VOL. 54 NO. 8, AUG. 2001 THE JOURNAL OF ANTIBIOTICS pp. 628 — 634

TMC-205 a New Transcriptional Up-Regulator of SV40 Promoter Produced by an Unidentified Fungus

Fermentation, Isolation, Physico-chemical Properties, Structure Determination and Biological Activities

MASAAKI SAKURAI*, JUN KOHNO, MAKI NISHIO, KOZO YAMAMOTO, TORU OKUDA†, KIMIO KAWANO and NORIYUKI NAKANISHI

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

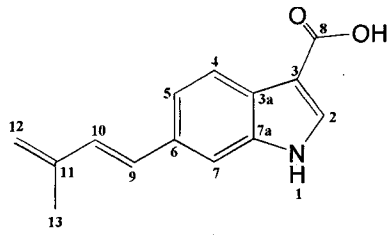
(Received for publication March 1, 2001)

A new transcriptional up-regulator designated TMC-205 was discovered from the fermentation broth of an unidentified fungal strain TC 1630 by using an SV40 promoter-luciferase reporter assay. Based on spectroscopic analyses, its structure was determined to be (*E*)-6-(3-methyl-1,3-butadienyl)-1*H*-indole-3-carboxylic acid. Expression of the luciferase activity was activated *ca.* 2-, 4-, and 6-fold by 1, 10, and 100 μM TMC-205, respectively. TMC-205 activated the transcriptional activity in a manner dependent on the presence of the enhancer element of SV40 in its promoter region.

Transcriptional modulators of genes involved in cell proliferation and differentiation might be useful as anti-tumor agents. 1-β-D-Arabinofuranosylcytosine (ara-C), one of the most effective agents in the treatment of human acute myelogenous leukemia, induced transcription of the c-*jun* gene through the AP-1 sites in the promoter region[1]. SOWA *et al.* reported that anti-tumor activity of trichostatin A (TSA) was attributable to the activation of $p21^{WAF1/Cip1}$ promoter through the Sp1 sites in the promoter region[2]. The AP-1 and Sp1 sites were contained in the enhancer and promoter region of SV40 DNA tumor virus so that these compounds activated expression from SV40 promoter[1~5]. Other examples of anti-tumor agents with the up-regulatory activity of SV40 promoter include azelaic bishydroxamic acid[6], FR901228[3,7~9], FR901463[10,11], FR901464[10,11] and FR901465[10,11]. Therefore the SV40 promoter seems to be a good tool for searching for transcriptional modulators with anti-tumor activities.

We have screened microbial metabolites for a transcriptional regulator using SV40 promoter-luciferase assay and discovered a new substance designated as TMC-205 (Fig. 1). This report describes the fermentation, isolation, structure determination and biological activities of TMC-205.

Fig. 1. Structure of TMC-205.



† Present address: Applied Life Science Research Center, Tamagawa University Research Institute, 1-1 Tamagawagakuen-6-chome, Machida-shi, Tokyo 194-8610, Japan.

* Corresponding author: sakura@tanabe.co.jp

Materials and Methods

Fermentation

A slant culture of the TMC-205 producing organism was inoculated into a 500-ml Erlenmeyer flask containing 70 ml of a seed medium consisting of glucose 1%, polypepton 0.5%, dried yeast 0.5%, V-8 juice 20%, apple juice 20% and $CaCO_3$ 0.5% in distilled water (pH 6.0 before autoclaving). The culture was incubated at 27°C for 4 days on a rotary shaker (220 rpm). Two milliliters of the seed culture were transferred into a 500-ml Erlenmyer flask containing 70 ml of a producing medium consisting of potato starch 2%, glucose 2%, High-pro meal 2%, yeast extract 0.5%, NaCl 0.25%, $MgSO_4 \cdot 7H_2O$ 0.02%, trace salt solution (composed of $ZnSO_4 \cdot 7H_2O$ 5%, $CuSO_4 \cdot 5H_2O$ 0.5%, $MnCl_2 \cdot 4H_2O$ 0.5%, $FeSO_4 \cdot 7H_2O$ 1%, $Na_2B_4O_7 \cdot 10H_2O$ 0.06%, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 0.04%, and $CoCl_2 \cdot 6H_2O$ 0.4%) 0.1%, $CaCO_3$ 0.32% in distilled water. The fermentation was carried out at 27°C for 11 days on a rotary shaker (220 rpm).

