Ac-Phe-Lys-PABC-doxorubicin via mitochondria-centered apoptosis involving reactive oxidative stress and the ERK1/2 signaling pathway in MGC-803 cells

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Abstract. Ac-Phe-Lys-PABC-doxorubicin (PDOX) is a smart doxorubicin (DOX) prodrug designed to decrease toxicities while maintaining the potent anticancer effects of DOX. The present study aimed to elucidate the molecular mechanisms of action of PDOX using MGC-803 gastric cancer cells as a model. The cells were treated with both PDOX and DOX, and cytotoxicities, cell cycle analysis, reactive oxygen species (ROS) generation, mitochondrial damage and ERK1/2 signaling pathway alterations were studied. Abundant cathepsin B expression was observed in the MGC-803 cells, and treatment with PDOX and DOX triggered dose-dependent cytotoxicity and resulted in a significant reduction in cell viability. IC₅₀ of PDOX and DOX was 14.9 and 4.9 µM, respectively. Both PDOX and DOX significantly decreased p-ERK1/2, increased ROS generation, reduced mitochondrial membrane potential, caused mitochondrial swelling and arrested the cell cycle at the G2/S phase, and these effects were more pronounced for PDOX than for DOX. PDOX and DOX have different mechanisms of action, particularly the mitochondria-centered intrinsic apoptosis involving reactive oxidative stress and the ERK1/2 signaling pathway.

Introduction

Doxorubicin (DOX, Adriamycin), an anthracycline isolated from Streptomyces strains, is one of the most efficacious anticancer drugs for the treatment of hematological malignancies and a broad range of solid tumors (1,2). However, the clinical application of DOX has long been limited by its dose-dependent toxicities to the heart, kidney, liver and bone marrow. To reduce these side effects, significant efforts have been made to develop DOX-prodrugs, such as PK1 and PK2, which remain inactive in blood and normal tissues but release DOX at the tumor site (3), and which have entered phase II/III clinical studies (4,5).

Cathepsin B (Cat B), a lysosomal cysteine protease in normal cells and tissues, is highly upregulated in malignant tumors and premalignant lesions at the mRNA, protein and activity levels (6). The active cleavage sites of Cat B cover a range of oligopeptides, including Arg-Arg, Ala-Leu, Phe-Arg, Phe-Lys, Ala-Phe-Lys, Gly-Leu-Phe-Gly, Gly-Phe-Leu-Gly and Ala-Leu-Ala-Leu (7-11). Therefore, several low- and high-molecular-weight DOX prodrugs through Cat B-cleavable oligopeptides, have been designed (4,5,12) and have demonstrated rapid and nearly quantitative DOX release in the presence of Cat B.

Based on the characteristics of Cat B, we designed and developed a smart DOX prodrug, Ac-Phe-Lys-PABC-DOX (PDOX), in which a Cat B-specific dipeptide (Phe-Lys) is introduced, containing a self-immolative spacer para-amino-benzylloxycarbonyl (PABC) to increase the distance between the dipeptide and DOX (13-16), so that the dipeptide can be directly accessible to the Cat B active site. PDOX remains inactive and stable in blood circulation and normal tissues. When PDOX reaches Cat B-enriched areas such as tumor sites, the dipeptide Phe-Lys is cleaved by Cat B, exposing the PABC spacer that is hydrolyzed spontaneously, releasing free DOX. An in vivo study in a nude mouse model of gastric cancer peritoneal carcinomatosis showed that, compared with free DOX, PDOX produced superior anti-metastasis effects in terms of the experimental peritoneal carcinomatosis index (ePCI) and body weight, and reduced toxicities to the liver, kidney and particularly the heart (16). However, the underlying mechanism remains unclear.

It has been reported that apoptosis plays a key role in the anticancer effects and toxicities of DOX (17), mainly via...
the mitochondria-centered intrinsic pathway (18,19), which involves the release of cytochrome c from the mitochondria (20,21). DOX promotes the generation of reactive oxygen species (ROS) in various cell types (22,23) and mitochondria are the major source of ROS production (24). The present study was aimed to investigate the in vitro mechanisms of action of PDOX with special focus on the mitochondria-centered intrinsic pathway.

Materials and methods

Drug preparation and cell culture. DOX (Pharmacia, Milan, Italy) and PDOX (synthesized by Y.-P.H.) were used. MGC-803 gastric cancer cells were cultured in 25 cm² tissue culture flasks at 37°C in 5% CO₂, 95% air and 100% humidity. Cells were grown in RPMI-1640 medium containing 10% newborn calf serum and 1% penicillin and streptomycin. Throughout the study, the medium was replaced every 2 to 3 days. The cells were passaged when grown to 90% confluence.

