Triptolide Enhances Chemotherapeutic Accumulation Through P-Gp Inhibition in Cisplatin-Resistant HNE1/DDP Nasopharyngeal Cancer Cells

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Abstract

Background: We have previously shown that triptolide (TPL) could synergize with cisplatin (DDP) in drug-resistant nasopharyngeal cancer cells (NPC). However, the underlying mechanism remains elusive. Therefore, we hypothesized that TPL could inhibit P-glycoprotein (P-gp) expression, reduce the chemotherapeutic agent efflux from tumor cells and increase their drug content, then by increasing drug efficiency against the tumor cells. So the study was performed to elucidate the mechanism of triptolide (TPL) sensitization with chemotherapeutics in drug-resistant nasopharyngeal cancer cells.

Methods and Results: Cell viability was analyzed by MTT. The apoptosis and intracellular concentration of doxorubicin/rhodamine123 were determined by flow cytometry. The expression of P-gp, MRP1, Caspase-3, CAP1, and CAP2 were measured by western blot. The ATP level was examined by Cell Titer-Glo Luminescent Cell Viability Assay kit. The results showed that TPL inhibited cell viability, increased apoptosis, and intracellular doxorubicin accumulation. Furthermore, TPL inhibited the rhodamine123 efflux, reduced the ATP level and the expression of P-gp, MRP1, and CAP1, while it increased the expression of CAP2 and Caspase-3.

Conclusions: The results of this study confirm the role of TPL in restoring the sensitivity of cancer cells to chemotherapeutics by down regulating P-gp expression and function in drug-resistant nasopharyngeal cancer cells.

Introduction

MDR (multidrug resistance) is a major impediment in cancer chemotherapeutics. The typical MDR mechanism is associated with increased P-glycoprotein (P-gp) expression, hastening active chemotherapeutics efflux from the tumor cells [1]. Tumor cells can easily escape from the chemical toxic effects mainly due to their highly-expressed membrane transporters. Chemotherapeutics eliminate the majority of cancer cells but also spare P-gp-overexpressing cells [2, 3]. These escaped cells become resistant to the chemicals used in the patients and other structurally dissimilar agents could also serve as MDR substrates. Therefore, inhibiting or blocking "molecular pumps" could be a potentially efficient strategy to reverse MDR during cancer treatment [4, 5].

Triptolide (TPL), a diterpenoid triepoxide, is an activity agent being extracted from the root of *Tripterygium wilfordii Hook F* (TWHF). Growing evidence demonstrates that TPL possesses several beneficial properties, such as antitumor, antimicrobial, anti-inflammatory, anti-rheumatic, anti-dementia, and cardiovascular protective effects [6, 7]. TPL efficiently inhibits tumor growth both *in vitro* and *in vivo*, and can improve the antitumor activities of cytotoxic agents [8, 9]. Our previous study also showed that TPL could synergize with cisplatin (DDP) in drug-resistant nasopharyngeal cancer cells (NPC) [10]. However, the potential underlying mechanism has not yet been fully elucidated. Moreover, it remains to be determined whether the anticancer effect of TPL is involved in regulating the expression and function of P-gp. Therefore, we hypothesized that TPL could disturb P-gp expression, decrease the chemotherapeutic

agent efflux, thereby increasing the drug content in tumor cells, and enhance drug efficiency against the tumor cells.

Materials And Methods

Materials

Triptolide (Tianjin Medical Co., Ltd, Tianjin, CHINA) was lysed in dimethyl sulfoxide (DMSO) as a storage solution and freshly diluted into $5 \text{ mmol} \cdot \text{L}^{-1}$ in RPMI 1640 medium before use. Cisplatin (Qilu Pharmacy Co., Ltd), Rhodamine123 (Rh123, fanbobiochemicals Co., Ltd), 3 (4, 5 dimethylthiazol-2-yl) 2, 5 diphenyltetrazolium bromide (MTT), Doxorubicin(Dox) (ZHEJIANG HISUN PHARMACEUTICAL CO., LTD), just as well as the β -actin mouse monoclonal antibody (1:1000) were acquired from Santa Cruz Biotechnology (Santa Cruz, USA). The P-glycoprotein, MRP1, CAP1, CAP2, and Caspase-3 antibodies (1:500) were obtained from Abcam (Abcam, Hong Kong).