General

UV spectrum was measured on a Shimadzu model UV-2200A spectrophotometer. IR spectrum was recorded on a JASCO model 100 infrared spectrophotometer. Mass spectra were obtained by using MStation 700 tandem type mass spectrometer (JEOL, Japan) equipped with an electrospray ionization source. Analytical HPLC were carried out on a HP1100 system (Hewlett Packard, USA). The ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX-400 NMR spectrometer at 30°C. The chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as an internal standard.

HPLC Analysis

The conditions for HPLC analysis were as follows: column, YMC-Pack ODS-AM-301-3 (4.6×100 mm); mobile phase, 50% CH_3CN - 10 mM phosphate buffer at pH 3.5; flow rate, 1.2 ml/minute; detection, UV at 299 nm. Under these conditions, TMC-205 was eluted at 3.2 minutes.

Cells and Culture Conditions

HeLa (human cervix adenocarcinoma) cells were maintained in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS). Stable transfectants H-144 cells were maintained in DMEM containing 10% FBS and 200 μg/ml of hygromycin.

Plasmids

pGL3-control (promoter with enhancer) and pGL3-promoter (promoter without enhancer) were purchased from Promega, and pREP4 was from Invitrogen.

Stable Transfection and Luciferase Assay

Stable transfection of HeLa cells with pGL3-control and pREP4 was carried out by using SuperFect™ Transfection Reagent (QIAGEN) as described by the manufacture's instructions. One of the hygromycin resistant clones obtained, HeLa-114 expressed the luciferase activity constitutively.

HeLa-114 cells were seeded into 96-well white cell culture microplates (1×10^4 cells per well) and incubated for 24 hours, and then samples were added to the cell culture at 10% v/v. After further 24-hour incubation, the cells were washed twice with 200 μl of PBS, and 20 μl of cell lysis buffer (Promega) and 50 μl of luciferase assay buffer (Promega) were added to the cells. The luciferase activities were measured using a luminometer (Berthold Microlumat LB96P).

Transient Transfection and Luciferase Assay

On the day before transfection, HeLa cells were seeded into 96-well microplates (5×10^3 cells per well) and incubated. Transient transfection of HeLa cells with pGL3-control or pGL3-promoter was carried out by using SuperFect™ Transfection Reagent (QIAGEN) as described by the manufacture's instructions. After 24-hour incubation, samples were added to the cell culture at 10% v/v. The cells were incubated for 24 hours, and the luciferase activities were measured as described above.

In Vitro Cytotoxic Activity Assay

The cells were cultured in the following media: HCT-116 and SK-Br-3, completed McCoy's 5A supplemented with 10% fetal bovine serum; B16 and HeLa, complete DMEM supplemented with 10% fetal bovine serum; HL-60, complete RPMI-1640 supplemented with 20% fetal bovine serum; WiDr and HT-29, complete DMEM supplemented with non essential amino acid solution and 10% fetal bovine serum; Jurkat, Raji and U937, complete RPMI-1640 supplemented with 10% fetal bovine serum; P388D1, complete RPMI-1640 supplemented with 5% fetal bovine serum.

The cells to be tested were seeded into 135 μl of culture medium per well of 96-well microtiter plates (1×10^4 cells per well). The serially diluted samples in 1% DMSO solution (15 μl) were added to each well of the plates. The cells were incubated for 72 hours, and then WST-1 and 1-

methoxy PMS (Dojindo) solution (25 μl) was added to each well. After 3-hour incubation, *in vitro* cytotoxic activity was evaluated by the colorimetrical determination method at 450 nm[12].

Results

Fermentation and Isolation

We were prevented from taxonomical studies of the producing fungal strain due to the fact that the strain did not sporulate on any medium tested so far.

