Protein Science Key Laboratory of the Ministry of Education[1], Department of Biological Sciences and Biotechnology, School of Medicine, Tsinghua University, Beijing, and School of Pharmaceutical Sciences[2], Jinan University, Guangzhou, People's Republic of China

Dendroflorin retards the senescence of MRC-5 cells

JINGHUA JIN[1], YU LIANG[1], HONG XIE[1], XUE ZHANG[1,2], XINSHENG YAO[2], ZHAO WANG[1]

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Zhao Wang, Protein Science Key Laboratory of the Ministry of Education, Department of Biological Sciences and Biotechnology, School of Medicine, Tsinghua University, 100084, Beijing, China
zwang@tsinghua.edu.cn

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The influence of dendroflorin, a potentially active compound extracted from *Dendrobium nobile*, on cell growth and cell cycles was assessed in a human embryo lung fibroblastic MRC-5 cell line. An MTT assay indicated that dendroflorin benefits cell proliferation. Additionally, we used flow cytometry to examine the cell cycles in senescent MRC-5 cells after treatment with dendroflorin, discovering that dendroflorin could trigger cells in the G1 phase to enter the S phase. Further examination of intracellular ROS contents using DCFH-DA suggested that dendroflorin helps ROS degradation, partly elucidating the mechanism of its effects. All of the above results suggest that dendroflorin is a potential candidate with anti-senescence activity.

The accumulation of senescent cells prognosticates the age-related decline in tissue/organ functions (Faragher and Kipling 1998). It is widely accepted that senescent cells possess a predominant characteristic whereby they cease division at the G1 phase and maintain a low rate of metabolism. It has been reported that supplementation of the growth medium of human fibroblast cells with hydrocortisone or dexamethasone to reduce the G1 phase leads to a substantial increase (30%) in the replicative lifespan (Cristofalo 1975; Grove and Cristofalo 1977). What's more, cultured cells can be induced to senesce under various conditions including serum starvation, addition of sublethal cell toxicants into medium and transformation of oncogenes, demonstrating that the process of senescence could be interfered with. Although the mechanism of cell senescence is very complicated, common consensuses have been reached. Accumulated reactive oxygen species (ROS) are considered the most likely cause of cell senescence (Finkel and Holbrook 2000) because of their remarkable impairment of macromolecules in cells, including proteins, DNA and telomeres (Kovtun et al. 2007). Oxidative DNA damages trigger p53-dependent G1 growth arrest, following the expression of $p21^{Waf\text{-}1/SDI\text{-}1/Cip1}$ (Chen et al. 1998) and cell senescence in normal human fibroblasts. Intracellular ROS levels are thus one of the best parameters used not only to judge, but also to predict the living state of the cells.

Efforts to discover active anti-aging substances have never ceased. A previous study showed that genus *Dendrobium* has anti-aging effects (Shi and Huang 1994), even though the active components have not been identified. Dendroflorin is a kind of fluorenone compound extracted from *Dendrobium nobile* (Talapatra et al. 1984), with a molecular weight of 258. Compounds extracted from *Dendrobium* species have been shown to possess various medical benefits (Fan et al. 2001). Dendroflorin might be such a component, and an anti-aging activity might be one of its biological properties.

Thus, we added dendroflorin into culture medium to check its influence on cell growth and cell cycles. It was observed that the viability of the cells with 24 h exposure to dendroflorin increased evidently at concentrations greater than 2 μM relative to the control (as shown in Fig. 1A), indicating that dendroflorin helped senescent cells proliferate. SA-β-Gal staining, which has been widely used as an apparent biomarker to identify senescent cells (Dimri et al. 1995), showed that the level of SA-β-Gal was obviously decreased after treatment of dendroflorin (data not shown), suggesting that a part of senescent cells were reversed from senescence. However, it remained unclear whether senescent cells could indeed continue to divide under treatment of dendroflorin. Therefore, to confirm whether senescent cells were indeed propelled to proliferate by dendroflorin, flow cytometry was introduced to check the cell cycle phase alteration of the senescent MRC-5 cells after treatment with dendroflorin. In our study, we observed that with the concentration of dendroflorin increased, the ratio of cells in the G1 period was reduced gradually. On the contrary, more cells were driven to enter the S phase, preparing for cell division (Fig. 1B). Although no substantial cell division was observed in the short period of treatment, these data provide reasonable evidence that the senescent MRC-5 cells had gained the potential to divide and proliferate.