Cells and cell culture

The HNE1/DDP cells were provided by the biochemical pharmacology experiment center of Bengbu Medical College (Bengbu, China). Briefly, cells were incubated in RPMI-1640 solution, supplemented with Newborn Calf Serum (10%) and streptomycin/penicillin (100 U/mL) at 37°C under 5% humidified CO_2 atmosphere. The HNE1/DDP cells were incubated in DDP ($1 \mu\text{g/mL}$)-containing culture medium in order to maintain their DDP resistance.

Cell viability assay

The HNE1/DDP cells were added in plates with 6,000 cells a well and cultured overnight for adherence. The supernatant was discarded and replaced by the fresh medium containing TPL (0, 12.5, 25, 50, 100 $\text{nmol} \cdot \text{L}^{-1}$) or doxorubicin (0.1, 0.2, 0.4, 0.8 $\mu\text{mol} \cdot \text{L}^{-1}$) or TPL (12.5 $\text{nmol} \cdot \text{L}^{-1}$) and doxorubicin (0.1, 0.2, 0.4, 0.8 $\mu\text{mol} \cdot \text{L}^{-1}$). The cells have been cultured for 24h or 48h. Following treatment, MTT (5 mg/mL) was added into the supernatant (15 μL) and maintained in culture for additional 4 h. The supernatant removed, 150 μL DMSO was added to each well and then incubated at 37°C for 30 minutes. Subsequently, the absorbance of all wells was detected at 570 nm with a Microplate Reader (BioTek, USA).

Apoptosis detection

HNE1/DDP cells were seeded in a 6-well plate with a density of 3×10^5 cells per well. After overnight incubation for attachment, cells were treated with different concentrations of TPL (0, 12.5, 25, and 50 $\text{nmol} \cdot \text{L}^{-1}$) for 24 h. Next, cells were digested with trypsin, washed with cold PBS, and fixed with alcohol solution overnight at 4°C . The fixed cells were washed twice with PBS, stained with a PI solution (50 mg/mL PI, 0.05% Triton X-100, and 0.1 mg/mL RNase A) for 30 mins in the dark. Finally, at least 6000 events per sample were acquired using a flow cytometer (BD, Becton, Dickinson, and Co., Piscataway, NJ).

Intracellular Dox content estimation

The cellular efflux and uptake of Dox with or without TPL were fixed using a flow cytometer (BD, USA). Briefly, HNE1/DDP cells were captured in the exponential growth phase and seeded onto 12-well plates at a density of 50,000 cells per well. Following 12 h of incubation, the wells were divided into 5 groups, each group comprising 3 wells. The five groups of cells were pretreated with TPL (0, 12.5, 25, 50 nmol·L⁻¹, respectively) for 2 h, then treated with Dox (0.2 μmol L⁻¹) with or without TPL (12.5, 25 and 50 nmol L⁻¹, respectively) for 8 h. Next, the cells were washed twice with PBS and incubated with fresh RPMI-1640 for 6 h. Subsequently, the cells were collected to analyze the Dox fluorescence. Baseline controls were also provided for the Dox efflux evaluation. Non-treated cells were used to set the baseline. We detected 10,000 events from each harvested sample. The net Dox uptake was presented as the fluorescence intensity difference between the treated and non-treated cells.

