Reversal of taxol resistance by cisplatin in nasopharyngeal carcinoma by upregulating thromspondin-1 expression

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Drug resistance often causes failure of chemotherapy in nasopharyngeal carcinoma (NPC). Thus, it is of great importance to overcome drug resistance by developing effective reversal therapies. The purposes of this study were to examine whether cisplatin could reverse the taxol-resistant phenotype of NPC cells, and to evaluate the role of the taxol-resistant gene (TXR1)/thrombospondin (TSP1) pathway in the reversal of taxol resistance. A drug (taxol)-resistant cell line, CNE-1/taxol, was established from a human NPC cell line, CNE-1. The sensitivity of both CNE-1 and CNE-1/taxol to cisplatin or paclitaxel was detected using the colony formation assay. Apoptotic death was measured by flow cytometry. The expression of the TXR1 and TSP1 was determined by RT-PCR and western blot. The growth inhibition rate in CNE-1/taxol cells in response to taxol was significantly increased when they were pre-treated with low-dose cisplatin. CNE-1/taxol cells were more sensitive to cisplatin than CNE-1 cells when exposed to 300–1500 nmol/l of cisplatin. An approximate seven-fold increase in TXR1 mRNA expression and an 8.9-fold decrease in TSP1 mRNA expression were observed in taxol-resistant cells compared with their parental cells. An 8.7-fold increase in TSP1 mRNA expression was observed in CNE-1/taxol cells exposed to 590 nmol/l of cisplatin for 24 h. An increase in TSP1 protein expression was obtained in a dose-dependent manner after CNE-1/taxol cells were exposed to cisplatin. However, there was no change in TXR1 mRNA expression after both CNE-1 and CNE-1/taxol cells were exposed to cisplatin. We conclude that cisplatin reverses drug resistance through the upregulation of TSP1 downstream of TXR1. *Anti-Cancer Drugs* 21:381–388

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Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy of epithelial origin with a multifactorial etiology. It is a rare cancer and has a high incidence among Southern Chinese and Southeast Asians [1]. The incidence rate of NPC is 15–50 cases per 100 000 people per year in the Cantonese region of Southern China [2]. Because the nasopharynx is poorly accessible and NPC is radiosensitive, radiotherapy is the most common therapeutic approach. Chemotherapy is also used to treat advanced carcinoma and to enhance the radiosensitivity. Although some patients initially respond to chemotherapy, the majority of patients with advanced NPC fail to respond to the treatments because of the development of drug resistance [3,4]. It is therefore necessary to improve clinical treatment efficacy and to develop methods to reverse drug resistance.

Taxol, a prototypic taxane compound, specifically binds to the β-tubulin subunit in microtubulin, which promotes the polymerization of tubulin and disrupts microtubule dynamics. Taxol is one of the most active agents used in the clinical treatment of breast, ovarian, lung, bladder, prostate, and head and neck cancers, and it is currently being used for advanced NPC [5–7]. Despite a dramatic response of susceptible tumors to initial treatment with taxol, the subsequent development of resistance limits its use as a long-term anticancer drug [8]. A widely accepted mechanism associated with taxol resistance is the increased expression of P-glycoprotein, which functions as an efflux pump to export taxol from the cells [9]. In addition, genetic mutations detected in tubulin could be the cause of taxol resistance because the mutated tubulin disrupts the binding of taxol to microtubules [10]. Taxol resistance gene (TXR1)-mediated thrombospondin (TSP1) repression has been recently described as a new mechanism of drug resistance [11,12].

P-glycoprotein inhibition has been attempted earlier to reverse drug resistance. Some molecules have proven to be effective in restoring the sensitivity of drugs that are susceptible to resistance. For instance, quinine homodimer Q2 [13] completely reverses P-glycoprotein-mediated taxol resistance through interaction with the drug binding site(s) of P-glycoprotein, while resveratrol combined with chemotherapeutic agents [14] produces a synergistic effect and reverses the multidrug-resistant phenotype through inhibiting the expression of P-glycoprotein and BCL-2 and/or promoting cell apoptosis.

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Many other molecules, including immunosuppressive agents, hormone-related drugs and steroids function as promising reversal agents [15–17]. However, successfully using these molecules in the clinic is a challenge.

In our earlier study, we reported that P-glycoprotein expression was undetectable, and that other members of the ATP-binding cassette superfamily were not significantly increased in a taxol-resistant NPC line [18]. Obviously, reversal targeting P-glycoprotein should not be effective in drug-resistant NPC cells. Interestingly, taxol-resistant NPC cells were much more sensitive to cisplatin than parental NPC cells when we tested the cytotoxicity of multiple drugs in resistant NPC cells [19]. Therefore, we proposed that cisplatin might be a reversal agent that restores taxol resistance in NPC.

