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Ardisinol III, a naturally occurring alkenylmethylresorcinol displayed cytotoxic effects in carcinoma cells

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Abstract

Background: Cancer is a serious health concern worldwide making the continuous discovery of new cytotoxic agents a challenging issue. In this study, the cytotoxicity of a naturally occurring alkenylmethylresorcinol, ardisinol III was evaluated in a panel of six human carcinoma cell lines and the normal fibroblasts.

Methods: The cytotoxicity of samples was evaluated by the neutral red uptake (NR) assay; the activity of caspases in breast adenocarcinoma MCF7 cells was detected by caspase-Glo assay; flow cytometry was used to analyze the cell cycle and mitochondrial membrane potential (MMP), and spectrophotometry was used to measure levels of reactive oxygen species (ROS).

Results: Ardisinol III had IC_{50} values below 10 µM in all the six tested carcinoma cell lines. The obtained IC_{50} values ranged from 0.88 µM (against SPC212 mesothelioma cells) to 8.36 µM (against hepatocarcinoma HepG2 cells). Ardisinol III was less toxic in normal CRL2120 fibroblasts and the selectivity indexes in all cell lines were above 6. This alkenylmethylresorcinol induced apoptosis in MCF7 breast adenocarcinoma cells, through activation of caspases 3/7 and 9, loss of MMP and increase ROS production.

Conclusions: Ardisinol III is a cytotoxic molecule that deserves to be further explored as a potential anticancer agent to combat human carcinoma.

Keywords: alkenylmethylresorcinol; ardisinol III; carcinoma; cytotoxicity; mode of action.

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Background

Cancer is a global health concern with about 14.1 million new cases and 8.2 million deaths reported in 2012 [1, 2]. Lung, breast, colon, prostate and liver cancers were reported as the most occurring cancers worldwide [3]. In 2008, about 1.4 million women were diagnosed with breast adenocarcinoma and approximately 459,000 deaths were recorded, making it the most occurring malignant diseases in females [4]. Colon cancer had the second highest mortality and morbidity rate [5]. Lung cancer kills more than one million people each year, ranking at the top in cancer-related mortalities in humans [6]. Prostate cancer is commonly diagnosed in men over the age of 50, and is the most common cancer diagnosed in North American men (about 28% of all cancers diagnosed), and the second most common cause of cancer death (10%) in 2012 [7, 8]. About 748,000 hepatocarcinoma cases and 696,000 deaths were reported worldwide in 2008 [9]. Chemotherapy remains the dominant mode of the treatment of various cancers. Natural products have long been source of several cytotoxic drugs such as paclitaxel [10], combretastatins [11], vinblastine and vincristine [12] and camptothecin [13]. It was previously demonstrated that African flora is a good reservoir of cytotoxic agents [14, 15]. In our continuous search of antiproliferative phytochemicals from African medicinal plants, this work was designed to evaluate the cytotoxicity and modes of action of ardisinol III, an alkenylmethylresorcinol isolated from the fruits of a Cameroonian medicinal plant, *Ardisia kivuensis* Taton (Myrsinaceae). This compound previously showed antioxidant activity [16]. To the best of our knowledge, this is the first report on the cytotoxicity of this ardisinol III.

Methods

Chemicals

The control drug, Doxorubicin 98.0% was purchased from Sigma-Aldrich (Munich, Germany). Ardisinol III $C_{23}H_{38}O_2$ (yellowish amorphous powder; m/z: 346.28) (Figure 1) was obtained from the Chemical Bank of Laboratory of Organic Chemistry, Department of Chemistry, University of Dschang. Its isolation from the fruits of *Ardisia kivuensis* Taton (Myrsinaceae) and its identification was previously reported [16].