Cellular Rh123 uptake and efflux estimation

The extracellular secretion of P-gp was evaluated by rhodamine 123 (Rh-123), a fluorescent P-gp substrate. Briefly, the cellular uptake and efflux of Rh123 with or without TPL were determined using a flow cytometer (BD, USA). The HNE1/DDP cells were captured and seeded onto 12-well plates at a density of 30,000 cells per well, then incubated overnight. The wells were divided into four groups and pretreated with TPL (0, 12.5, 25, 50 nmol·L⁻¹) for 4 h, followed by the removal of the supernatant. Next, Rh123 (0.5 μmol·L⁻¹) alone or in combination with triptolide (12.5, 25, and 50 nmol·L⁻¹, respectively) were supplemented, and the cells were incubated for an additional 4 h, treated the same way as described for the intracellular Dox content estimation.

Intracellular ATP measurement

The cells were seeded onto 6-well dishes at a density of 2×10^5 cells per well. After being incubated overnight, the wells were divided into five groups, each group comprising three wells, and treated with TPL at different concentrations (0, 12.5, 25, 50, and 100 nmol·L⁻¹, respectively) for 24 h. The cells were then collected and the ATP levels were determined using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luminescence levels were measured using a microplate reader.

P-gp, MRP1, Caspase-3, CAP1, and CAP2 expression level evaluation in HNE1/DDP cells

The cell suspensions were seeded onto 6-well dishes at a density of 7.5×10^6 cells per well. After overnight incubation for attachment, the cells were treated with different concentrations of TPL (0, 12.5, 25, and 50 nmol·L⁻¹, respectively) for 24 h. The cells were then harvested and lysed in lysis buffer (40 mmol L⁻¹ Tris (pH = 8), 0.1% NP40, 120 mmol L⁻¹ NaCl) on ice for 30 mins, and centrifuged for 30 mins at 12000 rpm. The protein quantity was assayed using a BCA Protein Assay kit. Equal protein quantities were electrophoresed, transferred onto polyvinylidene difluoride (PVDF) membranes, and incubated with the

primary antibodies overnight at 4 °C. After washing three times with TPBS, the membranes were exposed to the secondary antibodies at room temperature for 2 h. The blots were detected using the immobilon western chemiluminescent HRP substrate (Millipore).

Data analysis

Data were analyzed by ANOVA using the SPSS 17.0 software, presented as the mean $\pm$ standard deviation, and differences of $p < 0.05$ or 0.01 were regarded as statistically significant.

Results

TPL-mediated cytotoxicity and mediated with Dox the sensitization of Cisplatin-Resistant HNE1/DDP nasopharyngeal cancer cells

The research evaluated the growth of HNE1/DDP cells treated with TPL ($0-100\text{nmol}\cdot\text{l}^{-1}$) for (24 – 48 h). The MTT results showed that TPL exhibited a time- and dose-dependent inhibition effect on the HNE1/DDP cell proliferation (Fig. 1a). When combined with Dox, the inhibitory effect was significantly enhanced (Fig. 1b).

TPL induced apoptosis

To further investigate the cytotoxicity of TPL against the HNE1/DDP cells, cells were subjected to concentrations of TPL for 24 h, cell apoptosis was examined. Results were shown in Fig. 2, the potency of TPL on apoptosis was related to the dose in HNE1/DDP cells.

TPL enhanced the cellular accumulation of Dox

Cancer MDR is associated to enhanced efflux of drugs. Increasing the concentration of drugs is the main way to overcome it, which often leads to systemic toxicity. To determine whether TPL induced the intracellular accumulation of chemotherapeutic drugs in nasopharyngeal cancer cells, we evaluated the intracellular Dox concentration using flow cytometry. As shown in Fig. 3a, the intracellular Dox concentration increased in HNE1/DDP cells 4 h after the TPL treatment. Furthermore, the TPL induced Dox accumulation occurred dose-dependently in the HNE1/DDP cells.

TPL inhibited the efflux function of P-gp

Normally, P-gp is a molecular "pump" exhibiting extracellular substrate secretion. When P-gp is inhibited, its substrates accumulate in the cells. Rh123, a fluorescent low-molecular-weight P-gp substance, is generally used to detect the efflux function of P-gp. As shown in Fig. 3b, TPL increased the cellular concentration of Rh123 in the HNE1/DDP cells, especially in the middle- and high-dose groups ($p < 0.05$ or $p < 0.01$). The results indicated that TPL could inhibit the P-gp function, resulting in Rh123 or Dox accumulation in the HNE1/DDP cells.