In this study, we aimed to further verify that cisplatin restores the NPC taxol resistance phenotype, to examine the cytotoxicity of cisplatin and taxol using the colony formation assay, and to analyze the effect of drug combinations by combination index (CI)-isobologram [20,21]. We also show that the TXR1/TSP1 regulation pathway, a taxol-resistance-related signal pathway, is a possible mechanism by which to reverse the taxol-resistant phenotype.

Materials and methods

Materials

Taxol was obtained from Bristol–Myers Squibb (Princeton, New Jersey, USA). Bradford assay kits and Chemiluminescent Western Detection Kits were purchased from Bio-Rad (Hercules, California, USA). Annexin V-FITC apoptosis kits were purchased from BioVision, Inc. (Mountain view, California, USA). RNA extraction kits and RT-PCR kits were obtained from Life Technologies (Gaithersburg, Maryland, USA). Cisplatin and other reagents were purchased from Sigma (St. Louis, Missouri, USA). Mouse polyclonal antibodies against TSP-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Cell cultures and treatments

A human NPC cell line, CNE-1, was a gift from the Cancer Research Institute of Central South University (China). The cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C and then replated 48 h before use. The taxol-resistant cell subline (CNE-1/taxol) was established by exposing parental CNE-1 to gradually increasing concentrations of taxol. The initial taxol concentration was 0.5 nmol/l, and the final concentration was 5 nmol/l. The half-inhibition concentration (IC_{50}) was determined using the colony formation assay [22], and the resistance index of drug-resistant variants of sub-cell lines was defined as the IC_{50} of parental cells divided by the IC_{50} of resistant cells. All cells were subcultured at 5-day intervals.

Exponentially growing cells (1×10^6) of both CNE-1 and CNE-1/taxol were plated in 25 cm^2 cell culture bottles in a total volume of 10 ml of medium. The cells were then incubated with or without cisplatin and taxol for 24 h. They were then collected and RNA and protein were isolated for RT-PCR and western blot.

Cell growth inhibition

Cell growth inhibition was determined by a modified colony formation assay, as described earlier [19]. Six-hundred exponentially growing cells were plated in six-well cell culture plates in a total volume of 2 ml of medium. The various treatments were as follows.

The cells were incubated with 300–1500 nmol/l cisplatin for 24 h. After incubation, the drug-containing medium was replaced with fresh medium and the cells were continuously cultured for 8 days. The colonies were then fixed with 75% ethanol, stained with Giemsa and counted. The IC_{20}, IC_{50}, and IC_{80} values of cisplatin in both CNE-1 and CNE-1/taxol cells were determined.

The cells were incubated for 6 h with or without cisplatin at the IC_{20} of 530 nmol/l or 230 nmol/l, as determined for CNE-1 or CNE-1/taxol, respectively. Taxol was added then to final concentrations of 2, 4, 6, 8, and 10 nmol/l, with dimethyl sulfoxide as the vehicle control, and incubation continued for 24 h. The IC_{50} values of taxol for both NPC lines with or without csipaltin pretreatment were calculated.

The cells were incubated with variable ratios of taxol and csiplatin concentrations for 24 h (the ratio of taxol: cisplatin in group 1 was taxol ranging from 0.5 to 2.5 nmol/l and csipaltin ranging from 150 to 750 nmol/l; the ratio in group 2 was taxol ranging from 1 to 5 nmol/l and cisplatin ranging from 150 to 750 nmol/l; the ratio in group 3 was taxol ranging from 0.5 to 2.5 nmol/l and cisplatin ranging from 300 to 1500 nmol/l). Next, the colony formation assay was carried out. The IC_{50} was determined for each drug in both NPC lines. The CI-isobologram by Chou and Talalay was used to analyze the drug combination assays and the CI was calculated [20,21]. A CI of less than, equal to, and more than 1 indicates synergy, additivity, and antagonism, respectively [20,21].