Under the conditions described in Materials and Methods, the production of TMC-205 reached a maximum (3.6 mg/liter) at 11 days.

The purification procedure was performed in the dark, because TMC-205 is sensitive to light. The fermentation broth (7.0 liters) of the producing strain was extracted with 1-butanol (3.0 liters). The extract (2.2 liters) was concentrated *in vacuo* to dryness to yield a tan solid (6.4 g). This solid was purified by gel filtration on a Sephadex LH-20 column eluted with CH_2Cl_2 - CH_3OH (1 : 1). The eluate was monitored by bioassay and HPLC analysis. The active fractions were concentrated and subjected to reverse phase silica gel (Varian Bond Elut C18) column chromatography, followed by stepwise elution with 20 and 30% CH_3CN - 10 mM phosphate buffer at pH 3.5. The active fractions eluted with 30% CH_3CN were concentrated and extracted with 1-butanol to give a semi-pure powder (40 mg). Final purification of TMC-205 was achieved by preparative HPLC (column, YMC-D-ODS-5 30×250 mm; mobile phase, 40% CH_3CN - 20 mM phosphate buffer at pH 3.5; flow rate, 25 ml/minute; detection, UV at 290 nm; Rt, 37 minutes). The fraction containing TMC-205 was desalted by reverse phase silica gel (Varian Bond Elut C18) column chromatography. Pure TMC-205 (3.3 mg) was obtained as pale yellow powder.

Physico-chemical Properties

The physico-chemical properties of TMC-205 are summarized in Table 1. TMC-205 was soluble in DMSO, methanol, acetone and ethyl acetate, but insoluble in *n*-hexane and water. The molecular formula of TMC-205 was established as $C_{14}H_{13}NO_2$ on the basis of high-resolution ESI-MS, and ^1H and ^{13}C NMR spectral data.

Structure Determination

The ^{13}C and ^1H NMR data of TMC-205 obtained from DEPT, pulsed field gradient (pfg)-HMQC and pfg-HMBC spectra are shown in Table 2.

Table 1. Physico-chemical properties of TMC-205.

Appearance	Pale yellow powder
ESI-MS (*m/z*)	228 $(M+H)^+$, 250 $(M+Na)^+$
	226 $(M-H)^-$
HRESI-MS (*m/z*)	
Found	226.0897
Calcd.	226.0868
	for $C_{14}H_{12}NO_2$
Molecular formula	$C_{14}H_{13}NO_2$
UV λ_{max} (MeOH) nm (log ε)	205 (4.03), 210 (sh, 4.02)
	271 (4.27), 305 (4.15)
IR ν_{max} (KBr) cm^{-1}	3370, 1665, 1560, 1535, 1450, 1345
	1310, 1285, 1250, 1200, 1140, 1100
	1030, 955, 865, 810, 780, 755
TLC, Rf Value*	
CH_2Cl_2 - MeOH (9:1)	0.54

* Merck Kieselgel 60 F_{254} (Art. 5719)

Table 2. ^{13}C and ^{1}H NMR data for TMC-205 and indole-3-carboxylic acid.

Position	TMC-205		Indole-3-carboxylic acid
	^{13}C	^{1}H	^{13}C
2	132.8 d[a]	7.98 (1H, d, 2.7)[b]	132.1 d
3	107.6 s		107.3 s
3a	125.8 s		125.9 s
4	120.6 d	7.94 (1H, d, 8.5)	120.5 d[c]
5	119.5 d	7.39 (1H, dd, 8.5, 1.2)	120.9 d[c]
6	131.2 s		122.0 d[c]
7	110.5 d	7.52 (1H, brs)	112.1 d
7a	136.8 s		136.3 s
8	165.6 s		165.8 s
9	129.6 d	6.69 (1H, d, 16.1)	
10	129.7 d	6.96 (1H, d, 16.1)	
11	141.7 s		
12*cis*	116.6 t	5.16 (1H, brs)	
12*trans*		5.05 (1H, brs)	
13	18.4 q	1.95 (3H, s)	
1-NH		11.82 (1H, brs)	
COOH		11.88 (1H, brs)	
$^{1}J_{C\text{-}2,H\text{-}2}$	186.1 Hz		186.1 Hz

[a] Multiplicity.