In order to investigate the mechanism of dendroflorin on senescent cells, we examined one commonly accepted cause of senescence, intracellular ROS accumulation. The fluorescent dye DCFH-DA was used to measure ROS contents with flow cytometry. It was shown that compared with the control, the ROS contents were decreased evidently in cells incubated with 2 to 30 μM dendroflorin (Fig. 2). These results indicate that dendroflorin might act as an antioxidant, lowering ROS production or accelerating ROS removal. Interestingly, at a concentration of 1 μM, the ROS contents appeared to be even higher than control. We suspect that at such low concentrations, its antioxidating ability dose not balance the ROS production in the process of proliferation. As previously reported, ROS accumulation causes oxidative stress and several subsequent biochemical events such as p53 activation and $p21^{Waf\text{-}1/SDI\text{-}1/Cip1}$ upregulation (Chen et al. 1998), so dendroflorin should have corresponding effects on these molecules. Indeed, $p21^{Waf\text{-}1/SDI\text{-}1/Cip1}$ was confirmed to be down-regulated in a dose-dependent manner through incubation with high concentrations of dendroflorin (more than 2 μM), using RT-PCR and Western blotting (data not shown). However, when it came to low concentrations, for example 1 μM, $p21^{Waf\text{-}1/SDI\text{-}1/Cip1}$ and p53 were increased, while c-JUN was decreased.

Generally speaking, the effects of dendroflorin on MRC-5 cells could be divided into two aspects: to promote cell proliferation and to reduce ROS accumulation. Cell proliferation causes production of ROS, and dendroflorin itself

