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Nidurufin as a New Cell Cycle Inhibitor from Marine-derived Fungus *Penicillium flavidorsum* SHK1-27

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A new cell cycle inhibitor, nidurufin (**1**), was isolated from the marine-derived fungus *Penicillium flavidorsum* SHK1-27. An evaluation of antitumor activity indicated that **1** induced *in vitro* cell cycle arrest at G_2/M transition in the K562 cell line in a concentration and time-dependent manner, with an IC_{50} value of 12.6 μM.

Key words: *Penicillium flavidorsum* SHK1-27, Nidurufin, Cytotoxicity, Cell cycle inhibitor

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INTRODUCTION

Marine fungi constitute one of the richest sources of bioactive secondary metabolites (Blunt et al., 2010; Ebada et al., 2010). In continuation of our projects designed to screen for novel anticancer agents derived from marine fungi, the ethyl acetate extract of a fungus *Penicillium flavidorsum* SHK1-27 exhibited potential cytotoxicity against the K562 cell line. The bioactive constituents of this strain were investigated through a bioassay-guided isolation procedure. The investigation resulted in the isolation of 2 bioactive anthraquinone derivatives: nidurufin (**1**) (Murphy and Cava, 1984; Ren et al., 2007) and averantin (**2**) (Birkinshaw et al., 1966; Ren et al., 2007). **1** and **2** are secondary metabolites which commonly occur in certain strains of *Aspergillus flavus* and *A. parasiticus* (Murphy and Cava, 1984; Yabe et al., 2003). Several bioactivities such as antimicrobial activity (Maskey et al., 2003) and anticancer activity by nidurufin derivatives substituted with low alkyl and phosphono functional groups (Mizukami et al., 1997) have been reported. The present study provides the first example of nidurufin as a human myeloid leukemia K562 cell cycle inhibitor.

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MATERIALS AND METHODS

Marine fungus material

The producing strain was separated from marine sediment samples collected from Weizhou Island, China, in July 2004, and was frozen and kept at −20°C until fermentation. Taxonomically, the strain belongs to *Penicillium flavidorsum*, which has vegetative mycelia that are submerged within the first couple of millimeters on an agar surface. The orange conidiophores, which appear slightly curled, form directly from the submerged hyphae. This strain grows rapidly. The voucher specimen was deposited in the Laboratory of Natural Products Chemistry of the Ocean University of China.

General experimental procedures

Melting points were measured using a Yanaco MP-500D micro melting point apparatus and were uncorrected. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^{1}H-, ^{13}C-NMR, DEPT spectra, and 2D NMR were recorded on a JEOL Eclips-600 spectrometer using TMS as an internal standard and

chemical shifts were recorded as δ values. ESI MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semi-preparative HPLC was performed using an ODS column [Shin-pak ODS (H), 20 × 250 mm, 5 μm, 4 mL/min]. A flow cytometer (Becton-Dickinson, Vantage) was used to analyze inhibitory effects on cell cycles.

Isolation and purification

The producing strain was incubated on a rotary shaker at 120 rpm and 28°C for 9 days in one hundred 500 mL Erlenmeyer flasks, each containing 150 mL of liquid medium composed of mannitol 2%, glucose 1%, maltose 2%, monosodium glutamate 1%, corn syrup 0.1%, KH_2PO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.03%, yeast extract 0.3%, and sea-water; the final pH was adjusted to 6.5. The fermented broth was filtered through cheesecloth to separate the supernatant and the mycelia, and both fractions were extracted 3 times with EtOAc, respectively. Both EtOAc extracts demonstrated similar cytotoxicity on K562 cells. The extracts were combined and concentrated under reduced pressure to give a crude extract (27.2 g), which was further chromatographed over silica gel H by a stepwise elution with petroleum ether-$CHCl_3$-MeOH solvent to produce 4 fractions designated as A, B, C, and D, respectively. Among them, fractions B and D exhibited cytotoxicity on K562 cells. Fraction B (234 mg) was subjected to liquid chromatography over silica gel using petroleum ether-EtOAc (5:1 to 3:1) and Sephadex LH-20 chromatography with $CHCl_3$-MeOH (1:2), to give **1** (12.4 mg). Fraction D (272 mg) was subjected to liquid chromatography over silica gel with petroleum ether-EtOAc (3:1 to 1:1), and to repeated Sephadex LH-20 chromatography with $CHCl_3$-MeOH (1:1) to give **2** (11.0 mg).