Annexin V apoptosis assay

The percentage of apoptotic death was measured using the Annexin V-FITC apoptosis kit according to the manufacturer's instructions. Briefly, 1×10^5 cells were washed twice with cold phosphate buffered saline, and then washed twice with binding buffer containing 10 mmol/l HEPES, 140 mmol/l NaCl and 2.5 mmol/l CaCl at pH 7.4. The cells were resuspended with 100 µl of

binding buffer and Annexin V-FITC (titer from 0.1 to 1.0 μg), and incubated at room temperature for 10 min. Subsequently, 400 μl of binding buffer containing 1 μl of potassium iodide was added and the cells were incubated on ice for 15 min. The cells were analyzed by flow cytometry within 1 h.

Semi-quantitative RT-PCR analysis

Total RNA was prepared using the SV total RNA extraction kit (Life Technologies), and the RNA concentration was determined by ultraviolet spectrophotometry. Reverse transcription of 1 μg RNA was performed using an RT-PCR kit (Life Technologies) in a 20 μl reaction. The yield of cDNA was measured according to the PCR signal generated from the internal standard housekeeping gene β-actin, amplified from 18 to 24 cycles starting with 1 μl of 10 × dilution of the RT reaction. The volume of each cDNA pool was adjusted to give the same exponential phase PCR signal strength for β-actin after 20 cycles. Two microlitres of cDNA from 10 × dilution of the RT reaction was used for PCR. The primers were as follows: TXR1 (280 bp) forward, 5′-GCT TTC TTC ATT TTC TTC TG-3′; reverse, 5′-GTT CCA ATC CTG CCC A 3′; TSP1 (469 bp) forward, 5′-CCT GTG ATG ATG ACG ATG A -3′; reverse, 5′- CTG ATC TGG GTT GTG GTT GTA- 3′; β-actin (540 bp) forward, 5′-GGA CCT GAC TGA CTA CCT C-3′; reverse, 5′-TCA TAC TCC TGC TTG CTG-3′. All PCR reaction mixtures contained 1 × PCR buffer (200 mmol/l Tris–HCl, pH 8.4, 500 mmol/l KCl), 2.5 mmol/l $MgCl_2$, 0.8 mmol/l dNTP (Life Technologies), 0.2 μmol/ml of each primer, and 1 unit of platinum Taq DNA polymerase (Life Technologies). Reactions were performed using a PTC-200 PCR machine (MJ Research Inc, Massachusetts, USA) as follows: an initial denaturation for 10 min at 95°C followed by 45 s at 95°C, 45 s at 60°C, and 1 min at 72°C for 24 cycles for TXR1, 21 for TSP1, and 18 for β-actin. The PCR products were loaded onto ethidium bromide-stained 1.2% agarose gels. Images of the gels were acquired with a Cohu High Performance CCD camera (Cohu Inc. San Diego, California) and the quantification of the signals was determined using densitometric analysis (Quantity One, version 4, Bio-Rad).

Western blotting

After treatment, the cells were collected, rinsed twice with phosphate buffered saline and lysed in a buffer containing 20 mmol/l of HEPES (pH 7.4), 0.1 mol/l of NaCl, 0.1% deoxycholic acid, 1% NP-40, 1 mmol/l of EDTA, 1 mmol/l of EGTA, 10% glycerol, 1 mmol/l of PMSF, 10 μg/ml of aprotinin, 10 μg/ml of leupeptin, and 50 mmol/l of sodium fluoride at 4°C for 30 min. Insoluble material was removed by centrifugation. The protein concentrations were determined using the Bradford protein assay system (Bio-Rad). Twenty micrograms of protein was separated by 12% SDS–PAGE and then transferred to polyvinyl difluoride membranes (Millipore, Bedford, Massachusetts, USA) by electroblotting. After blocking with 5% non-fat dry milk, the blots were incubated with primary antibodies (for TSP1 and GAPDH) and developed with alkaline phosphorylase-conjugated secondary antibodies using a chemiluminescent substrate. A densitometer was used for the quantification of the signals on the films.

Statistical analysis

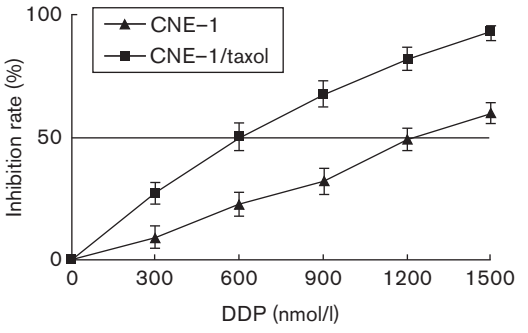
All experiments were performed at least three times. Data are mean ± SD. Statistical analysis was performed by the Student's *t*-test. A P value of less than 0.05 was considered statistically significant.

