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TMC-264, a Novel Inhibitor of STAT6 Activation Produced by *Phoma* sp. TC 1674

MASAAKI SAKURAI*, MAKI NISHIO, KOUZOU YAMAMOTO, TORU OKUDA†,
KIMIO KAWANO and TETSUO OHNUKI

Discovery Research Laboratory, Tanabe Seiyaku Co., Ltd.,
2-50 Kawagishi-2-chome, Toda-shi, Saitama 335-8505, Japan

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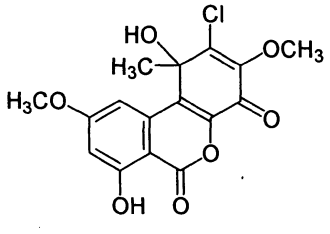
A novel inhibitor of STAT6 activation, named as TMC-264 (**1**), was discovered from the fermentation broth of *Phoma* sp. TC 1674. Based on spectroscopic analyses, TMC-264 was found to be a novel tricyclic polyketide with chloro-1*H*-dibenzo[*b*,*d*]pyran-4,6-dione. TMC-264 suppressed expression of IL-4 driven luciferase and germline Cε mRNA with IC_{50} values of 0.3 μM and 0.4 μM, respectively. TMC-264 exhibited a potent inhibitory activity against tyrosine phosphorylation of STAT6 with an IC_{50} value of 1.6 μM, whereas TMC-264 weakly inhibited tyrosine phosphorylation of STAT5 with an IC_{50} value of 16 μM, but did not inhibit the phosphorylation of STAT1 up to 40 μM. TMC-264 blocked formation of the complexes between phosphorylated STAT6 and STAT6 oligonucleotides in a dose dependent manner, while TMC-264 did not affect the formation of phosphorylated STAT1/STAT1 oligonucleotides complexes. These results suggested that TMC-264 selectively inhibited IL-4 signaling by interfering both of phosphorylation of STAT6 and binding of the phosphorylated STAT6 to the recognition sequence.

Signal transducer and activator of transcription 6 (STAT6) is a transcriptional factor that plays a critical role in interleukin-4 (IL-4) signaling. IL-4 induces phosphorylation of a tyrosine residue ^{641}Tyr of STAT6 by IL-4 receptor associated Janus family of tyrosine kinases, JAK1 and JAK3. The phosphorylated STAT6 forms a homodimer through its SH2 domain interactions. The homodimer translocates to the nucleus, and binds to the specific recognition sequences (TTCCCAAGAA) within the promoter region of germline Cε[1~3]. Expression of germline Cε mRNA is essential for the class switching of an immumogloblin isotype IgM to IgE in B cells[4~6]. The IgE produced induces release of inflammatory mediators in the mast cell[7].

STAT6-deficient ($STAT6^{-/-}$) mice abrogated IL-4 mediated functions including Th2 differentiation and Ig class switching to IgE[8~10], and abolished bronchial eosinophilic inflammation, airway hyperactivity, and lung damage *in vivo*[11,12]. Expression of STAT6 was markedly increased in nasal mucosa from atoppic allergic rhinitis after allergen challenge[13]. In subjects with severe asthma, STAT6 was expressed in the bronchial epithelium more than in those with mild asthma or normal controls[14].

Judging from these findings, it would be possible that selective inhibitors of STAT6 activation might prevent the development of allergic disease. As a result of screening for inhibitors of IL-4 signal transduction using an IL-4 driven luciferase assay system, we had discovered new naphtha-γ-pyrones (TMC-256A1 and C1)[15]. Further screening identified a novel inhibitor of STAT6 activation designated as TMC-264 (Fig. 1) from the fermentation broth of *Phoma*

Fig. 1. Structure of TMC-264.



† Present address: Tamagawa University Research Institute, 1-1 Tamagawa Gakuen-6-chome, Machida, Tokyo 194-8610, Japan.

* Corresponding author: sakura@tanabe.co.jp

sp. TC 1674. In this paper, we describe the taxonomy of producing strain, fermentation, and biological activities of TMC-264. The physico-chemical properties and structure determination will be reported in another paper[16].

