Chemotherapeutic Agents Increase PD-L1 Expression in A549 Lung Cancer Cell Line

Haniye Ajdary
Tabriz University of Medical Sciences
Sahar Safaei
Tabriz University of Medical Sciences
Shahryar Hashemzadeh
Tabriz University of Medical Sciences Faculty of Medicine
Tohid Kazemi
Tabriz University of Medical Sciences
Dariush Shanehbandi
Tabriz University of Medical Sciences
Amir Baghbanzadeh
Tabriz University of Medical Sciences
Darya Javadrashid
Tabriz University of Medical Sciences
Mohammad Amini
Tabriz University of Medical Sciences
Behzad Baradaran (baradaranb@tbzmed.ac.ir)
Tabriz University of Medical Sciences https://orcid.org/0000-0002-8642-6795

Research Article

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Abstract

Introduction:

With the highest mortality rate, lung cancer is one of the most prevalent malignancies worldwide. Increasing PD-L1 expression is one of the mechanisms of ineffective anti-tumor response and immune evasion, which causes poor prognosis and survival rate. Chemotherapy has its own limitations such as drug resistance and the escape of cancer cells from the immune system. The current research aimed to assess the effects of common chemotherapy agents used in lung cancer on PD-L1 expression in tumor cells in order to help for selecting appropriate treatment regimens to increase patient survival.

Methods

First, A549 cells were cultured, and the expression of PD-L1 on this cell line was measured by qRT-PCR. Then, the viability of cancer cells was examined by MTT test. PD-L1 gene expression was measured by qRT-PCR in A549 cell line, after treatment with chemotherapy agents.

Results

PD-L1 gene expression was increased after 24 and 48 hours after treatment with cisplatin, docetaxel, paclitaxel and carboplatin. The IC50 of the mentioned drugs 24 hours after treatment were evaluated 27, 38.79, 18.66, and 54.17 µg/ml respectively.

Conclusion

The current study shows that PD-L1 expression increases in response to carboplatin, docetaxel, cisplatin, and paclitaxel in A549 cell line. This indicates that chemotherapy agents cause immune evasion by increasing PD-L1 expression on cancer cells, which makes these cells resistant to the immune system. The results also showed that carboplatin, docetaxel, cisplatin, and paclitaxel at 48 hours after treatment had a greater effect on increasing PD-L1 expression than 24 hours after treatment.

1. Introduction

One of the most common cancers worldwide is cancer of lung, with the highest mortality among cancers [1]. In 2019, 228,150 cases of lung cancer and 142,670 cancer-related diseases were reported in the United States of America, being responsible for about 24% of all cancer deaths in the country [2]. Histologically, lung cancer is categorized into two key groups, small cell lung cancer cell and non-small cell lung cancer. Non-small cell lung cancer (NSCLC) accounts for nearly 85% of all lung cancer cases [3]. Smoking is a main risk factor for this cancer which includes about 80–90% of patients suffering from lung cancer. Nonsmoker individuals are 20 times less probable to develop lung cancer than those who

continue to smoke [4]. In addition, exposure to arsenic, asbestos, chromium, nickel, and radiation exposure comprising radon in households and mines, alcohol consumption, exposure to air pollution, chronic inflammation due to infection or other chronic disease also increase the risk of lung cancer [5, 6].

The treatment is chosen based on the histological and molecular type and stage of cancer. Surgery is currently the only viable and successful treatment option. Surgical resection is the preferred method for T1b and larger tumors [7]. However, 70% of patients bearing lung cancer have local invasion or metastasis at the time of diagnosis, who do not have the obligatory condition for surgery. Other treatment methods include chemotherapy alongside radiation for some locally advanced cancers, palliative chemotherapy and palliative radiation, and target therapy combined with chemotherapy for metastatic disease [7, 8]. Platinum-based chemotherapy is extensively acknowledged as the standard therapeutic strategy. Common chemotherapy regimens are: cisplatin and paclitaxel, cisplatin and gemcitabine, cisplatin plus docetaxel and carboplatin plus paclitaxel [5, 9, 10].

The average survival of patients bearing metastatic untreated cancer is only 4 to 5 months, and the one-year survival rate is only 10%. Cancer patients' overall survival has not enhanced significantly despite significant advances in cancer treatment using conventional techniques like chemotherapy, radiotherapy, and surgery over the past decade. The 5-year survival of patients' post-treatment remains only 17.4% [11, 12]. Among these methods, chemotherapy is widely used to hinder the cancer cells' growth of and apoptosis induction in them. Although chemotherapy does not directly kill immune cells due to cytotoxicity, it inhibits the immune response against tumor. So, resistance of cancerous cells to the immune system and immune evasion are major limitations of chemotherapy. During the last few years, as a complementary strategy, immunotherapy can be useful in eradicating cancer cells in combination with traditional chemotherapy [13, 14].