Nidurufin (1)

Crystalline powder with orange-red color (from $CHCl_3$ solution), m.p. 188-190°C; $[\alpha]_D^{20}$ +157° (*c* 0.05, MeOH); IR ν_{max} (KBr) cm^{-1}: 3409br, 3184br, 2925, 2853, 1725, 1659, 1606, 1566, 1467, 1407, 1255, 1149; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ 12.48 (1H, brs, OH-1), 12.06 (1H, s, OH-8), 7.09 (1H, s, H-5), 6.99 (1H, s, H-4), 6.58 (1H, s, H-7), 5.39 (1H, d, *J* = 3.7 Hz, H-1'), 3.77 (1H, brs, H-2'), 2.16-1.82 (2H, m, H-3'), 1.57 (2H, m, H-4'), 1.56 (3H, s, H-6'); ^{13}C-NMR (DMSO-d_6, 150 MHz): δ 188.8 (C-9), 180.8 (C-10), 165.3 (C-6), 164.3 (C-1), 159.9 (C-8), 158.4 (C-3), 134.9 (C-4a), 133.4 (C-10a), 115.0 (C-2), 108.9 (C-5), 108.7 (C-8a), 108.4 (C-9a), 108.1 (C-7), 107.4 (C-4), 101.5 (C-5'), 70.7 (C-1'), 63.5 (C-2'), 30.1 (C-3'), 27.2 (C-6'), 22.7 (C-4').

Averantin (2)

Crystalline powder with orange color (from $CHCl_3$ solution), m.p. 279-280°C, $[\alpha]_D^{20}$ −84° (*c* 0.0016, dioxane); IR ν_{max} (KBr) cm^{-1}: 3400br, 2978, 2896, 1624, 1610, 1579, 1472, 1391, 1317, 1165; ^{1}H-NMR (DMSO-d_6, 600 MHz): δ 12.69 (1H, s, OH-1), 12.09 (1H, s, OH-8), 7.07 (1H, d, *J* = 2.2 Hz, H-5), 7.06 (1H, s, H-4), 6.55 (1H, d, *J* = 2.2 Hz, H-7), 5.14 (1H, dd, *J* = 5.2 Hz, H-1'), 1.81-1.72 (2H, m, H-3'), 1.72-1.42 (2H, m, H-2'), 1.33-1.27 (2H, m, H-4'), 0.94 (2H, m, H-5'), 0.85 (3H, t, *J* = 6.6 Hz, H-6'); ^{13}C-NMR (DMSO-d_6, 150 MHz): δ 188.9 (C-9), 181.0 (C-10), 165.1 (C-8), 164.2 (C-1), 163.3 (C-6), 160.8 (C-3), 134.9 (C-4a), 132.9 (C-10a), 122.2 (C-2), 108.9 (C-5), 108.7 (C-9a), 108.4 (C-8a), 108.2 (C-7), 108.0 (C-4), 66.9 (C-1'), 35.4 (C-2'), 31.1 (C-3'), 24.8 (C-4'), 22.1 (C-5'), 14.0 (C-6').

Cell cultures

K562 cells were routinely maintained in RPMI-1640 medium supplemented with 10% FBS under a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

In vitro cytotoxicity assay

Cytotoxicity was measured by the sulforhodamine B (SRB) method as previously reported (Liu et al., 2005). Exponentially growing K562 cells were suspended in fresh RPMI-1640 medium at a density of 20×10^5 cells/mL. A 200 μL aliquot of the cell suspension was seeded into every well of a 96-well plate and 2 μL of compound sample was added into each well and cultured at 37°C for 24 h. The cells were fixed with cold 20% trichloroacetic acid on ice for 30 min, then washed with sterilized water and stained with 0.4% SRB dissolved in 1% acetic acid. The unbound SRB dye was removed and the protein-bound SRB dye was dissolved with 10 mM (pH 10.5) unbuffered Tris base [tris (hydroxymethyl) aminomethane] for determination of optical density. Absorbance of the solubilized SRB dye was measured at 540 nm with a SPECTRA MAX Plus spectrophotometer. The inhibition rates (IR%) were calculated using mean OD values from IR% = $(OD_{control} - OD_{sample})/OD_{control} \times 100\%$. The IC_{50} value, which was defined as the concentration of sample needed to reduce absorbance by 50% relative to the vehicle-treated control, was determined using the Bliss method. The same experiment was repeated 3 times to obtain a mean IC_{50} and its standard deviation.

