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Gamma-tocotrienol reverses multidrug resistance of breast cancer cells with a mechanism distinct from that of atorvastatin

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ABSTRACT

In addition to its antioxidant properties, γ-tocotrienol also has the ability to inhibit HMG-CoA reductase, which is the key enzyme in the mevalonate pathway for cholesterol biosynthesis. Statins, the competitive inhibitors of HMG-CoA reductase, display potent anticancer activity and reversal ability of multidrug resistance in a variety of tumor cells, which is believed to be due to their inhibition of HMG-CoA reductase. Here, we determined the role of the mevalonate pathway in γ-tocotrienol-mediated reversal of multidrug resistance in cancer cells. We found both γ-tocotrienol and atorvastatin effectively reversed multidrug resistance of MCF-7/Adr and markedly inhibited the intracellular levels of FPP and GGPP. Exogenous addition of mevalonate or FPP and GGPP almost completely prevented the reversal ability of atorvastatin but only partly attenuated the reversal effect of γ-tocotrienol on doxorubicin resistance. In addition, γ-tocotrienol actively inhibited the expression of P-gp and increased the accumulation of doxorubicin in cells, which led to the enhanced G2/M arrest and cell apoptosis. Taken together, γ-tocotrienol reversed the multidrug resistance of MCF-7/Adr with a mechanism distinct from that of atorvastatin. Instead of the mevalonate pathway, the inhibition of P-gp expression is a potential mechanism by which γ-tocotrienol reverses multidrug resistance in MCF-7/Adr.

1. Introduction

γ-Tocotrienol represents one of the eight natural isoforms that make up the family of vitamin E compounds and displays potent anticancer activity at treatment doses that have little or no effect on normal cell function and viability [1,2]. Although chemically very similar, tocopherols have a saturated phytyl chain, whereas tocotrienols have an unsaturated phytyl chain, attached to a chromane ring structure. Based on the number and location of the methyl groups on their chromanol rings, there are α-, β-,γ-, and δ-tocopherols and α-, β-,γ-, and δ- tocotrienols [3]. Both tocopherols and tocotrienols have well-known antioxidant capacity, which helps to prevent oxidative damage to polyunsaturated fatty acids [4]. However, tocotrienols possess powerful neuroprotective, anticancer, and cholesterol-lowering properties that are often not exhibited by tocopherols [5]. Emerging evidence suggests that tocotrienols are significantly more potent in suppressing growth and inducing cell death than tocopherols. The relative anticancer potency of vitamin E isoforms was characterized as follows: δ-tocotrienol ≥ γ-tocotrienol > α-tocotrienol > δ-tocopherol > γ- and α-tocopherol [2,6,7].

In addition to possessing antioxidant properties, tocotrienols influence cholesterol synthesis by directly regulating the expression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, principally through a posttranscriptional process involving accelerated degradation of the reductase protein [4,8,9]. HMG-CoA reductase is the key enzyme in the mevalonate pathway for cholesterol biosynthesis. It catalyzes the conversion of HMG-CoA to mevalonate, which subsequently produces isoprenoid intermediates such as farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), which are required for posttranslational modifications (isoprenylation) of various small GTP binding proteins, such as the Ras/Rho super family of proteins [10–12]. The isoprenylation of small G-proteins is essential for them to anchor to cell membranes and thus become activated following receptor activation, and subsequently participate in activating downstream mitogenic signaling pathways, such as the MAPK and Akt pathways [12,13].

Abbreviations: MDR, multidrug resistance; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; RI, Resistance index; IC_{50}, half-maximal inhibitory concentration; ROS, reactive oxygen species; ABC transporters, ATP-binding cassette transporters.

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It is clear now that HMG-CoA reductase activity is up-regulated in some malignant cells compared to that in their normal counterparts [14,15]. As a result, much attention has been focused on the development of agents that target and inhibit HMG-CoA reductase activity in cancer chemotherapy. Statins represent a class of such agents that act as competitive inhibitors of HMG-CoA reductase and display potent anticancer activity in a variety of cell culture and animal tumor models [16,17]. The cancer prevention activity reported for statins is believed to be owing to their inhibition of the isoprenylation of G-proteins and the subsequent alteration of downstream signaling pathways [18,19]. Since γ-tocotrienol is another competitive inhibitor of HMG-CoA reductase, it is hypothesized that inhibition of mevalonate synthesis may be ultimately responsible for mediating the growth inhibiting effects of γ-tocotrienol [15]. A previous study has revealed that the property of γ-tocotrienol as an HMG-CoA reductase inhibitor is related to its radioprotective effect. Deng et al. investigated the effect of γ-tocotrienol on preventing ovariectomy-induced bone loss and demonstrated that γ-tocotrienol could protect bone via the mevalonate pathway as an HMG-CoA reductase inhibitor [20,21].

Breast cancer is the most commonly occurring cancer in women, and the high rate of drug resistance limits the effectiveness of the chemotherapy [22]. Multidrug resistance (MDR) in cancer arises from increased copy number of the *mdr* genes and over-expression of the encoded membrane proteins [23]. The MDR proteins, of which P-glycoprotein (P-pg, also called ABCB1/MDR) is the most prominent member, cause efflux of the drugs from cancer cells. Therefore, new adjuvants that enhance the efficacy of chemotherapy in MDR cancer cells are urgently needed.

