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Research Paper

# Preclinical Efficacy and Safety Assessment of Artemisinin-Chemotherapeutic Agent Conjugates for Ovarian Cancer

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ABSTRACT

Artemisinin (ARS) and its derivatives, which are clinically used antimalarial agents, have shown antitumor activities. Their therapeutic potencies, however, are limited by their low solubility and poor bioavailability. Here, through a pharmacophore hybridization strategy, we synthesized ARS-drug conjugates, in which the marketed chemotherapeutic agents chlorambucil, melphalan, flutamide, aminoglutethimide, and doxifluridine, were separately bonded to Dihydroartemisinin (DHA) through various linkages. Of these, the artemisinin-melphalan conjugate, ARS4, exhibited most toxicity to human ovarian cancer cells but had low cytotoxicity to normal cells. ARS4 inhibited the growth and proliferation of ovarian cancer cells and resulted in S-phase arrest, apoptosis, and inhibition of migration; these effects were stronger than those of its parent drugs, DHA and melphalan. Furthermore, ARS4 modulated the expression of proteins involved in cell cycle progression, apoptosis, and the epithelial–mesenchymal transition (EMT). Moreover, in mice, ARS4 inhibited growth and intraperitoneal dissemination and metastasis of ovarian cancer cells without observable toxic effects. Our results provide a basis for development of the compound as a chemotherapeutic agent.

*Research in context:* Artemisinin compounds have recently received attention as anticancer agents because of their clinical safety profiles and broad efficacy. However, their therapeutic potencies are limited by low solubility and poor bioavailability. Here, we report that ARS4, an artemisinin-melphalan conjugate, possesses marked *in-vitro* and *in-vivo* antitumor activity against ovarian cancer, the effects of which are stronger than those for its parent drugs, Dihydroartemisinin and melphalan. In mice, ARS4 inhibits localized growth of ovarian cancer cells and intraperitoneal dissemination and metastasis without appreciable host toxicity. Thus, for patients with ovarian cancer, ARS4 is a promising chemotherapeutic agent.

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## 1. Introduction

Ovarian cancer is the sixth leading cause of cancer deaths in women in developed countries and eighth in developing countries (Torre et al., 2015). It is the most lethal gynecologic malignancy, due to the advanced stage of disease at diagnosis, its highly metastatic nature, and lack of effective therapeutic regimens (Jayson et al., 2014; Vaughan et al., 2011). Considerable efforts have been made in evaluating several classes of conventional chemotherapeutic agents, such as paclitaxel and platinum-based agents, for ovarian cancer therapy (Yap et al., 2009). However, the response rates are low, and clinical improvement is marginal, especially for patients with advanced stages of disease, largely due to late diagnoses, persistent dormancy, drug resistance, and cytotoxic side effects (Chen et al., 2013; Janzen et al., 2015; Yap et al., 2009). Therefore, it is necessary to develop new therapeutic agents for ovarian cancer.

We are committed to develop safer and more effective, natural products-based agents for therapy of ovarian cancer (Chen et al., 2009; Chen et al., 2011; Li et al., 2013a). Artemisinin (ARS), a natural sesquiterpene endoperoxide isolated from the plant *Artemisia annua* L, is widely used as an anti-malaria drug (Miller and Su, 2011). ARS and it derivatives also have broad anti-bacterial, anti-inflammatory (Shi et al., 2015), and anti-tumor activities (Firestone and Sundar, 2009). In our previous studies, we found that ARS derivatives, particularly dihydroartemisinin (DHA), exhibit activity against liver cancer cells

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and ovarian cancer cells *in vitro* and *in vivo* and sensitize cancer cells to conventional chemotherapeutic agents, such as gemcitabine and carboplatin (Chen et al., 2009; Hou et al., 2008). A reason for developing ARS and its analogs for cancer therapy is the safety profile of this class of compounds, which have been extensively used in the clinic (Lai et al., 2013). We and others have reported that the ARS compounds exert their anticancer effects by inhibiting cell proliferation, inducing cell cycle arrest and apoptosis, inhibiting angiogenesis, reducing cell migration and invasion, and modulating nuclear receptor responsiveness (Chen et al., 2009; Firestone and Sundar, 2009; Hou et al., 2008). However, their therapeutic potencies are limited by their low solubility and poor bioavailability (Steyn et al., 2011). To combat these shortcomings, ARS derivatives have been synthesized and evaluated for their anti-tumor activities; some demonstrated anti-tumor activity against cultured cancer cells (Blazquez et al., 2013; Buragohain et al., 2015; Crespo-Ortiz and Wei, 2012; Srivastava and Lee, 2015; Zuo et al., 2015). However, only a few of these compounds have been used in practice because of their low efficacy in animal models. Therefore, it is necessary to develop ARS derivatives with better biological activities.