Immunocytochemistry. Cells were transferred onto a coverslip in 6-well culture plates, grown for 2 days to reach 90% confluence and then fixed by ice-cold 4% paraformaldehyde for 30 min. Immunocytochemistry for Cat B was then performed following a standard method (25). Briefly, cells were first blocked with 2% bovine serum albumin (BSA) at 37°C for 30 min. Next, cells were incubated with a primary rabbit anti-human Cat B antibody (cat no. 3190-100, dilution 1:200; BioVision, Mountain View, CA, USA) at 4°C overnight. Cells were washed with Tris-buffered saline-Tween (TBST; pH 7.6) 3 times and then incubated with a peroxidase-labeled goat anti-rabbit IgG at 37°C for 30 min. The antibody reaction products were visualized with diaminobenzidine (Dako, Denmark). The images were captured using an Olympus BX51 fluorescence microscope equipped with an Olympus Micro DP 72 camera.

Cell viability study by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were passaged and 1x10⁶ cells in 100 μl medium were transferred into 96-well culture plates. After 24 h, the MGC-803 cells were treated with 0, 0.86, 1.72, 3.44, 6.88 and 13.76 μM of PDOX, or 0, 0.86, 1.72, 3.44, 6.88 and 13.76 μM DOX in medium for 24, 48, 72 and 96 h, respectively; then immediately incubated with 100 μl 0.5 mg/ml MTT at 37°C for 30 min. The antibody reaction products were visualized with diaminobenzidine (Dako, Denmark). The images were captured using an Olympus BX51 fluorescence microscope equipped with an Olympus Micro DP 72 camera.

Western blotting. MGC-803 cells (2x10⁵) in 2 ml of medium were transferred into 6-well culture plates and treated in the same way as above. The harvested cells were homogenated, centrifuged at 12,000 x g at 4°C for 30 min, and the protein concentration in supernatants was determined using the BCA assay.

A total of 100 μg proteins were separated by SDS-polyacrylamide gel electrophoresis (4% stacking and 10% separating gels) and then transferred overnight onto PVDF membranes. The membranes were blocked with 5% skimmed milk in 0.1 M PBS containing 0.05% (v/v) Tween. Next, they were immunoblotted with rabbit anti-human p-ERK1/2 antibody (cat no. 4370, dilution 1:200), rabbit anti-human ERK1/2 (cat no. 4370, dilution 1:200), rabbit anti-human cytochrome c (cat no. ab133504, dilution 1:1,000; Abcam, USA) for 2 h. Blots were then incubated with a peroxidase-conjugated sheep anti-rabbit IgG (dilution 1:8,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 h and developed using chemiluminescent detection with a Supersignal West Pico Assay kit (Thermo, USA) and autoradiography film.
Results

Cat B expression in MGC-803 cells. As shown in Fig. 1, immunocytochemical analysis revealed abundant Cat B expression in the cytoplasm of the MGC-803 cells.

Cytotoxic effects of DOX and PDOX on MGC-803 cells. To investigate the cytotoxic effects of DOX and PDOX, MGC-803 cells were treated with the indicated concentrations of DOX and PDOX for 24, 48, 72 and 96 h, respectively. As confirmed by the MTT assay, DOX and PDOX triggered dose-dependent cytotoxicity and resulted in a significant reduction in cell viability (Fig. 2). At 48 h, the rates of proliferation inhibition following treatment with 0.86, 1.72, 3.44, 6.88 and 13.76 µM of PDOX were 13.17±12.12, 15.10±5.46, 18.33±4.85, 34.99±2.27 and 54.86±0.93%, respectively; and following treatment with the same concentrations of DOX were 32.30±5.47, 34.39±5.81, 41.50±2.58, 52.35±9.25 and 59.97±10.29%, respectively (Fig 2B). At 48 h of treatment, the IC\textsubscript{50} concentration of PDOX (14.9 µM) was 3.04 times that of DOX (4.9 µM).

Effects of DOX and PDOX on the cell cycle distribution of MGC-803 cells. To study the effects of DOX and PDOX on the cell cycle, MGC-803 cells were treated with 4.9 and 14.9 µM PDOX or 4.9 and 14.9 µM DOX for 48 h, respectively. The percentages of cells at phases G1, G2 and S were 56.1, 18.7 and 25.5% in the control group; 37.0, 40.2 and 22.8% in the 4.9 µM DOX group; 29.2, 46.6 and 24.2% in the 4.9 µM PDOX group; 33.6, 41.7 and 24.7% in the 14.9 µM DOX group; and 14.4, 55.3 and 30.3% in the 14.9 µM PDOX group (Fig. 3). These results suggest that both PDOX and DOX arrested the cell cycle at the G2/S phase. Moreover, PDOX at IC\textsubscript{50} arrested more cells at the G2/S phase compared with DOX at IC\textsubscript{50}.

