Oxacyclododecindione, a Novel Inhibitor of IL-4 Signaling from *Exserohilum rostratum*

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Abstract In a screening program for new metabolites from fungi inhibiting the IL-4 mediated signal transduction, a novel chlorinated macrocyclic lactone, designated as oxacyclododecindione, was isolated from fermentations of the imperfect fungus *Exserohilum rostratum*. The structure was determined by a combination of spectroscopic techniques. Oxacyclododecindione inhibits the IL-4 induced expression of the reporter gene secreted alkaline phosphatase (SEAP) in transiently transfected HepG2 cells with IC_{50} values of 20~25 ng/ml (54~67.5 nM). Studies on the mode of action of the compound revealed that the inhibition of the IL-4 dependent signaling pathway is caused by blocking the binding of the activated STAT6 transcription factors to the DNA binding site without inhibiting tyrosine phosphorylation. The compound has no antibacterial or antifungal activity.

Keywords exserohilum, interleukin-4 signaling, inhibitor, new metabolite

Introduction

Asthma is a chronic inflammatory disorder of the airways, characterized by the accumulation of eosinophiles, neutrophils, lymphocytes and macrophages in the lung. Within the airway mucosa, $CD4^+$ Th2-type lymphocytes

(Th2 cells) and other inflammatory leukocytes release a range of inflammatory mediators that contribute to the remodeling of the airway wall, mucus hypersecretion, airway obstruction, and airway hyperreactivity [1, 2]. The Th2 cytokines, primarily IL-4, IL-5 and IL-13, control all the major components that characterize an inflammatory asthmatic response, including IgE isotype switching, mucus production, and the recruitment and activation of eosinophils. An essential biological activity for IL-4 in the development of allergic asthma is to drive the differentiation of native Th0 cells into Th2 cells and the production of IgE by B cells. The signaling pathway activated by IL-4 and IL-13 involves the recruitment of tyrosine kinases of the Janus family (JAK1-3, TYK2) to the receptor subunits, receptor dimerization, and phosphorylation of tyrosine residues in the cytoplasmatic domain of the IL-4R α . In various cell types, STAT6, a latent cytoplasmatic transcription factor of the signal transducer and activator of transcription family (STATs) is recruited to IL-4R α upon IL-4R α phosphorylation, becomes tyrosine phosphorylated, disengages from the IL-4R α , dimerizes by reciprocal SH2 domain interaction, migrates to the nucleus and binds to consensus sequences within promoters of IL-4 and IL-13 regulated genes [3, 4]. Studies using IL-4, IL-13, IL-4R α and STAT6 knockout animals have demonstrated the importance of this signal transduction pathway for the development of allergic

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inflammation. Therefore STAT6 might be an attractive target for the development of new therapeutics against various allergic conditions including asthma [5]. In a screening of fungal extracts inhibiting the IL-4 dependent signaling pathway, we found that *Exserohilum rostratum* produced a new metabolite with potent inhibitory activity on a STAT6 dependent reporter assay in transiently transfected HepG2 cells. In this paper the fermentation, structure elucidation and some biological properties of the compound, which we have named oxacyclododecindione, are described.

Materials and Methods

Producing Organism

Exserohilum rostratum strain IBWF99121 was isolated as a dark brown rhizomorph from a *Geastrum* species. The fungus was identified by PCR amplification and ITS sequence analysis of the ITS1-5.8 S rDNA-ITS2 region of nuclear DNA as described previously [6]. The strain is deposited in the culture collection of the IBWF e. V. (D-67663 Kaiserslautern, Germany). For maintenance on agar slants, the strain was kept on YMG medium (yeast extract 0.4%, malt extract 1.0%, glucose 1.0%, pH 5.5 and agar 1.5% for solid media).

Fermentation and Isolation of Oxacyclododecindione

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 YMG medium with aeration (3.0 liters air/minute) and stirring (120 rpm) at 22°C. The production of oxacyclododecindione was followed by the inhibitory effect of various concentrations of a crude extract of the culture fluid in the STAT6 dependent reporter gene assay as described below. The compound was isolated from the culture fluid by bioactivity-guided fractionation using the described IL-4 transcriptional reporter assay in HepG2 cells.

