Reports of Biochemistry & Molecular Biology
Vol.12, No.3, Oct 2023

Original article

www.RBMB.net

Resistance Improvement and Sensitivity Enhancement of Cancer Therapy by a Novel Antitumor Candidate onto A2780 CP and A2780 S Cell Lines

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Abstract

Background: To overcome cisplatin resistance, the cytotoxicity of a novel antitumor agent on two ovarian cancer cell lines sensitive and resistant to cisplatin was investigated.

Methods: MTT assay and flow cytometry were performed to assess the cytotoxicity of a novel water-soluble Pd (II) complex, [Pd(bpy)(pyr-dtc)]NO_3 (PBPD), on cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines. Furthermore, variations in the expression of drug resistance gene cluster of differentiation 99 (CD99), signal transducer and activator of transcription 3 (STAT3), octamer-binding transcription factor 4 (OCT4), and multidrug resistance mutation 1 (MDR1) were evaluated using Real-Time PCR.

Results: The IC50 values of PBPD in resistant cells were higher than those in sensitive cells. Furthermore, PBPD has a deadlier effect on sensitive cells compared to resistant cells, and the cell survival rate is reduced over time. Flow cytometry revealed that PBPD enhanced the population of living-resistant cells while driving them to apoptosis. PBPD, on the other hand, has a greater effect on the living cell population and has dramatically shifted the population toward apoptosis and necrosis in the sensitive cells. Furthermore, gene expression analysis showed that when sensitive and resistant cells were treated with cisplatin, all resistance genes increased significantly relative to the control. In contrast to OCT4, MDR1, STAT3, and CD99 resistance genes were not significantly elevated in sensitive cells treated with PBPD compared to the control. Thus, the expression of resistance genes in resistant cells treated with PBPD was lower than cisplatin.

Conclusions: As a result, PBPD is a promising anticancer agent for CDDP-resistant ovarian cancer.

Keywords: Chemotherapy, Cisplatin, Apoptosis, Drug Resistant, Ovarian Cancers, Organometallic Compound.

Introduction

With more than 23,000 new cases identified each year and more than 15,000 deaths in the United States alone, ovarian carcinoma is the leading cause of death among gynecological neoplasms (1,2). Chemotherapy resistance is a significant barrier to effective treatment of ovarian and other malignancies (3-5).

Ovarian cancer is known to have a less favorable outlook than other types of cancer affecting women. The standard approach for treating this condition involves surgical intervention, followed by additional chemotherapy, and subsequent re-administration of platinum-based chemotherapy if the cancer returns. This treatment regimen has demonstrated positive outcomes in a significant number of cases. However, the effectiveness of this potent

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Received: 18 June 2023; Accepted: 15 Sep, 2023

chemotherapeutic agent has been hindered by the development of resistance, both acquired and newly emerging, thereby limiting its overall success (1,2). Cisplatin (CDDP), a platinum-containing chemical, is commonly used to treat malignancies of the ovary, testicles, head and neck, lungs, and bladder (4). Despite potent anticancer activity of CDDP, long-term treatment is limited due to the development of resistance. Furthermore, certain cancers develop resistance to CDDP, reducing its potency (6), and cisplatin resistance, both inherent and acquired, limits the therapeutic use of this essential anticancer medication (7).

Advances in chemotherapy treatment for ovarian cancer can be achieved by identifying specific genes that play a role in regulating both intrinsic and acquired mechanisms of drug resistance. By targeting these genes, it is possible to develop a more potent and effective chemotherapeutic approach that can effectively combat this disease. This scientific approach holds promise for enhancing the ability to overcome drug resistance and improve overall treatment outcomes in patients with ovarian cancer (8). One of the most effective tactics in this sector is the development of new anticancer medicines based on CDDP and modification of the nature of the central metal ion. Because the chemistry of palladium (Pd) and platinum (Pt) is comparable, Pd(II) complexes are expected to have anticancer properties similar to those of platinum-based complexes (9,10). Pd(II) complexes are more cytotoxic than platinum-based medicines which are widely used in clinics (11,12). Furthermore, some studies have demonstrated the cytotoxic effects of Pd(II) complexes in cancer cell lines such as leukemia, skin, breast, liver, lungs, prostate, head and neck, and kidney. Therefore, the cytotoxic and apoptotic effects of a Pd(II) complex and some drug-resistance genes against CCDP-sensitive and -resistant ovarian cancer cells A2780 should be studied (13-15).