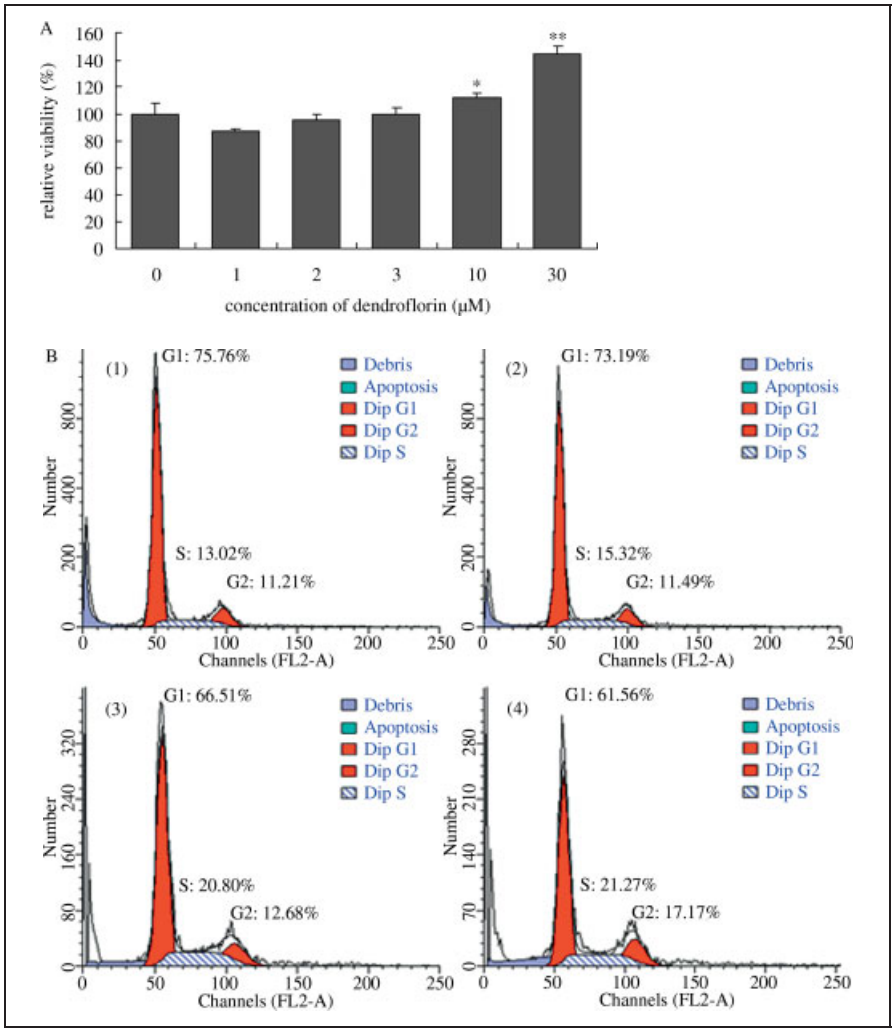

Fig. 1:
Effects of dendroflorin on cell proliferation (A) and cell cycle (B). (A) Histograms in the figure represent non-treated cells, cells treated with 1, 2, 3, 10 and 30 μM of dendroflorin, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$. Three independent assays were performed, and data shown are the mean ± S.D. Analyzed by one-tailed t test. (B) (1) Non-treated MRC-5 cells at late cell population doublings; (2), (3) and (4) represent cells treated with dendroflorin at 0.3, 1 and 3 μM, respectively. Two independent assays were performed and one typical result was presented

can reduce ROS contents. However, the ability to reduce ROS depends on the concentration. Therefore, at high concentrations (more than 2 μM) it benefited cell proliferation while at low concentrations (less than 1 μM), as intracellular ROS accumulates, no evident increase in cell viability was observed (Fig. 1A).

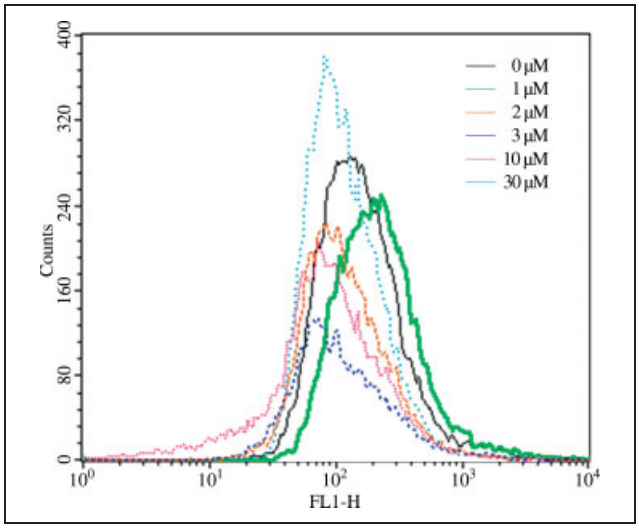

Fig. 2: Dendroflorin decreases the level of intracellular ROS. Different colors indicate dendroflorin at concentrations of 0, 1, 2, 3, 10, 30 μM as labeled in the legend. Experiments were performed three times independently, and a representative histogram for the ROS contents vs cell distribution was presented

Based on these facts, we conclude that dendroflorin has anti-senescence activity at least in MRC-5 cells. It benefits cell proliferation, while reducing the intracellular ROS contents. However, its effect depends on the concentration.

In summary, the results of our study show that dendroflorin can reduce ROS accumulation, promote MRC-5 cells from the G1 phase into the S-phase and thus improve proliferation of the cells. Although the precise mechanism of cell senescence is still unknown and the network of cell cycle regulation remains controversial, we have demonstrated that dendroflorin can protect cells from oxidative damage. We therefore speculate that dendroflorin might be a good candidate for regulating senescence, even though the precise underlying mechanism remains an open question and further studies are in progress.

Experimental

1. Chemicals

Dendroflorin was isolated as follows: using silica gel-filtration chromatography, ODS chromatography, Sephadex LH-20 column chromatography and reverse high pressure liquid chromatography, dendroflorin was purified from the ethyl acetate extract of 60% alcohol solution of *Dendrobium nobile*. DMSO was used as solvent to dilute dendroflorin.

2. Cell culture and treatment

Human embryo lung fibroblastic cell line MRC-5 cells were obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS. Cells were maintained in modified minimum essential medium (Gibco/BRL) and supplemented with 10% fetal bovine serum. MRC-5 cells undergo replicative senescence after multiple cell passages, assessed by the slowing of metabo-

lism and cessation of division within 7 days. All cultures were seeded at a cell density of $1 \times 10^4/cm^2$ unless otherwise noted, and were allowed to proliferate, undisturbed, for 7 days. Various concentrations of dendroflorin were added 24 h before examination with solvent as control, unless otherwise stated. The concentration of DMSO in cultures was less than 0.3% (v/v).

