MECHANISM OF DRUG RESISTANCE AND REVERSAL WITH LIGUSTRA-ZINE AND CYCLOSPORIN A IN CISPLATIN-INDUCED HUMAN EPITHELIAL OVARIAN CANCER RESISTANT CELL LINE 3AO/CDDP

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ABSTRACT

Objective: To investigate the mechanism of resistance and reversal effect of ligustrazine and cyclosporin A in cisplatin-induced multidrug resistance ovarian cancer cell line 3Ao/cDDP. Methods: Using the corresponding dose calculated from clinical chemotherapy at 30 mg cisplatin per cycle, we established 3Ao/cDDP with 3Ao exposed at regular intervals and repeatedly to high-level concentration of cisplatin at 10 μg/ml for 24 hours each time. Expressions of LRP, MRP, P-gp, GSTπ and TopoII were quantitatively detected with FCM. For drug resistance reversal, cyclosporin A and ligustrazine were administered singly or in combination at the maximal dose without cytotoxicity. Inhibition rates were determined by MTT assay. Results: 3Ao/cDDP was established after 4.5 months, with resistance factor 1.6 which was similar to clinical resistance degree. Low expression levels of MRP and P-gp were found in both 3Ao and 3Ao/cDDP ($P>0.05$), and LRP and GSTπ expression levels in 3Ao/cDDP were significantly higher than those in 3Ao ($P<0.005$ and $P<0.05$, respectively), and TopoII in 3Ao/cDDP was significantly lower vs 3Ao ($P<0.05$). The inhibition rate of cDDP was 20.807±0.015%, cDDP plus ligustrazine 27.421±0.07% ($P>0.05$ vs cDDP), cDDP plus cyclosporin A 49.635±0.021% ($P<0.01$ vs cDDP), and cDDP plus ligustrazine and cyclosporin A 58.861±0.014% ($P<0.01$ vs cDDP). Conclusions: 3Ao/cDDP, induced by cisplatin and established by imitating the characteristics of clinical chemotherapy for epithelial ovarian cancer, was an ideal model for investigation of cisplatin resistance *in vitro*. Cisplatin resistance in 3Ao/cDDP could be accounted for by higher LRP, GSTπ and lower TopoII expression and was not associated with MRP or P-gp. Ligustrazine had no significant reversal effect on cisplatin resistance, but cyclosporin A could reverse the resistance effectively.

Key words: Ovarian neoplasms, Drug resistance, Multiple, Cisplatin, Chemotherapy

Epithelial ovarian cancer is the leading cause of death among gynecologic malignancies. Chemotherapy plays an irreplaceable role in the treatment of patients. Protocols based on platinum compounds have significantly improved the overall response and clinical complete response but only less than a quarter of patients with advanced disease will be alive 5 years later.[1] Diagnosis at an advanced stage and development of resistance to chemotherapy, despite remarkable initial chemosensitivity, account for the poor overall prognosis.[2] To investigate and overcome drug resistance will be an effective pathway to improve survival.

Cisplatin has been the choice for ovarian cancer chemotherapy. The mechanisms of cisplatin resistance have not been completely elucidated and, up to now, are mainly investigated *in vitro* with cisplatin-resistant ovarian cancer cell lines, which are mainly induced by exposure to increasing concentration of cisplatin.[3, 4] But this inducement method of exposuring parent cell line to gradually increasing drug concentration has departed from the chemotherapy characteristics of repetition and intermission pattern in clinical practice. Meanwhile, study has been reported that several sublines with different resistant mechanisms can be acquired by different

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inducement ways with the same drug, the same ultimate concentration and just from the same parent cell line.[5]

Our study is designed to imitate the characteristics of clinical chemotherapy to establish cisplatin resistant cell line and detect expression of LRP, MRP, P-gp, GSTπ and TopoII, which have been found to be associated with drug resistance of other human tumors. Ligustrazine, abstracted from traditional Chinese herb, is also a kind of calcium channel antagonist with less toxicity than verapemil at the same dosage level cyclosporin A (CsA) and its analogues have been regarded as the most prospective reversal agent.[6] Therefore, we investigate their abilities to reverse cisplatin resistance.

MATERIALS AND METHODS

Monoclonal Antibodies

MRP monoclonal antibody (QCR-1) was kindly provided by Prof. Cole of Canada Queen University and LRP monoclonal antibody (LRP-56) offered by Prof. Scheper of Holland Free University. Monoclonal antibodies of P-gp, GSTπ, and TopoII were bought from Wuhan Boshide Biological Engineering Corp..