To overcome cisplatin resistance, cytotoxicity of a novel water-soluble Pd (II) complex, [Pd(bpy)(pyr-dtc)]NO3 (PBPD), was assessed in CDDP-sensitive and CDDP-resistant ovarian cancer cell lines using MTT and Flow cytometry. Real-time PCR was used to evaluate the expression variations of the resistance genes CD99 (Gene ID: 4267), STAT3 (Gene ID: 6774), OCT4 (ID: 5460), and MDR1 (ID: 23158) associated with drug resistance.

Materials and Methods

Phosphate buffer saline tablet (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO) were obtained from Sigma Aldrich. Penicillin-streptomycin, Eagle Dulbecco's modified medium, fetal bovine serum (FBS), L-glutamine, and sodium pyruvate were purchased from Gibco, US. Human ovarian cancer cell lines that were resistant to cisplatin (A2780CP) and sensitive to cisplatin (A2780) was provided from Pasteur Institute of Iran. The water-soluble palladium (II) complex, [Pd(bpy)(pyr-dtc)]NO_3, 2,2-bipyridine pyrrolidinethocarbamato palladium(II) nitrate, (PBPD) were synthesized in our laboratory (16), and their molecular structures are shown in Figure 1. All the other solvents and chemicals were used without further purification. Deionized water was utilized in all the experiments.

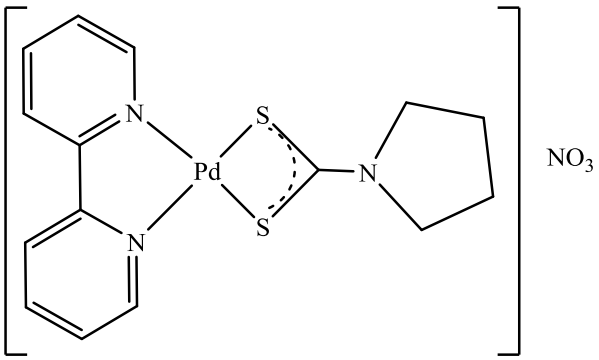

Fig. 1. The structure of PBPD complex.

Cell Culture

A2780CP and A2780 was used as human ovarian cancer cell lines of resistant to cisplatin and sensitive to cisplatin, respectively. Eagle Dulbecco's modified medium was used to cultivate the cells, which contained 10% fetal bovine serum and 4.5 g/L L-glutamine, sodium pyruvate, and 1% penicillin/streptomycin. Then, the cells were

kept in an incubator (325 cm flask) at 37 C with 5% CO_2.

MTT assay

2,2-bipyridine pyrrolidinethocarbamato palladium(I) nitrate, (Pd(bpy)(pyr-dtc)]NO_3, (PBPD) was produced in our laboratory, and its molecular structure is presented in Scheme 1(16), and their molecular structures are presented in Scheme 1. MTT assay was performed on human ovarian cancer cell lines A2780CP, which is resistant to cisplatin, and A2780, which is susceptible to cisplatin, after 24 and 48 hours of treatment. 10^4 cells were seeded in 96-well plates with 100 l of media per well from each resistant and sensitive cell line and cultivated for 24 hours at 37 C. MTT assay was used to analyze four groups of control (no drug treatment), cisplatin (2.5 g/ml) treatment, PBPD treatment onto resistant cells, and sensitive cells. Then, the cells were treated in separate wells with cisplatin (2.5 g/ml) and PBPD (5, 10, 20, 30, 40, and 50 M) for 24 and 48 hours. After incubation, 100 l of 3-(4,5-Dimethylthiazolyl)-2,5-diphenyltetrazolium bromide, MTT at a concentration of 5 mg/ml was added to each well, and the plates were incubated at 37 C for 4 hours. The medium was then withdrawn from each well, and 200 L of dimethyl sulfoxide was added and agitated for 20 minutes. Finally, the absorbance at 570 nm was measured using a spectrophotometer (Biorad, USA). Each well was tested three times. The percentage of viable cells was determined as follows (2):

Cell viability (%) $=(A_{treated}/A_{control}) \times 100$

Where $A_{treated}$ and $A_{control}$ are the absorbance values of the treated and untreated cells, respectively. GraphPad Prism version 9 software was used to determine the half-maximal inhibitory concentration (IC50) of the drug in sensitive and resistant cells, which was measured as the concentration of PBPD at which 50% of the cells were viable relative to the control.

