The p53 pathway is synergized by p38 MAPK signaling to mediate 11,11′-dideoxyverticillin-induced G_2/M arrest

Yi Chen[a,c], Ze-Hong Miao[a], Wei-Min Zhao[b], Jian Ding[a,*]

[a] *Division of Anti-tumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Science, Chinese Academy of Sciences, Shanghai, PR China*
[b] *Department of Phytochemistry, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, PR China*
[c] *Graduate School of the Chinese Academy of Sciences, PR China*

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Abstract The phytochemical 11,11′-dideoxyverticillin, derived from the fungus *Shiraia bambusicola*, has been shown to possess potent anticancer activity in vitro and in vivo. Here, we investigated the effect of 11,11′-dideoxyverticillin on cell cycle progression, and explored the potential mechanisms for this effect. A concentration- and time-dependent cell cycle blockade at G_2/M phase was observed in human colon cancer cells (HCT-116) following 11,11′-dideoxyverticillin treatment and was associated with marked increases in levels of p53, phospho-p53(ser20) and phospho-Chk2(Thr 68). When wild type p53 expression was specifically inhibited by RNA interference, HCT-116 cells treated with 11,11′-dideoxyverticillin failed to arrest in G_2/M and did not show increased phospho-Chk2(Thr 68). On the other hand, 11,11′-dideoxyverticillin treatment also elicited p38 MAP kinase activity and expression of phospho-p38 MAPK. Treatment with a specific p38 MAPK inhibitor (SB203580) successfully inhibited p38 MAPK and delayed the onset of G_2/M arrest induced by 0.5 μM 11,11′-dideoxyverticillin after approximately 6 h, but did not abolish the induction of G_2/M arrest. Additionally, SB203580 did not alter the levels of p53, phospho-p53 (ser20), or phospho-Chk2 (Thr68) proteins in 11,11′-dideoxyverticillin-treated cells. Together, these findings indicate that p53-mediated phosphorylation of Chk2 maybe plays a vital role in 11,11′-dideoxyverticillin-induced G_2/M arrest, and that p38 MAPK might accelerate this progression. Our work suggests a new possibility of interactions among p53, Chk2 and p38 MAPK signaling in G_2/M arrest.

Keywords: 11,11′-dideoxyverticillin; G_2/M arrest; p53; p38 MAPK; Chk2; Anti-tumor; HCT-116 cell

1. Introduction

Numerous anticancer drugs kill tumor cells by disrupting the cell cycle. Activation of checkpoints in the G_1 and G_2 phases leads to cell cycle arrest, which provides time for repair of damaged DNA or (in case of severe damage) activation of programmed cell death [1–3]. The G_2 checkpoint is intricately regulated by a myriad of protein factors. Among them, p53, checkpoint kinase 1 (Chk1), checkpoint kinase 2 (Chk2) and p38 mitogen-activated protein kinase (p38 MAPK) have been the most extensively investigated [4]. Continued research into cell cycle regulation in general and these factors in specific will allow us to better understand anticancer activities, and may lead to the identification of novel anticancer molecules.

The Chinese folk medicine fungus, *Shiraia bambusicola*, grows on bamboo found primarily in several provinces in the southern China. This fungus contains 11,11′-dideoxyverticillin, a potent small molecule compound (Fig. 1) capable of potently inhibiting tyrosine kinase activity, especially that of epidermal growth factor receptor (EGFR), in both molecular and cellular models. The compound also exhibits cytotoxicity against a broad spectrum of cancer cell lines in vitro, and has been shown to suppress angiogenesis and reduce secretion of VEGF from tumor cells [5,6].

Here, we further confirmed the G_2/M arrest elicited by 11,11′-dideoxyverticillin in HCT-116 cells, examined its effect on wild type p53 and Chk2, defined the role of p38 MAPK, and investigated the possible relationships among these signals.

