EFFECTS OF ARTEMISININ AND ITS DERIVATIVES ON GROWTH INHIBITION AND APOPTOSIS OF ORAL CANCER CELLS

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Abstract: Background. Artemisinin is of special biological interest because of its outstanding antimalarial activity. Recently, it was reported that artemisinin has antitumor activity. Its derivatives, artesunate, arteether, and artemeter, also have antitumor activity against melanoma, breast, ovarian, prostate, CNS, and renal cancer cell lines. Recently, monomer, dimer, and trimer derivatives were synthesized from deoxoartemisinin, and the dimers and the trimers were found to have much more potent antitumor activity than the monomers.

Methods. We evaluated the antitumor activity of artemisinin and its various derivatives (dihydroartemisinin, dihydroartemisinin 12-benzoate, 12-(2'-hydroxyethyl) deoxoartemisinin, 12-(2'-ethylthio) deoxoartemisinin dimer, deoxoartemisinin trimer) in comparison with paclitaxel (Taxol), 5-fluorouracil (5-FU), cisplatin in vitro.

Results. In this study, the deoxoartemisinin trimer had the most potent antitumor effect (IC50 = 6.0 μM), even better than paclitaxel (IC50 = 13.1 μM), on oral cancer cell line (YD-10B). In addition, it induced apoptosis through a caspase-3-dependent mechanism.

Conclusion. The deoxoartemisinin trimer was found to have greater antitumor effect on tumor cells than other commonly used chemotherapeutic drugs, such as 5-FU, cisplatin, and paclitaxel. Furthermore, the ability of artemisinin and its derivatives to induce apoptosis highlights their potential as chemotherapeutic agents, for many anticancer drugs achieve their antitumor effects by inducing apoptosis in tumor cells. ©2006 Wiley Periodicals, Inc. Head Neck 29: 335–340, 2007

Keywords: artemisinin; deoxoartemisinin; antitumor activity; apoptosis

Many anticancer drugs exert their cytotoxicity by inhibiting DNA synthesis and cell replication. However, side effects such as bone marrow suppression, gastrointestinal toxicity, and renal damage still remain as problems that need to be solved.1 Therefore, a possible approach for developing clinically applicable chemotherapeutic agents is to screen traditional medicinal plants that have been used for thousands of years for their anticancer activities with few side effects.2–4

For instance, artemisinin is an endoperoxide-containing sesquiterpene isolated from the Chinese herbal plant, Artemisia annua L, or sweet...
Artemisinin has been of special biological interest because of its outstanding antimalarial activity. Recently, it was reported that artemisinin also has antitumor activity. Its derivatives, such as artesunate, arteether, and artemeter, also have antitumor activity against melanoma, breast, ovarian, prostate, CNS, and renal cancer cell lines. Recently, monomer, dimer, and trimer derivative forms of artemisinin were synthesized from deoxoartemisinin. The dimers and the trimers were reported to have much more potent antitumor activity than the monomers.

Many studies have shown that the cellular responses to artemisinin and its derivatives occur in a multifactorial manner in nature. Artemisinin and its derivatives have antiproliferative and antiangiogenic effects. In addition, they also cause the tumor cells to undergo apoptosis by damaging target proteins and causing lipid peroxidation using carbon-centered radicals or reactive oxygen species.

In an oral cancer cell line, artemisinin was also found to be cytotoxic to oral malignant epithelial cells. Moreover, it killed these cells through apoptosis, not necrosis.

This study was designed to examine the effects of artemisinin and its derivatives on the growth of the oral squamous carcinoma cell line, YD-10B, and to determine whether the derivatives induce apoptosis through the activation of caspase-3, which is known to be the general mediator of apoptosis.

In this study, 3 commonly used chemotherapeutic agents, 5-fluorouracil (5-FU), cisplatin, and paclitaxel (Taxol, Bristol-Myers Squibb), were used as the control.

**MATERIALS AND METHODS**

**Materials.** Artemisinin and its derivatives were synthesized at the Department of Chemistry, Yonsei University. Three commonly used chemotherapeutic drugs (5-FU, cisplatin, paclitaxel) were also used as the control (Figure 1).