Cell line and Main Chemicals

3Ao, human epithelial ovarian mucous cystadenocarcinoma cell line, was grown in RPMI-1640 supplemented with 20% FCS at 37°C and 5% carbon dioxide.

Paclitaxel (TAX) and Teniposide (VM-26) were obtained from Bristol-Myers Squibb Pharmaceutical Co., USA Cisplatin (cDDP) was purchased from Qilu Pharmaceutical Factory, Shandong. CsA from Switzerland Sandoz Pharmaceutical Factory and ligustrazine from Beijing No. 4 Pharmaceutical Factory. Carboplatin (CBP), Cyclophosphamide (CTh), 5-Fluorouracil (5-Fu), Methotrexate (MTX), Vincristine (VCR), Adriamycin (ADM) and Etoposide (VP-16) were respectively obtained from different pharmaceutical corporations.

Toxicity Assays

We evaluated cytotoxicity by MTT assay. 5×10^4 cells/well were seeded in 96-well plate and treated for 26 hours with different concentrations of cDDP and other drugs. 20 μl of MTT (5 mg/ml, Sigma) were added and incubated for 4 hours. DMSO (150 μl) was then added and optical density (OD) values were determined using EL-309 instrument (Japan) (λ=570 nm, 630 nm).

Establishment of Cisplatin-resistant Cell Line 3Ao/cDDP

Cisplatin concentration in medium was caltimated according to the following formula[7]: μg/ml=(60×D)/(5000)×2×10^3. "60" stands for average adult body weight (Kg), "D" for clinical dosage mg/kg·d^{-1}, "5000" for average adult blood volume (ml), "2" for divided by blood corpuscle relative cubage 50%, "10^3" for changing mg to μg. Approximate cisplatin 10 μg/ml was figured out corresponding clinical minimal dose 30 mg. 3Ao cells in logarithm-growth phase were incubated in medium containing 10 μg/ml cisplatin for 24 h, and then cisplatin was withdrawn. Cells were rinsed three times with PBS to culture in no-drug medium until new cells clone formed. This same process was performed six times through 4.5 months until the cell line IC50 was stable with MIT assay. The resistant cell line was named 3Ao/cDDP. All the following experiments were carried out after two weeks culture without cisplatin. Cell cycle profiles were analysed with FCM. The growth-doubling time was calculated by cytometry.

Expression of LRP, MRP, P-gp, GSTπ and TopoII

FCM was employed. Before detection adjust the instrument to stablize the coefficient of variability within 2%. Twelve 3Ao/cDDP and 3Ao samples were available for analysis by FCM. Cells were washed twice in PBS and incubated for 15 minutes at 37°C in PBS containing 1% (v/v) BSA (PBS/BSA). Cells (10^7) were pelleted and incubated on ice for 1 hour with 2 μl monoclonal antibodies or mouse matched-isotype control (IgG2b). Antibody binding was detected with fluorcscein isothiocyanate-labeled rabbit anti-mouse immunoglobulins (Dako). Fluorescence was analyzed on a FACscan flow cytometer (Becton Dickinson). Ten thousand events were recorded and analyzed by Cell Quest Plot software.

Cisplatin Resistance Reversal of Ligustrazine and CsA

Cytotoxicity Test of Ligustrazine and CsA

Ligustrazine concentration ladder was 5, 10, 20, 100, 200, 2000, 20000 (μg/ml); CsA concentration ladder was 0.0125, 0.125, 1.25, 12.5, 125, 1250 (μg/ml). Cytotoxicity test employed MTT assay and cell control and blank control were set up at the same time. Cell inhibition rates at corresponding different ligustrazine or CsA concentrations were calculated. Semi-logarithm equations were obtained with logarithm values of different concentrations as X and with cell inhibition rates as Y:

CsA: $Y=0.2572+0.1778X$ ($r=0.956$)
Ligustrazine: $Y=-0.2814+0.3002X$ ($r=0.949$)

Then the maximal concentrations of CsA and ligustrazine without cytoxicity were worked out when *Y* value was zero-40 ng/ml and 5 μg/ml, respectively.

Resistance Reversal

Different groups were divided-- A: cell control; B: cDDP; C: CDDP+CsA; D: cDDP+ligustrazine; E: cDDP+CsA+ligustrazine; F: blank control. 3×10^4 cells/well were seeded in a 96-well/plate, treated after 24 h with 10 μl of cDDP (500 μg/ml) and 10 μl of CsA and ligustrazine and their ultimate concentrations were sure to be 40 ng/ml and 5 μg/ml respectively. Then 20 μl of MTT (5 mg/ml, Sigma) were added to each well and incubated in the dark for 4 h. 150 μl DMSO (Sigma) was then added and OD values were determined.