Effects of DOX and PDOX on ROS generation of MGC-803 cells. ROS generation of MGC-803 cells was significantly
increased by PDOX when compared with that by DOX (Fig. 4). Compared with the control, ROS levels were increased by 1.20-fold (P>0.05), 1.95-fold (P<0.05), 2.09-fold (P<0.05) and 2.15-fold (P<0.05) following treatment of 4.9 µM DOX or PDOX, 14.9 µM DOX or PDOX, respectively. Meanwhile, compared with DOX at IC_{50}, PDOX at IC_{50} significantly increased ROS generation (1.53-fold, P<0.05).

Effects of DOX and PDOX on the mitochondria in MGC-803 cells. ΔΨm of MGC-803 cells was significantly decreased following treatment with both DOX and PDOX (Fig. 5A). Compared with the control, fluorescence intensity was increased by 1.04-fold (P<0.05), 1.07-fold (P<0.001), 1.08-fold (P<0.01) and 1.10-fold (P<0.001) after treatment with 4.9 µM DOX or PDOX and 14.9 µM DOX or PDOX, respectively. Meanwhile, compared with DOX at IC_{50}, PDOX at IC_{50} significantly increased fluorescence values (1.53-fold, P<0.01).

Results of transmission electron microscopy revealed that PDOX and DOX induced mitochondrial swelling, particularly in cells treated with PDOX at IC_{50} (Fig. 5B).

Effects of DOX and PDOX on ERK1/2 phosphorylation and cytoplasmic cytochrome c release in MGC-803 cells. Phosphorylation of ERK1/2 was significantly reduced by both
PDOX and DOX treatments, and the effect of PDOX was much more marked. Similarly, cytoplasmic cytochrome c was increased following both PDOX and DOX treatments, and the effect of PDOX was more obvious (Fig. 6).

Discussion

DOX is one of the most commonly used chemotherapeutic drugs with proven efficacy, but is associated with serious side effects. To resolve this issue, the ‘smart’ DOX-prodrug PDOX was designed with increased *in vivo* anti-metastasis effects and reduced toxicities in gastric cancer peritoneal carcinomatosis (16).

The present study aimed to elucidate the molecular mechanisms of action of PDOX using MGC-803 cells as a model, since immunocytochemical analysis demonstrated that this cell line is rich in Cat B. Our results showed that: i) the direct cytotoxicity of PDOX on cancer cells did not exceed that of DOX since the IC₅₀ of PDOX was significantly higher than that of DOX; ii) PDOX induced a greater degree of mitochondria-centered intrinsic apoptosis than DOX, since more cytochrome c was released from the mitochondria into the cytoplasm following PDOX treatment; iii) PDOX promoted more ROS production and significantly inhibited p-ERK1/2 to a greater degree than DOX. Therefore, treatment with both PDOX and DOX caused mitochondrial swelling and arrested the cell cycle at the G2/S phase; moreover, the effects of PDOX...
on mitochondria and the cell cycle were more marked than DOX.

Previous studies found that the two N-(2-hydroxypropyl) methacrylamide copolymer-bound DOX-prodrugs (PK1 and HYD) and free DOX greatly differ in regards to their anti-proliferative effect and cell death signals in EL-4 cancer cells; treatment with free DOX greatly increased p38 phosphorylation, while PK1 increased it slightly; PK1 also significantly increased ERK phosphorylation, while the free DOX slightly decreased it (27). Based on our study and on the results from the PK1 study, we conclude that antitumor mechanisms of action of a prodrug may be different from the original drug itself. Molecular modifications could bring new active groups, structural changes, drug metabolic alterations, which together may account for the different mechanisms of action from free DOX. This also suggests that the prodrugs may have a different antitumor spectrum. Therefore, more studies are needed to investigate the molecular mechanisms of action and the antitumor spectrum.

In conclusion, the present study found that PDOX has different mechanisms of action, particularly the mitochondria-centered intrinsic pathway involving reactive oxidative stress and the ERK1/2 signaling pathway. The new knowledge gained from this study may aid in the development of PDOX as a ‘smart’ molecular targeting drug against cancer metastasis.

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References