2. Materials and methods

2.1. Agents

11,11′-dideoxyverticillin was prepared from *Shiraia bambusicola Henn*. The fungus was powdered and refluxed three times with petroleum ether for 1 h each time. The defatted residue was then refluxed twice with 95% ethanol for 2 h each time. The combined ethanol extract was evaporated in vacuo; the aqueous residue was chloroform extracted three more times, and then subjected to column chromatography over silica gel with a petroleum ether-acetone gradient (4:1 → 1:1) as an eluent, to yield white 11,11′-dideoxyverticillin crystals (⩾ 98% pure). The crystals were dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO remaining below 0.1% (v/v) in all 11,11′-dideoxyverticillin treated groups. DMSO 0.1% (v/v) was used as a vehicle control throughout the study.

The p38 MAPK inhibitor, SB203580 (Calbiochem, Darmstadt, Germany), was dissolved in DMSO at a concentration of 50 mM, and stored at −20 °C in the dark.

2.2. Cell culture

HCT-116 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in McCoy's 5A medium (Gibco, Grand Island, NY, USA) with 10% heat-inactivated fetal

*Corresponding author. Fax: +86 21 50806722.
E-mail address: jding@mail.shcnc.ac.cn (J. Ding).

Abbreviations: Chk1, checkpoint kinase 1; Chk2, checkpoint kinase 2; p38 MAPK, p38 mitogen-activated protein kinase; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; PBS, phosphate buffered saline; PI, propidium iodide

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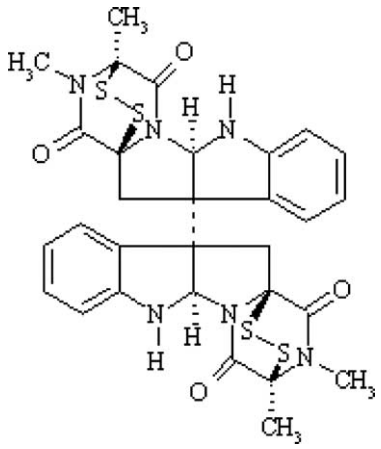

Fig. 1. Chemical structure of 11,11′-dideoxyverticillin.

bovine serum (FBS, Gibco), 100 kIU/L benzylpenicillin, and 100 mg/L streptomycin. Cells were incubated under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment.

2.3. p53 siRNA transfection

Cells (0.8×10^5) were incubated overnight in 24-well dishes in McCoy's 5A medium containing 10% FBS. On day 2, the medium was replaced with fresh medium containing 50 nM p53 siRNA (Cell Signaling Technology, Beverly, MA, USA; 5′-CUACUUCCUGAAAACAACGTT) and Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Forty-eight hours after transfection, cell lysates were prepared for Western blot analysis for detection of p53 expression and cell cycle analysis.

2.4. Cell cycle analysis

The distribution of cells at different stages in the cell cycle was estimated by flow cytometric DNA analysis as described previously [7]. Briefly, 5×10^5 cells were incubated overnight in 6-well dishes in medium containing 10% FBS, then for different times with or without various concentrations of 11,11′-dideoxyverticillin, or with SB203580 pretreatment for 30 min, in HCT-116 cells or in transfected cells. Cells were harvested, washed twice with cold phosphate buffered saline (PBS, pH 7.4), and fixed with 70% ethanol/30% PBS at 4 °C. The fixed cells were incubated with 0.5 ml PBS containing 10 μg/ml RNase for 30 min at 37 °C, then stained with 20 μg/ml propidium iodide (PI, Sigma, St. Louis, MO, USA) for 30 min in the dark at room temperature, and finally analyzed on a FACS cytometer (Calibur™, Becton Dickinson, USA). A minimum of 1×10^4 cells/sample was evaluated, and the percentage of cells in each cell cycle phase was calculated using the CELLQUEST and ModFITLT software packages (Becton Dickinson).

