30 μg/ml, 120 μM), compound S14-95 (30 μg/ml, 67 μM), or *S*-curvularin (30 μg/ml, 100 μM) were isolated as described previously (Ohlsen et al., 1993).

For the detection of tyrosine (Tyr701)-phosphorylated STAT1 and nonphosphorylated STAT1, the PhosphoPlus STAT1 (Tyr701) antibody kit (New England Biolabs) was used as described by the manufacturer. Briefly, 50 μg of the nuclear protein samples was separated by SDS-polyacrylamide gel electrophoresis (7.5%). The proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by electroblotting (Bio-Rad). Blots were blocked for 30 min with 3% (w/v) lowfat milk powder, 0.05% (v/v) Tween 20, and 10% goat serum in TBS (10 mM Tris-HCl pH 7.4, 150 mM NaCl, w/v) at room temperature. After three washes in TBS containing in 1% BSA and 0.1% (v/v) Tween 20, the blots were incubated with the polyclonal rabbit anti-pTyr^{701}STAT1 antibody

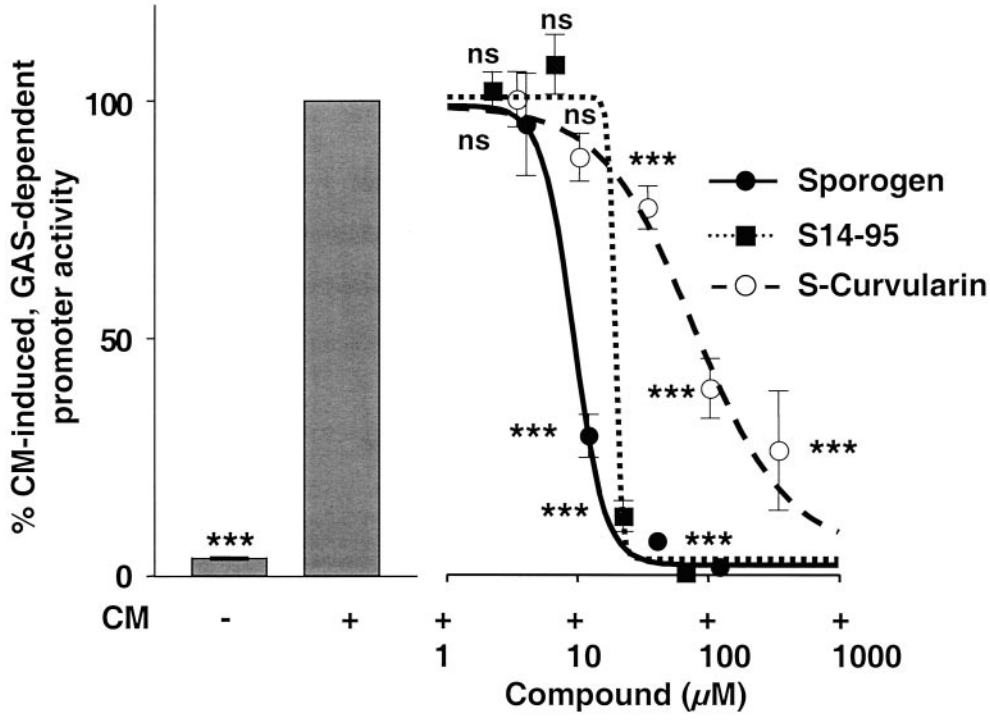

Fig. 3. Effect of sporogen, S14-95, and *S*-curvularin on GAS-dependent cytokine-induced reporter gene expression in stably transfected A549/8-pGASLuc cells. A549/8-pGASLuc cells stably transfected with pGAS-Luc (Stratagene), a construct containing five GAS elements in front of a TATA box driving a luciferase reporter gene, were preincubated in serum-free medium with or without sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM) for 18 h. Then cells were incubated with a CM containing 100 U/ml IFN-γ, 10 ng/ml TNF-α, and 100 U/ml IL-1β for another 4 h in the presence or absence of the respective compounds. Cell extracts were prepared and luciferase activity and protein content determined. Columns and symbols (means ± S.E.M.; $n = 10$) represent the relative luciferase activities (percentage of the CM effect) in extracts of A549/8-pGASLuc cells, incubated with the different compounds (***, $p < 0.001$; ns, not significant versus CM). Half-maximal inhibition was achieved with 2.3 μg/ml (9.4 μM) sporogen, 8.1 μg/ml (19.6 μM) S14-95, and 23 μg/ml (79.9 μM) *S*-curvularin.