Results

Growth of taxol-resistant NPC cells is inhibited by cisplatin

The effect of taxol on both CNE-1 and CNE-1/taxol cells was examined by exposing them to different concentrations of taxol. The IC_{50} value of taxol was 1.33 nmol/l for CNE-1 and 11.24 nmol/l for CNE-1/taxol, as determined by the colony formation assay and the growth-inhibition curve. The CNE-1/taxol cells were 8.43-fold less sensitive than CNE-1 cells in response to taxol. To investigate the effect of cisplatin on CNE-1/taxol and CNE-1, the cells were exposed to different doses of cisplatin (300, 600, 900, 1200, and 1500 nmol/l), and the cell viability was determined. The growth of both cell lines was inhibited in a dose-dependent manner when exposed to 300–1500 nmol/l of cisplatin (Fig. 1). The IC_{50} and IC_{20} values of cisplatin were 1270 ± 52 and 530 ± 34 nmol/l in CNE-1 cells, and 590 ± 23 and 230 ± 15 nmol/l in CNE-1/taxol cells. These results indicate that the growth inhibition rate was significantly higher in CNE-1/taxol cells than in CNE-1 cells ($P < 0.05$), and that CNE-1/taxol cells were more sensitive than CNE-1 cells to treatment with cisplatin.

Fig. 1



Growth inhibitory curve of cisplatin in CNE-1 and CNE-1/taxol cells determined by the colony formation assay (Mean ± SD, $n = 9$).

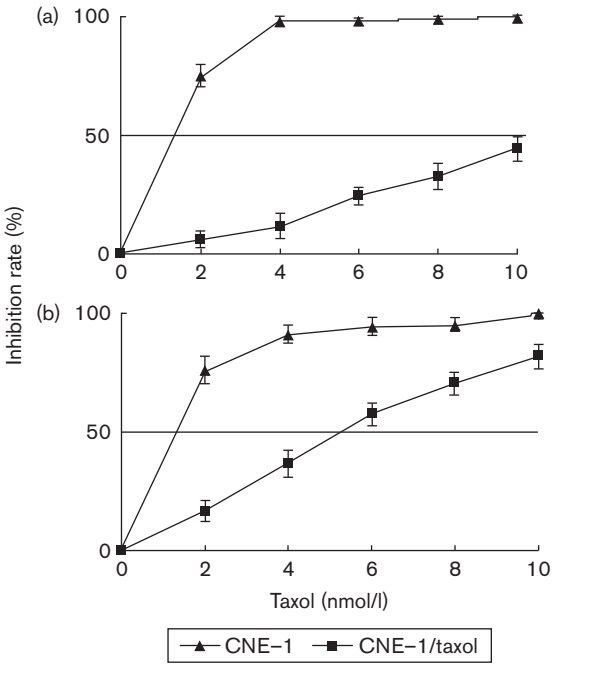
Low-dose cisplatin partially reverses the taxol-resistant phenotype of NPC

To further determine whether cisplatin was able to reverse the taxol-resistant phenotype in NPC cells, both CNE-1/taxol and CNE-1 cells were pretreated with cisplatin at the IC_{20} for 6 h, and the cells were subsequently exposed to different doses of taxol for another 24 h. The growth inhibition rates and IC_{50} values were then determined by the colony formation assay. Results showed that the growth inhibition rates of taxol in CNE-1 cells remained unchanged. However, the rate significantly increased in CNE-1/taxol cells (Fig. 2) if the cells were pretreated with low-dose cisplatin. The IC_{50} of taxol in CNE-1/taxol cells increased from 11.24 ± 0.51 to 5.34 ± 0.29 nmol/l, and the resistance index decreased from 8.43 ± 0.42 to 4.02 ± 0.26 nmol/l (Table 1).

Growth inhibitory effects of drug combination

To evaluate whether the concurrent use of cisplatin and taxol produced additive or synergistic effects in both CNE-1 and CNE-1/taxol cells, the CI-isobologram [20,21] was used to analyze the drug combination assays. As shown in Table 2, the CIs of cisplatin and taxol at different ratios in CNE-1/taxol cells were less than 1, and peaked to 0.558 when the cells were exposed to a ratio of 2 : 1 (taxol:cisplatin). In contrast, the CIs of CNE-1 cells were close to 1 (Table 2). This result indicated that the coadministration of taxol and cisplatin produced the synergistic effect in taxol-resistant NPC cells, but only additive effects in parental NPC cells.