γ-Tocotrienol is always co-administered with other anticancer agents, such as statins [12,24,25], celecoxib [26,27], or tamoxifen [28], resulting in synergistic antiproliferative effects on mammary tumor cell growth. Therefore, with the combination of γ-tocotrienol and doxorubicin together to treat multidrug resistant cells of human breast cancer MCF-7/Adr, the dosage of doxorubicin could be decreased and the resistance of MCF-7/Adr would be reversed by γ-tocotrienol. Up to now, most researches have focused on the expression and function of ATP-binding cassette (ABC) transporters (principally P-gp) to elucidate the mechanism underlying MDR reversal. However, the role of mevalonate pathway in MDR reversal is still unknown.

In the present study, we hypothesized whether γ-tocotrienol can reverse MDR of human breast cancer cell MCF-7/Adr and whether the reversal effect was mediated by the mevalonate pathway via HMG-CoA reductase inhibition. Statins have been shown to display potent anticancer activity through the inhibition of HMG-CoA reductase activity. Thus, atorvastatin was simultaneously used as a positive control to identify the role of the mevalonate pathway in the process of MDR reversal.

2. Materials and methods

2.1. Reagents and antibodies

γ-Tocotrienol and atorvastatin were kind gifts from Dr. Qiang Fu (Center for Osteoporosis & Metabolic Bone Diseases, University of Arkansas for Medical Sciences, Little Rock, Arkansas, USA), and were dissolved in methanol at a concentration of 50 mM as a stock solution. Mevalonic acid, FPP, GGPP, FTI-277, and GGTI-2133 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Doxorubicin hydrochloride, obtained from Zhejiang Hisun Pharmaceutical Co., LTD. (China), was dissolved in saline and further diluted in culture medium. Primary antibodies against β-actin (ACTBD11B7: sc-81178) and Mdr-1 (G-1: sc-13131) were purchased from Santa Cruz (CA, USA).

2.2. Cell lines and cell culture

The human breast cancer cell line MCF-7 was purchased from the cell library of biochemistry and cell biology, Chinese Academy of Sciences Institute. The cells were cultured in minimum essential medium (MEM, Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Sijiqing, Zhejiang, China), 0.11 g/l sodium pyruvate, 0.01 mg/ml bovine insulin, 100 U/ml penicillin, and 100 μg/ml streptomycin. A human breast cancer cell line resistant to doxorubicin (MCF-7/Adr) was purchased from Shanghai Bogoo Biotech and cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Both cell lines were cultured at 37 °C in a 5% CO_2 incubator. To maintain drug resistance, doxorubicin (1 μg/ml) was supplied to MCF-7/Adr cells at regular intervals. Cells in the logarithmic growth phase were used for further experiments.

2.3. Determination of multidrug resistance

The multidrug resistance of MCF-7/Adr cells to doxorubicin was determined using a CellTiter-Blue Cell Viability Assay kit (Promega, USA). MCF-7/Adr cells and the parent MCF-7 cells were seeded into 96-well plates (Corning Inc., USA) and incubated with various concentrations of doxorubicin for 48 h. A total of 20 μl of CellTiter-Blue Reagent was added to each well, and the cells were incubated at 37 °C for 4 h to allow the dark blue resazurin to be reduced into pink resorufin. Finally, the surviving cells were measured with a fluorescence reader with excitation 560 nm and emission 590 nm filter pair. Each group consisted of five parallel wells. After plotting the dose-response curve, IC_{50} was calculated. The degree of resistance was calculated by resistance index (RI), which was obtained from the following formula: IC_{50} of MCF-7-Adr cells/IC_{50} of MCF-7 cells [22,29]. All assays were performed in triplicate. The IC_{50} values were determined by GraphPad Software.

2.4. Reversal effect assay

The ability of γ-tocotrienol or atorvastatin to reverse multidrug resistance of MCF7/Adr was measured using the CellTiter-Blue Cell Viability Assay kit. MCF-7/Adr cells were seeded in 96-well plates at a density of 8×10^3 cells/well. Combinations of different concentrations of doxorubicin with γ-tocotrienol (25 μM) or atorvastatin (10 μM) diluted in RPMI-1640 were then added. The cells were incubated in a humidified incubator in 5% CO_2 at 37 °C for 72 h, and the quantity of viable cells was determined using a CellTiter-Blue Reagent. Each group consisted of five parallel wells. The reversal potency was obtained from the fluorescence readings of the cytotoxic drug in the presence of the test drugs compared to that of the fluorescence readings of cytotoxic drug alone.

2.5. Apoptosis analysis

Apoptotic cells were detected by measuring DNA fragmentation using the TdT-mediated dUTP nick-end labeling (TUNEL) method (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. MCF-7/Adr cells were seeded in 6-well plates at a density of 4×10^5 cells/well with or without doxorubicin, alone or in combination with γ-tocotrienol or atorvastatin for 48 h. Cells were trypsinized and fixed with 4% paraformaldehyde for 30 min and then washed with PBS twice. Samples were treated with cytopermeabilization solution (0.2% Triton X-100) and then washed again with PBS. A mixture of 2 μl TdT (terminal deoxynucleotidyl transferase) enzyme and 48 μl fluorescein-labeled dUTP was added to each sample. Finally, the samples were incubated for 1 h at 37 °C in a container to ensure proper moisture and darkness.