[b] Proton number, multiplicity and coupling constants in Hz.

[c] May be exchangeable.

The ^{13}C NMR spectrum displayed 14 signals composed of $CH_3\times1$, $CH_2=\times1$, $-CH=\times6$, $>CH=\times5$ and carbonyl $C\times1$. The ^{1}H NMR spectrum showed the presence of two D_2O exchangeable protons (δ 11.82 and 11.88). The DQF-COSY and pfg-HMBC experiments clarified the connectivity of one methyl and all sp^2 carbons and protons except for carbonyl carbon as shown in Fig. 2.

The presence of an Indole nucleus was demonstrated by the following observations: i) HMBC correlations from NH proton (δ 11.82) to C-3a (δ 125.8), and from H-2 (δ 7.98) to C-3 (δ 107.6) and C-3a, ii) NOESY correlation from NH-proton to H-7 (δ 7.52), iii) the characteristic large coupling constant $^{1}J_{C\text{-}2,H\text{-}2}=186.1$ Hz.

HMBC correlation network of the olefinic protons H-9 (δ 6.69) and H_2-12 (δ 5.16 and 5.05), and methyl proton H-13 (δ 1.95) along with ^{1}H-^{1}H coupling between H-9 and H-10 showed the presence of the 3-methyl-1,3-butadienyl moiety. The configuration of the double bond between H-9 and H-10 was assigned as *E* on the basis of the large coupling

Fig. 2. 2D NMR experiments of TMC-205.

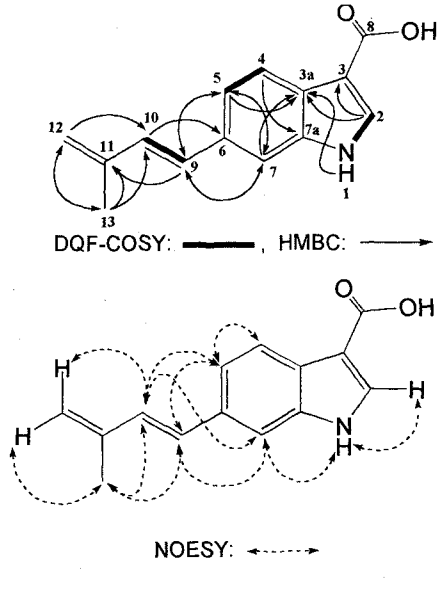


Fig. 3. Time course of the induction of luciferase expression by TMC-205.

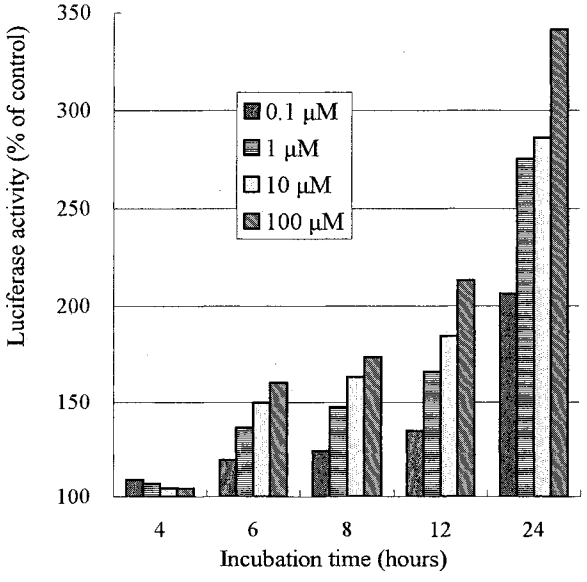
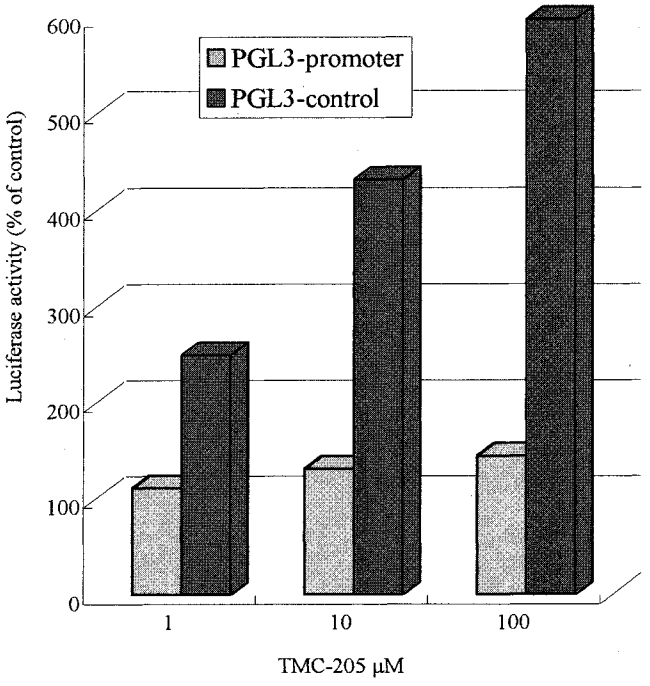