Fig. 2



The growth inhibitory curve of taxol in NPC cells with or without pre-treatment of cisplatin. (a) NPC cells without pre-treatment; (b) CNE-1 with pretreatment of 530 nmol/l cisplatin (IC_{20}), and CNE-1/taxol with 230 nmol/l cisplatin (IC_{20}) (Mean ± SD, $n = 9$).

Table 1 The IC_{50} of taxol in both CNE-1 and CNE-1/taxol cells pretreated with low-dose cisplatin

	No cisplatin		Pre-treated by cisplatin	
	IC_{50} (nmol/l)	RI	IC_{50} (nmol/l)	RI
CNE-1	1.33 ± 0.05		1.32 ± 0.04	
CEN-1/taxol	11.24 ± 0.51*	8.43 ± 0.42	5.34 ± 0.29*	4.04 ± 0.26**

RI, resistance index, the IC_{50} of parental cells divided by the IC_{50} of resistant cells.
*$P < 0.01$, comparison of IC_{50}.
**$P < 0.01$, comparison of RI alteration.

Table 2 CIs of taxol and cisplatin at different ratios in both CNE-1 and CNE-1/taxol cells

	Group 1 (1 : 1)	Group 2 (2 : 1)	Group 3 (1 : 2)
CNE-1	0.977 ± 0.012	1.008 ± 0.015	1.015 ± 0.011
CNE-1/taxol	0.682 ± 0.026	0.558 ± 0.027	0.767 ± 0.022

The equation as follows was used to calculate the CIs:

$$CI = \frac{CA,x}{ICx,A} + \frac{CB,x}{ICx,B}$$

where CA,x and CB,x are the concentrations of drugs A and B used in combination to achieve x% drug effect, and ICx, A and ICx, B are the concentrations of the single agents alone required to achieve the same effect. CI, combination index.

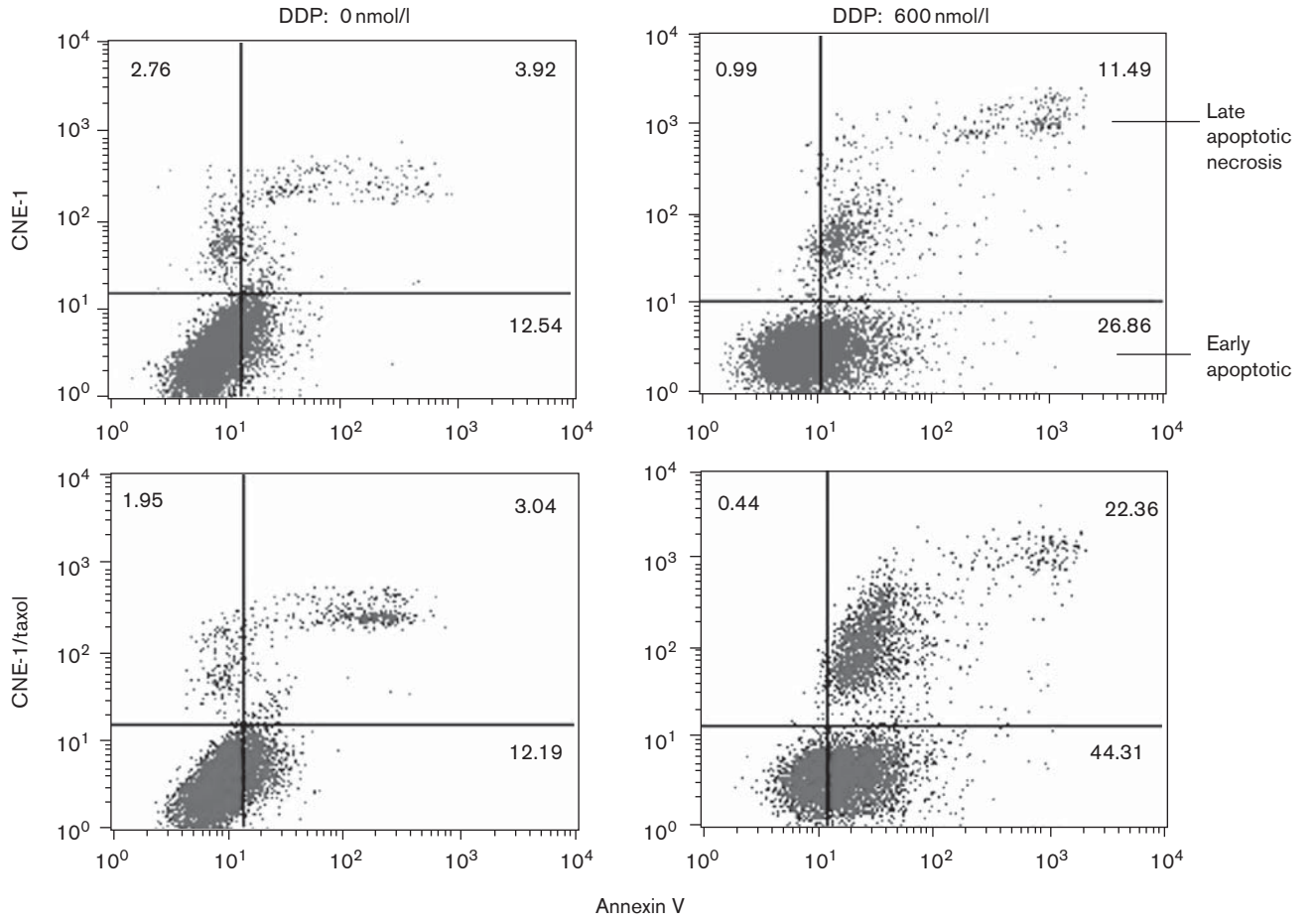
Induction of programmed cell death by cisplatin

