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Original article

Antroquinonol blocks Ras and Rho signaling via the inhibition of protein isoprenyltransferase activity in cancer cells

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ABSTRACT

Antroquinonol is the smallest anticancer molecule isolated from *Antrodia camphorata* thus far. The ubiquinone-like structure of Antroquinonol exhibits a broad spectrum of activity against malignancies in vivo and in vitro. However, the mechanism of action of Antroquinonol remains unclear. Here, we provide evidence that Antroquinonol plays a role in the inhibition of Ras and Ras-related small GTP-binding protein functions through the inhibition of protein isoprenyl transferase activity in cancer cells. Using cell line-based assays, we found that the inactive forms of Ras and Rho proteins were significantly elevated after treatment with Antroquinonol. We also demonstrated that Antroquinonol binds directly to farnesyltransferase and geranylgeranyltransferase-I, which are key enzymes involved in activation of Ras-related proteins, and inhibits enzymes activities in vitro. Furthermore, a molecular docking analysis illustrated that the isoprenoid moiety of Antroquinonol binds along the hydrophobic cavity of farnesyltransferase similar to its natural substrate, farnesyl pyrophosphate. In contrast, the ring structure of Antroquinonol lies adjacent to the Ras-CAAX motif-binding site on farnesyltransferase. The molecular docking study also showed a reasonable correlation with the IC_{50} values of Antroquinonol analogues. We also found that the levels of LC3B-II and the autophagosome-associated LC3 form were also significantly increased in H838 after Antroquinonol administration. In conclusion, Antroquinonol inhibited Ras and Ras-related GTP-binding protein activation through inhibition of protein isoprenyl transferase activity, leading to activation of autophagy and associated mode of cell death in cancer cells.

1. Introduction

Natural products have traditionally been an important source of organic chemicals and pharmaceuticals. More than 20 new drugs derived from natural sources have been launched on the market during the past 10 years [1]. We adopted an anticancer drug screen model to test the anticancer effects of crude extracts of Chinese herbs, plants, and fungi collected in Taiwan. Anticancer activities were identified in *Antrodia camphorata*, a species of fungus endemic to Taiwan [2]. Antroquinonol is a new chemical entity isolated from the mycelium of *A. camphorata* that showed interesting anticancer and anti-inflammatory activities [3–5]. Previous studies have indicated that signaling molecules, such as PI3K, AMPK, and mTOR, participated in Antroquinonol-induced cancer cell death, whereas Nrf2 and NF-kB were involved in the anti-inflammatory effects of Antroquinonol [3,4,6]. However, the precise mechanism of action remains unknown.

Yu et al. reported that Antroquinonol caused a mobility shift of the Ras protein on immunoblots [7], suggesting that Antroquinonol-mediated Ras expression and activation in cancer cells. Ras proteins have been considered drug targets due to gain-of-function mutations in approximately 30% of all human cancers [8]. Ras undergoes posttranslational processing to become a biologically active protein that is embedded in the plasma membrane. Prenylation is mediated by isoprenyltransferase such as farnesyltransferase (FTase) and geranylgeranyltransferase-1 (GGTase-1),

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and is the first committed step for Ras processing [9]. FTase, for example, covalently transfers a 15-carbon isoprenyl group from farnesyl pyrophosphate (FPP) to the cysteine residue of the conserved CAAX motif in the C-terminus of Ras [10].

FTase inhibitors (FTIs) represent a new class of anticancer agents that inhibit Ras prenylation. Several FTIs that mimic the structures of the FTase substrates, farnesyl pyrophosphate (FPP) and CAAX tetrapeptides, have been developed and are currently being evaluated in clinical trials [11]. However, the results are not entirely promising. Two main questions driving preclinical and clinical studies of FTIs are whether Ras can be alternatively prenylated by GGTase-1 and whether inhibition of FTase can affect proteins other than Ras. Evidence indicates that most FTIs specifically inhibit FTase but not GGTase-1 in vitro and in vivo [12–14]. As a result, alternative prenylation of K-Ras and N-Ras by the attachment of a 20-carbon geranylgeranyl chain by GGTase-I has been reported in FTI-treated cells [15,16]. Furthermore, cells with wild-type or mutant Ras were sensitive to FTIs in vitro and in vivo [13,17]. Collectively, these observations indicate that prenylated proteins other than Ras might also contribute to the anticancer activities of FTIs. Thus, further investigation of an active prenyltransferase inhibitor that blocks the growth of Ras-dependent and Ras-independent cancer cells is warranted.

In this study, we used molecular docking techniques combined with biochemistry, molecular biology, and medicinal chemistry approaches to elucidate the mechanism of action of Antroquinonol. Our results suggest that Antroquinonol prevents posttranslational prenylation of the Ras and Ras-related proteins through inhibition of the enzyme isoprenyltransferase, ultimately causing cancer cell death.