After 48 hours the culture fluid was separated from the mycelium by filtration. The culture fluid was extracted with EtOAc, dried over Na₂SO₄ and concentrated *in vacuo* (40°C). The crude extract (3.3 g) was fractionated by subsequent chromatography on silica gel (Merck 60, eluent: cyclohexane:EtOAc, 70:30 v/v). The enriched product (310 mg) was further purified by preparative HPLC (Merck LiChrosorb RP18; column 250×25 mm, flow 15 ml/minute; gradients: H₂O - MeCN 30~35% in 30 minutes, 35~40% in 15 minutes, 40~100% in 5 minutes) to yield 12.1 mg pure oxacyclododecindione. Using UV detection at 210 and 233 nm, the Retention time (Rt) of the

fractions containing oxacyclododecindione was 24.5 minutes.

Spectroscopy

¹H-NMR (400 MHz) and ¹³C-NMR (100.6 MHz) were recorded at 25°C with a Bruker Avance-II spectrometer equipped with an inverse multinuclear 5.0 mm probehead and a z-gradient coil. The spectra were measured in CD₃CN and the chemical shifts were referenced to the residual solvent signal ($\delta_{\text{H}}=1.94$ ppm, $\delta_{\text{C}}=1.32$ ppm). Standard pulse sequences for gs(gradient-selected)-COSY, gs-HSQC, gs-HMBC, and gs-NOESY experiments were used. The refocussing delays for the inverse heterocorrelations were set to 3.45 and 62.5 ms, corresponding to $^1J_{\text{C,H}}=145$ Hz and $^nJ_{\text{C,H}}=8$ Hz, respectively. The mixing time for the gs-NOESY was 1 second. Processing of the data was performed with the MestreNova Software (Mestrelab Research). APCI-MS spectra were measured from a solution of the analyte in acetonitrile/water with a Hewlett Packard MSD 1100 using an evaporator temperature of 400°C, a drying gas temperature of 350°C at a flow of 6.0 liters/hour (N₂). In positive ionization mode, the capillary voltage amounted to 3.5 kV, the corona discharge current was 4 μ A. In negative ionization mode, the capillary voltage amounted to 2.2 kV, the corona discharge current was 6 μ A. ESI-HRMS data were measured from a solution of the analyte in acetonitrile with a Waters Q-TOF-Ultima 3 equipped with a LockSpray interface (tri-*n*-octylamine as external reference). IR and UV spectra were measured with a Bruker IFS48 FTIR spectrometer and a Perkin-Elmer Lambda-16 spectrophotometer, respectively. The optical rotation was measured with a Perkin-Elmer 241 polarimeter at 25°C.

Oxacyclododecindione (1)

Oxacyclododecindione (**1**) was obtained as a slightly brown amorphous solid. $[\alpha]_{\text{D}}^{25}=+24.5$ ($c=0.41$, CH₃CN). IR (KBr): $\nu=3428$ (broad), 2927, 1718, 1625, 1438, 1372, 1298, 1258, 1168, 1058 cm⁻¹. UV: λ_{max} (MeOH)=233 nm ($\log \epsilon=4.13$), 290 nm ($\log \epsilon=3.41$). ¹H-NMR, COSY (400 MHz, CD₃CN): $\delta=6.46\sim6.57$ (m, 2H, H-6, H-11), 4.72 (q, $J=6.4$ Hz, 1H, H-15), 3.52 (d, $J=16.8$ Hz, 1H, H_a-2), 3.24 (broad d, $J=16.8$ Hz, 1H, H_b-2), 2.37~2.50 (broad m, 1H, H_a-12), 2.20~2.32 (broad m, 1H, H_b-12), 1.97~2.05 (m, 1H, H_a-13), 1.82 (s, 3H, 10-CH₃), 1.45~1.54 (broad m, 1H, H_b-13), 1.06~1.08 (m, 6H, 14-CH₃, 15-CH₃), contained in this multiplet: 1.08 (s, 3H, 14-CH₃), 1.07 (d, $J=6.4$ Hz, 3H, 15-CH₃) ppm. ¹³C-NMR, HSQC, HMBC (100.6 MHz, CD₃CN): $\delta=199.5$ (C-9), 169.2 (C-1), 154.6 (broad, 2C, C-5, C-7), 154.4 (broad, C-11), 136.5 (broad, C-10), 132.7 (C-3), 122.7 (broad, C-8),