2.5. Western blot analysis

Cell lysates were prepared in lysis buffer (2 mM sodium orthovanadate, 50 mM NaF, 20 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 5 mM sodium pyrophosphate, 10% glycerol, 0.2% Triton X-100, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml aprotinin) on ice for 30 min. Samples were clarified by centrifugation at 4 °C for 15 min at $13\,000 \times g$, and then equal amounts of protein were separated on SDS–polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes. The blots were incubated overnight at 4 °C with the following antibodies: anti-p53 antibody, anti-phospho-p53(ser20) antibody, anti-p38MAPK antibody, anti-phospho-p38 MAPK antibody, anti-Chk1 antibody, anti-phospho-Chk1(Ser 345) antibody, anti-Chk2 antibody and anti-phospho-Chk2(Thr 68) antibody (all diluted 1:1000 and obtained from Cell Signaling Technology, Beverly, MA, USA). Bands were visualized using horseradish peroxidase-conjugated secondary antibodies (Calbiochem, Darmstadt, Germany, diluted 1:2000) followed by enhanced chemiluminescence detection (ECL, Pierce Biotech, Rockford, IL, USA).

2.6. p38 MAP kinase assay

The p38 MAP kinase assay was carried out using the appropriate kit (Cell Signaling Technology) according to the manufacturer's instructions. In brief, 1×10^7 cells were incubated overnight in 6-well dishes in medium containing 10% FBS, then treated for different times with or without various concentrations of 11,11′-dideoxyverticillin, with or without pretreatment with SB203580 for 30 min. Cells were washed with ice-cold PBS and lysed in 500 μl of lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, and 1 mM PMSF). The lysates were sonicated and centrifuged. The resulting supernatants (containing 200 μg total protein) were incubated with an immobilized phospho-p38 MAPK antibody (Thr180/Tyr182) with gentle shaking overnight at 4 °C. The beads were washed twice with 500 μl of lysis buffer and twice with 500 μl of kinase buffer (25 mM Tris pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$), and kinase reactions were carried out in the presence of 200 μM ATP, and 2 μg of ATF-2 at 30 °C for 30 min. Phosphorylation of ATF-2 at Thr71 was selectively measured by Western blotting (Cell Signaling Technology) using a chemiluminescent detection system (Cell Signaling Technology).

3. Results

3.1. Treatment with 11,11′-dideoxyverticillin arrests HCT-116 cells in G_2/M phase

The effect of 11,11′-dideoxyverticillin treatment on cell cycle progression of HCT-116 cells was examined at varying doses and time points by flow cytometry. Treatment with 11,11′-dideoxyverticillin resulted in a concentration- and time-dependent increase in the G_2/M phase cell population and a corresponding decrease in the G_1 and S phase populations (Fig. 2). Treatment with 0.12 μM 11,11′-dideoxyverticillin for 6 h increased the percentage of cells in the G_2/M phase from 15.90% (control) to 16.44%, and treatment of the same duration with 1.0 μM 11,11′-dideoxyverticillin further increased the G_2/M population to 27.48% (Fig. 2(b)). Treatment with 0.5 μM 11,11′-dideoxyverticillin increased the G_2/M population within as little as 1 h, and this population continued to increase with time; at 6 h, the percentage of the cells at G_2/M phase was 30.33% and at 10 h it was 37.99%, significantly different from that of the control (18.73%) (Fig. 2(a)).

3.2. Treatment with 11,11′-dideoxyverticillin enhances levels of p53 and phosphorylated p53 (Ser20)

Treatment with 11,11′-dideoxyverticillin may elicit G_2/M arrest *via* triggering G_2/M checkpoint protein, such as p53 [8–10]. Although the significance of individual residues to p53 activation is not entirely clear, phosphorylation of the serine residue at position 20 appears to be essential to p53 stabilization [11,12]. Accordingly, we investigated whether 11,11′-dideoxyverticillin affected the expression of p53 and phospho-p53(ser20) proteins in HCT-116 cells. Treatment with 11,11′-dideoxyverticillin for 4 h increased the levels of p53 and phospho-p53(ser20) in a dose-dependent fashion (Fig. 3(a)). The same trend was observed when cells were exposed to 0.5 μM 11,11′-dideoxyverticillin for increasing durations (Fig. 3(b)). Notably, the increase in phospho-p53(ser20) seemed to lag slightly behind that of p53, though the levels of both p53 and phospho-p53(ser20) rose time- and dose-dependently (Fig. 3). These data suggest that 11,11′-dideoxyverticillin can promote p53 expression and/or stabilization, and indicate that p53 signaling may participate in 11,11′-dideoxyverticillin-triggered G_2/M arrest.