diluted 1:700 in the same solution at 4°C overnight. After washing in TBS containing 3% (w/v) lowfat milk powder and 0.1% (v/v) Tween 20, the blots were incubated with the horseradish peroxidase-conjugated second antibody (Sigma Chemie) diluted 1:3000 for 1 h at room temperature. The blots were washed three times with TBS containing 0.1% (v/v) Tween 20 and then the immunoreactive proteins were detected using an enhanced horseradish peroxidase luminol chemiluminescent reagent (PerkinElmer Life Sciences) according to the manufacturer's instruction. After detection of the phosphorylated STAT1 protein, the same blot was stripped (stripping buffer, 62.5 mM Tris-HCl pH 7.4, 2% SDS, and 100 mM 2-mercaptoethanol) and reincubated with a polyclonal rabbit anti-STAT1 antibody 1:700 using the same procedure as described above for the detection of total STAT1 protein.

Calculations. All data are presented as mean ± S.E.M. Differences between means were tested for statistical significance using factorial analysis of variance followed by Fisher's protected least significant difference test as the post hoc test (StatView software; SAS Institute, Cary, NC). Concentrations of compounds producing half-maximal inhibition were determined using Prism 3 software (GraphPad Software Inc., San Diego, CA).

Results

Isolation of Sporogen, S14-95, and *S*-Curvularin from *Penicillium* Species. Screening of approximately 1000 culture fluids and mycelial extracts of basidiomycetes, ascomycetes, and imperfect fungi for compounds inhibiting the inducible expression of the GAS/ISRE-driven SEAP reporter gene resulted in the isolation of the new fungal metabolite S14-95 (mol. wt. 450 g/mol) as well as sporogen (mol. wt. 248 g/mol) and *S*-curvularin (292 g/mol; for structures, see Fig. 1) from fermentations of three *Penicillium* species. Transfection of HeLa S3 cells with an SEAP reporter plasmid containing multiple GAS/ISRE sites in front of a minimal promoter and stimulation with 10 ng/ml IFN-γ resulted in an 8- to 10-fold increase of SEAP expression compared with the uninduced control. Sporogen, S14-95, and *S*-curvularin inhibited the IFN-γ-induced GAS/ISRE-dependent expression of the reporter gene SEAP in a concentration-dependent manner (Fig. 2).

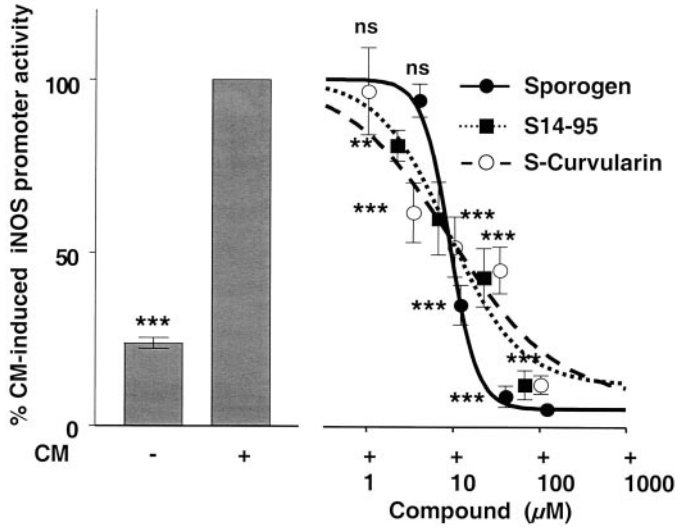

Fig. 4. Effect of sporogen, S14-95, and *S*-curvularin on cytokine-induced human iNOS promoter activity in stably transfected A549/8-pNOSII(16)Luc cells. A549/8-pNOSII(16)Luc cells stably transfected with pNOSII(16)Luc (de Vera et al., 1996), a construct containing a 16-kb fragment of the human iNOS promoter in front of a luciferase reporter gene, were preincubated in serum-free with or without sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM) for 18 h. Then cells were incubated with a CM for another 4 h in the presence or absence of the respective compounds. Cell extracts were prepared and luciferase activity and protein content determined. Columns and symbols (means ± S.E.M.; $n = 18$) represent the relative luciferase activities (percentage of the CM effect) in extracts of A549/8-pNOSII(16)Luc, incubated with the different compounds (***, $p < 0.001$; ns, not significant versus CM). Half-maximal inhibition was achieved with 2.3 μg/ml (9.2 μM) sporogen, 3.9 μg/ml (8.9 μM) S14-95, and 2.5 μg/ml (9.5 μM) *S*-curvularin.