Materials and Methods

Materials

Dulbecco's modified Eagles' medium (DMEM) and RPMI1640 medium were purchased from SIGMA (St. Louis, MO). Human recombinant IL-4, IFN-γ and GM-CSF were from R&D systems (Minneapolis, MN). Antibodies to STAT6, STAT1, and IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides used for electrophoretic mobility shift assay (EMSA) were chemically synthesized by Amersham Biosciences (Piscataway, NJ). The DNA sequences of the nucleotides are as follow: wild type STAT6 recognition sequence (wt-st6), gatccacttcccaagaacaca; mutated STAT6 recognition sequence (mt-st6), gatccactagccaactacaga; wild type STAT1 recognition sequence (wt-st1), gatccgtatttcccagaaaaggaaca; mutated STAT1 recognition sequence (mt-st1), gatccgtattagccactaaaggaaca. The annealed oligonucleotides were labeled by using Klenow fragment (TaKaRa; Kyoto, Japan) and [α-^{32}P] dCTP (Amersham Biosciences).

Taxonomic Studies

For identification of the strain TC 1674, cultures grown on malt extract agar (MEA), Miura medium (LCA), oatmeal agar (OA) and Czapek-yeast extract agar (CYA) were observed after incubating at 25°C for 10 to 14 days. The color name used in this study was taken from Munsell color system[17].

Fermentation

The producing strain TC 1674 was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of the seed medium consisting of 1.0% glucose, 0.5% Polypepton (Nihon Pharmaceutical Co.; Osaka, Japan), 0.5% dried yeast (Asahi Brewery Co.; Tokyo, Japan), 20% V-8 juice (Campbells; New Castle, CO), 20% apple juice (Kirin Tropicana Inc.; Tokyo, Japan), and 0.5% $CaCO_3$ and cultivated at 27°C for 4 days on a rotary shaker (220 rpm). One milliliter of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 70 ml of the production medium consisting of 5.0% glucose, 5.0% Diaion HP-20 (Mitsubishi Kasei; Tokyo, Japan), 1.25% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.072% $Ca(NO_3)_2 \cdot 4H_2O$, 0.01% NaCl, 0.005% $FeSO_4 \cdot 7H_2O$. Fermentation was carried out at 27°C for 8 days on a rotary shaker (220 rpm). Packed cell volume (PCV) was measured by centrifugation at 3,000 rpm for 10 minutes.

HPLC Analysis

Analytical HPLC was carried out on a Hewlett-Packard HP-1100 equipped with a diode array detector. The condition for HPLC analysis was as follows. Column: Capcell Pak C18 UG120 (4.6 i.d.×150 mm, Shiseido Co., Tokyo, Japan). Mobile phase: aqueous CH_3CN, gradient: 15% from 0~2 minutes, 15~40% from 2~5 minutes, 40% from 5~11 minutes, 40~55% from 11~17 minutes. Flow rate: 1.2 ml/minute. Detection: UV absorption at 254 nm. TMC-264 (**1**) eluted at 11.2 minutes.

Cells and Culture Conditions

Stable transfectants HeLa IL-4/STAT6 (2-21)[15], HeLa IFN-γ/STAT1 (1-26)[15], and HeLa H-144[18] cells were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS) and 200 μg/ml of hygromycin. DND39 (human Burkitt lymphoma B-cell line) cells were kindly provided by Fujisaki Cell Center (Hayashibara Biochemical Laboratories; Okayama, Japan) and were cultured in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, 0.1 g/liter streptomycin and 100,000 U/liter penicillin G. TF-1 (human erythroleukemic cell line) cells were kindly provided by Dr. TOSHIO KITAMURA (The Institute of Medical Science, University of Tokyo) and were maintained in RPMI 1640 medium supplemented with 10% FBS, 0.1 g/liter streptomycin and 100,000 U/liter penicillin G and 3 ng/ml GM-CSF. HeLa (human cervix adenocarcinoma) cells were maintained in DMEM supplemented with 10% FBS, 0.1 g/liter streptomycin and 100,000 U/liter penicillin G.

