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Anticancer activity of arborinine from *Glycosmis parva* leaf extract in human cervical cancer cells

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ABSTRACT

Glycosmis parva is a small shrub found in Thailand. Ethyl acetate (EtOAc) extract from its leaves has been shown to exert anticancer effects *in vitro*; however, the compound responsible for this activity has not been isolated and characterized. In this study, we demonstrate that arborinine, a major acridone alkaloid in the EtOAc fraction, decreased proliferation and was strongly cytotoxic to HeLa cervical cancer cells without significantly affecting normal cells. The compound also inhibited tumor spheroid growth much more potently than chemotherapeutic drugs bleomycin, gemcitabine, and cisplatin. In addition, unlike cisplatin, arborinine activated caspase-dependent apoptosis without inducing DNA damage response. We further show that arborinine strongly suppressed cancer cell migration by downregulating expression of key regulators of epithelial-mesenchymal transition. Taken together, our data provide important insights into the molecular mechanism of arborinine's anticancer activity, supporting its potential use for treating cervical cancer.

1. Introduction

Cervical cancer is one of the most common gynecological malignancies, causing 10–15% of all female cancer-related deaths worldwide. The availability of vaccines and screening tests has decreased cervical cancer incidence and mortality rates. However, in developing countries—where 85% of cases occur—the incidence remains high [1, 2]. Current cervical cancer treatment can yield cures in 60–90% of women at an early-stages of the disease. However, a majority of patients are diagnosed in the advanced stage, whereby prognosis remains poor [3, 4]. Chemotherapy is often the treatment of choice for advanced, recurrent, or metastatic cervical cancer [4], yet, present chemotherapeutic regimens suffer from non-specific cytoxicity and drug resistance [5]. Therefore, effective anticancer agents with reduced side effects are urgently needed.

Plants represent sources of potentially usable compounds and have a long history in cancer treatment [6]. Moreover, such bioactive compounds are readily available, considered to be less toxic than current chemotherapy, and can be administered as part of dietary intake for chemoprevention [7, 8]. Plant-derived agents with high specificity towards cancer cells but no toxicity to normal cells thus hold great potential for further testing in clinical trials [8].

Glycosmis parva Craib (Rutaceae) is a wild small shrub found in Thailand [9]. The leaf extract from this plant represents a rich source of acridone alkaloids and sulfur-containing propanamide derivatives, shown to exert antimalarial, antiviral, and anticancer effects [10, 11]. Ethyl acetate (EtOAc) extract from *G. parva* leaves has been documented to possess anti-cancer activity by inducing apoptosis and cell cycle arrest in a human colorectal cancer cell line [11]. Further separation of the EtOAc fraction led to identification of arborinine as the major alkaloid and (+)-(*S*)-deoxydihydroglyparvin as the major sulfur-containing propanamide [10]. We hypothesized that either arborinine or (+)-(*S*)-deoxydihydroglyparvin were the main contributors to its anticancer property.

In the present study, we purified and investigated the cytotoxic activities of arborinine and (+)-(*S*)-deoxydihydroglyparvin on cervical cancer cells as compared to normal cells. The compound with

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highly selective anticancer activity was further analyzed for its ability to inhibit multicellular tumor spheroid growth, in comparison to commonly used chemotherapeutic drugs. In addition, we examined the effect of the compound on apoptosis, DNA damage, cancer cell migration, and epithelial-mesenchymal transition (EMT).

2. Materials and methods

2.1. Extraction and structure characterization

Arborinine and (+)-(*S*)-deoxydihydroglyparvin were isolated from the leave extract of *G. parva* collected in Wang Nam Khiao, Nakhon Ratchasima province, Thailand. Dried powdered leaves were macerated with methanol and the crude extract was partitioned with hexane, EtOAc, and water. EtOAc extract was passed through a chromatographic column using silica gel as stationary phase. Arborinine-containing fractions were then eluted with a gradient solvent system of dichloromethane and methanol. Yellow needle-like crystals of arborinine were obtained by recrystallization of the fractions using a dichloromethane-acetone mixture. (+)-(*S*)-deoxydihydroglyparvin was isolated from the EtOAc fraction using Sephadex LH20 with 50% dichloromethane in methanol. A white solid was obtained after further purification with a silica gel chromatographic column using 40% acetone in hexane as eluent. The compounds were then characterized by NMR for structural identification using a Bruker AVANCE III HD 500 MHz instrument.