Real-time PCR

After exposing the sensitive and resistant cells to 30 M of PBPD and the cisplatin for 24 hours, total RNA was extracted from the treated and control cells using the RiboEx (GeneALL, Korea) kit according to the protocol. The quality of the extracted RNA was evaluated using a 2% agarose gel, and the absorbance value was measured at 260 nm, 260 nm/280 nm, and 230 nm/260 nm. The cDNA synthesis procedure (SMOBIO kit) was then started with 1000 ng of RNA from each sample. The cDNA synthesis kit included oligo(dt) and random hexamer primers. A specific primer design was done using the Primer3 software (table 1) for the desired gene expression level, then Amplicon master mix (Denmark) and Real-time PCR step one plus machine were utilized, and the GAPDH gene was chosen as the internal control gene. A specific concentration of cDNA was combined with forward and reverse synthetic primers in the master mix and real-time PCR process. Finally, when the cycles were completed, melting curve analysis was utilized to determine the purity of the PCR products. The multiple expression of genes was determined by 2-ΔΔCT.

Table 1. Primer used in the present study.

Gene	Tm	GC%	Length	sequence	PCR size (bp)
STAT3-F	61.41	52.17	23	GCTGACTACACTGGCAGAGAAAC	189
STAT3-R	60.57	54.55	22	CCCATGATGTACCCTTCGTTCC	
OCT4-F	61.55	63.16	19	CAGTGCCCGAAACCCACAC	155
OCT4-R	61.56	57.14	21	GACCCAGCAGCCTCAAAATCC	
MDR1-F	60.34	57.14	21	CAGAGGGGATGGTCAGTGTTG	111
MDR1-R	61.21	52.38	21	TCGTGGTGGCAAGCAATACAG	
CD99-F	60.27	52.38	21	GTGCTGGGGATGACTTTGACT	65
CD99-R	60.57	52.38	21	TGGTCGTGGGTCGTCATTTTC	
B2M-F	61.58	52.38	21	AGATGAGTATGCCTGCCGTGT	106
B2M-R	60.29	45.45	22	TGCGACATCTTCAAACCTCCAT	

Apoptosis induction

Flow cytometry was used to examine the effect of PBPD-induced apoptosis on resistant cells, A2780CP, and sensitive cells, A2780. 105 resistant and sensitive cells were cultured independently, and each cell received 30 M PBPD. After 24 hours, the cells were scraped with Trypsin EDTA, and the extent of apoptotic induction was determined by flow cytometry with Annexin-V labeling.

Statistical analysis

Graph pad prism version 9 software and the Two-way ANOVA statistical method were used to perform the statistical analysis of the data obtained from the studied tests. In statistical tests, a 95% confidence interval and P<0.05 were considered, and the tests were repeated three times.

Results

Cell Viability evaluation by MTT assay

The viability of sensitive and resistant cells against PBPD was evaluated at 24 h and 48 h by MTT assay (Fig. 2). The results showed that the lethality rate in both cell lines increased with an increase in drug concentration. However, the lethality rate in the sensitive cells (Fig. 2A) was higher than that in the resistant cells (Fig. 2B); this increase was statistically significant at P<0.05.

In addition, the IC50 value of PBPD was 29.72 µM after 24 h and 19.34 µM after 48 h in the resistant cells and, was 9.594 µM after 24 h and 5.94 µM after 48 h in the sensitive cell (Fig.2 C). Therefore, the efficacy of PBPD on sensitive cells was higher than that on resistant cells.