Fig. 4. Effect of the TMC-205 on luciferase expression from SV40 promoter with or without enhancer element.



constant $^{3}J_{H\text{-}9,H\text{-}10}$=16.1 Hz. HMBC correlations from H-10 to H-6, from H-9 to H-5 and H-7 indicated that the (*E*)-3-methyl-1,3-butadienyl moiety was attached to the indole moiety at C-6. Finally, the remaining carbonyl carbon at δ 165.6 and D_2O exchangeable proton at δ 11.88 were assigned to be carboxyl group, which was attached to quaternary carbon C-3. The ^{13}C NMR data of the indole-3-carboxylic acid moiety in TMC-205 was in good agreement with those of authentic indole-3-carboxylic acid except for C-6. The gross structure was also supported by NOE network (Fig. 2). The structure of TMC-205 was thus established to be (*E*)-6-(3-methyl-1,3-butadienyl)-1*H*-indole-3-carboxylic acid.

Biological Properties

As shown in Fig. 3, the up-regulatory activity of TMC-205 in the HeLa-114 cells was first detected after 6-hour incubation and continued to increase to 24-hour as far as tested. The activity was dependent on the concentration of TMC-205 up to 100 μM.

In order to confirm that the induction of luciferase activity by TMC-205 was due to the up-regulation through SV40 promoter, we analyzed the effect of TMC-205 by transient transfection of HeLa cells. As shown Fig. 4, TMC-205 was able to activate transcription of pGL3-control containing the intact SV40 promoter with enhancer element, but pGL3-promoter lacking the SV40 enhancer element could not.

The cytotoxic activities of TMC-205 were determined against various tumor cell lines (Table 3). TMC-205 showed weak to moderate cytotoxicities to these cell lines.

Discussion

In this study, we isolated a new transcriptional regulator, TMC-205 from an unidentified fungal strain, and demonstrated that TMC-205 was a new indole alkaloid containing 3-methyl-1,3-butadienyl unit. TMC-205 activated the transcriptional activity in pGL3-control containing the SV40 enhancer and promoter region, but pGL3-promoter lacking the SV40 enhancer element could not. This data suggested that the mechanism of up-regulatory activity by TMC-205 involved transcription factors binding at the SV40 enhancer element. Searching a database of transcriptional factors (TFSEARCH) identifies binding sites for various transcriptional factors in the SV40 enhancer element such as AP-1, Sp1, HSF, Oct-1, GATA-1, GATA-2, NF-κB and C/EBP. Further studies are needed for

Table 3. Cytotoxicities of TMC-205 against tumor cells *in vitro*.

Cell lines	IC50 (μM)
HCT-116 human colon carcinoma	64
SK-BR-3 human breast adenocarcinoma	203
HeLa human cervix adenocarcinoma	157
B-16 murine melanoma	86
WiDr human colon adenocarcinoma	146
HT29 urinary bladder carcinoma	139
HL-60 human premyelocytic leukemia	143
THP1 human monocytic leukemia	143
Raji Burkitt's Lymphoma	67
Jurkat human lymphoma	52
MOLT-4 human acute lymphoblastic leukemia	90
P388D1 murine lymphoid neoplasm	87
U937 human histiocytic lymphoma	103

making it clear which transcription factor is involved in the up-regulatory mechanism of TMC-205.

