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Widdrol induces cell cycle arrest, associated with MCM down-regulation, in human colon adenocarcinoma cells

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ABSTRACT

Widdrol, an odorous compound extracted from *Juniperus chinensis*, has been shown to inhibit the *in vitro* growth of in human cancer cells. This study was conducted on cultured human colon adenocarcinoma HT29 cells to elucidate the possible mechanisms by which widdrol exerts its anti-proliferative activity, which until now has remained poorly understood. It was found that widdrol induces accumulation of sub-G1 phase and arrests in the G1 phase of the cell cycle. Induction of G1 arrest by widdrol was correlated with induction of Chk2, p53 phosphorylation and CDK inhibitor p21 expression as well as inhibition of cyclin E, cyclin-dependent kinase (CDK2) and retinoblastoma protein (pRB). Moreover, mini-chromosome maintenance (MCM) proteins were markedly down-regulated in HT29 cells treated with widdrol. Altogether, these results show widdrol possesses potential anti-cancer activity against colon adenocarcinoma cells by inhibiting their proliferation and inducing cell cycle G1 arrest.

1. Introduction

Cell cycle control mechanisms serve major regulatory functions for cell growth. Many cytotoxic agents and/or DNA-damaging agents induce apoptosis by arresting the cell cycle [1,2]. In fact, the anti-cancer properties of many anti-cancer agents act through the induction of cell cycle arrest and/or apoptotic cell death. Cell cycle checkpoints ensure the maintenance of genomic integrity by protecting dividing cells from the potentially fatal consequences of DNA damage, the detection of which relies on a cascade of enzymes that convey the signal(s) generated by different genotoxic stresses that block key cell cycle transitions until DNA repair has occurred [3].

Mini-chromosome maintenance (MCM) proteins, originally identified from yeast *Saccharomyces cerevisiae*, are essential regulators in eukaryotic DNA replication [4]. Several studies have recently reported that MCM proteins are highly expressed in malignant human cancer cells and precancerous cells but not in non-proliferating normal cells, whether originating from either tissue or organ [5–10]. MCM proteins are good diagnostic indicators for a wide variety of cancers as their misexpression is regarded as a feature of cell cycle deregulation that encourages tumorigenesis in cells.

MCM proteins and E2F seem to be related as the promoter region of MCM genes contains several E2F binding sites and MCM proteins are up-regulated in late G1 by E2F. Furthermore, it has been suggested MCM proteins could be the rate-limiting components in the E2F-mediated G1/S transition [11–13]. The progression of eukaryotic cells through the cell cycle is orchestrated by the sequential activation and inactivation of cyclin-dependent kinases

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(CDKs), which are associated with their respective cyclin subunits [14,15]. In addition, cell cycle progression is also regulated by the relative cellular concentrations of CDK inhibitors. The Cip/Kip family contains proteins such as p21/WAF1 and p27/KIP that bind to cyclins/CDK complexes and prevent kinase activation, subsequently blocking the progression of the cell cycle at the G1/S or G2/M phases [16,17].

Many studies have shown that phytochemicals in medicinal and edible plants are important pharmacological agents that possess anti-inflammatory/anti-oxidant and anti-carcinogenic activities [18,19]. Anti-MCM molecules, which down-regulate the activity or expression of MCM proteins, are potential targets for anti-cancer drug development [20]. Therefore, to search for anti-MCM molecules we screened several medicinal plants based on ethnomedical information. Among them, we isolated as a candidate the active compound from *Juniperus chinensis* and identified it as widdrol.

Widdrol, a crystalline, natural substance, is an odorant derivative contained in various plants such as the *Juniperus* sp., including *J. chinensis* [21,22]. Widdrol has been found to possess anti-fungal activity against *Botrytis cinerea* [23]. However, currently the anti-cancer properties of widdrol are not well understood. In this study, we therefore evaluated widdrol-induced growth inhibition to determine if it is related to the down-regulation of MCM expression and induction of cell cycle arrest in human colon adenocarcinoma HT29 cells. Furthermore, the expression levels of several important genes strongly associated with the cell cycle signal transduction pathway were measured to elucidate the anti-cancer mechanism of widdrol.