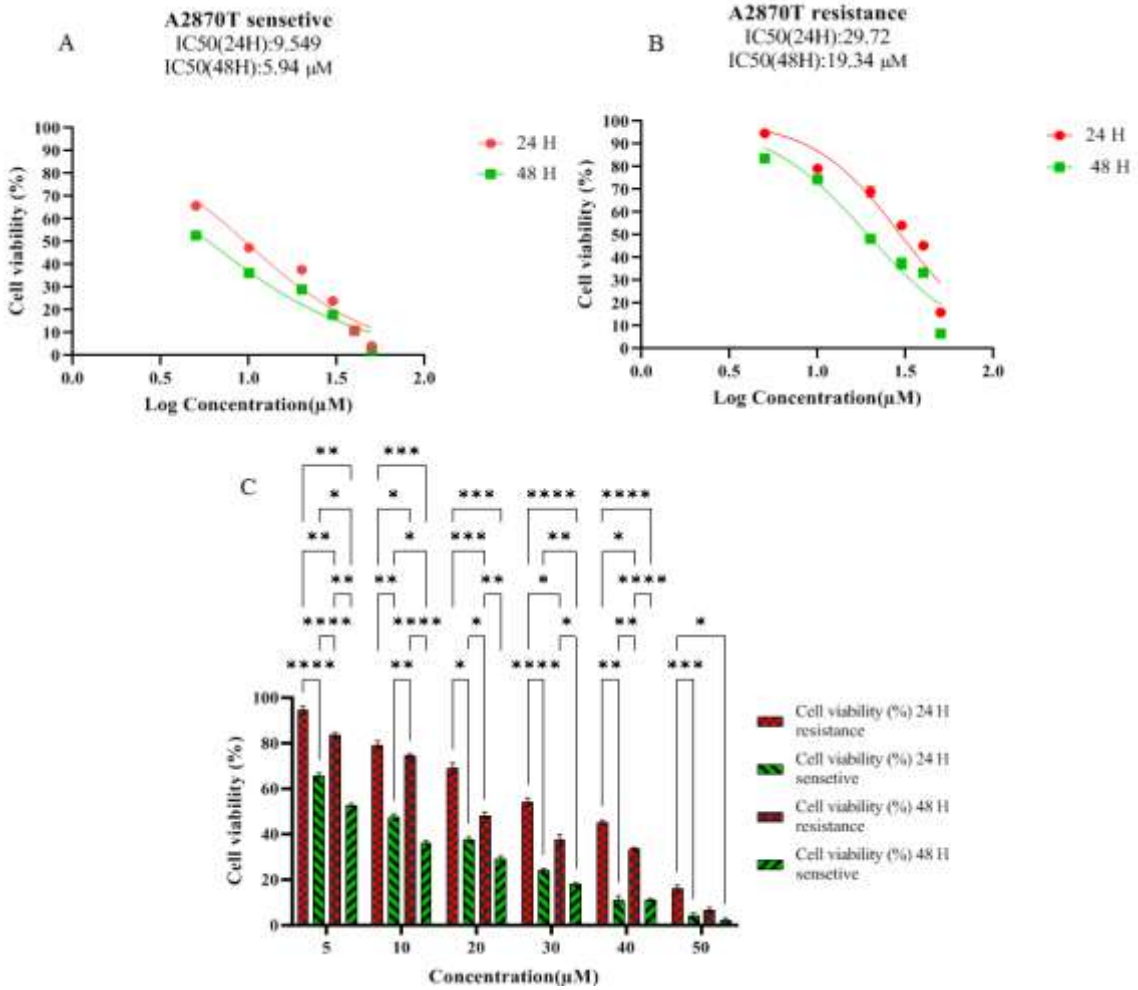

Fig. 2. The survival graphs in terms of concentration logarithms for sensitive (A) and resistant (B) cells at 24 h and 48 h. The comparison of survival in different concentration groups was made by the two-way ANOWA statistical test and Tukey's multiple comparisons tests (C). This statistical difference between the groups is indicated by * that * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001.

Gene expression by real time PCR

As shown in Figure 3, the changes in the expression of CD99, STAT3, OCT4, and MDR1 drug resistance genes in the samples treated with the new palladium drug increased compared to the control cells, but in comparison with the samples treated with the cisplatin drug, they increased less than the control. This shows that the new drug reduced the expression of resistance genes more than cisplatin did, and according to the graph, this reduction was statistically significant at $P<0.05$. The results demonstrated that the gene expression of MDR1, OCT4, CD99, and STAT3 in sensitive cells treated with cisplatin compared to control was 0.318±5.04, 0.43±6.115, 0.36±2.9, and 1.22±10.23, respectively. The control has increased by 0.98±11.37, 0.99±9.34, 0.18±3.98, and 1.24±15.39, respectively.