All the experiments were performed using the YD-10B cell line. In Table 1, the characteristics of the YD-10B cell line are summarized.

**Cell Culture.** YD-10B, a human oral squamous carcinoma cell line was cultured in Dulbecco's...
modified Eagle's medium (DMEM) and F-12 media (Gibco BRL, NY, USA) at a ratio of 3:1 supplemented with 10% fetal bovine serum (FBS), 100 IU/mL, and 100 μg/mL streptomycin. The cells were maintained as monolayers in a plastic culture plate at 37°C in a humidified atmosphere containing 5% CO2.

**Growth Inhibition of YD-10B Cells by Artemisinin and its Derivatives.** The cells were plated into a 96-well plate at a density of 3 × 10^3/well, and treated with a series of artemisinin derivatives after overnight incubation. All the artemisinin derivatives tested were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration in the culture medium was less than 0.1%, and the controls were treated with DMSO alone. The cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazonium bromide (MTT) assay. Briefly, the cells were grown in the media in the presence or absence of the test compound for 72 hours. They were then placed in a 200 μL culture medium containing 0.5 mg/mL MTT, for 3 hours. The resulting MTT-formazan product was dissolved in the same volume of DMSO. The amount of formazan was determined by measuring the absorbance at 570 nm.

**DNA Fragmentation Analysis.** Following the treatment with 75 μM artemisinin for 6, 12, and 24 hours, approximately 5 × 10^6 cells were collected in a lysis buffer containing 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% sodium dodecyl sulfate (SDS), and 0.5 mg/mL proteinase K. The DNA was extracted with an equal volume of a phenol–chloroform–isoamylalcohol mixture (25:24:1, v/v/v) and precipitated with pure ethanol. The DNA was resuspended in Tris-ErDA buffer (pH 8.0) containing 5 μg/mL DNase free RNase and was incubated at 37°C for 2 hours. The DNA fragments were visualized on 1.8% agarose gels in the presence of 0.5 μg/mL ethidium bromide.

**Western Blot Analysis of Caspase-3 Activation.** Western blot analysis was performed to determine whether caspase-3 is involved in the apoptosis induced by artemisinin and its derivatives. The treated YD-10B cells were washed twice with ice-cold phosphate buffered saline (PBS), lysed in a buffer solution, containing 60 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 14.1 mM 2-mercaptoethanol, and 0.1% bromophenol blue on ice for 30 minutes. Then, they were vigorously vortexed before centrifugation at 12,500g for 20 minutes. Fifty micrograms of the total protein, as determined by BioRad Protein Assay, was resolved in 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and it was transferred to a microcellulose membrane using a semi-dry transfer system (BioRad). The membrane was then blocked with 5% fat-free dry milk-PBST buffer (PBS, 0.1% Tween-20) for 1 hour at room temperature, and then it was washed 3 times in PBS. The membrane was incubated first with anti-caspase-3 (1:1000, Cat. No. 65906E, PharmMingen, CA) antibody in PBS for 2 hours and then with the horse-radish peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (1:2000, Zymed biotechnology, San Francisco, CA, USA) for 1 hour. The proteins were visualized using chemiluminescence ECL (Amersham, USA) system.

**RESULTS**

**Artemisinin and its Derivatives Induced Reduction of Cell Survival.** MTT assay was initially performed to analyze the effects of artemisinin and its derivatives on the viability of YD-10B cells. Figure 2 shows that the YD-10B cells have different cytotoxic and apoptosis profiles when treated with each artemisinin derivative and commonly used chemotherapeutic agents. After 72 hours of treatment, all drugs, except artemisinin and 12-(2’-hydroxyethyl) deoxoartemisinin, inhibited the proliferation of YD-10B cells in a time- and dose-dependent manner. Although artemisinin and 12-(2’-hydroxyethyl) deoxoartemisinin had no effect on the suppression of cell proliferation within 72 hours, they both inhibited the proliferation of these cells after 72 hours at a concentration of 100 μM. In particular, the trimer derivative, deoxoartemisinin trimer, appeared to be the most potent suppressor (IC₅₀ [concentration required for 50% cell growth inhibition] = 6.0 μM).