Immune checkpoints are regulators of the immune system that keep tissues from harm when the immune system responds to a pathogenic infection. These molecules are essential to maintain balance and autoimmunity prevention [15]. The expression of immune checkpoints proteins by tumor cells is an essential mechanism of tumor resistance to the immune system, which causes the immune system to escape and an insufficient anti-tumor response[16]. PD-L1 (Programmed Death Ligand 1), as a participant of the CD28 family and a vital immune checkpoint receptor, is expressed on the surface of active T, B, and NK cells and has an important role in tumor escape from the immune system. Besides PD-L1, the major PD-1 ligand is expressed in various tumors, including NSCLC, breast, gastric, colorectal, papillary thyroid, and testicular cancers. PD-L1 which is expressed on tumor cells, binds to PD-1 which is expressed on T cells and inhibits a cytotoxic T-cell response and T-cell–induced anti-tumor activity [17–19]. According to numerous studies, high expression of PD-L1 in lung cancer promotes metastasis and invasion and weakens the prognosis of these patients [11, 20, 21]. Thus, blocking the PD-L1 and PD-1 pathways via PD-1 / PD-L1 blockers results in sustained anti-tumor responses and improves prognosis in many cancers, including lung cancer [17, 19, 22].

The effects of chemotherapy in lung cancer patients on immune response are not yet fully understood. Also, due to the evidence of the influence of amplified the expression of PD-L1 on the lung cancer prognosis and its resistance against the immune system and inhibition of T cells, there is a need to investigate the interactions of common therapies and PD-L1 expression.

Since chemotherapy is still utilized as the initial line of therapy for lung cancer, a deeper comprehension of the impact of chemotherapy agents on immune response against the tumor, especially in immune evasion, is important in improving the effectiveness of immunotherapy and chemotherapy combined. In the current research, particularly, the effects of common chemotherapy drugs used in lung cancer on expression of PD-L1 in tumor cells were investigated in order to help select appropriate treatment regimens to optimize treatment, increase patient survival, observe more improvement, and a better prognosis for lung cancer.

2. Methods

2.1. Cell culture

First, A549, the lung cancer cell line, was purchased from Pasteur Institute Cell Bank then cultured in RPMI 1640 medium enriched with 10% Fetal Bovine Serum (FBS, Gibco, USA) and was preserved in humid incubator containing 5% CO2 with temperature of 37 ° C.

2.2. MTT assay

A certain amount of suspension containing 15× 10^3 cells, A549, were distributed into per well of 96-well plate and incubated at 37 ° C, 95% humidity, and 5% carbon dioxide for 24 hours to allow cells to attach to the bottom of the plate. Cells were then treated with different concentrations of drugs including paclitaxel, docetaxel, cisplatin, and carboplatin. MTT assay was performed 24 and 48 hours after incubation of cells with drugs.

For this purpose, the supernatant was removed, and then 50 µL of MTT solution (2 mg/ml MTT in PBS) with 100 µl of complete culture medium were added to each well, and the plate incubated for 4 hours. After incubation, the solution was removed, and 200 µl of dimethyl sulfoxide (DMSO) was added to each well, and after incubation for 30 minutes, the ELISA reader measured the optical density of cells at 570.620 nm.

2.3. RNA extraction and qRT-PCR

Total RNA was extracted using RiboEx reagent (Geneall, Korea) according to the manufacturer's instructions. The expression of all genes was assessed using SYBR Premix Ex Taq II (TAKARA, Japan) and the Applied Biosystems StepOnePlus™ Real-Time PCR System (Life Technologies, Carlsbad, USA). GAPDH was used as an internal control to normalize the expression of the candidate gene using $2^{\triangle\triangle Ct}$ formula. All primers were blasted using the Primer Blast section of the NCBI website before experiments (http://www.nchi.nlm.nih.gov) (Table 1).

Table 1
shows the sequence of forward and reverse primers. The sequence of the primers is as follows:

Gene	Forward/Reverse	Sequence
GAPDH	FW	CAAGATCATCACCAATGCCT
	RV	CCCATCACGCCACAGTTTCC
PD-L1	FW	TGCCGACTACAAGCGAATTACTG
	RV	CTGCTTGTCCAGATGACTTCGG

2.4. Statistical analysis

For statistical analysis of the results obtained from the experiments, Graph pad prism 6 was used. To define the variable changes amongst control and drug-transfected groups, one-way ANOVA analyses were done. P value < 0.05 was considered statistically significant.

3. Results

3.1. Determination of IC50 of chemotherapy agents

First, the dose-dependent cytotoxic effect of these drugs on cancer cells was measured using MTT assay. Figures 1 to 4 demonstrate the effective doses of each drug on the A549 cell line. The obtained data were interpreted using GraphPad prism software, and the IC50 of the drugs was calculated according to the table below (Table 2).