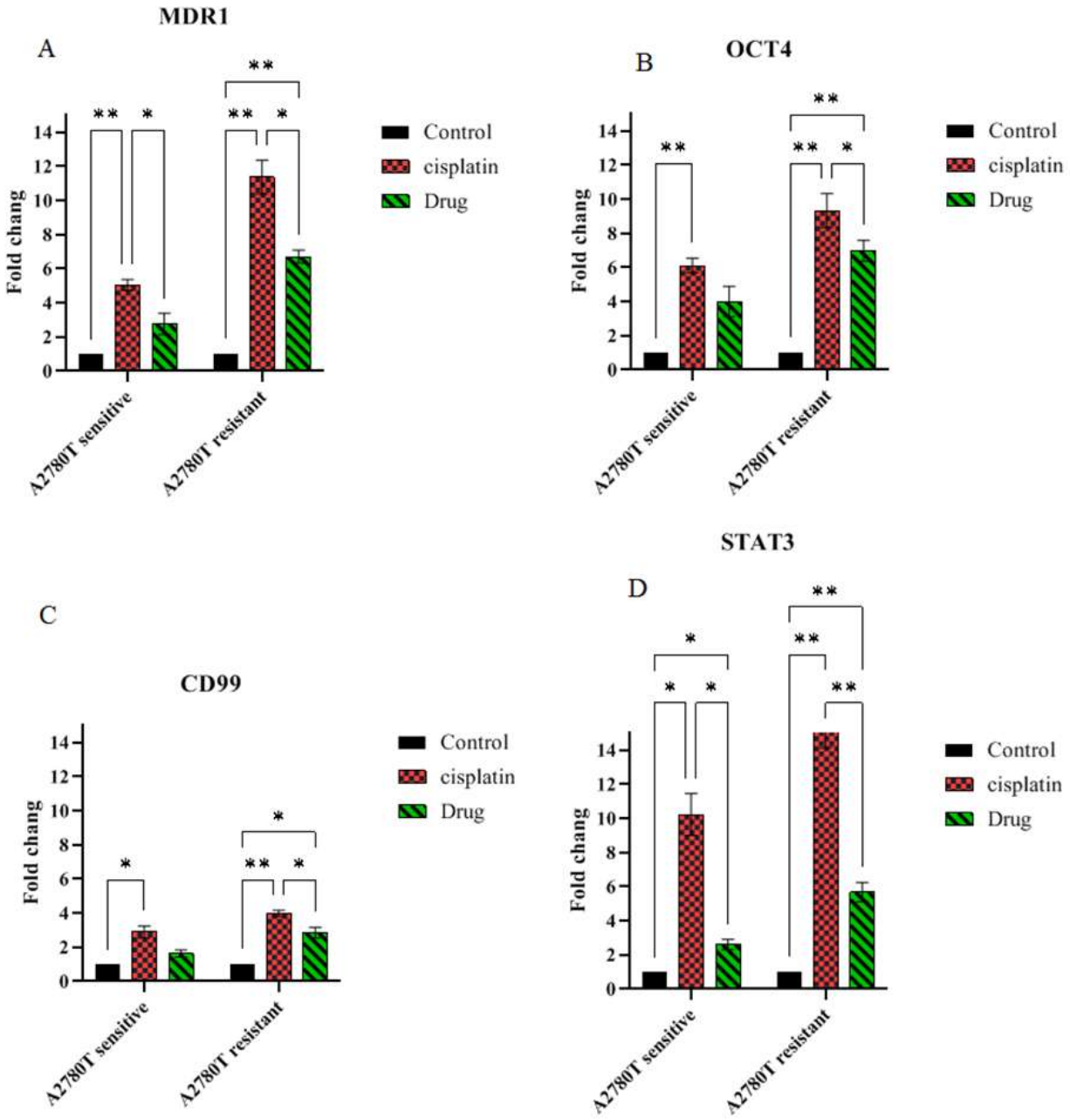

Fig. 3. Changes in MDR1 gene expression (A), OCT4 gene expression (B), CD99 pression (C), and STAT3 gene expression (D) in sensitive and resistant cells under the influence of PBPD and cisplatin. The statistical difference between the groups was determined by * $P<0.05$, ** $P<0.01$.

In addition, the changes in the expression of CD99, STAT3, OCT4, and MDR1 drug resistance genes in the samples affected by PBPD increased compared to the control cells, but compared to the samples treated with cisplatin, they increased less than the control. This result showed that the new drug reduced the expression of resistance genes compared to cisplatin.

Apoptosis induction by flow cytometry

Fig. 4A shows the effect of PBPD on the resistant cell; the new palladium complex, via affecting the resistant cells, causes an increase in the population of apoptotic cells and a decrease in the population of living cells compared to the control cells, which shows the effect of apoptosis induction of PBPD on resistant cells. As shown in Fig. 4B, the effect of PBPD on the sensitive cells shows that the drug sent a large population of sensitive cells to apoptosis, especially secondary apoptosis. $P<0.05$ is statistically significant between the control group and the drug-affected group in resistant and sensitive cells in the number of viable and apoptotic cells. The results of flow cytometry showed that PBPD, with its effect on resistant and sensitive cells, caused an increase in the population of apoptotic cells and a decrease in the population of living cells compared to the control cells, indicating the effect of the drug on inducing apoptosis. It is noteworthy that in sensitive cells, the most significant effect was observed on secondary apoptosis.

Discussion

The MTT results in the current study showed that when drug concentration increased, the lethality rate increased in both cells, although the lethality rate was higher in sensitive cells than in resistant cells.