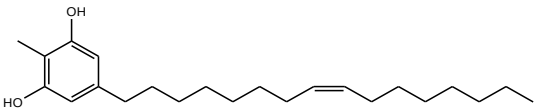

Figure 1. Chemical structures of ardisinol III

Cell culture

Seven cell lines including six human carcinoma and normal fibroblasts were tested. They were: A549 human non-small cell lung cancer (NSCLC) cell line, SPC212 human mesothelioma cell line, DLD-1 colorectal adenocarcinoma cell lines, Caco2 colorectal adenocarcinoma cells, HepG2 hepatocarcinoma cells, MCF-7 breast adenocarcinoma cells and the normal CRL2120 human skin fibroblasts. A549 cells (provided by Prof. Dr Tansu Koparal; Anadolu University, Eskisehir, Turkey) were obtained from the Institute for Fermentation, Osaka (IFO, Japan); SPC212 cells (provided by Dr. Asuman Demiroğlu Zergeroğlu; Gebze Technical University, Kocaeli, Turkey) cells, DLD-1 cells, HepG2 cells, MCF-7 (provided by Prof. Dr. Tansu Koparal (Anadolu University, Eskisehir, Turkey) and CRL2120 skin fibroblasts were obtained from American Type Culture Collection (ATCC); Caco2 cells were obtained from the ŞAP Institute of Turkey (Ankara); DMEM medium (Sigma-aldrich, Munich, Germany) medium was used to maintain cells as a monolayer and was supplemented with 10% fetal calf serum and 1% penicillin (100 U/mL)-streptomycin (100 µg/mL) in a humidified 5% CO_2 atmosphere at 37 °C.

Neutral red (NR) uptake assay

The cytotoxicity of ardisinoll III and doxorubicin (positive control) were performed by NR uptake assay as previously described [17-20]. NR uptake assay is cheaper and more sensitive than other cytotoxicity tests and is based on the ability of viable cells to incorporate and bind the supravital dye NR in the lysosomes [21]. Dimethylsufoxide (DMSO) at less than 0.1% final concentration was used to dilute the tested samples. DMSO at 0.1% was used as the solvent control. Briefly, cells were seeded at 1×10^4 cells in each well of a 96-well cell culture plate; Samples were tested in a total volume of 200 µL. After 72 h incubation in humidified 5% CO_2 atmosphere at 37 °C, the medium was removed, followed by coloration with medium containing 50 µg/mL NR [19, 20]. ELx 808 Ultra Microplate Reader (Biotek) equipped with a 540 nm filter was used to measure the absorbance. Each experiment was performed three times, with three replicates each. The viability was evaluated based on a comparison with untreated cells. The IC_{50} values represent the sample's concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel [22].

Flow cytometry for cell cycle analysis and detection of apoptotic cells

The effect of ardisinol III in the distribution of cell cycle in MCF7 cells was performed by flow cytometry using BD cycletest™ Plus DNA Kit Assay (BD Biosciences, San Jose, USA) as previously described [19]. Briefly, MCF-7 cells (3 mL, 1×10^5 cells/mL) were seeded into each well of 6-well plates and allowed to attach for 24 h (humidified 5% CO_2 atmosphere at 37 °C). Cells treated with ¼×IC_{50}, ½×IC_{50} and IC_{50} concentrations of ardisinol III and doxorubicin, as well as untreated cells (control) were then grown in 6-well plates for 72 h. The BD FACS Aria I Cell Sorter Flow Cytometer (Becton-Dickinson, Germany) was then used for cell cycle analysis. For each sample, 10^4 cells were counted. For PI excitation, an argon-ion laser emitting at 488 nm was used. Cytographs were analyzed using BD FACSDiva™ Flow Cytometry Software Version 6.1.2 (Becton-Dickinson).

Caspases activity

Caspases activity in MCF-7 cells was detected using Caspase-Glo 3/7 and Caspase-Glo 9 Assay kits (Promega, Mannheim, Germany) as previously reported [23-25]. Cells were treated for 6 h (humidified 5% CO_2 atmosphere at 37 °C) with ardisinol III and doxorubicin at their ½ × IC_{50} and IC_{50} values and with DMSO as the solvent control. The BioTek Synergy™ HT multi-detection microplate reader was used to measure the luminescence and Caspases activity was expressed as the percentage of the untreated control. All experiments were performed at least three times and in triplicate.

Analysis of mitochondrial membrane potential (MMP)

The MCF7 cells were treated with ardisinol III and doxorubicin, and the integrity of MMP was analyzed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Biomol, Hamburg, Germany) staining as previously reported [23-25]. Cells (3 mL, 1×10^5 cells/mL) treated for 72 h (humidified 5% CO_2 atmosphere at 37 °C) with different concentrations (¼×IC_{50}, ½×IC_{50} and IC_{50}) of ardisinol III, and doxorubicin (drug control) or DMSO (solvent control) were incubated with JC-1 staining solution for 30 min according to the manufacturer's protocol as reported previously [19]. Cells were then measured in a BD FACS Aria I Cell Sorter Flow Cytometer (Becton-Dickinson, Germany). The JC-1 signal was measured at an excitation of 561 nm (150 mW) and detected using a 586/15 nm band-pass filter. The signal was analyzed at 640 nm excitation (40 mW) and detected using a 730/45 nm bandpass filter. Cytographs were analyzed using BD FACSDiva™ Flow Cytometry Software Version 6.1.2 (Becton-Dickinson). All experiments were performed at least three times and in triplicate, and the most illustrative data were shown.

