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Tyrosyl-DNA Phosphodiesterase 1 Inhibitor from an Anamorphic Fungus

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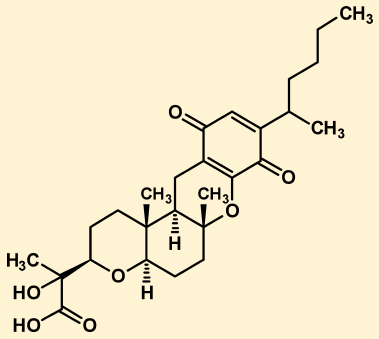
Supporting Information

ABSTRACT: Tyrosyl-DNA phosphodiesterase 1 (Tdp1) is an enzyme that catalyzes hydrolysis of 3′-phosphotyrosyl bonds and is involved in repair of irreversible topoisomerase I (Top1)−DNA covalent complexes. Tdp1 inhibitors are regarded as potential cancer therapeutics in combination with Top1 inhibitors, which are currently used to treat human cancers. While screening for Tdp1 inhibitors, we discovered a novel compound, JBIR-21 (**1**), from the culture of an anamorphic fungus, RF-13305. The structure of **1** was established by extensive NMR and MS analyses. Compound **1** showed inhibitory activity against Tdp1 (IC_{50} value, 18 μM) and cytotoxic activity against cancer cell lines (IC_{50} values, 3.5−13 μM). Compound **1** also exhibited antitumor activity in a mouse xenograft model without adverse effects.



Human tyrosyl-DNA phosphodiesterase (Tdp1) is a member of the phospholipase D superfamily.[1] Tdp1 catalyzes hydrolysis of the phosphodiester bond between a catalytic tyrosine of topoisomerase 1 (Top1) and a 3′-end phosphate of DNA. Tdp1 appears to be responsible for repairing this unique protein−DNA linkage in cells, where the damage occurs naturally or can be induced with Top1 inhibitors.[2] The sensitivity of cancer cells to DNA-damaging agents is related to intrinsic deficiencies in DNA repair and checkpoint mechanisms. The capacity of cancer cells to recognize DNA damage and initiate DNA repair is a key mechanism for resistance to chemotherapy. Therefore, targeting DNA-repairing enzymes may potentiate the cytotoxicity of currently available DNA-damaging agents, and Tdp1 is a known drug target for the treatment of cancer. Previous work has suggested that Tdp1 inhibitors act synergistically with the Top1 inhibitor camptothecin during combined anticancer therapy. It was also proposed that therapeutic selectivity may be achieved by combining inhibitors of both Top1 and Tdp1 since a significant number of tumors have defective DNA repair and checkpoint pathways.[3]

At present, several Tdp1 inhibitors have been characterized.[2] The aminoglycoside antibiotic neomycin B has also been examined as a potential Tdp1 inhibitor based on its ability to target members of the phospholipase D superfamily.[4] In addition, recent high-throughput screening has identified furamidine,[3] several phosphotyrosine mimetics,[5] 1*H*-indole-3-acetic acid derivatives,[6] and C21-substituted progesterone derivatives[7] as Tdp1 inhibitors. However, no antitumor activity of these inhibitors in animal models has been reported. In the course of a screening program for Tdp1 inhibitors from a natural product library, we isolated a novel Tdp1 inhibitor designated as JBIR-21 (**1**) from the culture of an unidentified anamorphic fungus, RF-13305.

A total of 29 904 diverse natural product samples were screened employing a previously reported assay system.[8,9] After performing reproducible and dose-dependent tests, we ultimately selected a crude fungal sample with inhibitory activity toward Tdp1.

The producer was statically cultured for 14 days at 23 °C in 500 mL Erlenmeyer flasks (10 flasks), each containing 100 mL of a production medium. The culture was extracted with 1 L of EtOH, filtered, and concentrated *in vacuo*. After lyophilization, the residue was dissolved in $CHCl_3$, and then the soluble fraction was subjected to sequential silica gel flash column chromatography.