Sporogen, S14-95, and *S*-Curvularin Inhibited GAS-Dependent Reporter Gene Expression in A549/8-GAS-Luc Cells. For further analysis of the isolated compounds on cytokine-induced iNOS expression, we used the human epithelial alveolar cell line A549/8 as the model system. To validate the effects of sporogen, S14-95, and *S*-curvularin on GAS-dependent gene transcription also in this cell line, we stably transfected the plasmid pGAS-Luc (Stratagene) in A549/8 cells. This plasmid contains five STAT1 binding sites (GAS sites) in front of a TATA box driving a luciferase reporter gene. In stable transfected A549/8-pGASLuc cells luciferase activity was induced more than 30-fold by incubation with CM (Fig. 3). Incubation of A549/8-pGASLuc cells with sporogen, S14-95, and *S*-curvularin resulted in a concentration-dependent inhibition of GAS-dependent CM-induced luciferase expression, validating the data described above (Fig. 3).

Sporogen, S14-95, and *S*-Curvularin Inhibited Human iNOS Promoter Activity in A549/8-pNOS(16kb)Luc Cells. To analyze the effect of sporogen, S14-95, or *S*-curvularin on human iNOS promoter activity, we incubated A549/8 cells stably transfected with pNOSII(16)Luc (de Vera et al., 1996; Hausding et al., 2000) with different concentrations of sporogen, S14-95, or *S*-curvularin. The plasmid pNOSII(16)Luc contains a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene. CM incubation of the stably transfected A549/8-pGAS-Luc cells (Fig. 4) resulted in a 5-fold enhancement of the luciferase expression as described previously (Hausding et al., 2000). Sporogen, S14-95, or *S*-curvularin inhibited the CM-induced iNOS promoter activity in A549/8-pNOSII(16)Luc cells in a concentration-dependent manner (Fig. 4).

Sporogen, S14-95, and *S*-Curvularin Inhibited Human mRNA Expression and Nitrite Production in A549/8 Cells. Sporogen, S14-95, or *S*-curvularin inhibited human iNOS mRNA expression induced by CM treatment of A549/8 cells for 8 h in a concentration-dependent manner (Fig. 5). The three compounds also inhibited CM-induced nitrite production (Fig. 6).

Sporogen, S14-95, and *S*-Curvularin Did Not Change Cell Proliferation of A549/8 Cells. To determine whether the inhibition of iNOS expression was a result of a toxic effect on A549/8 proliferation, the CellTiter 96 AQ_{ueous} one solution cell proliferation assay, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (Cory et al., 1991), was used. Incubation of A549/8 cells with sporogen, compound S14-95, or *S*-curvularin did not reduce the tetrazolium dye-to-formazan conversion compared with untreated cells (Fig. 7).

Sporogen and S14-95 Only Slightly Inhibited Human 3.5-kb eNOS Promoter Activity in ECV-3.5kb-eNOS Cells. *S*-Curvularin Had No Effect on eNOS Promoter Activity. To determine whether the inhibition of iNOS expression was a result of the inhibition of general transcription factors, the effect of sporogen, compound S14-95, or *S*-curvularin on a constitutive active promoter was analyzed. For this purpose hu-

man ECV304 cells (Kobayashi et al., 1991) were stably transfected with the plasmid pNOS III-Hu-3500-Luc-neo (Li et al., 1998) to generate the cell line ECV-pNOS III-Hu-3500-Luc-neo. This plasmid contains a 3.5-kb fragment of the human eNOS promoter cloned in front of a luciferase reporter gene. This promoter displayed a significant constitutive activity in the stably transfected cells (Fig. 8). Incubation of ECV-pNOS III-Hu-3500-Luc-neo cells with the highest concentrations of sporogen, compound S14-95, or *S*-curvularin resulted in only a moderate reduction in eNOS promoter activity (control cells 100%; sporogen: 30 μg/ml, 120 μM, 67.4 $\pm$ 9.1%, $n = 12$; S14-95: 30 μg/ml, 67 μM, 64.8 $\pm$ 7.3%, $n = 12$; *S*-curvularin: 100 μg/ml, 340 μM, 104.0 $\pm$ 6.7%, $n = 12$).

