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A semisynthetic taxane Yg-3-46a effectively evades P-glycoprotein and β-III tubulin mediated tumor drug resistance in vitro

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ABSTRACT

Tumor resistance, especially that mediated by P-glycoprotein (P-gp) and β-III tubulin, is a major obstacle to the efficacy of most microtubule-targeting anticancer drugs in clinics. A novel semisynthetic taxane, 2-debenzoyl-2-(3-azidobenzyl)-10-propionyldocetaxel (Yg-3-46a) was shown to be highly cytotoxic to breast cancer cell lines MCF-7 and MCF/ADR which overexpressed P-gp via long term culture with doxorubicin, and cervical cancer cell lines Hela and Hela/βIII which overexpressed βIII-tubulin via stable transfection with TUBB3 gene. siRNA transfection experiments also confirmed that Yg-3-46a can circumvent P-gp and β-III tubulin mediated drug resistance. In addition, its cytotoxicity was lower than that of paclitaxel in the human mammary cell line HBL-100 and the human telomerase-immortalized retinal pigment epithelium cell line (hTERT-RPE1), suggesting a better safety margin for this compound in vivo. It exhibited more potent microtubule polymerization ability than paclitaxel in vitro, and also induced G_2/M phase arrest in MCF-7/ADR cells. Moreover, it was found to induce apoptosis in MCF-7/ADR cells through the caspase-dependent death-receptor pathway by enhancing levels of Fas and FasL, and activating caspase-8 and 3. Yg-3-46a was found to be a poorer substrate of P-gp compared to paclitaxel, in both binding and ATPase experiments, which is likely responsible for its ability to circumvent P-gp mediated multidrug resistance (MDR). All of these results indicate that Yg-3-46a is a novel microtubule-stabilizing agent that has the potential to evade drug resistance mediated by P-gp and β-III tubulin overexpression.

1. Introduction

Paclitaxel, a plant-derived natural product [1], has been used for the treatment of breast cancer, ovarian cancer, Kaposi's sarcoma, as well as many other cancers, including non-small cell lung, prostate, and cervical cancers [2–4]. It is a microtubule (MT) stabilizing agent, binding to β-tubulin and thus inhibiting cell proliferation by disrupting normal mitotic spindle formation and inducing cell apoptosis [5,6].

Despite the prominent success of paclitaxel, its clinical use is severely restricted by intrinsic and acquired tumor cell resistance [7,8]. Many tumor drug resistance mechanisms have been revealed [9–11], although only some of these have been confirmed clinically, including overexpression of P-glycoprotein (P-gp, ABCB1, MDR1) and the β-III isotype of tubulin [12–14].

Drug resistance mediated by P-gp overexpression is also known as multidrug resistance (MDR), a phenomenon by which resistance to one drug is associated with cross-resistance to other structurally unrelated drugs [15]. MDR is often linked to the overexpression of drug efflux pumps, especially those in the ATP-binding cassette (ABC) family of transporters [16,17], among which the best known is P-gp, encoded by the *abcb1/mdr*1 gene [18]. P-gp is an ATP-dependent broad-spectrum drug efflux pump, which can decrease the intracellular drug concentration, thereby reducing drug-mediated cytotoxicity. Numerous studies have confirmed the relevance of P-gp expression in clinical resistance to cancer chemotherapy [19–21]; e.g. vinblastine, vincristine, doxorubicin and paclitaxel are all affected by P-gp mediated drug resistance [22].

The β-III isotype of tubulin is usually expressed in neurons of the central and peripheral nervous systems. In non-neoplastic tissues it is rarely detectable, whereas many tumor cells resistant to paclitaxel overexpress β-III tubulin, including epithelial tumors of the ovary, breast, uterine cervix, prostate, stomach, as well as other organs and tissues [23,24]. There are some hypotheses proposing why β-III tubulin overexpression results in tumor cell resistance to paclitaxel. For example, β-III tubulin can increase the dynamic instability of MTs, thus counteracting the stabilization induced by paclitaxel [10], or it may affect paclitaxel binding to β-tubulin in MTs, although the exact mechanism is still not fully elucidated.

To develop novel taxane-based MT stabilizing agents capable of overcoming drug resistance mediated by P-gp and β-III tubulin

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overexpression, a series of new synthetic taxanes has been designed, synthesized and tested in our labs, among which Yg-3-46a, 2-debenzoyl-2-(3-azidobenzyl)-10-propionyldocetaxel (Fig. 1B) is the most active [25], and was thus selected for further studies.

In this paper, the in vitro anticancer activity of this compound has been evaluated in the breast cancer cell line MCF-7 and its P-gp-overexpressing drug-resistant counterpart MCF-7/ADR (Fig. 2A), as well as in the human cervical cell line Hela and the drug-resistant cell line Hela/βIII derived by β-III tubulin gene transfection (Fig. 2B). Furthermore, the mechanism for its effectiveness in drug-resistant tumor cells was also explored.