The molecular formula of **1** was established as $C_{27}H_{38}O_7$ based on the HRESIMS data. The IR spectrum suggested the presence of carbonyl groups of carboxylic acid (1725 cm^{-1}) and conjugated ketone (1645 and 1605 cm^{-1}). The ^1H and ^{13}C

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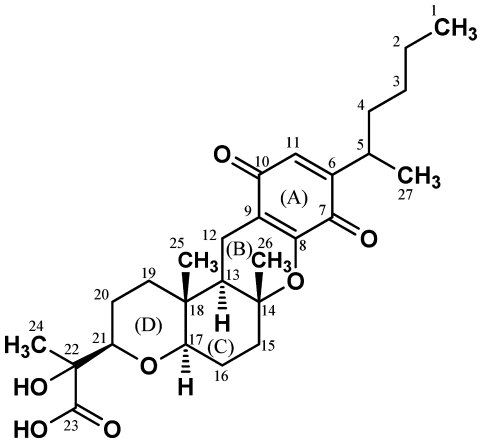

NMR spectroscopic data for **1** are shown in Table 1. The structure of **1** was determined from a series of 2D NMR analyses,

Table 1. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) Spectroscopic Data for 1 in $CDCl_3$

	δ_C	δ_H (multiplicity, J in Hz)
1	13.9	0.84, t (7.0)
2	22.6	1.24, m
3	29.3	1.28−1.12, m
4	35.62	1.46, ddt (13.0, 7.0, 1.5); 1.34, m
5	31.3	2.89, sextet (7.0)
6	151.8	
7	181.8	
8	152.2	
9	117.5	
10	187.5	
11	131.1	6.36, d (0.5)
12	16.3	2.50, dd (18.5, 5.0); 2.10, dd (18.5, 13.0)
13	46.6	1.42, dd (13.0, 5.0)
14	80.2	
15	37.1	2.15, dt (13.0, 3.0); 1.77, m
16	25.0	1.73, m; 1.55, ddt (12.5, 12.0, 3.0)
17	84.6	3.16, dd (12.0, 3.0)
18	35.56	
19	36.1	1.93, ddd (13.0, 4.5, 2.5); 1.21, m
20	19.9	1.75, m; 1.61, dddd (14.0, 12.0, 10.0, 4.5)
21	81.6	3.70, dd (12.0, 3.0)
22	76.1	
23	178.2	
24	21.5	1.36, s
25	12.1	0.89, s
26	20.8	1.25, s
27	19.3	1.07, d (7.0)

including HSQC, double-quantum filtered COSY, and constant time HMBC[10] spectra.

Proton spin coupling between methylene protons H_2-12 (δ_H 2.50, 2.10) and methine proton H-13 (δ_H 1.42) and two sequences from methylene protons H_2-15 (δ_H 2.15, 1.77) to oxymethine proton H-17 (δ_H 3.16) through methylene protons H_2-16 (δ_H 1.73, 1.55) and from methylene protons H_2-19 (δ_H 1.93, 1.21) to oxymethine proton H-21 (δ_H 3.70) through methylene protons H_2-20 (δ_H 1.75, 1.61) were observed in the DQF-COSY spectrum (Figure 1). HMBC correlations from methyl protons H_3-25 (δ_H 0.89) to a methine carbon C-13 (δ_C 46.6), oxymethine carbon C-17 (δ_C 84.6), quaternary carbon C-18 (δ_C 35.56), and methylene carbon C-19 (δ_C 36.1) and from methyl

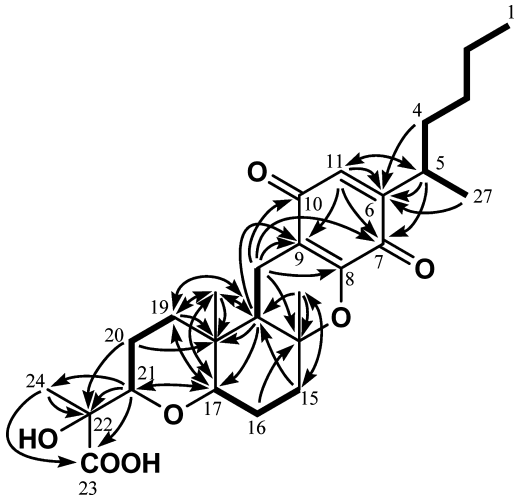

Figure 1. Key correlations observed in 2D NMR spectra of **1** (bold lines show ^{1}H−^{1}H DQF-COSY results, and arrows show HMBC results).

protons H_3-26 (δ_H 1.25) to C-13, oxygenated quaternary carbon C-14 (δ_C 80.2), and methylene carbon C-15 (δ_C 37.1) established a connectivity among these three proton systems and a cyclohexane moiety. In addition, ^{1}H−^{13}C long-range coupling from the methylene protons H_2-21 to C-17 elucidated a tetrahydropyran moiety. Thus, a chromene substructure was established as shown in Figure 1. Furthermore, long-range couplings from methyl protons H_3-24 (δ_H 1.36) to oxygenated methine carbon C-21 (δ_C 81.6), hydroxy quaternary carbon C-22 (δ_C 76.1), and carboxyl carbon C-23 (δ_C 178.2) indicated that a 2-hydroxypropanoic acid moiety is substituted at position C-21 in the chromene moiety.