Statistical Analysis

We performed student T-test, Chi-square test and variance analysis to calculate the significance of differences between the various cohorts. Differences were considered statistically significant when $P<0.05$.

RESULTS

Establishment of Resistant 3Ao/cDDP

We imitated the characteristics of clinical chemotherapy of epithelial ovarian cancer to establish the cisplatin-resistant cell line 3Ao/cDDP, with 3Ao exposured to high-dose cisplatin 10 μg/ml for 24 h every time. It took us 4.5 months to make it. IC50 (3Ao/cDDP)=226.4±0.02, IC50 (3Ao)=139.5±0.01, RF= 1.6 ($P<0.01$). During the experiment, we found that the intervals between every withdrawal and new clone formation were 25, 23, 16, 11, 4, 3 days, which took on a gradually shortened trend. The growth-doubling time of 3Ao/cDDP was 23.8±0.3 (h) and 3Ao 24.9±0.1 (h) ($P>0.05$). Cell cycle kinetics and DNA, RNA concentration: See Table 1. Analysis results of drug resistance spectrum suggested that 3Ao/cDDP had resistance not only to cisplatin, but also to other nine drugs which were being used in clinical practice (Table 2 and Figure 1).

Expression of LRP, MRP, P-gp, GSTπ and TopoII

FCM can analyse and classify cells rapidly and exactly with better accuracy, repeatability and sensitivity. Data statistical analysis employed T-test. Compared with 3Ao, expression of P-gp and MRP in 3Ao/cDDP was not significantly increased ($P>0.05$); LRP and GSTπ expression levels were elevated significantly ($P<0.005$); and TopoII was significantly decreased ($P<0.05$) (Table 3 and Figure 2).

Cisplatin Resistance Reversal of Ligustrazine and CsA

Maximal reversal concentration was employed to reverse cisplatin resistance. Values were representative of at least 3 independent experiments. Inhibition rates of all groups were determined (Table 4).

Table 1. Cell cycle kinetics of 3Ao and 3Ao/cDDP

Cell line	G_0±G_1 (%)	G_2±M (%)	S (%)	DNA	RNA
3Ao	47.2±1.1	15.1±1.5	39.9±1.9	46.9±1.5	30.4±0.9
3Ao/cDDP	38.4±1.4*	24.1±1.9*	42.0±1.7**	55.1±0.7*	49.5±0.5*

Vs 3Ao *$P<0.01$ **$P<0.05$

Table 2. Resistance spectrum of 3Ao/cDDP

Drug	IC50 (μg/ml)		RF
	3Ao	3Ao/cDDP	
cDDP	139.5±0.01	226.4±0.02	1.62
CBP	101.41±0.02	210.3±0.01	2.07
TAX	64.8±0.7	101.1±0.6	1.56
MTX	1013.0±0.1	3500.0±0.7	3.45
VCR	222.4±0.3	449.9±0.9	2.02
VP-16	905.3±0.02	2193.0±0.04	2.42
VM-26	965.1±0.06	1216.0±0.04	1.26
ADM	950.2±0.03	1881.6±0.01	1.98
5-FU	764.1±0.3	10875.1±0.8	14.21
CTX	281.2±0.2	1612.0±0.5	5.73