Table 2
IC50 of the mentioned drugs.

drug	IC50(24)	IC50(48)
CARBOPLATIN	54.17	13.20
CISPLATIN	27	22.5
PACLITAXEL	18.66	8.3
DOCETAXEL	38.79	7.3

3.2. The PD-L1 gene expression was increased after treatment with chemotherapy agents

After IC50 measurement, we assessed the effects of chemotherapeutic agents on PD-L1 gene expression in A549 lung cancer cell line. The cells were treated by the obtained doses of chemotherapeutic agents. The expression of PD-L1 gene was evaluated by qRT-PCR method and analyzed by PRISM software. The T-Test was used for standard distribution, and the Mann-Whitney test was used for data with the

abnormal distribution. To compare the gene expression before and after the drug treatment, the primary amount was considered the control value and the amount of 1, and then the other values were measured according to it. The PD-L1 gene expression in response to Carboplatin, Docetaxel, cisplatin, and Paclitaxel are shown in Fig. 5 in A549 cell line.

Discussions

Despite significant advances in cancer treatment using conventional methods over the past decade, there has been no significant improvement in patients' overall survival [11]. Recently, with the introduction of new molecular and immunological therapies such as immunotherapy and targeted therapy, the effectiveness of treatment has improved significantly [23]. As a complementary approach, immunotherapy can be accommodating in eradicating cancer cells in combination with chemotherapy [24]. As chemotherapy is still the first line of treatment, it is important to have an understanding of how chemotherapy agents affect the patient's immune system. Although chemotherapy does not directly cause apoptosis of immune cells due to cell toxicity, it inhibits the tumor-reactive immune responses. Thus immune escape and immunoresistance are the main restrictions of chemotherapy. However, the exact mechanism of cancer immunoresistance has not yet been fully elucidated [25].

Previous studies suggested that amplified expression of PD-L1 may encourage lung cancer metastasis and invasion, which leads to the dismal prognosis of patients suffering from lung cancer. [26] Recently, the effect of different chemo-therapeutic mediators on expression of PD-L1 has been widely investigated in many cancers. However, there are conflicting results among these studies. This paper specifically studied the effects of common chemotherapy agents used in lung cancer on PD-L1 expression. Our study results showed that the level of PD-L1 gene expression was increased in response to carboplatin, docetaxel, cisplatin, and paclitaxel in A549 cell line. It indicates that chemotherapy drugs increased PD-L1 level of cancer cells, making these cells resistant to the immune system. Our results also showed that these agents have a greater effect on increasing PD-L1 expression in 48 hours than 24 hours after treatment, which may help determine the correct time of starting immunotherapy agents.

Among many studies that have assessed the influence of chemotherapeutic agents on expression of PD-L1 in lung cancer, most of them have similar results with our study, but there are also contradictory results among them.

Sakai et al. showed that the rate of PD-L1 TPS (tumor proportion score) in post-chemotherapy biopsies was increased compared to pre-chemotherapy biopsies in a study with 17 NSCLC patients [27].

In another study, Fonaki and colleagues studied the association between chemotherapy agents and expression of PD-L1 levels in NSCLC. Chemotherapy with carboplatin or cisplatin-based diets increased TGF-β expression and thus increased the EMT process and the progression of cancer. This study was conducted on A549 cell line and clinical samples taken from patients treated with these drugs and underwent surgery afterwards. PD-L1 expression and EMT status were measured by RT-PCR and

immunohistochemistry. This study demonstrated that chemotherapy increased TGF-β and increased EMT, and PD-L1 expression [28].

In a retrospective study, Jungon Shin et al. assessed 86 NSCLC patients who had undergone therapeutic resection after at least one cycle of platinum-based NACT (neoadjuvant chemotherapy) and measured PD-L1 TPS using immunohistochemistry in pre and post-NACT biopsy specimens. The comparison presented a significant upsurge in PD-L1 expression after NACT with platinum-based agents [29].

Yang Wang and colleagues assessed the alteration in PD-L1 expression in non-metastatic NSCLC patients. The whole blood from 38 patients was collected before, during, and after radiation or chemoradiation. Immunofluorescence and qPCR were used to assess PD-L1 expression in CTCs through the course of treatment. Among these patients, in 7 out of 8 were treated with paclitaxel and carboplatin, expression of PD-L1 was increased after treatment [30].

Rivia Rojocco measured the expression of PD-L1 in bronchoscopic biopsy specimens of different varieties of lung cancer. Expression of PD-L1 was reduced in 24.4% of patients and augmented in 7.32% of the patients post chemotherapy. Decreases in PD-L1 expression were detected only in patients receiving a combination of cisplatin-gemcitabine, whereas a similar change was not observed in the carboplatin-paclitaxel receiving group. As a result, the deviations in expression of PD-L1 was not statistically significant [31].