Measurement of reactive oxygen species (ROS)

The MCF7 cells (3 mL, 1×10^4 cells/mL) treated for 24 h (incubated in humidified 5% CO_2 atmosphere at 37 °C) with different concentrations (¼×IC_{50}, ½×IC_{50} and IC_{50}) of ardisinol III, and doxorubicin (drug control) or DMSO (solvent control)) were analyzed for ROS production with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFH-DA) (Sigma-Aldrich) using OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence) as recommended by the manufacturer, Cell Biolabs Inc. (San Diego, USA) [19]. The fluorescence was measured using SpectraMax® M5 Microplate Reader (Molecular Devices, Biberach, Germany) at 480/530 nm. All experiments were performed at least three times and in triplicate.

Results

The cytotoxicity of Ardisinol III and doxorubicin in a panel of carcinoma cells was determined by NR uptake assay. The selectivity index (S.I.) was determined as the ratio of IC_{50} value in the CRL2120 normal fibroblasts divided by the IC_{50} in the cancer cell lines. The obtained IC_{50} values and S.I. are summarized in Table 1.

The IC_{50} values ranged from 0.88 µM (against SPC212 mesothelioma cells) to 8.36 µM [against hepatocarcinoma HepG2 cells] for ardisinol III and from 0.07 µM (against SPC212 cells) to 1.01 µM (against A549 lung cancer cells) for doxorubicin. In normal CRL2120 fibroblasts, ardisinol III had low cytotoxic effects, with an IC_{50} value of 51.91 µM (Table 1). The S.I. in all cell lines were above 6, showing that this compound is less toxic in normal fibroblast and could be suitable as a potential anticancer drug. Interestingly, the S.I. of ardisinol III in all cell lines was better than that of the reference drug, doxorubicin (Table 1).

The effects of ardisinol III and doxorubicin on cell cycle distribution of MCF7 cells are depicted in Figure 2. The tested alkenylmethylresorcinol modified the distribution of cell cycle phases in MCF7 cells, with dose-dependent increase of cells in sub-G0/G1 phase. This compound induced cell cycle arrest in G0/G1 meanwhile doxorubicin induced arrest in G2/M. Treated cells progressively underwent apoptosis, with percentages in sub-G0/G1 phase ranged from 12.1% (¼ × IC_{50}) to 17.3% (IC_{50}) for ardisinol III, and from 27.6% (¼ × IC_{50}) to 60% (IC_{50}) for doxorubicin. In non-treated cells, the percentage of cells in sub-G0/G1 phase was 3.1% (Figure 2).

Breast adenocarcinoma MCF7 cells were treated with ardisinol III and doxorubicin, and their influences on caspases 3/7 and 9 activities were evaluated (Figure 5). The two molecules activated caspases 3/7 and 9 in MCF7 cells at their IC_{50} and ½-

fold IC_{50} values. Optimal activations with ardisinol III were observed when cells were treated with IC_{50}, and were 5.03-fold and 3.82-fold respectively for caspases 3/7 and 9 (Figure 3).

The involvement of ardisinol III and doxorubicin in the integrity of MMP in MCF7 cells was evaluated. Data depicted in Figure 4 show that the studied alkenylmethylresorcinol altered the MMP in MCF7 cells in a dose-dependent manner upon 72 h treatment. The degree of alteration ranged from 9.8% (¼ × IC_{50}) to 17.8% (IC_{50}); Doxorubicin also altered MMP in the range of 20.7% (¼ × IC_{50}) to 26.0% (IC_{50}).

The effects of ardisinol III and doxorubicin after 24 h treatment on ROS levels were evaluated in MCF7 cells. Results are depicted in Figure 5. Ardisinol III and doxorubicin induced increases in ROS levels respectively by 3.28-fold and 2.40-fold as compared to non treated cells.

Discussion

Cancer still remains a serious health concern worldwide despite the progress in various type of therapy. In this study, we targeted some commonly occurring and most killing cancers such as breast, colon, lung and liver cancers [3]. According to the National Cancer Institute (NCI), molecules with IC_{50} values around or below 4 µg/mL or 10 µM [14, 26, 27] can be considered as potential cytotoxic substances. Ardisinol III had IC_{50} values below 10 µM in all the six tested carcinoma cell lines. Importantly, IC_{50} values below 1 µM were obtained in lung SPC212 human mesothelioma cell line, indicating the good cytotoxic potential of the tested compound. The good selectivity of ardisinol III makes it compatible for cancer chemotherapy. Though this compound was less active than the reference drug, it was found that ardisinol III was also more selective in all tested cell lines than doxorubicin. This is an indication this alkenylmethylresorcinol could be more appropriate in the treatment of carcinoma.