Cell cycle progression analysis

A flow cytometry assay was performed according to the previously reported method (Dhulipala et al., 2004; Ren et al., 2006). Cells were plated at a density

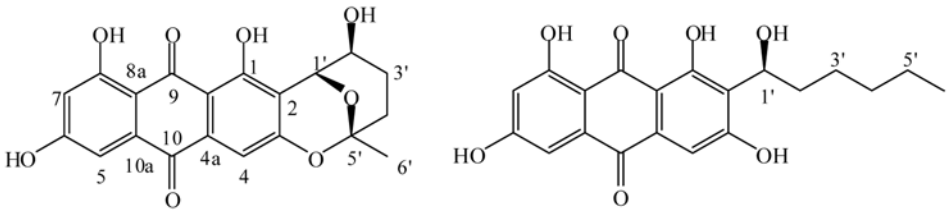

Fig. 1. The structures of **1** and **2**

of 20×10^5 cells/mL in the absence or presence of the indicated concentrations of compounds **1** or **2**, and cultured at 37°C for 24 h. The treated cells were washed twice in PBS and stained with 150 μL propidium iodide (PI) in water solution at 4°C for 30 min under light-proof conditions. The cell cycle distribution was determined by flow cytometry analysis using WinCycle™ (Beckman-Coulter) computer software.

RESULTS AND DISCUSSION

Compound **1** was obtained as a crystalline powder with orange-red color and a m.p. of 188-190°C. It showed a positive color reaction with the ferric chloride reagent, indicating **1** has a phenolic structure. The positive and negative ESI MS of **1** gave *quasi*-molecular ion peaks at *m/z* 385 $[M+H]^+$ and *m/z* 767 $[2M-H]^-$, respectively, which indicated a molecular weight of 384 and a formula of $C_{20}H_{16}O_8$. The ^{1}H- and ^{13}C-NMR including DEPT spectra depicted **1** as possessing 2 carbonyls (δ_C 188.8 and 180.8), 12 aromatic carbons including 4 oxygen-bearing carbons, 5 quarternary carbons, and 3 methines (δ_C 107.4, δ_H 6.99; δ_C 108.1, δ_H 6.58; δ_C 108.9, δ_H 7.09), as well as 1 acetal carbon (δ_C 101.5), 2 oxymethines (δ_C 70.7, δ_H 5.39; δ_C 63.5, δ_H 3.77), 2 methylenes (δ_C 22.7, δ_H 1.57; δ_C 30.1, δ_H 1.82 and 2.16), and 1 methyl (δ_C 27.2, δ_H 1.56). Two proton signals of chelated phenolic hydroxyl (δ_H 12.48 and 12.06) were also observed. The structure of **1** was finally determined as nidurufin by ^{1}H ^{1}H COSY and HMBC experiments, as well as by comparison with the reported data (Murphy and Cava, 1984). Furthermore, **1** is an optically active compound and has a specific rotation of +157° (*c* 0.05, MeOH), similar to the reported value (+165°, *c* 0.07, MeOH) for nidurufin (Lee et al., 2010), which confirmed its structure.

Compound **2** showed the same color reaction with the ferric chloride reagent as observed in the test for **1**, indicating it was phenolic in structure. **2** also gave IR absorption and NMR signal patterns similar to those of **1**, indicating that it was an anthraquinone derivative. Further, by comparing its spectral and physiochemical data with those reported values (Aucamp and Holzapfel, 1970; Shier et al., 2005), compound **2** was identified as averantin.

The cytotoxicity assay was carried out *in vitro* on K562 cells by the SRB method. **1** and **2** showed cytotoxicity with IC_{50} values of 12.6 and 27.7 μM, respectively. Compared with the positive control consisting of C-DDP (C-DDP IC_{50} value on K562 cell is 0.078 μM), **1** and **2** exhibited moderate cytotoxicity on K562 cells.

Additionally, we studied the effects of **1** and **2** on the cell cycle distribution of K562 cells by flow cytometric analysis. **1** increased the percentage of the G_2/M phase to some extent at the concentrations of 1.0 μM to 100.0 μM (shown in Fig. 2), while **2** had no effect on K562 cell cycle progression at the same concentrations. The lowest concentration of **1** which had an effect on the cell cycle was 5.0 μM. After 17 h of treatment with 5.0 μM **1**, the cell population in the G_2/M phase increased from 10.6% to 26.6% compared to control, whereas in cells treated with 50.0 μM **1**, the G_2/M percentage increased to 89.4%. The increase of cell population at the G_2/M phase was correspondingly accompanied by a decrease of cell population in the G_0/G_1 and S phases of the cell cycle. Although the concentration reached a value as high as 100.0 μM, the G_2/M percentage remained unchanged. The findings are summarized in Fig. 2. The effect of **1** on the K562 cell cycle indicated that **1** induced a G_2/M block in a concentration-dependent fashion. Within the range of 5.0 to 50.0 μM, higher concentrations of **1** produced, greater increases in the G_2/M population.