Pharmacophore hybridization, a classical medicinal chemistry strategy, is used widely in drug discovery (Fisher et al., 2014; Romagnoli et al., 2014; Solomon et al., 2010). As described herein, we introduced the pharmacophores of marketed anti-cancer agents into the scaffold of ARS to prepare derivatives by the pharmacophore hybridization strategy. Nine ARS-drug hybrids were designed and synthesized. Compared with the parent drugs, most of the hybrids produced marked cytotoxicity to cancer cells. Of these, the ARS-melphalan conjugate, ARS4, was most toxic to human ovarian cancer cells but had low cytotoxicity to normal cells. ARS4 inhibited the growth and proliferation of ovarian cancer cells A2780 and OVCAR3 and resulted in S-phase arrest, apoptosis, and migration inhibition. These effects were greater than those for its parent drugs, DHA and melphalan. Exposure of cells to ARS4 modulated the expression of proteins involved in cell cycle progression, apoptosis, and the epithelial–mesenchymal transition (EMT). Moreover, in mice, ARS4 inhibited local growth and intraperitoneal dissemination and metastasis of ovarian cancer cells without any appreciable host toxicities. Based on its preclinical efficacy and safety, we conclude that the ARS-melphalan conjugate ARS4 is active as an anti-ovarian cancer agent.

## 2. Materials and Methods

### 2.1. Chemistry

The reagents (chemicals) were purchased from commercial companies and used without further purification unless otherwise stated. Analytical thin-layer chromatography (TLC) was with HSGF 254. All target products were characterized by their NMR, LRMS and HRMS spectra. Chemical shifts are reported in parts per million (ppm, $\delta$) downfield from tetramethylsilane. Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), quartet (q), multipet (m) and broad (br). Low- and high-resolution mass spectra (LRMS and HRMS) were obtained with electric, electrospray, and matrix-assisted laser desorption ionization (EI and ESI) produced by Finnigan MAT-95 and LCQ-DECA spectrometers.

### 2.2. Compounds and Reagents

ARS4 was synthesized from DHA and melphalan at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, PR China). The chemical structure is shown in Fig. 4A. DHA and carboplatin with purities >98% were purchased from Sigma. Carboplatin (PARAPLATIN) was from NOVAPLUS. The test drugs were dissolved in DMSO and in Cremophor EL:Ethanol:Saline (5:5:90, v/v/v) for *in-vitro* and *in-vivo* study, respectively. Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Laboratories. The ECL Plus system was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK).

### 2.3. Cell Lines and Cell Culture

Human ovarian epithelial adenocarcinoma cell lines A2780 and OVCAR3 and human ovarian epithelial cell line IOSE144 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). For luciferase stable labeling, A2780 cells were infected by lentivirus expressing firefly luciferase gene obtained from Dr. Dong Xie (Institute for Nutritional Sciences, SIBS, CAS). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37 °C in a humidified atmosphere with 5% $CO_2$. All cell culture supplies were obtained from Invitrogen-Gibco Co (Grand Island, NY, USA).

### 2.4. Cell Viability

Cell viability was determined with CCK-8 kits, as described previously (Li et al., 2013a; Li et al., 2013b). Briefly, cells were seeded in 96-well plates and were treated either with compounds at serial concentrations or for various times. After 24–72 h of treatment, 10 μl of CCK-8 solution was added to each well, followed by incubation for 3 h. For the determination of IC50 of different compounds, 48 h of incubation was conducted. The absorbance was measured at an optical density of 450 nm using an ApectraMax microplate reader, and the percentage of cell viability was calculated relative to vehicle-treated cells.

### 2.5. Cell-Cycle and Apoptosis Analysis

PI staining was used to detect the effects of ARS-related compounds on cell cycle distribution (Li et al., 2013a; Li et al., 2013b). In brief, after incubation with the compounds, A2780 and OVCAR3 cells were harvested and fixed with 70% ethanol, followed by RNase digestion and staining with PI (50 μg/ml). The cell-cycle distribution was determined with a BD FACS Caliber flow cytometer by quantification of cell DNA contents. Cell apoptosis was determined with Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection Kits (BioVision). The floating and trypsinized adherent cells were collected by centrifugation and suspended in binding buffer. Annexin V-FITC and PI were added to the cells before analysis by fluorescence-activated cell sorting (FACS) using a FACSCan flow cytometer (Becton Dickinson). Cell that stained positive for early apoptosis (Annexin V-FITC$^+$/PI$^-$) and for late apoptosis (Annexin V-FITC$^+$/PI$^+$) were combined for the final analysis.

### 2.6. Caspase 3/7 Apoptotic Activity Assay

Caspase-3/7 activity was measured using Caspase-Glo® 3/7 Assay (Promega) assay according to manufacturer's instructions. Routinely, A2780 and OVCAR3 cells were plated 4000/well in 96-well plates, and were treated 5 μM of ARS4 or DHA or melphalan for 24 h. After incubation, 100 μl of Caspase-Glo® 3/7 Reagent was added. Plates were incubated for 1 h prior to reading luminescence in EnSpire Luminescence Microplate Reader (PerkinElmer Inc., USA). No cell sample was used as a background control for luminescence. Caspase 3/7 activity is represented as relative Caspase 3/7 luminescence.

### 2.7. Cell Migration

For cell migration assay, A2780 and OVCAR3 cells were pretreated with indicated concentration of ARS4 for 12 h. Later, Cells were resuspended in serum-free 1640 medium and seeded onto Transwell with an 8 μm microporous membrane (Corning Costar, NY, USA) in 24-well plates. Culture medium with 10% fetal bovine serum was used as a chemoattractant in the lower compartment. Within 12 h, non-invasive cells were carefully removed, and the cells that had migrated to the

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