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CHM-1 induces apoptosis via p38-mediated upregulation of DR5 expression in human ovarian cancer SKOV3 cells

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ABSTRACT

Ovarian cancer is a leading cause of death due to neoplasm of the female genital tract. Treatment for advanced-stage disease remains limited, and an effective drug for ovarian cancer is urgently needed today. In the present study, MTT assay was used to evaluate the antiproliferative effect of the 2-(substituted phenyl)-6,7-methylenedioxyquinolin-4-one derivatives for developing new anti-ovarian cancer drugs. CHM-1 was the most active compound, and it exhibited potent antiproliferative activity against human ovarian cancer cells. CHM-1 inhibited the growth of SKOV3 cells and induced apoptosis in a concentration-dependent manner, but it was less cytotoxic to human diploid skin fibroblast Detroit 551 cells. The western blot experiments showed that CHM-1 caused the upregulation of death receptor (DR) 5 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Interestingly, CHM-1-mediated cellular apoptosis was found to be closely involved with the p38-mediated upregulation of DR5 expression. In an SKOV3 subcutaneous xenograft model, both CHM-1 and its phosphate, CHM-1-P caused a significant dose- and time-dependent tumor regression. Furthermore, CHM-1 inhibited tumor growth and prolonged the lifespan in the SKOV3 ip1/luc orthotopic xenograft model. Intravenous administration of CHM-1-P significantly prolonged the survival time in the SKOV3/ICR-Foxn1nu orthotopic xenograft model. Based on their excellent antitumor activity with the interesting mechanism of action, CHM-1 and CHM-1-P were considered new anti-ovarian cancer drug candidates.

1. Introduction

Ovarian cancer remains a major health problem for women because it is the most lethal neoplasm of the female genital tract. Worldwide, ovarian cancer is the sixth common malignancy as well as the seventh leading cause of cancer-related deaths in women (Boyle and Levin, 2008; Permuth-Wey and Sellers, 2009). The high mortality rate is attributed to the fact that nearly 75% of cases are diagnosed at an advanced stage, when the disease is widespread. This is because early-stage ovarian cancer is often asymptomatic. To date, despite aggressive ovarian cancer management, predominantly involving surgery followed by chemotherapy, the overall prognosis for patients remains poor (Modugno, 2003). Therefore, an effective drug for ovarian cancer is required.

Many chemotherapeutic drugs eradicate cancer cells by inducing apoptosis, and regardless of their primary targets, many drugs are similar in terms of the cellular response to the induced apoptosis (Ferri and Kroemer, 2001; Kim et al., 2002). Defects in apoptosis signaling not only contribute to tumorigenesis but are associated with the development of tumor chemoresistance. Apoptosis, or so-called programmed cell death, is a form of cell death executed by caspases. It plays a central role in multi-cellular organisms during early development during the sculpting of body parts and in adulthood for controlling cell numbers (Daniel and Korsmeyer, 2004). Apoptosis can be triggered in a cell through either an extrinsic or intrinsic pathway. The extrinsic pathway is initiated by the engagement of death receptors [Fas/CD95, tumor necrosis factor (TNF) receptor, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor] on the cell membrane. The binding of ligands

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(FasL, TNF, and TRAIL) to their respective death receptors (Fas/CD95, TNF-R, and TRAIL-R) activates membrane-proximal caspases (caspases-8 and 10), which in turn cleave and activate effector caspases such as caspases-3 and 7 (Cotter, 2009; Lavrik et al., 2005). In the intrinsic pathway, death signals act directly or indirectly on the mitochondria, resulting in the release of mitochondrial proteins, such as cytochrome *c*. Following mitochondrial release, cytochrome *c* works together with 2 other cytosolic protein factors, namely, apoptotic protease activating factor-1 (Apaf-1) and procaspase-9, to form a high-molecular weight caspase-activating complex termed the "apoptosome." Once assembled, the apoptosome processes and activates procaspase-9 as the initiator caspase, which in turn proteolytically initiates the apoptotic caspase cascade (Adrain and Martin, 2001; Budihardjo et al., 1999; Daniel and Korsmeyer, 2004).

The TRAIL receptors death receptor 4 (DR4; also termed TRAILR1) and death receptor 5 (DR5; also termed Apo2, TRAIL-R2, TRICK2, or Killer/DR5) are capable of transducing apoptotic signals (Kelley and Ashkenazi, 2004). Recently, these death receptors have attracted much more attention because their ligand TRAIL is a promising candidate for therapy for many forms of cancer, as it selectively triggers apoptosis in transformed cells (Ashkenazi, 2002; Hall and Cleveland, 2007). The interaction of TRAIL with DR4 and DR5 leads to the recruitment of the adaptor protein FADD and initiator caspase-8 to the death-initiating signaling complex (DISC), resulting in enzymatic activation of caspase-8, which in turn activates the downstream caspase cascade (Muzio et al., 1996). It is confirmed that the induction of DR4 and/or DR5 accounts for the induction of apoptosis and/or enhancement of TRAIL-induced apoptosis by certain stimuli, including some cancer therapeutic agents (Jin et al., 2005; Kim et al., 2006; Liu et al., 2004). Increasing knowledge on the relationship between apoptosis and carcinogenesis has enabled the design of novel approaches that exploit this process to treat cancer. Any therapeutic strategy aimed at specifically triggering apoptosis in cancer cells has potential therapeutic effects.