Sporogen, S14-95, and *S*-Curvularin Inhibit the Activation of STAT1 in A549/8 Cells. To study the effect of sporogen, compound S14-95, or *S*-curvularin on STAT1 acti-

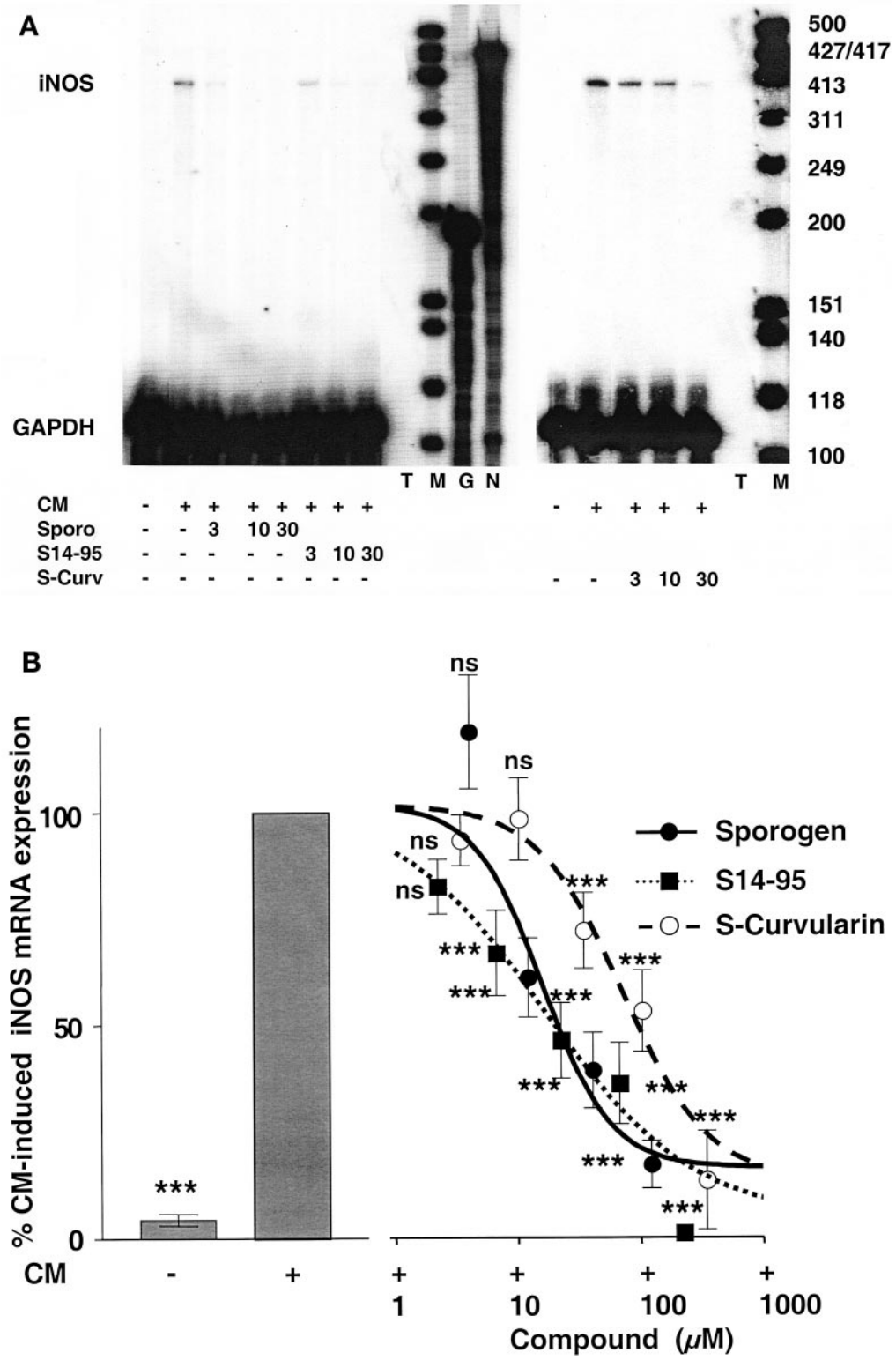

Fig. 5. Effect of sporogen, S14-95, and *S*-curvularin on cytokine-induced human iNOS mRNA expression in epithelial A549/8 cells. A, representative RNase protection assay performed with total RNA from human A549/8 cells incubated for 8 h in serum-free medium alone or in serum-free medium containing a CM in the presence or absence of sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–100 μg/ml, 2–222 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM). Cells had been preincubated for 18 h in serum-free medium with or without the compounds listed above. Experiments were performed using antisense RNA probes for human iNOS and GAPDH (for normalization). The positions of the protected iNOS and GAPDH fragments are indicated. (T, tRNA lane, negative control; M, molecular weight standard, ΦX174-restricted with *Hin*fI; N, iNOS antisense probe; G, GAPDH antisense probe). B, densitometric analyses of six different gels similar to the one shown in A. Columns and symbols (means $\pm$ S.E.M.) represent relative iNOS mRNA levels at the different concentrations of sporogen, compound