2. Materials and methods

2.1. Materials

Paclitaxel is a commercial product from Guilin Huiang Biopharmaceutical Co., Ltd., with purity better than 99%. Yg-3–46a was synthesized in our laboratory (Supplemental data 1), with purity better than 98% (Supplemental data 2). All compounds were dissolved in DMSO. Verapamil (VRL), propidium iodide (PI), RNase A and monoclonal antibody specific for β-actin were purchased from Sigma–Aldrich (St. Louis, MO, USA). Lipofectamine RNAiMAX Reagent, P-gp siRNA (5′-GCGAAGCAGUGGUUCAGGUTT-3′), TUBB3 siRNA (5′-UCUCUUCAGGCCUGACAAUTT-3′), negative control (5′-UUCUCCGAACGUGUCACGUTT-3′) were purchased from Invitrogen Trading (Shanghai) Co., Ltd. The antibodies for P-gp, α-tubulin, goat anti-mouse IgG H&L (ChromeoTM488) secondary antibody and DAPI were purchased from Abcam (UK). The other antibodies were purchased from Cell Signaling Technology (USA). Tubulin Polymerization Assay Kit (Porcine tubulin fluorescence based, Cat. # BK011P) was purchased from Cytoskeleton (USA). Pgp-Glo™ Assay Systems was purchased from Promega (USA). The Annexin V-FITC/PI Apoptosis Kit was purchased from Korad Biotech Technology (China).

2.2. Cell lines and culture

The human breast cancer cell line MCF-7 and its doxorubicin-resistant counterpart MCF-7/ADR (Supplemental data 3) were provided by Xiangya Hospital, Central South University. Hela and Hela/βIII cells were a generous gift from Dr. Richard Ludeña at the University of Texas. The human mammary line HBL-100 cell was provided by Cell Resource Center, IBMS, CAMS/PUMC. The human telomerase-immortalized retinal pigment epithelium cell line hTERT-RPE1 was a generous gift from Dr. Changjun Zhun at Tianjin Normal University. MCF-7 and MCF-7/ADR were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. To maintain the cell drug resistance phenotype, 1 μg/mL doxorubicin was added to the culture of MCF-7/ADR. Hela, Hela/βIII and HBL-100 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, and Hela/βIII cell line was additionally supplemented with 0.5 mg/mL G418 sulfate. hTERT-RPE1 was cultured in DMEM-Ham's F12 supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

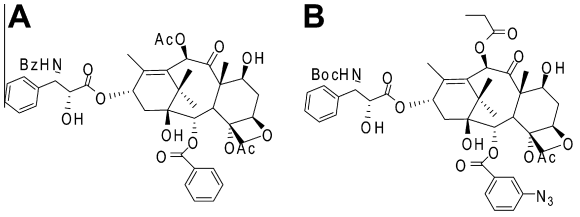

Fig. 1. Chemical structures of paclitaxel and Yg-3-46a. (A) Paclitaxel and (B) Yg-3-46a.

2.3. MTT assay

Cell viability after Yg-3-46a treatment was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 3000 cells per well were seeded in 96-well plates and incubated under normal conditions for 24 h. Cells were treated with different concentrations of the test agent for 72 h, then 20 μL of 5 mg/mL MTT solution was added to the wells for 4 h at 37 °C until crystals were formed. After the medium was removed, 150 μL of DMSO was added to each well. The plates were gently agitated until the color reaction was uniform and the absorbance was measured at 570 nm with a microplate reader. Vehicle-only treated cells served as the indicator of 100% cell viability. The 50% inhibitory concentration (IC_{50}) was defined as the concentration that reduced the absorbance of the vehicle-only treated wells by 50% in the MTT assay.

2.4. siRNA transfection

Cells (5×10^4) were dispensed in 6-well plates, and after growth for 24 h were then transfected with the indicated siRNAs (P-gp siRNA: 100 nM; TUBB3 siRNA: 100 nM) using Lipofectamine RNAiMAX Reagent according to the manufacturer's protocol. Silencing was examined 48 h after transfection by Western blotting. siRNA-transfected cells were plated in 96-well plates (3×10^3) and treated with different concentrations of the test agents. Then the IC_{50} of Yg-3-46a in these manipulated cells was determined using the MTT assay.

2.5. In vitro tubulin polymerization assay

In vitro tubulin polymerization was determined using the tubulin Polymerization Assay Kit from Cytoskeleton Inc. (USA), containing porcine brain tubulin (>99% purity). The procedure followed the manufacturer's instructions. In brief, tubulin proteins (100 μg) were suspended with 50 μL of G-PEM buffer (80 mM PIPES, 2 mM $MgCl_2$, 0.5 mM EGTA, 1.0 mM GTP and 15% glycerol, pH 6.9) in the absence or presence of the test agents at 4 °C. The sample mixture was transferred to a pre-warmed 96-well plate (37 °C), and the polymerization of tubulin was measured by the change in fluorescence intensity (ex = 370 nm, em = 445 nm) every 1 min for 60 min at 37 °C using a fluorescence plate-reader (Infinite F200 PRO, Tecan, Männedorf, Switzerland).

2.6. Immunofluorescence assay

Changes in MT morphology and cell nuclei were observed by immunofluorescence assay. Cells (3×10^3) were dispensed in 24-well plates, and then incubated with or without the test agents for 24 h. After washing with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 20 min and blocked with 5% bovine serum albumin (BSA)-0.3% Triton X-100 in PBS for 1 h at 37 °C. Then the cells were incubated with primary antibody at 4 °C overnight, followed by incubating with goat anti-mouse IgG H&L (Chromeo™488) secondary antibody for 1 h at 37 °C and DAPI for 10 min in the dark. Images were acquired with a fluorescence microscope (Olympus IX70, Japan).