A sequence from methyl protons H_3-1 (δ_H 0.84) to another methyl proton, H_3-27 (δ_H 1.07), through methylene protons H_2-2 (δ_H 1.24), H_2-3 (δ_H 1.28−1.12), and H_2-4 (δ_H 1.46, 1.34) and methine proton H-5 (δ_H 2.89), which was in turn long-range coupled to the two aromatic carbons C-6 (δ_C 151.8) and C-11 (δ_C 131.1) and carbonyl carbon C-7 (δ_C 181.8), was observed. Therefore, the alkyl side chain substructure was proved to be substituted at the position of C-6, as shown in Figure 1. ^{1}H−^{13}C long-range couplings from the methine proton H-13 to aromatic carbon C-9 (δ_C 117.5) and from methylene protons H_2-12 to aromatic carbons C-8 (δ_C 152.2) and C-9 established the sequence from C-8 to C-12 as shown in Figure 1. Finally, long-range couplings from aromatic proton H-11 (δ_H 6.36) to C-7 and C-9 and from the methylene protons H_2-12 to C-7 and carbonyl carbon C-10 (δ_C 187.5) indicated the existence of a quinone structure. According to the nine index of hydrogen deficiency deduced by the molecular formula of **1**, one more ring structure is required to complete the final structure. Methylation with TMS-diazomethane and methyl iodide (see Supporting Information) resulted in 22-OMe and 23-COOMe, establishing the ether linkage between C-8 and C-14. Thus, the planar structure of **1** was determined as shown in Figure 1.

The relative configurations were assigned on the basis of coupling constants and the analyses of differential nuclear Overhauser effect (NOE) spectra (Figure 2). The large coupling constants between H-12 (β-axial)/H-13 ($J_{H\text{-}12,H\text{-}13}$, 13.0 Hz), H-16 ($\beta$-axial)/H-17 ($J_{H\text{-}16,H\text{-}17}$, 12.0 Hz), and H-20 ($\beta$-axial)/H-21 ($J_{H\text{-}20,H\text{-}21}$, 12.0 Hz) suggested that H-13, H-17, and H-21 are in α-axial locations (Table 1). The NOEs among H-17, H-13, H-15,

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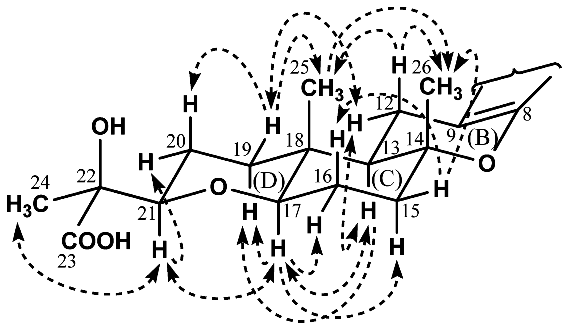

Figure 2. Key nuclear Overhauser effects for rings B, C, and D of **1**.

H-19, and H-21 indicated these protons are located in the same α-axial orientation. The NOEs among H-12 (β-axial) and the methyl protons H_3-25 and H_3-26 also revealed that the methyl groups on C-18 and C-14 are in the β-axial orientation and that rings B, C, and D are all in chair conformations. Thus, the structure of **1**, including relative configuration, was established.

Herein, we isolated a new merosesquiterpenoid including a *p*-quinone structure, **1**, from the culture of RF-13305. Compound **1** has a new carbon framework that is a 28-nor derivative of cochlioquinones previously isolated as phytotoxins from fungi such as *Bipolaris* spp.[11] Although many cochlioquinones have been reported, to the best of our knowledge, **1** represents the first 23-carboxylic acid derivative of cochlioquinone to be isolated from natural sources.