2.2. Cell culture

Human primary dermal fibroblasts (HDFa; PCS-201-012), HPV-16 E6/E7 transformed ectocervical Ect1/E6E7 (CRL-2614), and HPV-positive HeLa (CCL-2) and SiHa (HTB-35) cell lines were purchased from the American Type Culture Collection (ATCC). Primary fibroblasts were grown in Fibroblast Basal Medium (PCS-201-030, ATCC) supplemented with Fibroblast Growth Kit-Serum-free (PCS-201-040, ATCC). HeLa and SiHa cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; HyClone). Ect1/E6E7 cells were grown in Keratinocyte serum-free medium (SFM) with Keratinocyte-SFM Supplement (Gibco, Thermo Fisher Scientific). All cells were maintained at 37 °C in a 5% CO_2 incubator.

2.3. Cytotoxicity evaluation by MTS cell viability assay

Cells were seeded in 96-well plates and grown in culture medium. After 24 h of incubation, cells were treated with dimethyl sulfoxide (DMSO) or arborinine at the indicated concentration for 48 h. Cell viability was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega). Cell viability was determined by comparison with the DMSO control. Assays were performed in triplicate in three independent experiments.

2.4. Cell proliferation assay using real-time cell analysis (RTCA)

Cell viability and proliferation rate were measured in real-time using an xCELLigence RTCA dual-plate instrument (ACEA Biosciences). Cells were trypsinized, counted, and resuspended in their culture medium. Briefly, 50 μL of the medium was added to the E-plate followed by background measurement. Subsequently, cells were seeded into 100 μL of culture medium in the E-plate. The impedance value was automatically read every 30 min. When cells reached log growth phase, they were treated with 50 μL of the indicated concentration of arborinine or DMSO (solvent control). Impedance signals were recorded every 30 min for another 96 h. Cell proliferation rate was calculated by determining the slope of the line between two given time points. Experiments were performed in triplicate on two independent occasions.

2.5. Multicellular tumor spheroid assay

HeLa cells were seeded in 200 μL of complete growth medium in an ultra-low attachment 96-well plate (Corning, Thermo Fisher Scientific). Cells were cultured for 7 days with 50% medium changes every 2–3 days. Tumor spheroids were then treated with the indicated concentration of arborinine, cisplatin, gemcitabine, bleomycin, or DMSO for another 48 h. Cells were photographed using an Olympus Is71 inverted fluorescence microscope and the number of viable cells within tumor spheroids was quantified using the Cell-Titer-Glo® 2.0 assay (Promega). All assays were performed in triplicate.

2.6. Caspase-3/7 assay

HeLa cells were seeded in a 96-well plate and grown in DMEM with 10% FBS. Twenty-four hours after plating, cells were treated with cisplatin, arborinine, or DMSO at the indicated concentration for another 24 h. Caspase-3/7 activity was determined using the Caspase-Glo® 3/7 assay (Promega) in a Synergy™ H1 Multi-Mode Reader (BioTek). Assays were performed in triplicate during two independent experiments.

2.7. Real-time cell migration assay

Cell migration was assessed in real time using the RTCA system and a CIM-16 plate (ACEA Biosciences) as described previously with some modifications [12]. Briefly, 175 μL DMEM +4% FBS with DMSO or the indicated concentration of arborinine was added to the lower chamber of the CIM-16 plate. Then, 50 μL SFM was added to the upper chamber. For a negative control, SFM was added to both the lower and upper chambers. The plate was incubated at 37 °C and 5% CO_2 for 1 h, and background signal was measured. Next, DMSO or arborinine at the indicated concentration in 50 μL SFM was added to the upper chamber. Finally, 40,000 cells in 50 μL SFM were seeded into each well. The impedance, displayed as cell index, was recorded every 15 min for up to 30 h. Assays were performed in triplicate on two separate occasions.

2.8. Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from samples using the Quick-RNA Plus kit (Zymo) and concentrated by the RNA Clean & Concentrator kit (Zymo). The amount of total RNA was quantified on a NanoDrop One UV–Vis Spectrophotometer (Thermo Scientific). Total RNA was converted to cDNA using the iScript™ cDNA synthesis kit (Bio-Rad). The synthesized cDNA was used as a template for qPCR, performed in a Bio-Rad CFX96 Touch using a SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). PCR conditions were as follows: 98 °C for 30 s, 40 cycles of 98 °C for 5 s and 60–62.5 °C for 30 s, followed by melting curve analysis, from 65 to 96 °C with increments of 0.5 °C per cycle. Primers and annealing temperatures (T_a) are listed in Supplementary table 1. For each experiment, five controls including reverse transcription control (qHsaCtlD0001001), positive PCR control (qHsaCtlD0001003), DNA contamination control (qHsaCtlD0001004), RNA quality control (qHsaCtlD0001002) (Bio-Rad), and no template control (NTC) were run alongside any assay to assess the quality of the sample and assay.

Expression of target genes was normalized internally to at least two reference genes (GAPDH, U6, or RPS13) with the lowest average expression stability (M-value) as determined by GeNorm

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