S14-95, or *S*-curvularin (**, $p < 0.01$; ***, $p < 0.001$; ns, not significant versus CM). Half-maximal inhibition was achieved with 5.1 μg/ml (16.2 μM) sporogen, 7.4 μg/ml (16.7 μM) S14-95, and 25.7 μg/ml (72.3 μM) *S*-curvularin.

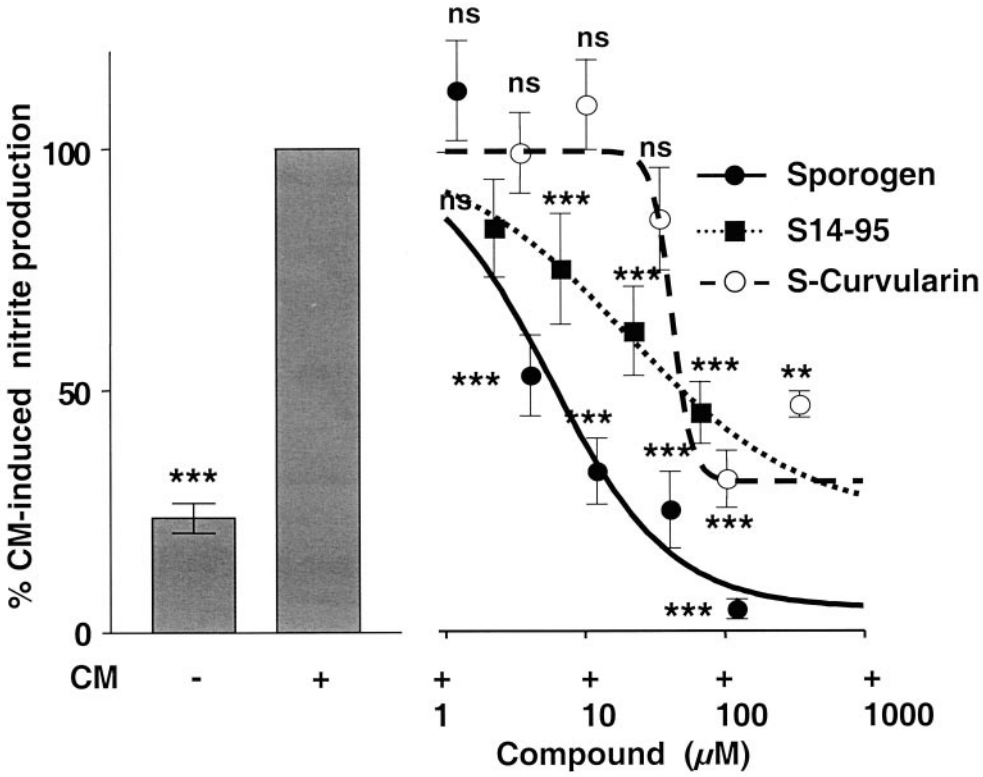

Fig. 6. Effect of sporogen, S14-95, and *S*-curvularin on cytokine-induced nitrite production in human epithelial A549/8 cells. Statistical analysis of nine different Griess assays performed with supernatants form untreated A549 cells or cells stimulated for 24 h with a CM in the presence or absence of sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM). Cells had been preincubated for 18 h in serum-free medium with or without these compounds. Columns and symbols (means ± S.E.M.) represent the relative nitrite levels determined under the conditions indicated (100% corresponds to 400 pmol/ml/24 h; **, $p < 0.01$; ***, $p < 0.001$ versus CM). Half-maximal inhibition was achieved with 1.4 μg/ml (5.8 μM) sporogen, 9.1 μg/ml (20.5 μM) S14-95, and 14.1 μg/ml (45.6 μM) *S*-curvularin. In a cell line expressing iNOS constitutively, none of the compounds produced any inhibition of nitrite production ($n = 6$; data not shown), thereby excluding a nonspecific effect of the compounds on the Griess assay.