In addition, as shown in Fig. 3, the cell cycle inhibitory effect of **1** was studied in detail as a function of time. When the K562 cells were treated for 8 h at a concentration of 10.0 μM, the cell population at the G_2/M phase increased to 29.6%, from a prior level of 10.4%. The increase in the G_2/M population was evidently differentiated from the control cells. With the extension of experimental time, the cells in G_2/M phase gradually increased and the cells in G_0/G_1 and S phases decreased correspondingly. These changes became maximal with treatment for 36 h, and the G_2/

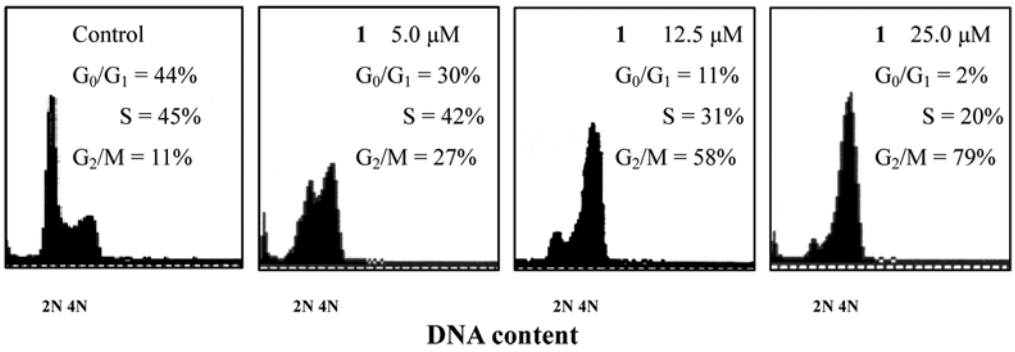

Fig. 2. K562 cells were treated with **1** for 17 h at the concentrations indicated. The percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis as described in Materials and Methods. It showed that 1 induced a G_2/M arrest in a concentration-dependent fashion.

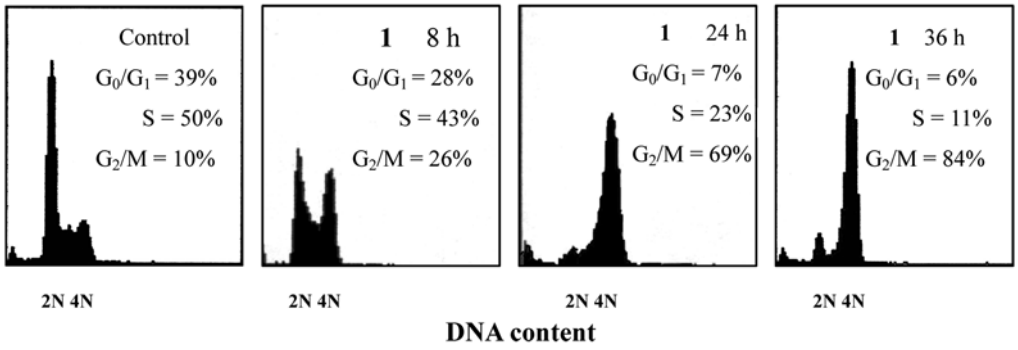

Fig. 3. K562 cells were treated with 10.0 μM **1** for indicated time. The percentage of cells in each phase of the cell cycle was determined by flow cytometric analysis as described in Materials and Methods. It showed that **1** induced a G_2 /M arrest in a time-dependent manner.

M population reached 84.2%, while there was no further change for the ensuing 48 h of observation. The above inhibitory effect showed that **1** caused G_2/M arrest in a time-dependent manner.

In the present study, polyhydroxy-anthraquinone derivatives **1** and **2** were isolated from the secondary metabolites of *P. flavidorsum* SHK1-27, and their antitumor activities were assayed by the SRB method and flow cytometric analysis using K562 cells. **1** inhibited the cell cycle of K562 cells at the G_2/M phase in a concentration and time-dependent manner. To our knowledge, this is the first report describing the inhibitory effects of polyhydroxy-anthraquinones on a human cell cycle, and the present result supports nidurufin as a new G_2/M inhibitor in K562 cells. In conclusion, the ability of **1** to block the progression of the K562 cell cycle suggests that its potential for impeding human tumor cell proliferation needs to be studied in detail.

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