TPL inhibited ATP generation in HNE1/DDP cells

P-gp and MRP1 are members of membrane transport proteins, mediating active substrate export. In the lack of energy (ATP), the function of P-gp and MRP1 would be interrupted. We had proved TPL inhibited the efflux function of P-gp by detecting the levels of Dox and Rh123. The function of P-gp disruption related to short of ATP should be uncovered. So the level of ATP was detected in this study. Our results showed that TPL significantly reduced the ATP level in a dose-dependent manner (Fig. 4a).

TPL altered the MRP1, P-gp, Caspase-3, CAP2, and CAP 1 expressions in HNE1/DDP cells

Drug resistance is mainly related to P-gp and MRP overexpression. In the study the expression of P-gp was detected in cisplatin-resistant and sensitive HNE1 cells. Results indicated that the expression of P-gp in resistant cell was great higher than that in sensitive cell (Fig.4 b). So inhibiting P-gp and MRP could overcome cancer's drug resistance. Finally, we investigated the effect of TPL on the cell membrane proteins (MRP1 and P-gp) in nasopharyngeal cancer cells. As shown in Fig 4 c, TPL dramatically reduced the expression of MRP1 and P-gp. Moreover, TPL also inhibited the MDR-related proteins, suggesting that MDR reversion might owe to its inhibition effect on the expression of P-gp and MRP1. Adenylate cyclase-associated protein 1 and 2 (CAP1 and CAP2) regulate both actin filaments and the Ras/cAMP pathway, and have been found play a role in cell motility and in the development of certain types of cancer. In mammal, CAP1 gene encodes an actin monomer-binding protein which is thought to facilitate cancer processes, but CAP2 is thought to blocking tumor growth. Our preliminary work had proved TPL could trigger ROS generation and activate the mitochondrial pathways to induce apoptosis. By activating the mitochondrial pathways, TPL could regulate the proteins of Bcl-2, Mcl-1, Bax and Caspase-9, which conctroll cell apoptosis. In the experiment, CAP1, CAP2, and Caspase-3 were assayed, which regulate apoptosis, too. As shown in Fig. 4c, TPL could regulate the expression of Caspase-3, CAP1, and CAP2.

Discussion

P-glycoprotein (P-gp), is a classic member of the MDR protein family, identified by Ling and co-workers in multidrug-resistant Chinese hamster ovary cells [11]. P-gp transmits a broad spectrum of chemotherapeutics in an ATP-dependent manner [12]. In cancer cells, the P-gp overload leads to the reduction of the intracellular drug concentrations, abating the cytotoxicity of chemotherapeutic drugs, such as anthracyclines (e.g., Dox), taxanes (e.g., taxol), and vinca alkaloids (e.g., vincristine) [13]. P-gp overexpression reduces the concentration of intracellular compounds, reducing the cytotoxicity of chemotherapeutic drugs [14]. Therefore, P-gp inhibitors/blockers have a promising potential for cellular bioavailability for a broad spectrum of chemotherapeutics.

In this research, the intracellular accumulation of Dox was detected. The results showed that TPL could induce Dox accumulation in HNE1/DDP cells. Whether the effect was due to TPL inhibiting the function of P-gp? Then, Rh123 was used to evaluate the excretion function of P-gp. As we know, Rh123 is one substrate of P-gp. Normally, cells excrete Rh123 to extracellular with the help of P-gp, which lower the level of Rh123. The results demonstrated that by inhibiting P-gp, TPL increased Rh123 intracellular

concentration. So could draw a conclusion that TPL could inhibit the function of P-gp, then reducing the amount of Rh123 to extracellular.