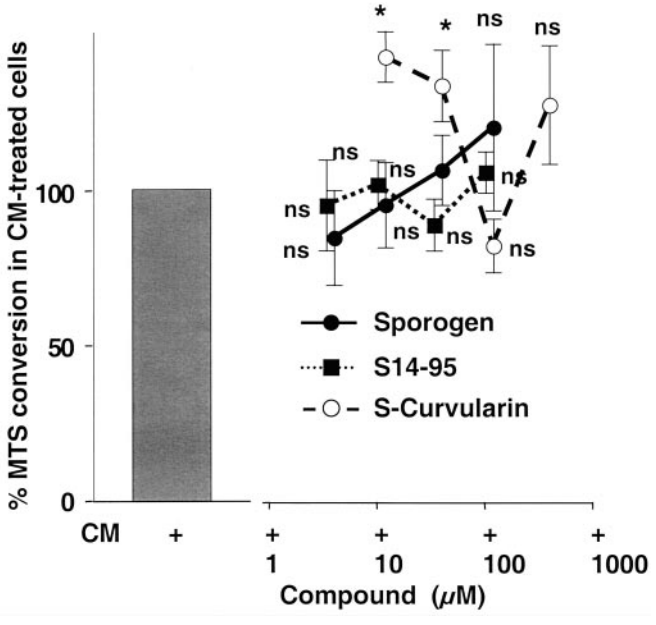

Fig. 7. Effect of sporogen, S14-95, and *S*-curvularin on cell proliferation of human A549/8 cells. A549/8 cells were incubated for 18 h in medium without FCS and phenol red. Then cells were incubated for additional 8 h with 100 μl of medium containing 5% FCS and the CM in the presence or absence of sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (3–100 μg/ml, 10–340 μM). Twenty microliters of the CellTiter 96 AQ_{ueous} one solution cell proliferation agens were added and the cells were incubated for 1 h. The optical density measured at 490 nm represents the amount of MTS-to-formazan conversion, which represents the number of viable cells. Columns and symbols (means ± S.E.M.; $n = 15$) represent relative MTS conversion rates (percentage of only CM-treated cells) at different concentrations of the compounds listed above (*, $p < 0.05$; ns, not significant versus CM).

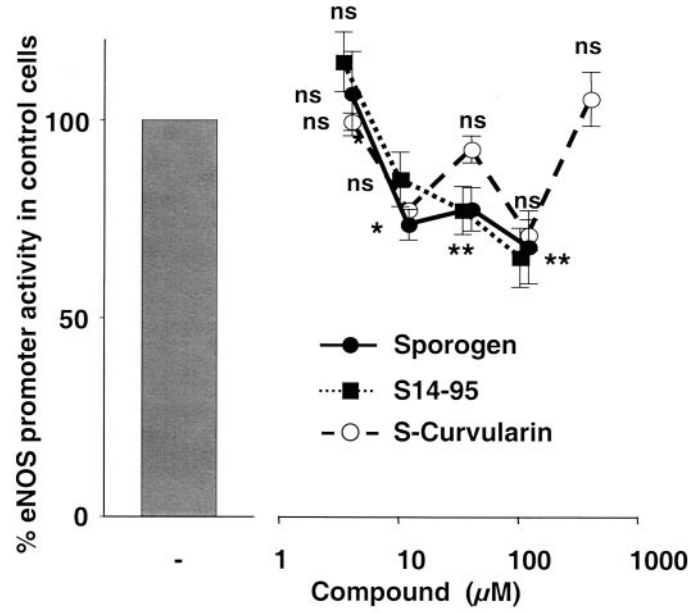

Fig. 8. Effect of sporogen, S14-95, and *S*-curvularin on constitutive human eNOS promoter activity in stably transfected ECV-pNOS III-Hu-3500-Luc-neo cells. ECV-pNOS III-Hu-3500-Luc-neo cells stably transfected with pNOS III-Hu-3500-Luc-neo, a construct containing a 3.5-kb human eNOS promoter fragment in front of a luciferase reporter gene (Li et al., 1998), were preincubated with serum-free medium for 18 h. Then cells were incubated with or without sporogen (1–30 μg/ml, 4–120 μM), compound S14-95 (1–30 μg/ml, 2–67 μM), or *S*-curvularin (1–100 μg/ml, 3–340 μM) for additional 6 h in serum-free medium. Cell extracts were prepared and luciferase activity, and protein content of the extracts was determined. Columns and symbols (means ± S.E.M.; $n = 15$) represent relative luciferase activities (percentage of control cells) in the presence of the different concentrations of the compounds (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant versus control).