Flow cytometry results showed that the new palladium drug, with its effect on resistant and sensitive cells, increased the population of apoptotic cells and decreased the population of living cells compared to the control cells, indicating the effect of the drug on inducing apoptosis. The largest influence was observed in cells sensitive to secondary apoptosis. According to Tanaka et. al., results, apoptosis was not reduced by PBPD compared to that in parent cells, although apoptosis induced by PBPD was reduced. These findings suggest that the resistance mechanism of Pd(II) complexes differs from that of Pt(II) complexes. Interestingly, the results of Tanaka's study were inconsistent with those of the present study, which could be due to the different cell lines used in the two studies (17). It is likely that the palladium medication used in this study can trigger the apoptotic pathway by lowering the expression of the CD99 gene, while also preventing drug resistance. The resistance genes CD99, STAT3, OCT4, and MDR1 were significantly increased in sensitive and resistant cells treated with cisplatin compared to the controls, indicating that the drug causes resistance in cells. MDR1, STAT3, and CD99 resistance genes were not significantly elevated in sensitive cells treated with the new drug compared with the control; however, the OCT4 gene was. Resistance gene expression increased significantly in resistant cells treated with the new drug. Thus, the expression of resistance genes in resistant cells treated with the new drug decreased dramatically when compared to cisplatin, indicating that this drug can increase the expression of drug-resistant genes in resistant cells compared to cisplatin. Hence, this new agent is more effective against cell resistance than cisplatin.

In response to cytokines and growth factors, STAT3 forms homo- or heterodimers and is transported to the cell nucleus where it functions as a transcriptional activator. STAT3 is phosphorylated by the receptor-associated kinases (JAKs). STAT3 is activated specifically in response to ligands, such as interferons, epidermal growth factor (EGF), and IL-6. STAT3 regulates the expression of a wide range of genes in response to cellular stimuli, and hence plays an important role in a variety of cellular processes, such as cell development and death.