In the present work, one of the sensitive cell lines, MCF7, was randomly selected as a model for the mechanistic studies. It was suggested that the percentage of induction of apoptosis between 10-20% at up to two-fold IC_{50} of a phytochemical can be considered as moderate [14]. Hence, ardisinol III moderately induced apoptosis (17.3% at IC_{50}; Figure 2) in MCF-7 cells. Caspases are central regulators of apoptosis and their activation is considered moderate in the range of 4-fold to 8-fold when a compound is tested at up to two-fold IC_{50} [14]. Herein, the activation of caspases 3/7 (effector caspases; >4-fold activation) and 9 (initiator caspases; 3.82-fold) (Figure 3) is an indication that intrinsic mitochondrial pathway could be involved in the cytotoxic effect of ardisinol III [28]. Mitochondria play a central role in cellular metabolism as main ATP source, and during ATP biosynthesis, ROS are generated. Mitochondria-targeting compounds kill cancer cells due to their ability to initiate mitochondrial outer membrane permeabilization [29, 30]. When tested at up to two-fold IC_{50}, a strong MMP alteration of a compound is considered in the range of 20-50% [14]. In the present study, ardisinol III induced up to 26.0% alteration of MMP at IC_{50} (Figure 4). Also, more than 3-fold increase in ROS production was obtained when MCF-7 cells were treated with ardisinol III at IC_{50} (Figure 5). These data indicate that MMP alteration as well as increase ROS production could be considered as other modes of ardisinol III-induced cell death in MCF-7 cells.

The cytotoxicity of this compound is reported for the first time. This study therefore, provides data on the potential of alkenylmethylresorcinol derivative as cytotoxic compound. Also, ardisinol III has differential effects depending on the cell type involved. This might be due to genetic background of each tested cell line that could result in differential metabolic activities [31, 32]. It should be noted that only activation of caspases, loss of MMP and increase ROS production are not enough to identify cell apoptosis. Therefore, additional studies including detection of other molecules related to apoptosis such as BCL2, BAX, PARP, etc., will be performed.

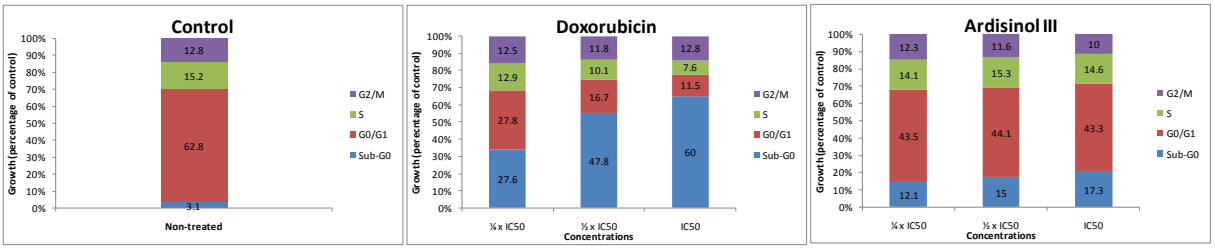

Figure 2. Distribution of cell cycle in MCF-7 cells treated with ardisinol and doxorubicin for 72 h. IC_{50} values were 3.22 µM (ardisinol III) and 0.35 µM (doxorubicin). Data are mean of three experiments.

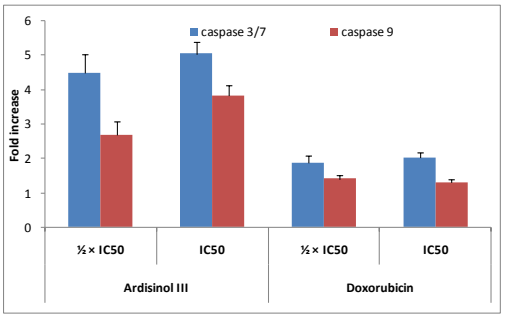

Figure 3. Activation caspases 3/7 and 9 in MCF-7 cells treated with ardisinol and doxorubicin for 6 h. IC_{50} values were 3.22 µM (ardisinol III) and 0.35 µM (doxorubicin). Data are mean of three experiments.