2. Materials and methods

2.1. Cell lines and cell culture

Human lung cancer (A549 and H838), liver cancer (HepG2 and Hep3B), and leukemia (K562 and THP-1) cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). A549 was cultured in Dulbecco's modified Eagle's medium. H838, K562 and THP-1 were cultured in RPMI-1640 medium. HepG2 and Hep3B were cultured in MEM. All cells were cultured at 37 °C in 5% CO_2 in culture media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL streptomycin and penicillin. For treatment, cells were seeded in six-well plates at 6.25×10^5 cells/well. On the following day, the media was changed to serum-free media, and cells were serum-starved for 24 h. Antroquinonol [4] was dissolved in DMSO and diluted to the required concentration in serum-free medium. Cultures were then treated with diluted Antroquinonol as indicated. After treatment, cells were washed with cold phosphate-buffered saline (PBS) and lysed using RIPA buffer containing phosphatase and protease inhibitors. Cell culture media and related reagents were purchased from Invitrogen (Rockville, MD).

2.2. Immunoblot analysis

Proteins were resolved on 12.5% SDS-polyacrylamide gels. Blots were blocked with 3% bovine serum albumin (BSA) and probed with a 1:1000 dilution of antibodies LC3B (Novus Biologicals, Cambridge, UK), Ras, GAPDH, or Rho (Millipore, Temecula, CA). Secondary antibodies were conjugated to horseradish peroxidase, which was detected using a 3,3′-diaminobenzidine substrate kit (Vector Laboratories, Burlingame, CA). The immunoreactive bands were quantified by densitometry using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

2.3. CCK-8 cell viability assay

Cell viability was measured using Cell Counting Kit-8 (CCK-8, Enzo Life Sciences, Farmingdale, NY). After treatment, CCK-8 solution was added to each well and incubated for 4 h. The concentration of formazan was measured with a spectrophotometer at an absorbance wavelength of 450 nm. Cell viability was expressed as a percentage of the corresponding control.

2.4. SDS-PAGE-based prenyltransferase assay

In vitro prenylation reactions were performed in 20 μL reaction buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, and 20 μM GDP) mixed with 3 μg FTase (Jena, Germany), 25 μM NBD-FPP, and 2 μg H-Ras^{GST} in the presence or absence of various concentrations of Antroquinonol. Reactions were incubated for 2 h at 37 °C and quenched by SDS-PAGE sample buffer. The mixtures were resolved by 15% SDS-PAGE. The gel was scanned using a Typhoon 9400 scanner (GE Healthcare, UK) (excitation laser, 473 nm; emission cutoff filter, 510 nm) followed by staining with Coomassie blue. The fluorescent bands were quantified using Image-Pro Plus software.

2.5. Immunofluorescent and DAPI staining

Cells were seeded onto glass coverslips. After an overnight incubation, cells were treated with Antroquinonol for 24 h. After treatment, cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked in 3% BSA and then incubated with a LC3B antibody at room temperature. After washing, cells were incubated with a fluorescein isothiocyanate-conjugated secondary antibody (Life Technologies, Scotland, UK) at room temperature for 60 min. Cells were mounted with Dapi-Fluoromount-G™ (SouthernBiotech, Birmingham, USA) and visualized by confocal fluorescence microscopy using a Zeiss LSM 780 plus ELYRA S.1.

2.6. Molecular docking

The amino acid sequence for FTase (Accession no.: 1JCQ_A) was downloaded from the National Center for Biotechnology Information protein database. A CDOCKER–A CHARMm-based molecular docking algorithm was applied to predict and assess the interaction between Antroquinonol and the FTase CAAX box [18]. In order to limit bias, all user-adjustable parameters were kept at their default settings.

2.7. Kinetics and affinity detection by BIAcore

The kinetics and affinity of protein FTase and GGTase-I against Antroquinonol respectively were measured using BIAcore T200 (Uppsala, Sweden, GE). Recombinant FTase and GGTase-I proteins were expressed using Baculovirus expression system and purified by Ni column. Proteins FTase and GGTase-I were immobilized on the Series S Sensor Chip CM5 using a standard amine coupling method. A scouting procedure was performed to establish an appropriate concentration range of analyte and monitor association/dissociation time for affinity measurement. The experiment was performed at 25 °C. Different concentrations of Antroquinonol were finally injected to the reference, FTase and GGTase surfaces at a flow rate of 30 μL/min. In each injection, association and dissociation phases were monitored for 240 s and 120 s respectively. One buffer-only injection was included for double reference. The carry-over injections refer to buffer injections that follow the sample injections during each cycle were set as control. All