Luciferase Assay

HeLa IL-4/STAT6 (2-21) and HeLa IFN-γ/STAT1 (1-26) cells expressed IL-4 driven luciferase and IFN-γ driven luciferase activities under the promoter having the binding sequences of STAT6 and STAT1, respectively, and were treated with TMC-264 in the presence of IL-4 (10 ng/ml) and IFN-γ (10 ng/ml) for 6 hours, respectively. H-144 cells, which expressed constitutively the SV40 driven luciferase activity, were treated with TMC-264 for 6 hours. The luciferase reporter assays were carried out as described previously[15,18].

Measurement of Germline Cε mRNA Expression

DND39 cells were treated with TMC-264 in the presence

of IL-4 (10 ng/ml) for 4 hours. The assay for analysis of germline Cε mRNA expression was performed as described previously[15].

Phosphorylation Assays

DND39 cells and TF-1 cells were cultured in RPMI1640 containing 0.5% FBS for 16 hours, and treated with TMC-264 in the presence of IL-4 (40 ng/ml), and GM-CSF (25 ng/ml), respectively for 15 minutes. HeLa cells were cultured in DMEM containing 0.5% FBS for 48 hours and treated with TMC-264 in the presence of IFN-γ (10 ng/ml) for 15 ninutes. The cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 30 mM $Na_4P_2O_7$, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton-X100, 1 mM Pefablock (Roche Diagnostics; Basel, Switzerland) and 10 μg/ml leupeptin) for 30 minutes on ice. The lysate was centrifuged at 12,000 rpm for 20 minutes at 4°C, and the supernatant was obtained. The total protein content of the supernatant was determined by using Protein Assay CBB Solution (Nacalai Tesque; Kyoto, Japan) as described by the manufacture's instructions. Proteins of the supernatants (10 μg) were resolved on 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred to polyvinylidene difluoride membranes (Immunoblot PVDF membrane) (Bio-Rad; Hercules, CA). The membranes were treated for 1 hour with Blockace (Dainippon Seiyaku; Tokyo, Japan), and incubated with Phospho-STAT Antibodies (New England Biolabs; Beverly, MA) in TTBS (20 mM Tirs-HCl (pH 7.5), 154 mM NaCl, 0.05 % Tween20) containing 5% BSA overnight at 4°C or the STAT antibodies in TTBS containing 0.1% BSA for 1 hour at room temperature. After washing in TTBS, the membranes were incubated with horseradish peroxidase labeled anti-rabbit Ig Ab (Pharmingen; San Diego, CA) in TTBS containing 0.1% BSA for 1 hour at room temperature, washed with TTBS and exposed to an enhanced chemiluminescence detection system (Amersham Biosciences). Percentage of inhibition was calculated by the relative intensity (phospho-STATs/STATs) measured by using bological imager (Quantity One, PDI, inc.; Huntington, NY).

Electrophoretic Mobility Shift Assay (EMSA)

DND39 cells and TF-1 cells were cultured in RPMI1640 containing 0.5% FBS for 16 hours in the presence of IL-4 (40 ng/ml), and GM-CSF (25 ng/ml) respectively for 15 minutes. HeLa cells were cultured in DMEM containing 0.5% FBS for 48 hours in the presence of IFN-γ (10 ng/ml) for 15 minutes. The cells were centrifuged at 1,000 rpm for 5 minutes at 4°C. The cellular pellet was suspended in buffer A (20 mM Hepes (pH 7.4), 1 mM EDTA, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 1 μg/ml leupeptin, 0.1 mM PefaBlock, and 1 mM DTT) and centrifuged at 3,000 rpm for 30 seconds at 4°C. The cellular pellet was resuspended in buffer A containing 0.2% NP-40 and centrifuged at 10,000 rpm for 30 seconds at 4°C. The pellet of nuclei was suspended in buffer A containing 420 mM NaCl and 20% glycerol, incubated for 30 minutes at 4°C, and centrifuged at 15,000 rpm for 20 minutes at 4°C. The supernatant was collected, and the amount of nuclear proteins was determined using the Protein Assay CBB Solution.

