Biochemical Pharmacology 79 (2010) 1261–1271

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Chamaecypanone C, a novel skeleton microtubule inhibitor, with anticancer activity by trigger caspase 8-Fas/FasL dependent apoptotic pathway in human cancer cells

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ARTICLE INFO

Article history:
Received 13 October 2009
Accepted 15 December 2009

Keywords:
Chamaecypanone C
Microtubule inhibitor
Anticancer
Drug resistance
Fas/FasL

ABSTRACT

Microtubule is a popular target for anticancer drugs. Chamaecypanone C, is a natural occurring novel skeleton compound isolated from the heartwood of *Chamaecyparis obtusa* var. *formosana*. The present study demonstrates that chamaecypanone C induced mitotic arrest through binding to the colchicine-binding site of tubulin, thus preventing tubulin polymerization. In addition, cytotoxic activity of chamaecypanone C in a variety of human tumor cell lines has been ascertained, with IC_{50} values in nanomolar ranges. Flow cytometric analysis revealed that chamaecypanone C treated human KB cancer cells were arrested in G_2–M phases in a time-dependent manner before cell death occurred. Additional studies indicated that the effect of Chamaecypanone C on cell cycle arrest was associated with an increase in cyclin B1 levels and a mobility shift of Cdc2/Cdc25C. The changes in Cdc2 and Cdc25C coincided with the appearance of phosphoepitopes recognized by a marker of mitosis, MPM-2. Interestingly, this compound induced apoptotic cell death through caspase 8-Fas/FasL dependent pathway, instead of mitochondria/caspase 9-dependent pathway. Notably, several KB-derived multidrug resistant cancer cell lines overexpressing P-gp170/MDR and MRP were sensitive to Chamaecypanone C. Taken together, these findings indicated that Chamaecypanone C is a promising anticancer compound that has potential for management of various malignancies, particularly for patients with drug resistance.

1. Introduction

Chemotherapy for the treatment of cancer was introduced into the clinic more than fifty years ago. Most chemotherapeutic anticancer drugs used in the clinic today include agents that target the cell cycle in order to inhibit the hyperproliferation state of tumor cells and – subsequently – to induce apoptosis, which is the desired outcome of chemotherapy [1]. Otherwise, the primary hurdle for effective cancer chemotherapy has been the intrinsic or acquired resistance of cancer cells to a variety of anticancer agents

Abbreviations: CA-4, combretastatin A-4; CCD, charge-coupled device; DIC, differential interference contrast; DMSO, dimethyl sulfoxide; FITC, fluorescent isothiocyanate; GFP, green fluorescent protein; HRP, horseradish peroxidase; MAP, Microtubule-associated protein; MDR, multidrug resistance; MRP, multidrug resistance-associated proteins; PI, propidium iodide; SPA, scintillation proximity assay; VP16, etoposide.

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doi:10.1016/j.bcp.2009.12.017

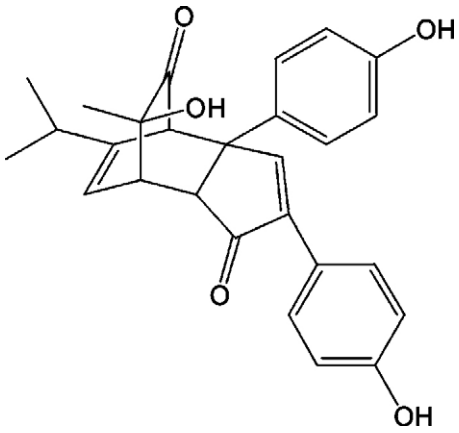

Fig. 1. Chemical structure of Chamaecypanone C.

with distinct chemical structures or mechanism of action, a phenomenon known as multidrug resistance (MDR). The classical form of MDR involves the overexpression of drug efflux transporters such as P-glycoprotien (P-gp170/MDR) and multidrug resistance-associated proteins (MRPs) in the cell membrane, which pump anticancer drugs out of the cells, resulting in low intracellular drug concentrations [2,3]. Therefore, there is necessary to discover the novel chemotherapeutic agents which could overcome MDR.

Historically, plants were a traditional source of medicinal agents. As modern medicine has evolved, numerous useful drugs were developed from lead compounds discovered from plants [4]. For examples, many current chemotherapeutic drugs, including bleomycin, doxorubicin, mitomycin, vinblastine, vincristine, etoposide (VP16), topotecan, irinotecan, paclitaxel and combretastatins, are natural products or their derivatives [5]. Thus, pharmacologically active compounds from plants represent an important pool for new investigative drugs [6–10].