vation, Western blots using an antibody that detects the tyrosine (Tyr701) phosphorylation of STAT1 (corresponds to STAT1 activation; Darnell et al., 1994; Ihle et al., 1994; Ihle, 1995) were performed. Incubation of A549/8 for 5 min with CM resulted in a marked enhancement of STAT1 tyrosine phosphorylation (Fig. 9). Coincubation of CM-treated A549/8 cells with sporogen (120 μM), S14-95 (67 μM), or *S*-curvularin (100 μM) significantly reduced this STAT1 tyrosine phosphorylation (Fig. 9).

Discussion

An aberrant expression of iNOS leading to inappropriate NO production has been implicated in human autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, asthma, colitis, psoriasis, lupus erythematosus, neurodegenerative diseases (like Alzheimer's disease or Parkinson's disease), as well as transplant rejection, tumor development, and septic shock (for review, see Kröncke et al., 1998). One possible way to inhibit excessive NO production by iNOS is specific enzyme inhibition. Today, 1400W identified by Garvey et al. (1997) and GW273629 or GW274150 identified by Young et al. (2000) seem to be promising compounds displaying a relatively high selectivity for iNOS (Garvey et al., 1997; Young et al., 2000; Alderton et al., 2001).

An alternative approach to block iNOS-dependent NO production is the suppression of iNOS induction. In contrast to eNOS and neuronal NOS, which are constitutively expressed, expression of iNOS has to be induced (Kleinert et al., 2000). The transcription factors involved in the expression of the three NOS isoforms differ markedly (Kleinert et al., 2000). Therefore, it seems likely that specific inhibitors of iNOS expression can be generated. In recent years, several studies described the dependence of iNOS induction on the IFN-γ-JAK-STAT pathway (Shiraishi et al., 1997; Kleinert et al., 1998b, 2000; Dell'Albani et al., 2001; Ganster et al., 2001; Ohmori and Hamilton, 2001). Therefore, we sought to isolate new compounds that target this pathway, thereby blocking cytokine-induced iNOS expression in human cells.

More than 30% of the drugs sold worldwide are based on natural products. Nature has been proven to be an outstanding source for new and innovative drugs. Secondary metabolites from plants, animals, fungi, and microorganisms display an enormous structural diversity and provide new lead structures for drug development (Grabley et al., 2000). Several natural compounds, including curcumin and various polyphenols, have been described as inhibitors of iNOS expression. The anti-inflammatory properties of these chemically diverse compounds have been mainly attributed to an inhibition of the activation of transcription factor NF-κB, which participates in the regulation of expression of immediate early genes involved in immune, acute phase, and inflammatory responses (Surh et al., 2001).

As a primary screening system, we used a cell-based reporter gene assay to monitor 1000 mycelial fungal extracts for their inhibitory activity of an artificial GAS/ISRE-dependent promoter reporter gene construct transiently transfected in human cells (Fig. 2). Extracts with inhibitory activity were further screened for the ability to inhibit the cytokine-induced activity of a 16-kb iNOS promoter stably transfected human epithelial A549/8 cells. This screening resulted in the isolation of three different compounds, namely, sporogen, compound S14-95, and *S*-curvularin (for structures, see Fig. 1). Although sporogen and *S*-curvularin have been isolated from cultures of *Aspergillus oryzae, Hansfordia pulvinata* as well as *Alternaria, Curvularia*, and *Penicillium* species (Hyeon et al., 1976; Tanka et al., 1984), they have not been reported as inhibitors of the cytokine-induced iNOS expression. All three compounds were able to reduce the cytokine-induced GAS-dependent gene expression in a concentration-dependent manner in stably transfected A549/8-pGASLuc cells (Fig. 3), confirming the transient transfection assay used initially. Sporogen, compound S14-95, and *S*-curvularin inhibited human iNOS promoter activity (Fig. 4),