The nuclear proteins (3 μg) were incubated with a sample in a 20 μl reaction mixture containing 20 mM Hepes (pH 7.9), 2 mM EDTA, 5 M NaCl, 10% glycerol, 0.2% NP-40, 2 mg/ml BSA, and 3 μg of poly (dI-dC) for 10 minutes on ice. The reaction mixture was incubated with the radio-labeled DNA probe for 30 minutes at room temperature. As controls of the experiment, competition and supershift assays were carried out. Competition assay were performed by addition of a 100-fold molar excess of the unlabelled wt-st6, wt-st1, mt-st6, or mt-st1 to the reaction. In supershift analysis, the antibody (2 μg) to STAT6, STAT1, or IgG was added to the reaction mixture, and the mixture was incubated for 60 minutes on ice prior to adding the radio-labeled DNA probe. Nucleoprotein complexes were resolved on 5% polyacrylamide gels containing 0.5X TBE: 1X TBE consisted of 89 mM Tris-borate and 1 mM EDTA (pH 8.0). The gels were run at 60 V/cm for 17 hours at 4°C, dried, and autoradiographed.

Results

Taxonomy

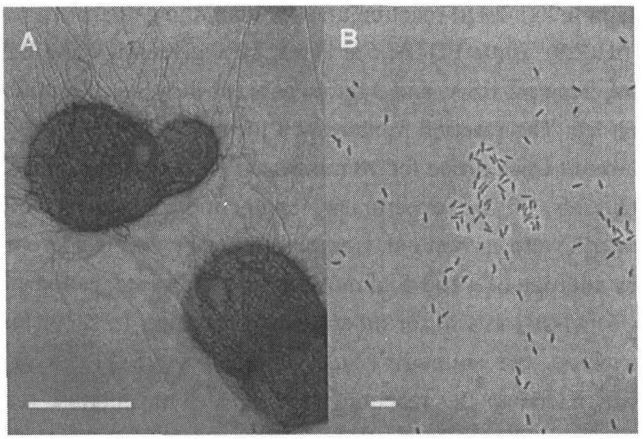
The producing strain TC 1674 was isolated from a soil sample collected at Toda-shi, Saitama, Japan.

Colonies of the strain TC 1674 on MEA, LCA, OA, or CYA, grew moderately, showing funiculose or zonate, occasionally floccose with thick mycelial mat, pale yellow, grayish yellow, grayish brown, or grayish green (Munsell 10Y9/2, 5Y8/2, 10YR5/2 or 5GY6/4) in the center. The margins were plane, immersed, zonate or funiculose, grayish beige, beige, or cream (Munsell 10YR3/6, 5Y8/2, 10YR8/2 or 10YR9/2). The reverse was grayish yellow, grayish brown, or dark yellowish brown (Munsell, 10YR3/6, 5Y8/2, 10YR6/2-2/2). The pigment was produced in MEA and CYA, brownish orange or yellowish brown. Abundant dark brown pycnidia were produced half immersed on LCA and OA, globose to subglobose, pseudoparenchymatous,

solitary or sometimes aggregated, 110~180 μm in diameter, with one or two ostioles, 17~27 μm in diameter (Fig. 2A). Conidia were 1-celled, cylindrical, hyaline, smooth-walled, 2.7~6.3×1.3~3.6 μm, L/W ratio 1.4~3.1 (average 4.0×1.6 μm, L/W 2.5) (Fig. 2B).

Globose pycnidia with hyaline, aseptate conidia without appendages were characteristic of the genus *Phoma*[19,20]. *Phoma* is one of the largest and most widely distributed Coelomycetes often associated with plants. Most species were described on natural substrata, so that a soil isolate is hardly identified to the species level. We therefore identified TC 1674 as *Phoma* sp. TC 1674.