113.6 (broad, C-4), 103.6 (C-6), 77.5 (C-15), 73.1 (C-14), 39.1 (C-2), 37.1 (broad, C-13), 27.4 (14-CH₃), 26.0 (broad, C-12), 14.3 (15-CH₃), 10.4 (broad, 10-CH₃) ppm. APCI-MS (negative): m/z (%) = 369.1 (34) [³⁷Cl-M-H]⁻, 367.1 (100) [³⁵Cl-M-H]⁻. APCI-MS (positive): m/z (%) = 371.1 (29) [³⁷Cl-M+H]⁺, 369.1 (85) [³⁵Cl-M+H]⁺, 353.1 (34) [³⁷Cl-M-OH]⁺, 351.1 (100) [³⁵Cl-M-OH]⁺, 243.1 (14), 241.1 (50). ESI-HRMS: calcd. for C₁₈H₂₁³⁵ClNaO₆ [M+Na]⁺ 391.0924, found 391.0933.

Biological Assays

HepG2 (ATCC HB 8065) cells were maintained in DMEM-medium supplemented with 10% fetal calf serum (FCS), 65 µg/ml penicillin G and 100 µg/ml streptomycin sulfate. The assays for antimicrobial activity, cytotoxicity as well as macromolecular syntheses in HepG2 cells were carried out as described previously [7].

The IL-4 inducible reporter plasmid was constructed by cloning seven copies of a CAAT enhancer binding protein (C/EBP) binding site and a STAT6 binding site (C/EBP-N4: 5'-GAT CCC GCT GTT GCT CAA TCG ACT TCC CAA GAA CAG-3') [8] immediately upstream of a thymidine kinase promoter driven secreted alkaline phosphatase (SEAP) reporter gene [9]. The STAT6 expression plasmid was constructed by inserting the STAT6 cDNA, obtained by RT-PCR of total RNA isolated from THP-1 cells, into the pcDNA3.1/V5-His-TOPO vector (Invitrogen, Karlsruhe, Germany). The plasmid pRL-CMV for normalizing transfection efficiency was obtained from Promega (Dual-Luciferase-Reporter-Assay). Transient transfections of HepG2 cells were accomplished by electroporation as described previously [9]. For induction of SEAP expression, the cells were treated with 10 ng/ml IL-4 with or without test compounds and activity of the SEAP in the culture medium was determined 24 hours after transfection using the Phospha-Light chemoluminescent assay (TROPIX, MA) with a luminometer according to the manufacturer's instructions.

For western-blotting, HepG2 cells were starved for 16 hours in DMEM medium containing 0.5% FCS, treated for 1 hour with test compounds and induced with 50 ng/ml IL-4 for 30 minutes. Total cell extracts were prepared using RIPA detergent buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na₂HPO₄ × 2 H₂O, 1.5 mM KH₂PO₄, pH 7.4, 1.0% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 1.0 µM Na₃VO₄, complete protease inhibitor cocktail 1:50 (Roche Diagnostics, Mannheim, Germany) and cell extracts (50–100 µg protein) were subjected to 10% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with an antibody specific for the phosphorylated form (Y641) of STAT6 (New England Biolabs, Frankfurt, Germany) and

then with the appropriate secondary antibody conjugated to horseradish peroxidase. Immunoreactive proteins were visualized by the enhanced chemoluminescent detection system (ECL system, Amersham International, Freiburg, Germany). After stripping of the corresponding membrane, the blots were reprobed with an anti-STAT6 antibody and developed as described above. For DNA binding assay, HepG2 cells were starved for 16 hours in DMEM medium containing 0.5% FCS, treated for 1 hour with test compounds and induced with 50 ng/ml IL-4 for 30 minutes. Extract preparation and DNA binding conditions for gel mobility shift assays have been described previously [10]. The C/EBP-N4 STAT6 oligonucleotide was used as EMSA probe.