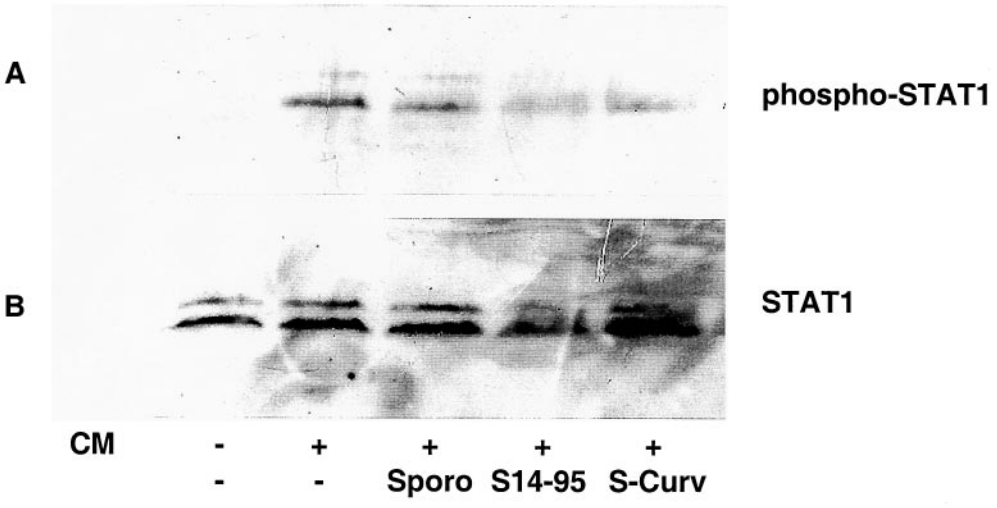

Fig. 9. Effect of sporogen, S14-95, and *S*-curvularin on cytokine-induced activation of STAT1α in human A549/8 cells. A549/8 cells were preincubated for 18 h with DMEM medium (without FCS and phenol red) in the presence or absence of sporogen (Sporo; 30 μg/ml, 120 μM), compound S14-95 (30 μg/ml, 67 μM), or *S*-curvularin (*S*-Curv; 30 μg/ml, 100 μM). Then cells were incubated for 5 min with or without a CM in the presence or absence of the fungal metabolites. Nuclear extracts were prepared and 50 μg of nuclear proteins was analyzed for STAT1 tyrosine-(Tyr701) phosphorylation by Western blotting using the PhosphoPlus STAT1 (Tyr701) antibody kit as described under *Materials and Methods*. The blots shown are representative for four experiments showing the same results. A, Western blot using a STAT1-tyrosine-(Tyr701)-phosphate-specific antibody. B, same blot (after stripping) using a STAT1 antibody, which detects phosphorylated and nonphosphorylated STAT1.

iNOS mRNA expression (Fig. 5), and iNOS-related nitrite production (Fig. 6) in human A549/8 cells. Interestingly, the slopes of the calculated inhibition curves varied between the three inhibitors. This may reflect different targets (e.g., different binding sites, different inhibition mechanisms) involved in the inhibition of the INF-γ-JAK-STAT pathway by these compounds.

As shown in Figs. 7 and 8, this inhibitory effect did not result from toxic effects on A549/8 cell proliferation or inhibition of general transcription factors. Sporogen, compound S14-95, and *S*-curvularin inhibited the cytokine-dependent tyrosine-(Tyr701) phosphorylation of STAT1, but did not change the STAT1 protein content in the cells. Because this tyrosine (Tyr701) phosphorylation is essential for dimerization, nuclear transfer, and hence activation of STAT1, this corroborates the data obtained with the primary screening system. Sporogen was the most potent inhibitor of the three compounds with respect to GAS-dependent promoter activity, human iNOS promoter activity, iNOS mRNA expression, and NO production (Figs. 2–6). However, the effect of sporogen on STAT1 tyrosine phosphorylation (Fig. 9) was less pronounced than the effects of compound S14-95 and *S*-curvularin. Therefore, it seems reasonable to speculate that besides inhibition of STAT1 tyrosine phosphorylation, sporogen may have additional mechanisms of action (e.g., inhibition of STAT1 dimerization or STAT1 transactivation activity).

In summary, we have established a screening system able to identify fungal secondary metabolites, which inhibit IFN-γ-JAK-STAT-dependent activation of the human iNOS promoter. Using this system, we isolated three novel compounds, namely, sporogen, compound S14-95, and *S*-curvularin. These compounds may serve as lead structures for the development of transcriptionally based inhibitors of iNOS-dependent NO production.

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