Fig. 2. Photomicrograph of pycnidia and conidia of strain TC 1674.



A, pycnidia, scale = 100 μm

B, conidia, scale = 10 μm

Fermentation

A typical time course of production of TMC-264 is shown in Fig. 3. The pH of broth gradually rose with the progress of fermentation. The production of TMC-264 measured by HPLC began at the late exponential growth phase on day 2, and reached a maximum of 0.5 mg/ml on day 8.

Table 1. Effects of TMC-264 on the IL-4 driven, IFN-γ driven, and SV40 promoter driven luciferase expressions.

compound	IC50 (μM)		
	IL-4 / STAT6	IFN-γ / STAT1	SV40
TMC-264	0.3	5.8	35

IL-4 (2-21) and IFN-γ (1-26) cells were treated with TMC-264 in the presence of IL-4 (10 ng/ml) and IFN-γ (10 ng/ml) for 6 hours, respectively. H-144 cells were treated with TMC-264 for 6 hours.

Fig. 3. Time course of fermentation of TMC-264 by *Phoma* sp. TC 1674.

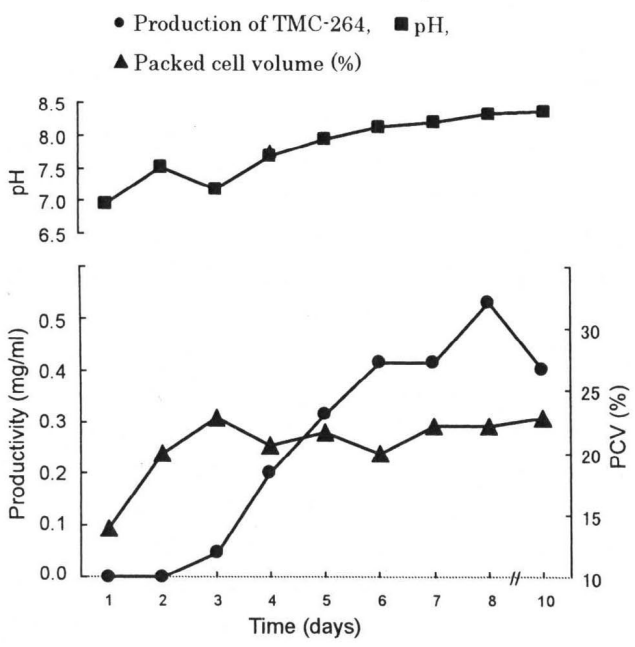
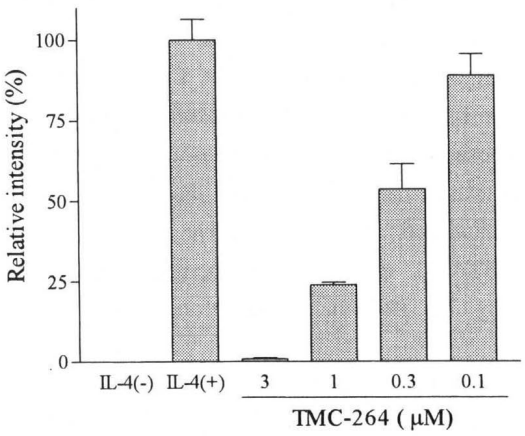
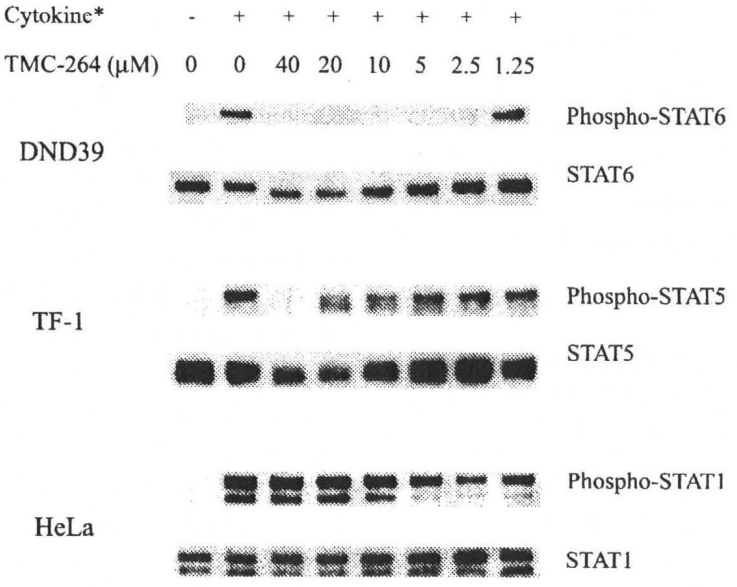