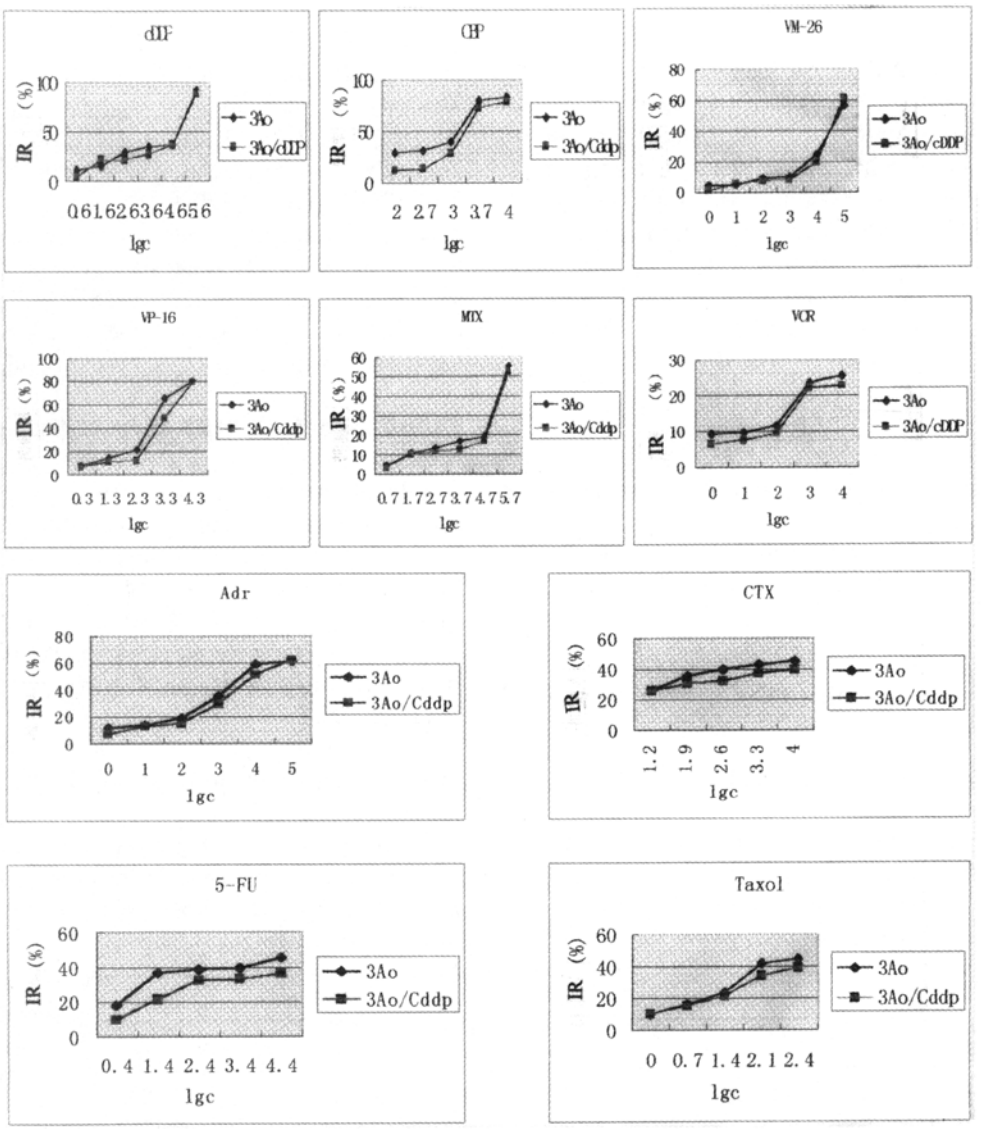

Fig. 1. Resistance spectrum of 3Ao/cDDP (inhibition rate, HI)

Table 3. Detection of LRP, MRP, P-gp, GSTπ and TopoII

Index	3Ao positive rate (%)	3Ao/cDDP positive rate (%)
LRP	9.4±0.9	30.1±0.1
MRP	7.8±0.6	8.6±0.3
P-gp	2.5±0.3	2.9±0.5
GSTπ	11.9±0.6	29.8±0.3
TopoII	20.2±0.3	8.5±0.1

$^{*}P>0.05$ $^{**}P<0.005$ $^{***}P<0.05$ vs 3Ao

Evidently, the inhibition rate of cDDP+ ligustrazine group showed no significant difference compared with that of cDDP group, which suggested ligustrazine could not reverse cisplatin resistance although it had showed slightly higher inhibition rate 27.421±0.07(%) than 20.807±0.015(%). And only groups including CsA (C and E) suggested significant reversal effect on cisplatin resistance

(P<0.01), but from the variance analysis result, CsA and ligustrazine had not indicated synergetic effect in spite of higher inhibition rate when they were administered simultaneously (P>0.05).