The translocation of membrane phospholipid phosphatidylserine (PS) serves as the hallmark of apoptotic death, because when an apoptotic event occurs, PS is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Measuring PS by Annexin V-FITC can indicate the incidence of apoptotic cell death. We used Annexin V-FITC facilitated by flow cytometry to monitor apoptotic cell death when both CNE-1 and CNE-1/taxol cells were exposed to different doses of cisplatin for 24 h. As shown in Fig. 3 and Table 3, apoptotic death was detected in both cell lines in a dose-dependent manner when the cells were exposed to 300–1500 nmol/l of cisplatin. However, the percentage of apoptotic events that occurred in CNE-1/taxol cells was significantly higher than that in CNE-1 cells for all of the doses used (Table 3).

Expression of TXR1 and TSP1 in taxol-resistant NPC cells

The TXR1/TSP1 regulation pathway has been recently described to be involved in taxol resistance in cancer [11]. To elucidate the mechanism of taxol resistance of NPC cells, we examined the expression of TXR1 and TSP1 in both CNE-1 and CNE-1/taxol cells at both the transcriptional and translational levels using RT-PCR and western blot. An approximately 6.5-fold increase of TXR1 mRNA expression was observed in CNE-1/taxol

Fig. 3



Induction of apoptosis. Lower left quadrant: annexin V and PI negative cells represent viable cells. Lower right quadrant: annexin V positive and PI negative cells indicate early apoptotic cells. Upper right quadrant: annexin V and PI-positive cells represent necrotic or late apoptotic cells.

Table 3 Percentage of early apoptotic events in both CNE-1 and CNE-1/taxol cells exposed to cisplatin

	Cisplatin concentration (nmol/l)					
	0	300	600	900	1200	1500
CNE-1	12.54 ± 2.34	19.22 ± 3.58	26.86 ± 2.87	34.33 ± 4.67	40.72 ± 6.12	46.74 ± 6.34
CNE-1/taxol	12.19 ± 1.98*	33.46 ± 4.33*	44.31 ± 3.33*	53.87 ± 6.71*	67.75 ± 5.11*	74.57 ± 6.53*

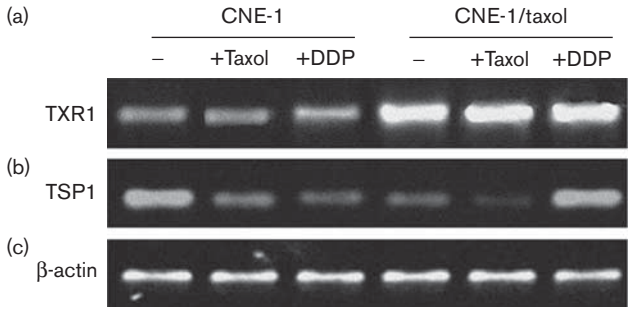
*$P<0.05$.

cells compared with its parental NPC cells (Fig. 4). In contrast, TSP1 mRNA expression in CNE-1/taxol cells was 8.9-fold decreased when compared with that in CNE-1 cells (Fig. 4). The expression of TSP1 protein was also detected to be 5.6-fold decreased in CNE-1/taxol cells (Fig. 5).