Acknowledgments

We wish to thank Ms. NAOKO FUKUI for NMR measurements, Dr. NORIKO OHASHI for mass measurements. We are grateful to Mr. SIGERU YANO and Ms. MIEKO KOITABASHI for their excellent technical assistance. We are also grateful to Dr. TETSUO OHNUKI for encouragement during this study.

References

1) BRACH, M. A.; F. HERRMANN & D. W. KUFE: Activation of the AP-1 transcription factor by arabinofuranosylcytosine in myeloid leukemia cells. Blood 79: 728~734, 1992
2) SOWA, Y.; T. ORITA, S. MINAMIKAWA, K. NAKANO, T. MIZUNO, H. NOMURA & T. SAKAI: Histone deacetylase inhibitor activates the WAF1/Cip1 gene promoter through the Sp1 sites. Biochem. Biophy. Res. Comm. 241: 142~150, 1997
3) NAKAJIMA, H.; Y. B. KIM, H. TERANO, M. YOSHIDA & S. HORINOUCHI: FR901228, a potent antitumor antibiotic, is a novel histone deacetylase inhibitor. Exp. Cell. Res. 241: 126~133, 1998
4) JONES, N. C.; P. W. RIGBY & E. B. ZIFF: *Trans*-acting protein factors and the regulation of eukaryotic transcription: lessons from studies on DNA tumor virus. Gene & Dev. 2: 267~281, 1988
5) GIDONI, D.; J. T. KADONAGA, H. BARRERA-SALDANA, K. TAKAHASHI, P. CHAMBON & R. TJIAN: Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. Science 230: 511~517, 1985
6) PARSONS, P. G.; C. HANSEN, D. P. FAIRLIE, M. L. WEST, P. A. C. DANOY, R. A. STURM, I. S. DUNN, J. PEDLEY & E. M. ABLETT: Tumor selectivity and transcriptional activation by Azelaic bishydroxamic acid in human melanocytic cells. Biochemical Pharmacology 53: 1719~1724, 1997
7) UEDA, H.; H. NAKAJIMA, Y. HORI, T. FUJITA, M. NISHIMURA, T. GOTO & M. OKUHARA: FR901228, A novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. I. Taxonomy, fermentation, isolation, physico-chemical and biological properties, and antitumor activity. J. Antibiotics 47: 301~310, 1994
8) SIGEMATSU, N.; H. UEDA, S. TAKASE & H. TANAKA: FR901228, a novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. II. Structure determination. J. Antibiotics 47: 311~314, 1994
9) UEDA, H.; T. MANDA, S. MATSUMOTO, S. MUKUMOTO, F. NISHIGAKI, I. KAWAMURA & K. SHIMOMURA: FR901228, A novel antitumor bicyclic depsipeptide produced by *Chromobacterium violaceum* No. 968. III. Antitumor activities on experimental tumors in mice. J. Antibiotics 47: 315~323, 1994
10) NAKAJIMA, H.; B. SATO, T. FUJITA, S. TAKASE, H. TERANO & M. OKUHARA: New antitumor substances, FR901463, FR901464 and FR901465. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. J. Antibiotics 49: 1196~1203, 1996
11) NAKAJIMA, H.; Y. HORI, H. TERANO, M. OKUHARA, T. MANDA, S. MATSUMOTO & K. SIMOMURA: New antitumor substances, FR901463, FR901464 and FR901465. II. Activities against experimental tumors in mice and

mechanism of action. J. Antibiotics 49: 1204~1211, 1996

12) ISHIYAMA, M.; H. TOMINAGA, M. SHIGA, K. SASAMOTO, Y. OHKURA & K. UENO: A combined assay of cell viability and *in vitro* cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol. Pharm. Bull. 19: 1518~1520, 1996