Table 4. Reversal of cisplatin resistance with ligustrazine and CsA

Groups	OD values (λ=570 nm)	Inhibition rate (%)	P (vs B)
A	0.5897±0.01		
B	0.4676±0.023	20.807±0.015	
C	0.2971±0.014	49.635±0.021	<0.01
D	0.4281±0.067	27.421±0.07	>0.05
E	0.2426±0.03	58.861±0.014*	<0.01

blank control OD value: 0.004 *Variance analysis: E vs C (P>0.05)

A: cell control; B: cDDP; C: cDDP+CsA; D: cDDP+ligustrazine; E: cDDP+CsA+ligustrazine

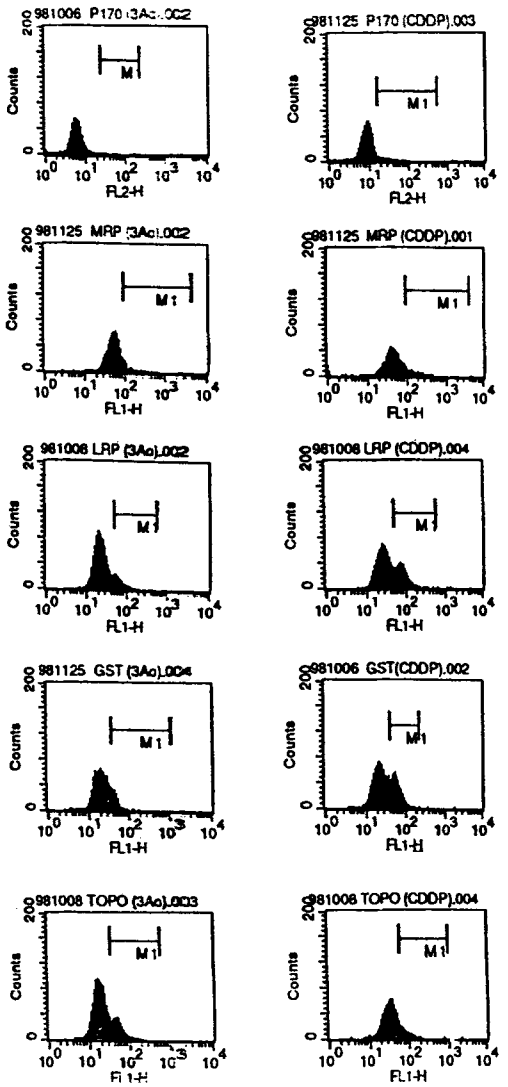

Fig. 2. P-gp, MRP, LRP, GSTπ and TopoII expression of 3Ao(left) and 3Ao/cDDP(right) with FCM

DISCUSSION

Many papers have been presented about different cisplatin-resistant cell lines,[4, 8] but they had always induced resistance by exposure parent cell line to increasing concentration of the target chemotherapeutic drug, as had completely deviated from clinical chemotherapy pattern. In the present study we established cisplatin-resistant cell line 3Ao/cDDP, imitating clinical protocols administered at regular intervals and repeatedly and at the corresponding dose 10 μg/ml against 30 mg. RF of 3Ao/cDDP is 1.6, which is similar to clinical resistant degree.[9] According to Yang,[5] our cell line 3Ao/cDDP should be the most ideal model to investigate cisplatin resistance *in vitro*. During the inducing process of resistance, we found the intervals between withdrawal and new clone formation shortened gradually. This phenomenon, which reflected the fundamental biological characteristic of cell against drug attack, can not be observed in the concentration-increasing model. This shortening intervals probably suggest that in clinical practice we should administer the next chemotherapy cycle as early as possible before cancer cell recovers growth. Certainly, chemotoxicity should also be taken into account, including cisplatin half-life time, possible toxicity accumulation and arrest degree of bone marrow. However, with the application of G-CSF, M-CSF, GM-CSF and gene transfer technology, chemotoxicity will be overcome in the near future. Under the premise, an interval-shortening chemotherapy pattern might be able to be given. Tumor growth-doubling time and its proliferation characteristic *in vivo* are to be investigated further.

Resistance to cisplatin has been shown to be multifactorial,[10] and specific biochemical resistance pathways include (1) decreased in drug accumulation,

(2) increased inactivation in the cytosol via detoxification enzymes such as glutathione S-transferases (GSTs) or by direct binding to nonprotein thiols such as glutathione, and (3) increased activity of DNA repair enzymes that remove lethal DNA adducts. P-gp, as the first efflux pump found in 1976, was reported to lead to MDR to bulky natural products. For a long time the relationship between P-gp and ovarian cancer drug resistance has been controversial. But recent reports have suggested that P-gp seems to be irrelevant to cisplatin resistance.[3] Our present results also demonstrated that (Table 4, $P>0.05$). Like P-gp, MRP also belongs to ATP-binding cassette proteins superfamily and also functions as a transmembrane efflux pump. But its accurate molecular mechanism remains open. Our study has demonstrated cisplatin resistance has nothing to do with MRP, which is compatible with Hamaguchi,[11] who had not found MRP gene amplification or overexpression with Northern and Southern hybridization blotting in cisplatin-resistant ovarian cancer cell line.