Effects of cisplatin on TXR1/TSP1 regulation pathway

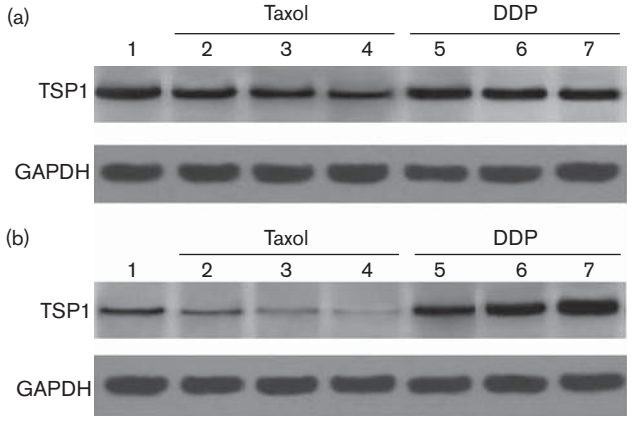
To further elucidate the mechanism of the reversal of taxol resistance by cisplatin, we treated both CNE-1 and CNE-1/taxol cells with cisplatin for 24 h and examined the changes of TXR1 and TSP1 expression. An 8.7-fold increase in TSP1 mRNA expression was observed in CNE-1/taxol cells exposed to 590 nmol/l cisplatin (Fig. 4). At the protein level, increased TSP1 protein expression was observed in a dose-dependent manner when CNE-1/taxol cells were exposed to 230 (IC_{20}), 590 (IC_{50}), and 1190 (IC_{80}) nmol/l of cisplatin for 24 h (Fig. 5). No significant changes were detected for TSP1 at either the mRNA or protein level when CNE-1 cells were exposed to cisplatin (Figs 4 and 5). In addition, there were no changes observed in TXR1 mRNA expression when both

Fig. 4



Representative gel image of semi-quantitative RT-PCR for: (a) taxol-resistant gene (TXR1) (280 bp) at 24 cycles. (b) Thrombospondin (TSP1) (469 bp) at 21 cycles. (c) β-Actin (540 bp) at 18 cycles. CNE-1, + taxol: treated with 1.33 nmol/l (IC_{50}) taxol, + DDP: treated with 1270 nmol/l (IC_{50}) cisplatin; CNE-1/taxol, + taxol: treated with 11.24 nmol/l (IC_{50}) taxol , + DDP: treated with 590 nmol/l (IC_{50}) cisplatin. DDP, cisplatin.

Fig. 5



Representative image of thrombospondin (TSP1) protein expression as detected by western blot. (a) CNE-1, parental nasopharyngeal carcinoma (NPC) cells: lanes 1, 2, 3, and 4 represent the addition of taxol at doses of 0, IC_{20}, $IC_{50,}$ and IC_{80} (0, 0.53, 1.33, and 2.44 nmol/l); lanes 5, 6, and 7 represent the addition of cisplatin at doses of IC_{20}, $IC_{50,}$ and IC_{80} (530, 1270, and 2006 nmol/l). (b) CNE-1/taxol, taxol-resistant NPC cells. Lanes 1, 2, 3, and 4 represent the addition of taxol at doses of 0, IC_{20}, $IC_{50,}$ and IC_{80} (0, 5.05, 11.24, and 18.40 nmol/l). Lanes 5,6, and 7 represent with the addition of cisplatin at doses of IC_{20}, $IC_{50,}$ and IC_{80} (230, 590, and 1190 nmol/l).

CNE-1 and CNE-1/taxol cells were exposed to cisplatin at doses of 1270 nmol/l and 590 nmol/l, the IC_{50} values for CNE-1 and CNE-1/taxol cells, respectively.

We also examined the changes in TXR1 and TSP1 expression when both cell lines were treated with taxol. A dose-dependent decrease in TSP1 protein expression was observed when CNE-1/taxol cells were treated for 24 h with taxol at doses of the IC_{20}, $IC_{50,}$ and IC_{80} (5.05, 11.24, and 18.40 nmol/l) (Fig. 5). There was a slight decrease in TSP1 protein expression in CNE1 cells exposed to a dose at the IC_{80} (18.40 nmol/l) of taxol (Fig. 5). There was no significant change observed in TXR1 mRNA expression in either CNE-1 or CNE-1/taxol cells after treatment with taxol compared with controls without taxol (Fig. 4).