Results and Discussion

Microbiology

Strain IBWF99121 was isolated as a dark brown rhizomorph. However, spore formation could not be induced on various media. Therefore the strain was identified by ITS sequence comparison. The ITS sequence was 100% identical to *Exserohilum rostratum* ITS1-5.8 S rDNA-ITS2 (GenBank accession no AJ853741.1). Oxacyclododecindione (**1**) was isolated from the culture fluid extract by bioactivity-guided fractionation as described in the experimental section.

Structure Elucidation

APCI-MS and ESI-HRMS data indicated a composition of C₁₈H₂₁ClO₆, requiring 8 unsaturations. Taking into account the two carbonyl groups, the olefinic double bond and the benzene ring, the molecule should be bicyclic. As only 18 carbon-bound protons could be found in the HSQC and the ¹H-NMR spectrum, three OH groups were contained. None of these had a sharp resonance. Many of the proton and carbon signals were broadened, an effect frequently observed in medium sized rings. The two diastereotopic methylene protons H_a-2 and H_b-2 exhibited a large geminal coupling constant of 16.8 Hz typical for a CH₂CO group. H_a-2 showed HMBCs with the lactone carbonyl (C-1), C-3,

Fig. 1 Structure of oxacyclododecindione (**1**) including the atomic numbering scheme.

C-4 and C-8. Correlations between H-15, C-1 and C-15 were indicative for the lactone moiety with a methyl substituted carbinol center. The neighboring quaternary center (C-14) carried a hydroxy group and a second methyl group which was almost isochronous to the one bound to C-15. Consequently, strong HMBCs were found between 14-CH₃/15-CH₃ and C-14 as well as C-15. Further contacts

to C-13 and even C-12 in combination with those between H_a-13 and C-14 as well as C-15 established the northeastern part of the molecule. The third methyl group showed correlations to C-10, C-9 and C-11. Unfortunately, the signal of H-11 overlapped with that of H-6 and is so broad that no HMBC crosspeaks could be found downfield from H-15 in f₂. However, clear COSY correlations

Fig. 2 Effect of oxacyclododecindione on IL-4 signaling.

(A) HepG2 cells were transiently transfected with a STAT6 or a CMV-promoter dependent reporter construct and stimulated with 10 ng/ml IL-4 for 24 hours with or without oxacyclododecindione. Control (100%): stimulation only. The expression of the reporter genes was determined as described in Material and Methods. Results represent the mean $\pm$ SEM of at least three independent experiments.

(B) Effect of oxacyclododecindione on STAT6 phosphorylation. HepG2 cells were pretreated for 1 hour in the presence or absence of test compound and stimulated with 50 ng/ml IL-4 for 30 minutes. Subsequently total cell extracts were prepared and equal amounts of protein (80 μ g) analyzed by western blotting for STAT6 (Tyr641)-phosphorylation with a phospho-STAT6 antibody. The same blot was stripped and reprobed using a STAT6 antibody, which detects phosphorylated and nonphosphorylated STAT6 protein.