Chamaecyparis obtusa var. *formosana* Rehd. (Taiwan hinoki; Cupressaceae) is a type of timber which is highly available in Taiwan. We have previously investigated the chemical components of the heartwood of this plant and found various monoterpenes, sesquiterpenes, diterpenes and lignans [11–14]. Other than those phytochemicals, a novel skeleton compound, chamaecypanone C (Fig. 1), had been elucidated as a dimeric of monoterpene and norlignan with tricycle[5.2.2.$0^{2,6}$]undecane, also isolated from the heartwood of this plant [12]. Notably, this novel compound exhibited better growth inhibition properties with IC_{50} values ranging from 190 to 520 nM in three different human cancer cell lines, as compare to the clinically available anticancer drug VP16 [12]. However, the detailed molecular functions of this compound have not been dissected previously. In the present study, we investigated the mechanism of action of chamaecypanone C. We further determine the anticancer efficacy of this compound in various human cancer cells with multidrug resistant properties.

2. Materials and methods

2.1. Purification of Chamaecypanone C

The compound was isolated at the College of pharmacy, China Medical University (Taichung, Taiwan) with use of the method described by Chien et al. [12]. In brief, the air-dried slices of heartwood of *C. obtusa* var. *formosana* were extracted with acetone at room temperature. After evaporation of acetone, the extract was partitioned with an ethyl acetate–water mixture to give an ethyl acetate-soluble fraction and an aqueous phase. The ethyl acetate-soluble fraction was repeatedly chromatographed on silicon dioxide column and HPLC [Merck Lichrosorb Si 60, 100 × 10 mm i.d., ethyl acetate–dichloromethane (3:2)] to give chamaecypanone C. The chemical structure of chamaecypanone C was confirmed by ^{1}HNMR, ^{13}CNMR and EI-MS. The stock solution of chamaecypanone C was prepared in dimethyl sulfoxide (DMSO) and kept at −20 °C and diluted in PBS buffer to appropriate concentrations before every experiment.

2.2. Reagents

Colchicine, paclitaxel and vincristine were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies were obtained from following companies: α-tubulin (Sigma), Bcl2, Cdc2, Cdc25C, cyclin B1, Fas and horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phosphospecific MPM-2 (Upstate Biotechnology, Lake Placid, NY), PARP (Severigen, Gaithersburg, MD), Fas-L (Oncogene Research Products, Darmstadt, Germany), FasL neutralizing monoclonal antibody 4A5 (MBL, Nagoya, Japan) and fluorescent isothiocyanate (FITC)-conjugated secondary antibody (Ancell Corporation, Bayport, MN). Recombinant human Fas/Fc chimera was purchased from R&D System (Minneapolis, MN). Medium and reagents of cell culture were acquired from Invitrogen (Carlsbad, CA). Microtubule-associated protein (MAP)-rich tubulin and biotin-labeled tubulin were from Cytoskeleton, Inc. (Denver, CO). [^{3}H]colchicine, [^{3}H]paclitaxel, [^{3}H]vinblastine and streptavidin-labeled poly(vinyl toluene) scintillation proximity assay (SPA) beads were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Other chemicals not specified were from Sigma or Merck (Darmstadt, Germany) with standard analytical or higher grade.

2.3. Cell cultures

Human oral epidermoid carcinoma KB (According to ATCC comments, the KB cell line was originally derived from an epidermal carcinoma of the mouth but has now been shown to have HeLa characteristics), nasopharyngeal carcinoma HONE-1, gastric carcinoma TSGH, hepatocellular carcinoma Hep3B and HepG2 and lung adenocarcinoma CL1-0 and CL1-5 cells were maintained in RPMI 1640 medium supplied with 5% fetal bovine serum. All the above cell lines were procured from American Type Culture Collection (ATCC, Rockville, MD) except CL1-0 and CL1-5 which were gift from Dr. Pan-chyr Yang in National Taiwan University Hospital, Taipei, Taiwan, ROC. The drug resistant cell lines, including KB-TAX50, KB-7D, HONE1-CPT30 were maintained in complete medium containing an additional 50 nM paclitaxel, 7 μM VP16 and 100 nM camptothecin, respectively. KB-TAX50 cells were generated by paclitaxel-driven selection and displayed overexpression of P-gp170/MDR [6,8–10]. KB-7D cells were generated by VP16-driven selection, which displayed down-regulation of Top II and overexpression of MRP [15,16]. HONE1-CPT30 cells have a mutation at topoisomerase I (E418K) resulting in camptothecin resistance [17]. All drug resistant cells were incubated in the drug-free medium for three days before harvesting for the growth inhibition assay.

2.4. Growth inhibition assay

Cells in logarithmic growth phase were cultured at a density of 10,000 cells/mL/well in a 24-well plate. The cells were exposed to various concentrations of the test drugs for three generation times. At the end of the incubation period, cells were fixed and stained with 50% ethanol containing 0.5% methylene blue for 30 min. The