**DNA Fragmentation Analysis.** Internucleosomal DNA fragmentation is a hallmark of apoptosis. To further assess the pattern of cell death, more focus was placed on the deoxoartemisinin trimer, which was the most potent suppressor when compared with other compounds, and on the dihydroartemisinin among the monomers. Paclitaxel, which was more potent than 5-FU and cisplatin, was also selected as the control. In result, 25 μM of the deoxoartemisinin trimer caused fragmentation of the genomic DNA as early as 12 hours. Fragmentation after treatment with 100 μM dihydroartemisinin and 50 μM paclitaxel became evident at 24 hours and 48 hours, respectively (Figure 3).
Induction of Apoptosis Through a Caspase-3-Dependent Mechanism. Caspase-3 is believed to be a key protease that is activated during the early stage of apoptosis. The caspases are activated by sequential cascade of cleavage of their inactive forms. For instance, active caspase-3 proteolytically cleaves and activates other caspases as well as other relevant target molecules in the cytoplasm or nucleus. The cleavage of procaspase-3 was evaluated using Western blot analysis to determine whether caspase-3 is involved in the apoptosis induced by dihydroartemisinin, the deoxoartemisinin trimer, and paclitaxel.

Treatment of YD-10B cells with 100 μM dihydroartemisinin did not cause any time-dependent proteolytic cleavage of procaspase-3. In contrast, addition of 25 μM of the deoxoartemisinin trimer or 50 μM paclitaxel caused a time-dependent proteolytic cleavage of procaspase-3 with the gradual disappearance of the full-size 33-kDa molecule. Such proteolytic cleavage of procaspase-3 became apparent within 12 hours after the treatment with deoxoartemisinin trimer and within 24 hours after the treatment with paclitaxel (Figure 4).

DISCUSSION
Head and neck squamous cell carcinoma is a complex disease occurring in various organs, including the oral cavity, pharynx, and larynx. Tumors from such different sites have distinct clinical presentation patterns and outcomes, and they are generally treated with a wide variety of strategies, including surgery, radiotherapy, and chemotherapy. Currently, the standard treatment of choice for locally advanced squamous head and neck carcinoma is surgical removal and radiation therapy. The routine application of neoadjuvant chemotherapy is still controversial. Nevertheless, to improve poor prognosis of the disease, different treatment strategies, for instance the inclusion of chemotherapy, need to be considered. While having been used historically only for the palliation of recurrent or metastatic disease, chemotherapy is becoming part of the initial management of locally advanced squamous cell carcinomas of the head and neck. However, application of cancer chemotherapy is limited by the development of drug resistance in tumor cells as well as the adverse side effects of the drugs. To overcome
such limitations, the direction of the present research for novel antitumor agents has turned to natural products, especially plants used in traditional medicine. Camptothecin from *Camptotheca acuminate* and paclitaxel from *Taxus brevifolia* are outstanding examples of natural products used as chemotherapeutic agents. Moreover, paclitaxel has been reported as the best novel anticancer agent developed from natural products, showing particular efficacy against ovarian cancer.\(^{18}\)

Artemisinin, a natural product extracted from the Chinese herbal plant, *Artemisia annua* L., or sweet wormwood, is a widely used antimalarial drug that acts quickly and is eliminated quickly.\(^{19}\) In case of artemisinin, no cross-resistance with other drugs or serious adverse effects have been reported in humans. Novel semisynthetic and synthetic derivatives have been constantly invented.\(^{20,21}\) Deoxoartemisinin and its dimer and trimer forms have been synthesized from the mother drug artemisinin, and they have shown potent antitumor effects.\(^{9,22,23}\) In this study, various artemisinin derivatives were used to examine their potent antitumor effect in comparison with commonly used chemotherapeutic agents.