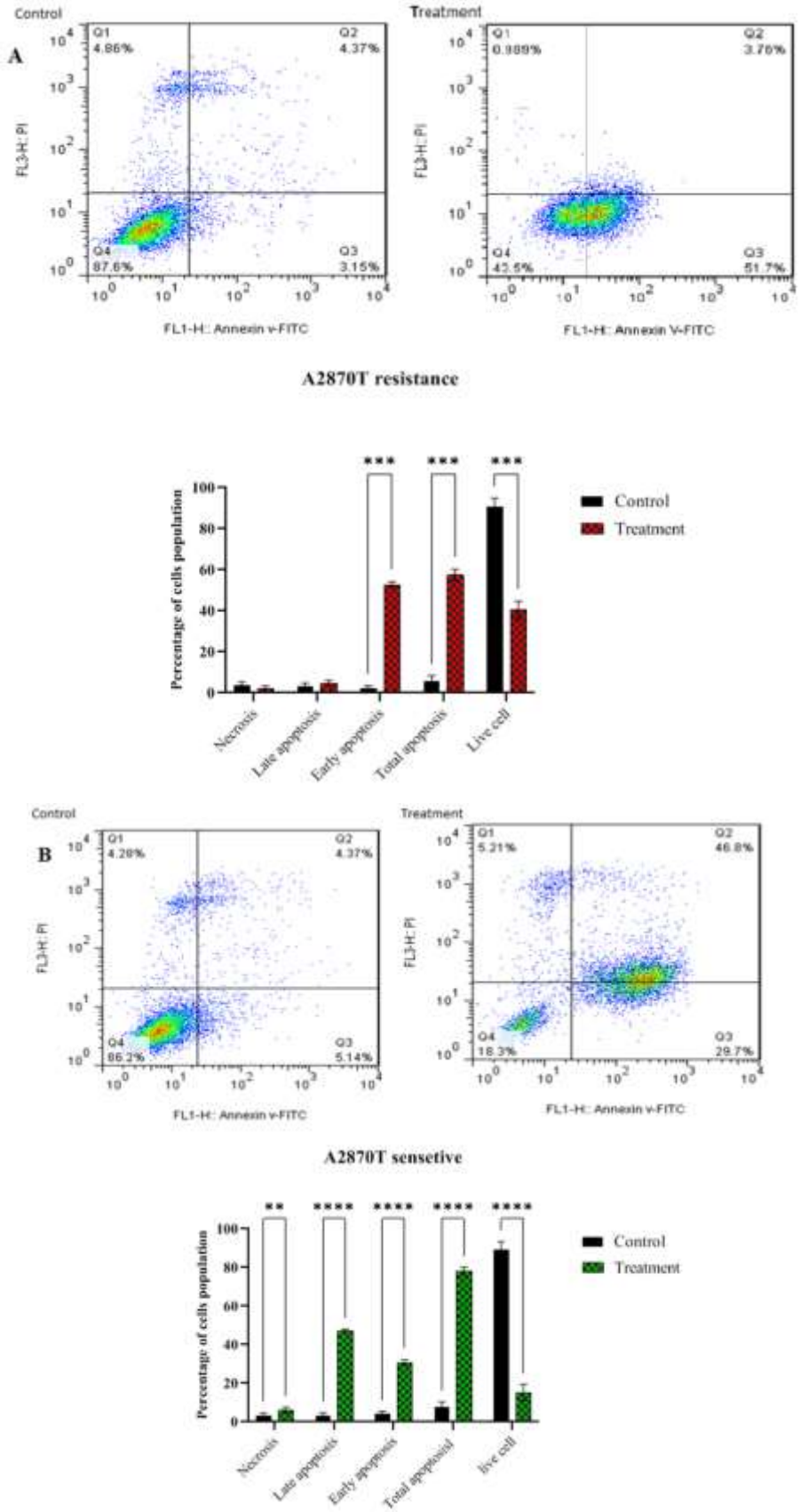

Fig. 4. The flow cytometry results of PBPD effect on resistant (A) and sensitive cells (B). The statistical difference between the groups was indicated by *, **P<0.01, ***P<0.001, ****P<0.0001.

Recent evidence has revealed that the signal transducer and activator of transcription-3 (STAT3) protein is a determinant of chemo-resistance and are linked to tumor recurrence in a variety of solid tumors. Han et al. discovered that high levels of STAT3 were linked to chemoresistance in human ovarian cancer cells. The use of siRNA technology to target STAT3 increases cisplatin-induced apoptosis in cisplatin-resistant ovarian cancer cells expressing high levels of STAT3. The molecular mechanism showed that interleukin-6 (IL-6) can activate STAT3 in cisplatin-sensitive ovarian cancer cells, resulting in cisplatin resistance. STAT3 siRNA therapy also inhibits IL-6-induced STAT3 phosphorylation, lowering IL-6's anti-apoptotic action. According to Hahn et. al., the combination of cisplatin and STAT3 siRNA caused the mitochondrial membrane potential to collapse, decreased Bcl-xL and Bcl-2 expression, and increased cytochrome C release and Bax expression. These findings imply that pharmacological STAT3 inhibition may be a promising treatment method for treating chemoresistance in ovarian cancer (18). This result confirms that palladium drugs can induce apoptosis by lowering the expression of the STAT3 gene, although the role of apoptotic genes and other genes will be investigated further.

Glycoprotein 1 (also known as multidrug resistance protein 1 (MDR1), ATP-binding cassette subfamily member B 1 (ABCB1), or cluster of differentiation 243 (CD243) is an important cell membrane protein that pumps many foreign substances out of cells. It is an ATP-dependent efflux pump with broad specificity. It is found in animals, fungi, and bacteria and most likely evolved as a defensive mechanism against potentially dangerous compounds.

In a subsequent clinical sample examination, Oyama et. al. found that CHD4 mRNA expression was considerably higher in platinum-resistant samples, implying that CHD4 overexpression provides platinum resistance to ovarian cancer cells, resulting in poor patient survival (19).

CHD4 deletion boosted cisplatin-mediated apoptosis in TOV21G ovarian cancer cells and improved cisplatin sensitivity in several ovarian cancer cell subtypes, consistent with these findings. CHD4 favorably influences the expression of the multidrug transporter MDR1 and its encoding protein p-glycoprotein, according to knockdown and expression studies.

Furthermore, according to the findings of Loh et. al., a lack of appropriate transfer of platinum-based medications can be the primary mechanism of drug resistance. On the other hand, drug transport proteins allow drugs to enter the cell. Of course, this comparison with the current findings may be inconclusive, and further research is needed to fully understand this issue (20).

The CD99 antigen (cluster of differentiation 99), also known as MIC2 or single-chain glycoprotein type 1, is a membrane protein that is strongly O-glycosylated and encoded by the CD99 gene in humans. This gene, which is expressed in all leukocytes but is most prevalent in thymocytes, is thought to promote T-cell adhesion (21). CD99 was shown to be substantially expressed in cisplatin-resistant ovarian cancer cells and tissues but not in cisplatin-sensitive ovarian cancer cells and tissues (22). Furthermore, CD99 overexpression results in cisplatin resistance. CD99 deletion, on the other hand, made ovarian cancer more sensitive to cisplatin. Previous studies have shown that CD99 is crucial for cancer growth, migration, and invasion. Manara MC et al., for example, demonstrated that CD99 promotes Ewing sarcoma metastasis via IGF-1R/RAS/Rac1 signaling (23). According to Cerisano et al., CD99 causes caspase-independent cell death and cell adhesion in Ewing sarcoma cells via actin and zyxin (24,25).