Just like above mentioned, GSTs may lead to resistance by increased drug inactivation. Human GSTs can be divided into four subtypes α, μ, π, θ. GSTπ, in particular, is the focus of many studies. Our results indicate higher expression level of GSTπ in 3Ao/cDDP vs 3Ao ($P<0.05$) and supports the status of GSTπ in MDR. However, different conclusions have also been presented.[12,13] We believe that different detection methods, different sample sources and sample sites, and different therapy protocols may be able to account for the different results. Therefore, research standards concerned should be figured out and complied with. Topoisomerase (including TopoI and TopoII) is a kind of nuclear enzyme and controls DNA space structure, duplication, breakage, repair and linkage. Altered topoisomerase phenotype in MDR primarily associated with drugs involving inhibition of topoisomerase II such as etoposide. But it is interesting in our study that TopoII expression in 3Ao/cDDP is significantly lower than 3Ao ($P<0.05$), indicating that TopoII also operates in cisplatin resistance.

Lung resistance protein (LRP) has been described as a 110 kDa protein that is overexpressed in several non-P-gp MDR cells lines of different histogenetic origin. In fact, LRP is human major vault protein (MVP) which is highly homologous to the major vault proteins of slime mold and rat.[14] Approximately 95% of LRP lies in the cytoplasm and 5% is linked with nuclear pore complex (NPC). LRP may display its functions by following mechanisms: (1) keep drugs which target DNA (such as platinum and alkylating agents, etc) out of nucleus, (2) transport diffusing drugs in cytoplasm into capsules and discharge them out of cell. Our investigation found that LRP expression in 3Ao/cDDP was significantly higher than 3Ao ($P<0.005$), demonstrating that LRP plays an important role in cisplatin resistance in epithelial ovarian cancer. Recently Izquierdo et al.[15] reported expression of LRP was significantly associated with chemo-sensitivity and prognosis. But its clinical significance of LRP can not be reached just according to limited studies and further investigations are needed in future.

In 1982, Tsuruo and his colleagues reported that verapamil could enhance vincristine- and adriamycin-induced cytotoxicity in P388 leukemia and its resistant sublines. Verapamil is a one of calcium channel blockers, which could decrease drug resistance or could restore drug sensitivity by interfering with the efflux function of P-gp. Recently Muller et al.[16] demonstrated that verapamil reversed resistance by down-regulation mechanism of mdr_1 gene transcription. But cardiovascular problems, such as hypotension and heart toxicity, have deadly limited its clinical use. Ligustrazine, also a kind of calcium channel blocker, abstracted from traditional Chinese herb, has lower toxicity than other agents. We believe that the ideal MDR modifier should has the strongest reversal activity and has no cytotoxicity to normal tissue. On this basis, we studied the reversal effect of ligustrazine at 5 μg/ml. But result indicated that ligustrazine could not reverse cisplatin resistance. 3Ao/cDDP resistance has nothing to do with P-gp, which explains the result. Certainly, our study also suggested that calcium channel blocker has no other main mechanism to exert reversal. CsA, an immunosuppressive agent that has been used extensively in organ transplantation, is now known to reverse P-glycoprotein-mediated multidrug resistance as efficiently as other prototype compounds of resistance modifiers. However, our result suggests that CsA can significantly enhance cytosensitivity to cDDP. In other words, the reversal activity of CsA does not depend on expression of P-gp. Mohammed et al.[17] found that the cisplatin-resistant cell displayed enhancement of c-fos and c-H-ras expression. CsA suppressed cisplatin-induced c-fos and c-H-ras oncogene expression and yielded an improved sensitivity to cisplatin. Unfortunately, we did not investigate the molecular mechanism. Further studies are to be needed. Also Mutch et al.[18] found that CsA , just like emetine and cycloheximide, decreased resistance to cDDP by affecting a protein synthesis-dependant resistant mechanism. Maybe these mechanisms exist during the reversal of 3Ao/cDDP with CsA.

In conclusion, 3Ao/cDDP is an ideal model for cisplatin resistance investigation *in vitro*. We demonstrate that P-gp and MRP have nothing to do with cisplatin resistance, and higher LRP, GSTπ and lower TopoII levels can account for cisplatin resistance. At the maximal concentration without cytotoxicity, ligustrazine can not increase cisplatin sensitivity, but CsA can. CsA exerts reversal effect by other mechanism independent of P-gp expression, as just needs further studies in future.

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