Table 1. Cytotoxicity of tested ardisinol III and doxorubicin in carcinoma and normal cell lines using neutral red assay

Cell lines	Compounds, IC_{50} values (in µM) and selectivity index* (in bracket)	
	Ardisinol III	Doxorubicin
A549	**6.28 ± 0.94** (8.26)	**1.01 ± 0.19** (0.59)
SPC212	**0.88 ± 0.06** (58.94)	**0.07 ± 0.01** (8.69)
DLD-1	**6.71 ± 0.57** (7.73)	**0.37 ± 0.05** (1.59)
Caco-2	**6.37 ± 0.71** (8.15)	**0.72 ± 0.13** (0.82)
MCF7	**3.22 ± 0.55** (16.12)	**0.35 ± 0.05** (1.69)
HepG2	**8.36 ± 0.22** (6.21)	**0.18 ± 0.03** (3.21)
CRL2120	51.91 ± 3.78	**0.59 ± 0.13**

(*): The selectivity index was determined as the ratio of IC_{50} value in the CRL2120 normal fibroblasts divided by the IC_{50} in the cancer cell lines. In bold: significant activity [14, 26, 27, 33]

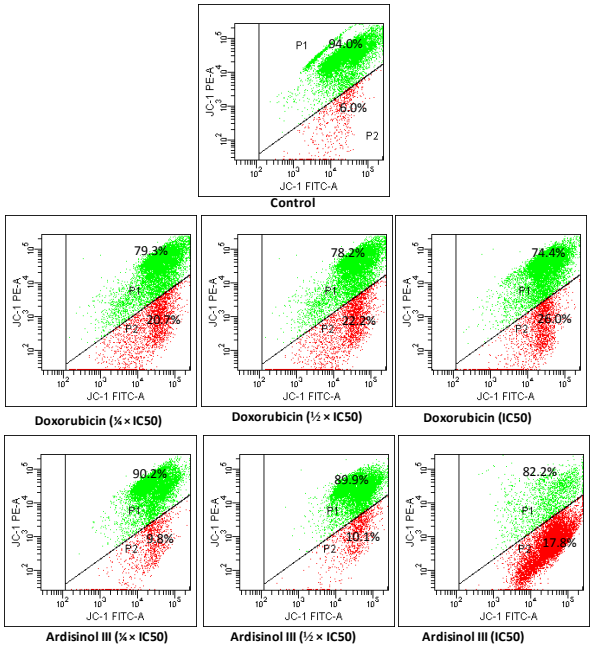

Figure 4. Effects of ardisinol and doxorubicin on MMP in MCF-7 cells for 72 h. IC_{50} values were 3.22 µM (ardisinol III) and 0.35 µM (doxorubicin). Data are most illustratives of three experiments.

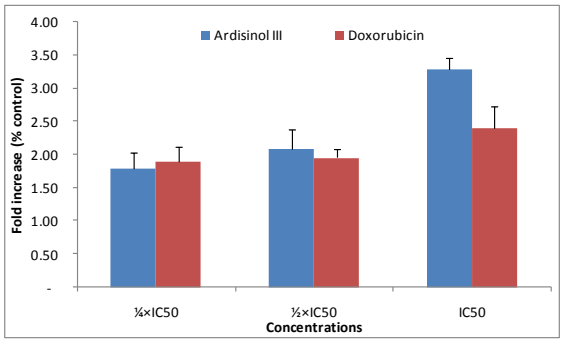

Figure 5. Generation of ROS in MCF-7 cells treated with ardisinol and doxorubicin for 24 h. IC_{50} values were 3.22 µM (ardisinol III) and 0.35 µM (doxorubicin). Data are mean of three experiments

Conclusions

The cytotoxicity and the selectivity of ardisinol III makes it a potential cytotoxic agent that could be used to fight against human carcinoma. This phytochemical induces apoptosis in MCF7 breast adenocarcinoma cells, mediated by the activation of caspase 3/7 and 9, loss of MMP and increase ROS production.

Abbreviations

BAX: Bcl-2-associated X protein
Bcl-2: B-cell lymphoma 2
DMSO: Dimethylsufoxide
H_2DCF = 2´,7´-Dichlorodihydrofluorescein
H_2DCFH-DA = 2´,7´-Dichlorodihydrofluorescein diacetate
MMP: Mitochondrial membrane potential
NR: Neutral red Uptake
PARP: poly(ADP-ribose) polymerase
ROS: Reactive oxygen species

Authors' Contribution

VK, BLN, YMMN, IC, RM, OK carried out the experiments. VK, PT and HS designed the study. VK wrote the manuscript. HS supervised the work and provided the facilities for the study. All authors read and approved the final manuscript.

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Conflict of interest

There is no conflict of interest.

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