2. Materials and methods

2.1. Chemicals

Widdrol used in this study was isolated from *J. chinensis* (Fig. 1). The identity of the widdrol was confirmed by ^{1}H NMR, ^{13}C NMR and GC–MS. Widdrol was dissolved in dimethylsulfoxide and stored at −20 °C before the experiments and dilutions were performed in culture medium.

2.2. Cell lines and culture

The human colon adenocarcinoma cell line HT29, human hepatocellular carcinoma cell line HepG2, human lung adenocarcinoma cell line A549, human bladder carcinoma cell line T24, human cervical adenocarcinoma cell line HeLa and human T-leukemia cell line Jurket were purchased from American Type Culture Collection (ATCC, Rockville, MD). HT29, A549, T24 and Jurket were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, Gemcell™, CA), and 0.1% gentamycin. HepG2 and HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose, Gibco) with the same supplement. Cells were grown at 37 °C in humidified conditions of 95% air and 5% CO_2.

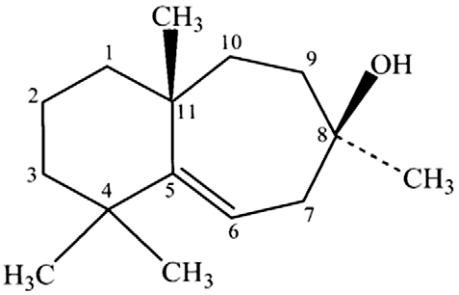

Fig. 1. Chemical structure of widdrol.

2.3. Growth inhibition

Measurement of cell viability was determined using a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzyme.

Anti-proliferative activity was determined by a trypan blue exclusion assay [24]. Briefly, cells were cultured in a 35 mm dish and exposed to widdrol for 7 days. The cells were trypsinized, washed with phosphate-buffered saline (PBS) and trypan blue dye solution was added to the cell suspension. Viable cells were counted with a hemocytometer.

2.4. Flow cytometry analysis of cell cycle

Cells were treated with 0.1% dimethyl sulfoxide (DMSO as vehicle control) or widdrol for 48 h. The cells were then harvested, washed once with cold PBS, fixed in ice-cold 80% ethanol and stored at 4 °C. Prior to analysis, the cells were washed again with PBS and suspended in 1 ml of a cold propidium iodide (PI) solution containing 100 μg/ml RNase A, 50 μg/ml PI, 0.1% (w/v) sodium citrate and 0.1% (v/v) NP-40. Following this cells were incubated on ice for an additional 30 min in the dark. Next, flow cytometry was conducted (FACS Caliber, Becton Dickinson, San Jose, CA) and the CellQuest software was used to determine the relative DNA content based on the presence of red fluorescence.

2.5. Microarray

Total RNA was extracted from the treated (68 μM widdrol) and control human cell line samples using TRI REAGENT (MRC, OH), according to the manufacturer's instructions. In this experiment, we used the Operon Human genome oligo 35 K microarray (OPHSV4, Operon Biotechnologies, GmbH) consisting of 35,159 oligos and representing about 33,005 genes, including ESTs. Microarray experiments were performed according to manufacturer's protocol. Each total RNA sample (30 μg) was labeled with either Cyanine (CY3) or Cyanine (Cy5)-conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction using SuperScrip ll (Invitrogen, Carlsbad, CA). The Cy3- and Cy5-labeled cDNA mixture was then concentrated by ethanol precipitation, followed by resuspension in 30 μl of hybridization solution (GenoCheck, Korea). The two labeled cDNAs were mixed, placed on a Operon Human genome oligo 35 K microarray (OPHSV4,