P-gp and/or MRP1 overexpression is the main reason for chemotherapeutic drug-resistance in tumor cells. If the expression of P-gp / MRP1 was interfered, cancer drug resistance would be overcome [15]. The fact that TPL could induce Dox accumulated in HNE1/DDP cell in certain degree contributed to its inhibition effect on the expression of MRP1 and P-gp. The fact that TPL could induce Dox accumulation in HNE1/DDP cell to a certain degree contributed to its MRP1 and P-gp expression inhibitory effect. Generally, both the expression and function of MRP1 and P-gp are ATP-dependent [16]. The lack of ATP inhibits MRP1 and P-gp function and expression. Mitochondria not only offer energy for the cells by ATP synthesis but also participate in various physiological functions in the cells [17–19], such as inducing apoptosis by regulating the mitochondrial membrane potential [20, 21]. Previous studies revealed that TPL could reduce the mitochondrial membrane potential and activate the mitochondrial apoptotic pathway [22], leading to apoptosis [23, 24], and overcoming cancer multidrug resistance [25–27].

Our preliminary work had proved TPL could trigger ROS generation and activate the mitochondrial pathways, then induce apoptosis [10]. By activating the mitochondrial pathways, TPL altered the expression of Bcl-2, Mcl-1, Bax and Caspase-9, which regulating apoptosis. CAP1, CAP2, and Caspase-3 are involved the mitochondrial apoptosis pathways. CAP1/CAP2 regulates the Ras/cAMP pathway, CAP1 is thought to facilitate cancer processes, and CAP2 is thought to blocking tumor growth. Caspase-3 participates in Caspase pathway. In this study, we detected apoptosis and certain apoptosis-regulating proteins. Our results indicated that TPL altered the expression of mitochondrial apoptotic pathway-related proteins CAP1, CAP2, and Caspase-3. The effect of TPL on CAP1, CAP2, and Caspase-3 might initiate the mitochondrial apoptotic pathway, followed by the down-regulation of ATP generation and even apoptosis induction or altering P-gp/MDR1 expression and function.

TPL could induce the accumulation of chemotherapeutic compounds in HNE1/DDP cells and enhance their chemotherapeutic effect. The underlying mechanisms involve the alteration of P-gp expression and function. The effect of TPL on P-gp might be related to its effect on ATP synthesis and the mitochondrial apoptotic pathways.

Declarations

Author contributions

Xiu Wang, Jing-jing Zhang conducted experiments. Yu-shuai Wang collected and analyzed the data. Xiu Wang and Jian-Chun Li designed and supervised the project. Xiu Wang wrote the manuscript. B Yu-shuai Wang edited the manuscript.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Figures

Triptolide enhances chemotherapeutic accumulation through P-gp inhibition in Cisplatin-Resistant HNE1/DDP nasopharyngeal cancer cells

Figure 1

HNE1/DDP cell viability a Cell viability following the TPL treatment. **p < 0.01 vs Con. b Cell viability after Dox/Dox treatment combined with TPL (12.5 nmol·L⁻¹). *p < 0.05, **p < 0.01

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Figure 2

Apoptosis - induced by TPL in the HNE1/DDP cells **p < 0.01 vs Con.

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Figure 3

Dox/Rh123 accumulation in the HNE1/DDP cells following the TPL treatment a TPL-mediated Dox accumulation in the HNE1/DDP cells * $p < 0.05$, ** $p < 0.01$ vs Dox b TPL induced the accumulation of Rh123 in the HNE1/DDP cells * $p < 0.05$, ** $p < 0.01$ vs Rh123

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Figure 4

Effect on ATP generation and the expression of MRP1, P-gp, Caspase-3, CAP1, and CAP2 in HNE1/DDP cells following the TPL treatment a ATP generation in HNE1/DDP cells following the TPL treatment. *p < 0.05, **p < 0.01 vs Con. b The expression of P-gp in HNE1/HNE1/DDP cell line. c The expression of MRP1, P-gp, Caspase-3, CAP 1, and CAP2 in HNE1/DDP cells following the TPL treatment

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