Compound **1** showed inhibitory activity against Tdp1 with an IC_{50} value of 18 μM. Therefore, the cytotoxic effects of **1** against four cancer cell lines were examined by the WST-8 colorimetric assay. Compound **1** exhibited cytotoxic effects against four cell lines—HeLa, NCI-H2052, HT-29, and Namalva—with IC_{50} values of 9.2, 13, 4.0, and 3.5 μM, respectively.

To verify whether **1** could inhibit tumor cell growth *in vivo*, we examined its antitumor activity against HT-29 cells in an animal xenograft model (Figure 3). HT-29 cells were selected

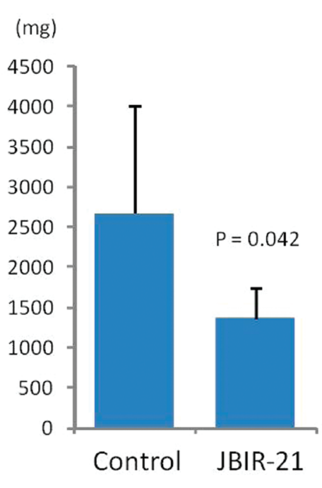

Figure 3. Inhibition of tumor growth in nude mice by administration of **1**. Each group comprised 6 mice, and the weights of their tumors were measured 21 days after the last administration of control (1.6% DMSO−PBS) or JBIR-21 (2 mg/kg).

for the xenograft experiments because they show successful engraftment and proliferation in the backs of nude mice. When the tumor volume reached 50 mm^3, **1** was injected intraperitoneally every other day for 19 days (10 total injections). Compared with the control mice (injected with PBS with 1.6% DMSO), treatment with **1** markedly prevented tumor growth (treatment-to-control ratio, T/C = 0.51), as demonstrated by differences in tumor volume 21 days after the final injection (Figure 3). Mice treated with **1** showed neither noticeable weight loss nor any other obvious adverse effects (data not shown). These results indicate that **1** selectively inhibits the proliferation of tumor cells *in vivo*, consistent with observations made in the cell culture studies. These results suggest that **1** is a promising lead compound for use as a cancer therapeutic without any side effects. Detailed studies of biological function, including the mechanism of action, are now underway.

■ EXPERIENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Horiba SEPA-300 polarimeter. UV and IR spectra were measured with a Beckman Coulter DU730 UV/vis spectrophotometer and a Horiba FT-720 spectrophotometer, respectively. NMR spectra were collected using a Varian NMR System 600 NB CL in $CDCl_3$ (δ_C 77.0, δ_H 7.26 ppm), with the residual solvent peak serving as the internal standard. HRESIMS data were recorded using a Waters LCT-Premier XE mass spectrometer. Reversed-phase MPLC was conducted on a Purif-Pack SI-60 column (Shoko Scientific). Other reagents and solvents were of the highest grade available.

Tdp1 Assay. Human Tdp1 protein and the substrate, a single thymidine nucleotide containing a 3′-phosphate-(4-methylumbelliferone), were prepared by the previously reported methods.[8,9] For performing screening, 7.5 μL of the Tdp1 protein solution (30 μg/mL, in 50 mM Tris-HCl pH 8.0 containing 80 mM KCl, 2 mM EDTA, 1 mM DTT, 40 μg/mL BSA) was dispensed into 384-well plates (784900, Greiner Bio-one) by using a Multidrop Combi dispenser (Thermo Fisher Scientific). The samples (0.5 μL) to be screened were added to the enzyme solution by using Multidispenser ADS-384-8 (BioTec). Subsequently, 2 μL of the substrate solution (100 μM) was added to 384-well plates by using a Multidrop Combi dispenser and incubated for 180 min at room temperature. After incubation, the fluorescence was measured using an EnVision reader (PerkinElmer, excitation filter: 340 nm, emission filter: 450 nm).

Our in-house natural product library was used as a source of samples for screening for inhibitors of Tdp1. The library contained 29 904 diverse samples of crude metabolites from actinomycetes (23 048 samples), fungi (6328 samples), other bacteria (88 samples), and synthetic compounds (440 samples). All samples were dissolved in DMSO.