Discussion

As drug resistance has been observed in cancer chemotherapy, significant efforts have been made to discover effective means to reverse it. The mostly widely studied reversal agents are molecules or drugs targeted at P-glycoprotein that are able to resensitize cancer cells by inhibiting the drug transporter. Some analogues of drugs or 'inclusive' anti-cancer drugs can also reverse the drug-resistant phenotype [23,24]. The epothilones, a class of microtubule targeting drugs, are active in multi-drug-resistant cancer cells and xenografts [25]. Ortataxel, a compound structurally related to docetaxel, seems to be promising as a selective inhibitor of taxol-resistant tumors that have increased P-glycoprotein expression [26]. The 'third generation taxoids', taxoids 19 and 14 g, exhibit excellent activities against taxol-resistant ovarian cancer cell lines with mutations in β-tubulin [27]. Combination chemotherapy with irinotecan hydrochloride (CPT-11) and mitomycin C has shown promising results in platinum-resistant ovarian cancer [28].

In our earlier study, we showed that taxol-resistant NPC cells were also resistant to vincristin, but not cisplatin. However, cisplatin was not found to reverse taxol resistance [19]. In this study, we investigated the reversal function of cisplatin in CNE-1/taxol cells and found that CNE-1/taxol cells were more sensitive to treatment with cisplatin than its parental cells, CNE-1. Particularly with a low dose pretreatment, cisplatin significantly increased the sensitivity to taxol in CNE-1/taxol cells, and partially reversed its resistant phenotype. This implied that cisplatin could be a reversal agent of taxol resistance in NPC cells.

It is expected that two drugs that individually produce overtly similar effects would yield a super-additive (synergistic) effect when they are present together. The synergism of two drugs has been documented to overcome multi-drug resistance in certain cancer cells [28]. Taxol and cisplatin are two cytotoxic drugs with unrelated structures and mechanisms. A synergistic effect was observed in ovarian cancer when a combination of these two drugs was used [29]. In this study, the effect of two-drug combinations was analyzed using CI-isobologram, and the synergistic effect was observed in taxol-resistant NPC cells. It is worth mentioning that this synergistic effect was only observed in taxol-resistant NPC cells, and not in its parental cells. This is indirect evidence that cisplatin reverses the taxol-resistant phenotype in NPC cells, and that the combination of taxol and cisplatin may be a potential treatment for taxol-resistant NPC.

The well-documented mechanism involving taxol resistance in cancer cells includes increased efflux of the drug mediated by enhanced expression of P-glycoprotein [30]. It has been reported that quinine homodimer Q2, the P-glycoprotein inhibitor, reverses P-glycoprotein-mediated taxol resistance in breast cancer cells [13]. Changes in β-tubulin isotype expression may also play a role in taxol resistance [31]. The mechanism of cisplatin reversing the taxol-resistant phenotype of NPC is unlikely to be linked to the enhanced expression of P-glycoprotein or the altered expression of the β-tubulin isotype. Our earlier study showed that the expression of P-glycoprotein was undetectable in both acquired taxol-resistant NPC and parental cells [18], and β-tubulin expression remained unchanged while parental cells transformed into resistant cells.

A recent study by Lih *et al.* [11] showed that TXR1 downregulated the expression of TSP1, the protein indicating anti-angiogenic and proapoptotic activity in oncogenesis. This is the same protein that decreases taxol-induced apoptotic incidence in prostate cancer cells. We observed an increased expression of TXR1 and a decreased expression of TSP1 in taxol-resistant NPC compared with its parental cells, which is consistent with the report by Lih *et al.* [11]. Our results imply that the taxol resistance of NPC may be associated with the TXR1/TSP1 regulation pathway, and that the taxol-resistant phenotype reversed by cisplatin may be associated with the TXR1/TSP1 pathway.

Interestingly, our results showed that treatment with cisplatin increased the expression of TSP1 in a dose-dependent manner at both the mRNA and protein levels in CNE-1/taxol cells, but the increased expression of TSP1 was not observed in the parental cells (CNE-1). Unexpectedly, cisplatin did not inhibit the expression of TXR1 in CNE-1/taxol cells, indicating that in taxol-resistant NPC cells, cisplatin may directly or indirectly regulate TSP1 downstream of TXR1.

In addition, a significantly increased amount of early apoptotic cells was detected when CNE-1/taxol cells were exposed to cisplatin. This is consistent with the differential expression of TSP1 in CNE-1/taxol and CNE-1 cells, implying that cisplatin reverses the taxol resistance of NPC by upregulating TSP1 and facilitating apoptosis.

Taken together, our findings suggest that the use of a combination of taxol and cisplatin may reduce the incidence rate of drug resistance in NPC. However, a direct clinical study may be warranted.

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