(C) Effect of oxacyclododecindione on STAT6 DNA binding. HepG2 cells were pretreated with or without oxacyclododecindione for 60 minutes and stimulated with 50 ng/ml IL-4 for 30 minutes. Subsequently total cell extracts were prepared and tested for DNA binding activity of STAT6 using a ³²P-labelled C/EBP-N4 oligonucleotide with a high affinity STAT6 binding site. The inducible STAT6 complex is indicated. A section of the fluorogram from a native gel is shown. Unlabelled probe: 100-fold molar excess of unlabeled probe; anti-STAT6: 1.0 μ g of STAT6 antibody was added to the reaction mixture.

between H-11 and H_a-12 as well as 10-CH₃ located H-11 in the β -position of a α -methyl substituted enone. The (*E*)-configuration of the olefinic double bond could be established by a NOE contact (NOESY) between 10-CH₃ and H_a-12. The chemical shifts of the chlorinated resorcine moiety compared well with those reported for (*S*)-4-chlorocurcularin [11], thus confirming the proposed structure. Neither the relative nor the absolute configuration of **1** could be determined from the existing data.

Biological Activity

In order to search for compounds and to characterize the influence of oxacyclododecindione on STAT6 activation, STAT6-dependent promoter reporter assays in the IL-4 responsive cell line HepG2 were carried out. The cells were transiently transfected with a SEAP reporter construct containing multiple STAT6 binding elements together with an expression vector encoding full length STAT6. Induction with 10 ng/ml IL-4 led to a 120~140 fold activation over the basal level of SEAP expression. As shown in Fig. 2A, oxacyclododecindione inhibited the inducible STAT6-dependent promoter activity in a dose dependent manner with IC₅₀-values of 20~25 ng/ml (54~67.5 nM). The constitutive activity of the cytomegalovirus promoter driven expression of the reporter gene renilla luciferase was not affected up to a concentration of 150 ng/ml (405 nM) oxacyclododecindione. To determine whether the inhibition of the IL-4 dependent reporter gene in HepG2 cells is due to an inhibition of tyrosine phosphorylation of the STAT6 transcription factor, western blots were performed with a phospho-STAT6 (Tyr641) antibody. As shown in Fig. 2B treatment of HepG2 cells with 50 ng/ml IL-4 resulted in a strong induction of tyrosine phosphorylation of the STAT6 protein. Pretreatment of HepG2 cells with oxacyclododecindione and stimulation with 50 ng/ml IL-4 showed no inhibition of the tyrosine phosphorylation of the STAT6 transcription factor up to a concentration of 500 ng/ml (1350 nM). These data suggest that oxacyclododecindione does not act as an inhibitor of one or several of the Janus kinases (Jak1-3 and Tyk2) responsible for the phosphorylation of the STAT6 transcription factor. We further characterized the mechanism involved in the inhibition of IL-4 signaling by analyzing the influence of the compound on the binding of activated (phosphorylated) STAT6 dimers to the high affinity C/EBP-N4 sequence. As shown in Fig. 2C treatment of HepG2 cells with IL-4 for 30 minutes resulted in a strong increase in STAT6 DNA binding, which was concentration-dependently inhibited by oxacyclododecindione. To show that the retarded band in IL-4 treated cells was indeed STAT6, we incubated the extracts with a STAT6 antibody. The antibody almost

completely abrogated the shifted band suggesting that the IL-4 activated (STAT6)₂·C/EBP-N4 complex contains STAT6 dimers. In addition, the IL-4 inducible DNA complex disappeared in the presence of a 100-fold excess of the unlabeled probe (Fig. 2C).

Cellular DNA-, RNA-, and protein syntheses were examined in HepG2 cells by determining the incorporation of (2-¹⁴C)-thymidine, (2-¹⁴C)-uridine and (1-¹⁴C)-leucine in TCA-insoluble fractions. Up to a concentration of 500 ng/ml of oxacyclododecindione, no effect on DNA-, RNA- and protein syntheses could be observed during a 24 hours incubation period, suggesting that the compound does not interfere with replication, transcription and translation in a general manner (data not shown). Cytotoxic properties of oxacyclododecindione were evaluated against HepG2 cells by measuring the reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) into a colored formazan product which is directly proportional to the living cells in culture. No cytotoxic activities against HepG2 cells could be observed up to 500 ng/ml (1350 nM) of the compound during a 24 hours incubation period (data not shown).

In summary we have identified a novel inhibitor of IL-4 signaling which may serve as lead structure for the development of transcriptionally based inhibitors of allergic diseases.

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