3. MTT assay

The method described by Chiba et al. (1998) was used in this study. In brief, the cells were seeded into 96 well microplates at a density of 2.5×10^3 cells/0.2 ml per well. 20 h later, the cells were in a subconfluent state and were exposed to dendroflorin, followed by 24 h incubation with 5% CO_2 at 37 °C. 25 µl MTT/PBS solution [3-(4,5-dimethythiazolzyl)-2, 5-diphenyl tetrazolium tromide, 10 mg/ml (Sigma)] was then added, and 4 h later, the formazan precipitate was dissolved in 0.2 ml DMSO. The absorbance values of each well were assayed spectrophotometrically at 490 nm on the spectrophotometer using a Microplate Reader (BIO-TEK, Rockville, MA, USA).

4. Flow cytometry analysis

Flow cytometry was introduced to measure the DNA contents and intracellular ROS levels using propidium iodide and DCFH-DA respectively. Cells were plated at a density of 1×10^5 cells/10 ml cell solution in 100 mm diameter dishes and supplemented with dendroflorin 20 h after cell seeding. After 24 h treatment, cells were harvested and washed in PBS after centrifugation, then resuspended in PBS solution with 0.1% RNase and 50 µg/ml propidium iodide or culture medium containing 10 µM DCFH-DA for 30 min. DNA or ROS contents were then determined by fluorescence-activated cell sorting on a Becton-Dickinson FACScan Flow Cytometry System (Becton Dickinson, Franklin Lakes, NJ, USA). The data were analyzed by CellFIT software.

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Key Laboratory of Marine Drugs, Chinese Ministry of Education, Institute of Marine Drugs and Food, Ocean University of China, Qingdao, People's Republic of China

A new diketopiperazine alkaloid isolated from an algicolous *Aspergillus flavus* strain

AIQUN LIN, YUCHUN FANG, TIANJIAO ZHU, QIANQUN GU, WEIMING ZHU

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Prof. Qianqun Gu, Institute of Marine Drugs and Food, Ocean University of China, 5 Yushan Road, 26603 Qingdao, P.R. China
guqianq@ouc.edu.cn, weimingzhu@ouc.edu.cn

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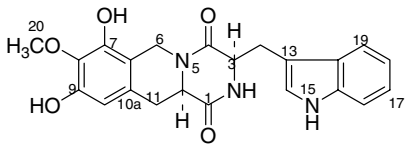
A new diketopiperazine alkaloid containing the uncommon amino acid L-7, 9-dihydroxy-8-methoxyphenylalanine (**1**), has been isolated from the algicolous *Aspergillus flavus* strain. The structure of **1** including the absolute stereochemistry was determined by spectroscopic data and chemical means. Compound **1** showed weak cytotoxicity against HL-60 cell lines with an IC_{50} value of 36.5 µg/ml.

Chemical studies of the metabolites of *Aspergillus* sp. have led to the discovery a variety of new natural products with cytotoxic and other biological activities (de Guzman et al. 1992; Cui et al. 1997), and even after investigations spanning over two decades, the genus still continues to provide metabolites with novel structures and interesting biological activities (Jian et al. 2004). In the course of searching for new antitumor compounds from marine-derived microorganisms, one new diketopiperazine (**1**) was isolated from the algicolous *Aspergillus flavus* strain, collected in Putian Pinghai, China. In this paper we describe the isolation and structure elucidation of **1**, and its cytotoxicity.



1

Compound **1** was obtained as amber amorphous solid. High-resolution ESI-MS supported the molecular formula $C_{22}H_{21}N_3O_5$ (m/z 408.1574 $[M + H]^+$, calcd. for 408.1559). The IR spectrum showed the presence of hydroxyl and amide carbonyl groups. The ^{13}C NMR and DEPT spectra displayed the characteristic structure of a diketopiperazine ring system, which include the two amide carbonyl groups at C-1 (δ_C 165.4) and C-4 (δ_C 164.0), two methine residues at C-3 (δ_C 55.7) and C-11a (δ_C 54.5) (Young et al. 2006). Careful analysis of the 1D NMR spectra indicated the presence of one disubstituded benzene ring, an N-H proton (δ_H10.82, s) and an aromatic proton (δ_H 6.91, d, J = 2.28) characteristic of a tryptophan residue (Santamaria et al. 1999), and one tetra-substituded phenylalanine residue (three of the substituted