Fig. 4. Effect of TMC-264 on induction of germline Cε mRNA expression by IL-4.



DND39 cells were treated with TMC-264 in the presence of IL-4 (10 ng/ml) for 4 hours. Detection of mRNAs was carried out with quantitative RT-PCR as described previously[15]. The relative intensity (germline Cε mRNA/GAPDH mRNA) was calculated and represented by percentage of the relative intensity of DND39 cells treated with IL-4 as 100%.

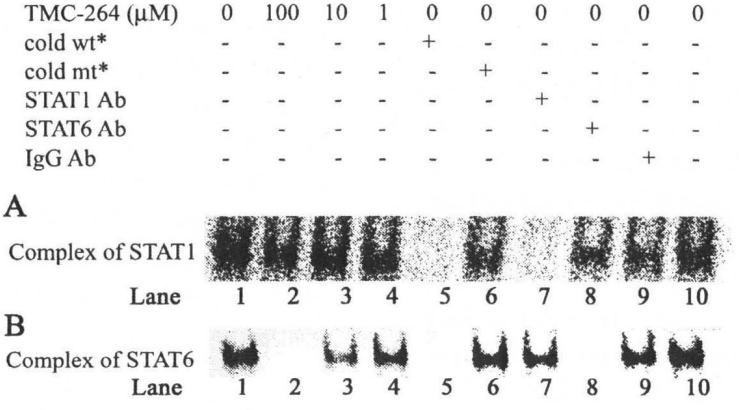
Fig. 5. Effect of TMC-264 on phosphorylation of STATs.



	%inhibition of phosphorylation						IC50
TMC-264 (μM)	40	20	10	5	2.5	1.25	(μM)
STAT6	100	100	100	100	95	29	1.6
STAT5	100	59	30	24	20	6.1	16

* DND39, TF-1, and HeLa cells were treated with TMC-264 in the presence of IL-4 (40 ng/ml), GM-CSF (25 ng/ml) and IFN-γ (10 ng/ml) for 15 minutes, respectively. Percentage of inhibition was calculated by the relative intensity (phospho-STATs/STATs).

Fig. 6. Effect of TMC-264 on binding of STAT6 to the DNA binding site.



* wt-st1 and mt-st1 were used in competition assay of STAT1 (panel A, lanes 5 and 6), whereas wt-st6 and mt-st6 were used in competition assay of STAT6 (panel B, lanes 5 and 6).

Biological Properties

Luciferase Assay

TMC-264 inhibited the IL-4 driven luciferase activity with an IC_{50} values of 0.3 μM (Table 1). To investigate the selectivities of TMC-264, we tested the inhibitory activities of this compound against the IFN-γ driven and the SV40 promoter driven expression of luciferase. TMC-264 inhibited weakly the IFN-γ driven and the SV40 promoter driven luciferase expression as compared to the IL-4 driven luciferase expression, with IC_{50} values of 5.8 μM and 35 μM, respectively.

Measurement of Germline Cε mRNA Expression

We tested the effect of TMC-264 on induction of germline Cε mRNA expression by IL-4 in DND39 cells. As shown in Fig. 4, TMC-264 dose-dependently inhibited the expression with an IC_{50} value of 0.4 μM.