Biological Material and Fermentation. An unidentified anamorphic fungi was isolated from plant residue of plant bark. To identify the strain, a partial sequence of rDNA and ITS region sequence was determined (accession number AB698827), and comparison with sequences in the DNA Data Base of Japan revealed that the closest phylogenetic neighbors of the strains were endphytic fungi, *Meliniomyces* spp. (AY762619, AY838789, and AY838785), with a sequence identity of 98%. The strain was cultivated in 500 mL Erlenmeyer flasks containing 50 g of brown rice, 6 g of lyophilized soybean fiber, and 100 mL of base medium (20% sucrose, 0.2% yeast extract) in static culture at 23 °C for 14 days.

Isolation. The cultures (10 flasks) were extracted with EtOH (1 L), filtered, and concentrated *in vacuo*. After lyophilization, the residue (31.3 g) was dissolved in $CHCl_3$, and then the soluble fraction was evaporated to dryness *in vacuo*. The residue (2.63 g) was purified using normal-phase medium-pressure liquid chromatography with stepwise elution of $CHCl_3$ and $CHCl_3$−MeOH (19:1) to yield **1** (509 mg) from the $CHCl_3$−MeOH eluate.

JBIR-21 (1): colorless oil; $[\alpha]^{25}_D$ +71.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 267 (3.59) nm; IR (KBr) ν_{max} 1725, 1645, 1605 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$), see Table 1; HRESIMS *m/z* 475.2722 [M + H]$^+$ (calcd for $C_{27}H_{39}O_7$, 475.2716).

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Methylation of 1. Compound **1** (5.2 mg) was dissolved in 1 mL of MeOH and then mixed with 200 μL of TMS−diazomethane solution. The solution was subsequently allowed to stand at room temperature for 2 h. The mixture was then diluted with MeOH, evaporated *in vacuo*, and subjected to preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column (20 i.d. × 150 mm) developed with 80% aqueous MeOH containing 0.1% formic acid (flow rate 10 mL/min) to give JBIR-21 methyl ester (t_R = 26.2 min, 4.2 mg). Furthermore, JBIR-21 methyl ester (4.2 mg) was dissolved in 500 μL of DMF and mixed with 0.5 mg of Ag_2O and 50 μL of methyl iodide. The solution was then stirred at 50 °C for 16 h. Next, the mixture was diluted with H_2O, extracted with EtOAc, evaporated *in vacuo*, and subjected to preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column (10 i.d. × 150 mm) developed with 80% aqueous MeOH containing 0.1% formic acid (flow rate 4 mL/min) to give 22-methoxy-JBIR-21 methyl ester (t_R = 17.0 min, 0.8 mg). The structure was confirmed by LC-MS and NMR analyses.

Growth Inhibition Assay. Human cancer cell lines used in this study were cervical carcinoma HeLa cells, malignant mesothelioma NCI-H2052 cells, colon adenocarcinoma HT-29 cells, and lymphoblastoid namalva cells. The cells were maintained in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (Gibco), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37 °C in a humidified incubator with 5% CO_2 atmosphere. The cytotoxicity of the cancer cell lines was assayed with the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt] colorimetric assay. The 384-well plates were seeded with aliquots of a 20 μL medium containing 1.0 × 10^3 cells per well and were incubated overnight before being treated with compounds at various concentrations for 48 h. Plates were incubated for 1 h at 37 °C after the addition of 2 μL of WST-8 reagent solution (cell counting kit; Dojindo) per well. The absorption of the formazan dye formed was measured at 450 nm. The vehicle solvent (DMSO) was used as a negative control.

***In Vivo* Studies with a Xenograft Model.** Five-week-old female nude mice of the KSN strain were purchased from Shizuoka Laboratory Animal Center. HT-29 cells (5 × 10^6 cells/0.1 mL) were injected subcutaneously into the back of the nude mice (age, 5 weeks). The drug treatment was initiated 7 days after inoculation when the volume of the tumors became approximately 50 mm^3. Compound **1** (2 mg/kg) dissolved in DMSO (final concentration, 1.6%) and phosphate-buffered saline was intraperitoneally administrated once every other day for 19 days (n = 6). Necropsy was performed on day 21 after the final administration of **1**, and tumor weight was determined. The tumor weight was analyzed for statistical significance by using two sample t tests. The experiments were carried out with the approval of the Institutional Ethical Committee for Animal Experiments of Aichi Cancer Center Research Institute.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR, DQF-COSY, HSQC, HMBC, and HRESIMS spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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