Treatment for advanced-stage ovarian cancer remains limited, and an effective drug is urgently needed. The goal of this study was to develop potential anti-ovarian cancer drug candidates. In the present study, we started by examining the cytotoxicity of 2-(substituted phenyl)-6,7-methylenedioxyquinolin-4-ones (2-PQs) against human ovarian cancer cell lines. The most promising compound, CHM-1, was evaluated for its action mechanism. Furthermore, CHM-1 and its phosphate (CHM-1-P) were evaluated for their in vivo antitumor activity with subcutaneous and orthotopic xenograft ovarian cancer nude mice models.

2. Materials and methods

2.1. Chemicals and reagents

2-(substituted phenyl)-6,7-methylenedioxyquinolin-4-ones (2-PQs) and CHM-1-P were synthesized in our laboratory. Cell culture materials were obtained from Invitrogen (Burlington, Ontario, Canada). Antibodies against poly(ADP)ribose polymerase (PARP), cleaved caspase-3, caspase-8, caspase-9, Fas/CD95, TRAIL, DR4, DR5, ERK1/2, JNK, and p-JNK were purchased from Cell Signaling Technology (Beverly, MA); those against p-ERK1/2, p38, and p-p38 and anti-mice and anti-rabbit antibodies conjugated to horseradish peroxidase, from Santa Cruz Biotechnology (Santa Cruz, CA); and β-actin antibody, propidium-iodide (PI), and 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole (SB203580), from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture

All cell lines were purchased from American Type Culture Collection (Manassas, VA). Hep3B, SKOV3, and SKOV3 ip1 cells were cultured in DMEM/F12. The normal human diploid skin fibroblast line Detroit 551 was cultured in DME. PC-3, HCT116, and H460 cells were cultured in RPMI-1640 medium with 2 mM L-glutamine and supplemented with 1 mM sodium pyruvate and 4.5 g/l glucose. All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen; Carlsbad, CA). Cells were incubated in 75-cm^2 flasks at 37 °C with 5% CO_2 and were passaged with 0.25% trypsin-EDTA when they reached 80% confluence.

2.3. Determination of cell viability by MTT assay

Cells were treated with various doses of compounds for the indicated times, and the MTT dye was then added to each well. After 4-h incubation, the growth medium was removed and the formazan crystals, generated by oxidation of the MTT dye by cell mitochondria, were dissolved in 0.04 N HCl in isopropanol. The absorbance was measured at 570 nm, and the cell survival ratio was expressed as a percentage of the control viability (Way et al., 2010).

2.4. FACScan flow cytometric analysis

Cells were treated with various agents for the indicated times, harvested by trypsinization, fixed with 70% (v/v) ethanol at 4 °C for 30 min, and washed twice with phosphate-buffered saline (PBS). After centrifugation, the cells were incubated with 0.1 ml of phosphate-citric acid buffer (0.2 M $NaHPO_4$ and 0.1 M citric acid (pH 7.8)) for 30 min at room temperature. Then, they were centrifuged and resuspended in 0.5 ml PI solution comprising Triton X-100 (0.1%, v/v), RNase (100 mg/ml), and PI (80 mg/ml). The percentage was analyzed with FACScan and the CellQuest software (Becton Dickinson; Mountain View, CA).

2.5. Western blotting

Cells were treated with various agents as indicated in the figure legends. After treatment, western blotting was performed as described previously (Lee et al., 2008). The expression levels of PARP, caspase-3, caspase-8, caspase-9, JNK, p-JNK, p38, p-p38, ERK1/2, p-ERK1/2, DR4, DR5, Fas/CD95, and β-actin were detected using specific antibodies in combination with enhanced chemiluminescence (ECL; Amersham; Arlington Heights, IL).

2.6. In vivo studies

The animals used in this had access to food and water ad libitum. Experimental procedures using animals were approved by the Institutional Animal Care and Use Committees of The National Health Research Institutes.

(A) Nude female BALB/c mice (18–20 g; 6–8 weeks of age) were purchased from The National Laboratory Animal Center, Taipei, Taiwan, and maintained in pressurized ventilated cages according to institutional regulations. Human ovarian cancer SKOV3 cells were cultured in DMEM/F12 with 10% heat-inactivated FBS and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Each nude mouse was subcutaneously inoculated with 3×10^6 SKOV3 cells in 0.5 ml PBS via a 24-gauge needle. After the appearance of a 100-mm^3 tumor nodule, 110 tumor-bearing mice were randomly divided into 10 groups. CHM-1 and CHM-1-P were administered by i.p. injection at 5, 10, 20, and 30 mg/kg on 5 days every week for 4 consecutive weeks.

The animals were weighed and the tumors were measured using calipers twice a week before, during, and after drug treatment. The tumor volume was calculated with the following formula: $1/2\ (L\times W^2)$, where L is the length and W is the width of the tumor (Tomayko and Reynolds, 1989). At the end of the experiments, the animals were euthanized with carbon dioxide followed by cervical dislocation.

(B) Five nude female BALB/c mice (18–20 g; 6–8 weeks of age) per group were inoculated with 3×10^6 SKOV3 ip1/luc human ovarian