Phosphorylation Assay & EMSA

We investigated the effect of TMC-264 for tyrosine phosphorylation of STATs in cytoplasm (Fig. 5). TMC-264 strongly inhibited tyrosine phosphorylation of STAT6 by IL-4 stimulation with an IC_{50} value of 1.6 μM in DND39 cells, whereas TMC-264 exhibited the weak inhibitory activity against tyrosine phosphorylation of STAT5 by GM-CSF stimulation with an IC_{50} value of 16 μM in TF-1 cells. TMC-264 did not affect tyrosine phosphorylation of STAT1 by IFN-γ up to 40 μM in HeLa cells.

We carried out EMSA to determine the effect of TMC-264 on binding of phosphorylated STAT1 or STAT6 to its recognition sequence (Fig. 6). It would be conceivable that nuclear extracts, which we used for EMSA, contained phosphorylated forms of STATs, because STATs translocates from cytoplasm to nucleus after phosphorylation[3]. As controls of the experiment, we confirmed that cold wt-st1 (panel A, lane 5) and antibody of STAT1 (panel A, lane 7) prevented the complex formation of STAT1, while cold mt-st1 (panel A, lane 6), antibody of STAT6 (panel A, lane 8) and IgG (panel A, lane 9) did not influence the reaction. In the same way, cold wt-st6 (panel B, lane 5) and antibody of STAT6 (panel B, lane 8) inhibited the complex formation of STAT6, whereas cold mt-st6 (panel B, lane 6), antibody of STAT1 (panel B, lane 7) and IgG (panel B, lane 9) did not. TMC-264 inhibited the complex formation of phosphorylated STAT6 and STAT6 oligonucleotides in a dose dependent manner (panel B, lanes 2~4), whereas TMC-264 did not inhibited the complex formation of phosphorylated STAT1 and STAT1 oligonucleotides up to 100 μM (panel A, lanes 2~4).

Discussion

In this study, we isolated a novel inhibitor of STAT6 activation, TMC-264 from the culture broth of *Phoma* sp. TC 1674. The structure was determined to be 2-chloro-4,6-dihydro-1,7-dihydroxy-3,9-dimethoxy-1-methyl-1*H*-dibenzo[*b*,*d*]pyran-4,6-dione[16].

TMC-264 strongly suppressed IL-4 signal transduction in the IL-4 driven luciferase assay system, whereas TMC-264 did weakly suppress the IFN-γ driven and the constitutive SV40 promoter driven luciferase expression. These data suggested that TMC-264 is a selective inhibitor of IL-4 signal transduction. In addition, we showed that TMC-264 inhibited germline Cε mRNA expression. This result inferred that TMC-264 might suppress the production of IgE, because germline Cε mRNA expression was essential for the production of IgE.

To analyze the inhibitory mechanism of TMC-264, we performed further experiments. First, we tested the effect of TMC-264 on tyrosine phosphorylation of STATs, since the phosphorylation of STATs was necessary for the activation and translocation to nucleus of STATs. It was found that TMC-264 strongly inhibited tyrosine phosphorylation of STAT6. On the contrary, these inhibitory activities of TMC-264 against STAT5 and STAT1 were weak or not detected at all, respectively. These results indicated that TMC-264 selectively inhibited tyrosine phosphorylation of STAT6. We next examined the effect of TMC-264 on the binding of phosphorylated STATs to its recognition sequence by EMSA, since the reaction was essential for the expression of germline Cε mRNA. TMC-264 inhibited the complex formation of phosphorylated STAT6 and its recognition sequence in a dose dependent manner, whereas TMC-264 did not affect the complex formation of STAT1. This data showed that TMC-264 inhibited binding of phosphorylated STAT6 to the recognition sequence as well as phosphorylation of STAT6.

The above results suggested that TMC-264 would act on both of STAT6 and phosphorylated STAT6, resulting in inhibition of phosphorylation of STAT6 and binding of phosphorylated STAT6 to the recognition sequence in a highly selective manner. TMC-264 might be a useful inhibition of IL-4 signaling for the treatment of allergic disease.

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