In 2004, Yamachika et al\(^ {11}\) reported the effect of artemisinin on cultured human papillomavirus (HPV) 16 immortalized and transformed human gingival epithelial (IHGK) cells. They examined the effect of artemisinin and 5-FU, and reported that cell cytolysis was both time- and dose-dependent. In addition, they showed that the cell death caused by 200 \(\mu\)M dihydroartemisinin was less evident after 8 hours but slightly evident after 24 hours since the time of treatment. However, when treated with 400 \(\mu\)M dihydroartemisinin, almost 40% of the cells were found to be dead within 24 hours and 63% within 48 hours. In this study, within 72 hours after the treatment with drugs, the proliferation of YD-10B cells was inhibited in a time- and dose-dependent manner in every case. The drugs used in this study were different from the ones Yamachika et al used. Therefore, it is impossible to make direct comparison between the results of these 2 studies. However, it is possible to compare and determine the effects of various artemisinin derivatives and chemotherapeutic agents on the actual oral squamous carcinoma cells, obtained by culturing a common cell line established from an oral squamous cell carcinoma patient. In this study, the deoxoartemisinin trimer showed the most potent antitumor effect (IC\(_{50}\) = 6.0 \(\mu\)M), even more effective than paclitaxel (IC\(_{50}\) = 13.1 \(\mu\)M).

Apoptosis, the programmed or physiological cell death, plays an important role in embryogenesis, homeostasis, and certain pathologic events. The biochemical hallmark of apoptosis is the appearance of a fragmentation pattern in chromatin, which indicates DNA cleavage at the linker regions between nucleosomes. It produces a characteristic pattern of DNA cleavage into 180-bp oligonucleosome, which generates an integer fragments (a DNA ‘ladder’) when the DNA from apoptotic cells is subjected to conventional gel electrophoresis.\(^{19}\) In DNA fragmentation analysis of this study, internucleosomal DNA fragmentation was evident after 12 hours since treatment with 25 \(\mu\)M of the deoxoartemisinin trimer. In contrast, internucleosomal DNA fragmentation became evident after 48 hours in case of treatment with 50 \(\mu\)M paclitaxel. Such results indicate the possibility of the deoxoartemisinin trimer having greater potency than paclitaxel.

The activation of a family of intracellular cysteine proteases, called caspases, plays a key role in the initiation and execution of apoptosis induced by various stimuli.\(^ {12,13}\) Among the several different members of caspases identified in mammalian cells, caspase-3 plays a direct role in the proteolytic cleavage of the cellular proteins responsible for cell death. In this study, cleavage of procaspase-3 by the deoxoartemisinin trimer and paclitaxel (Taxol) is demonstrated, indicating the potential of these compounds as potential anticancer agents.
for progression to apoptosis.\textsuperscript{12–14} It is synthesized as a 33-kDa inactive proenzyme requiring proteolytic activation. Although the detailed mechanism of the induction of apoptosis by artemisinin and its derivatives has not been determined,\textsuperscript{10} it was hypothesized that artemisinin and its derivatives induce apoptosis through the activation of caspase-3. In this study, the level of active caspase-3 did not increase studying treated cells. However, high level of the proenzyme of caspase-3 observed in the untreated tumor cells showed a pattern of gradual decrease in treated cells after administering the deoxoartemisinin trimer and paclitaxel. It suggests that artemisinin derivatives used in this study induce apoptosis through a caspase-3–dependent mechanism. In contrast, dihydroartemisinin did not cause the time-dependent proteolytic cleavage of procaspase-3, which suggests that the cellular responses to artemisinin and its derivatives occur in a multifactorial manner in nature. Such complex mechanism of artemisinin may be applied beneficially in treating otherwise drug-resistant tumors, and it may explain why the development of artemisinin resistance has not yet been encountered in cancer cell lines or malaria patients.\textsuperscript{10}

In conclusion, the deoxoartemisinin trimer had greater potency of antitumor effects on tumor cells than the other commonly used chemotherapeutic drugs, such as 5-FU, cisplatin, and paclitaxel. Furthermore, the induction of apoptosis by artemisinin and its derivatives suggests their potential as chemotherapeutic agents for many anticancer drugs function as antitumor agents by inducing apoptosis of tumor cells.

\textbf{REFERENCES}