Octamer-binding transcription factor 4 (OCT-4) is a POU (Pit-Oct-Unc) family transcription factor encoded by the POU5F1

gene. Expression of this gene causes phenotypic alterations in stem cell differentiation during mammalian embryo development. This gene plays an important role in defining the destiny of internal mass cells and embryonic stem cells, as well as in maintaining pluripotency during embryonic development. It has recently been discovered that OCT-4 not only maintains pluripotency in embryonic cells, but also has the potential to regulate cancer cell proliferation and may be present in adult germ cells in cancers such as pancreatic, lung, liver, and testicular germ cell tumors (26). Wang et al. also proposed that Octamer 4 (Oct4) messenger RNA levels were drastically raised in chemotherapy-resistant cancer cells due to Oct4 DNA demethylation regulation. Oct4 overexpression increased, whereas Oct4 knockdown decreased hepatocellular carcinoma cell resistance to chemotherapeutic treatments in vitro and in xenograft tumors. It has been discovered that the Oct4-TCL1-AKT pathway promotes cell proliferation in embryonic stem cells and cancer stem cells by suppressing apoptosis. This group showed that Oct4 overexpression stimulates TCL1, AKT, and ABCG2 to drive chemoresistance, which can be reversed by a PI3K/AKT inhibitor. As a result, a direct Oct4-TCL1-AKT-ABCG2 pathway or a combination of Oct4-TCL1-AKT with the AKT-ABCG2 pathway could represent a new possible mechanism involved in liver cancer cell chemoresistance. As a result, it may be concluded that a decrease in the expression of this gene can lead to apoptosis, which is supported by other studies, and this function of OCT4 contradicts the current study (27). Drug resistance in cancer cells is mediated by several intracellular processes. MRP1 and MDR1 have been identified as multidrug resistance-associated proteins that cause DDP resistance via distinct pathways in various carcinomas (28).

MDR1 and MRPs have been shown to contribute to DDP resistance, with silencing MDR1 and overexpression of MRPs being sufficient to reverse DDP resistance (29). STAT3, a nuclear transcription factor, has also been linked to MDR1 and MRPs activation in several malignancies. STAT3 inhibitors can regulate MDR1 and MRP1, and STAT3 may bind to bp -504 to -398 upstream of MRP1's TSS to affect its transcription (30). According to Fang's findings, the expression of MRP1 and MDR1 was higher in A549/DDP cells than in A549 cells, indicating that MRP1 and MDR1 are two critical determinants of DDP resistance. A549/DDP cells also have significantly higher levels of phosphorylated STAT3 than A549 cells (30).

The molecular mechanism findings further revealed that Oct4/Nanog targets the Stat3 pathway in HCC and promotes EMT. Co-expression of Oct4 and Nanog increased the expression of stem cell markers CD133, ALDH1, and BMi-1. Furthermore, exogenous Oct4 and Nanog expression greatly increased the expression of ABCG2 and MDR1, which are two key ABC transporters linked to drug resistance. STAT3 activation was found to play a mechanistic role in Oct4/Nanog-induced EMT and cell invasion in HCC. Furthermore, Oct4 has been found in embryonic stem cells to be required for its anti-apoptotic actions in response to stress, and these effects may be mediated through the activation of the STAT3 pathway. In fact, previous research has examined the biological functions of Oct4, Nanog, MDR1, and STAT3 (31).

MTT assay and flow cytometry were performed to assess the cytotoxicity of a new water-soluble Pd (II) complex, PBPD, on CDDP-sensitive and CDDP-resistant ovarian cancer cell lines to present a novel anticancer medication capable of overcoming cisplatin resistance. The results showed that resistant cells had higher IC50 values than the sensitive cells. Furthermore, PBPD has a more deadly effect on sensitive cells than on resistant cells, and the cell survival rate is reduced over time. Flow cytometry investigations revealed that PBPD had strong anticancer effects in both CDDP-sensitive and CDDP-resistant ovarian cancers, potentially triggering apoptosis. Furthermore, the expression of resistance genes in resistant cells treated with PBPD fell dramatically when compared to cisplatin, demonstrating that PBPD can lower the

expression of drug-resistant genes in resistant cells when compared to cisplatin. Therefore, PBPD may be a promising anticancer agent for CDDP-resistant ovarian cancers. Other elements, however, are implicated in the biology of CDDP resistance in ovarian cancer, which requires further investigation.

Acknowledgment
The authors gratefully acknowledge support from the Materials and Energy Research Center (MERC), Karaj, Iran, and Kharazmi University, Tehran, Iran, through a research grant (No. 781400002).

Funding
The funding was supported by Materials and Energy Research Center (MERC) and Kharazmi University.

Competing interests
Authors have declared no conflict of interest.

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