Fadi Nasr et al. in a case report research, evaluated the expression of PD-L1 in biopsies taken from a patient with lung squamous cell carcinoma pre- and post-treatment with carboplatin and gemcitabine. PD-L1 expression was decreased from 60% in the pre-treatment biopsy sample to 5% in the post-treatment sample [32].

The reason for the contradictory changes in PD-L1 in these studies is possibly due to dissimilarities in patients' features, the number of patients participating, the type of chemotherapy agents, and PD-L1 evaluation timing. As mentioned, NSCLC mutations affect PD-L1 expression changes. Therefore, it is noteworthy to determine the different mutations in the samples taken from patients, and the difference in these mutations can cause a difference in PD-L1expression.

Conclusions

Similar to other cancers, drug resistance is a challenge in the way of NSCLC treatment. The factors causing resistance and immune evasion of cancer cells could vary in different ways. In the current research, PD-L1 expression in A549 cell line was measured after treatment with carboplatin, docetaxel, cisplatin, and paclitaxel chemotherapeutic agents. The PD-L1 expression was augmented in response to these treatments, which pointed out the effect of these drugs on immune evasion through this upregulation in PD-L1 expression. So, it is maybe better to use chemotherapy with immune check inhibitors for further investigations.

Declarations

Funding

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Conflicts of interest

The authors declare that there is no conflict of interest.

Availability of data and material

The data that support the findings of this study are available on request from the corresponding author.

Code availability

Not Applicable.

Authors' contributions: HA and SS contribute equally to the Data collection, performing the analysis and writing the paper, SH, TK, and DS are the Advisor professors of this project, AB, DJ, and MA also contributed to data collection and analysis tools and read and edited the paper, BB is the corresponding professor who conceived and designed the analysis, supervised all the steps of original work and read and edited the paper.

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Figures

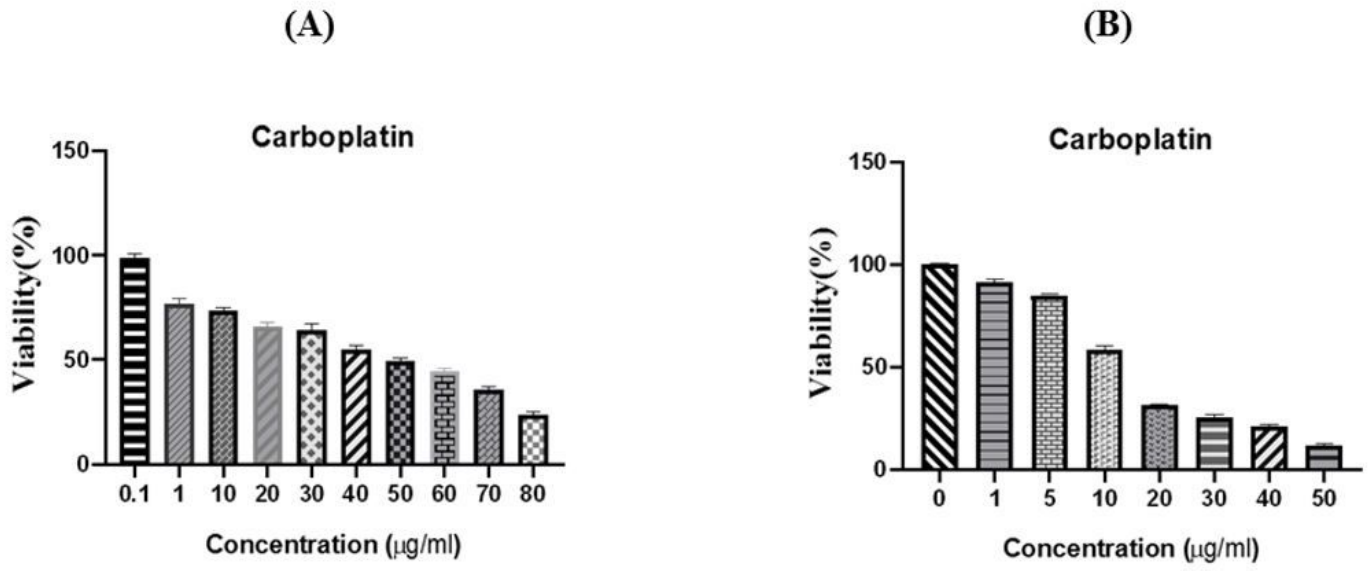

Figure 1

A) The effect of different carboplatin doses on the viability of A549 cells and its IC50 after 24 hours. B) The effect of different carboplatin doses on the viability of A549 cells and its IC50 after 48 hours.

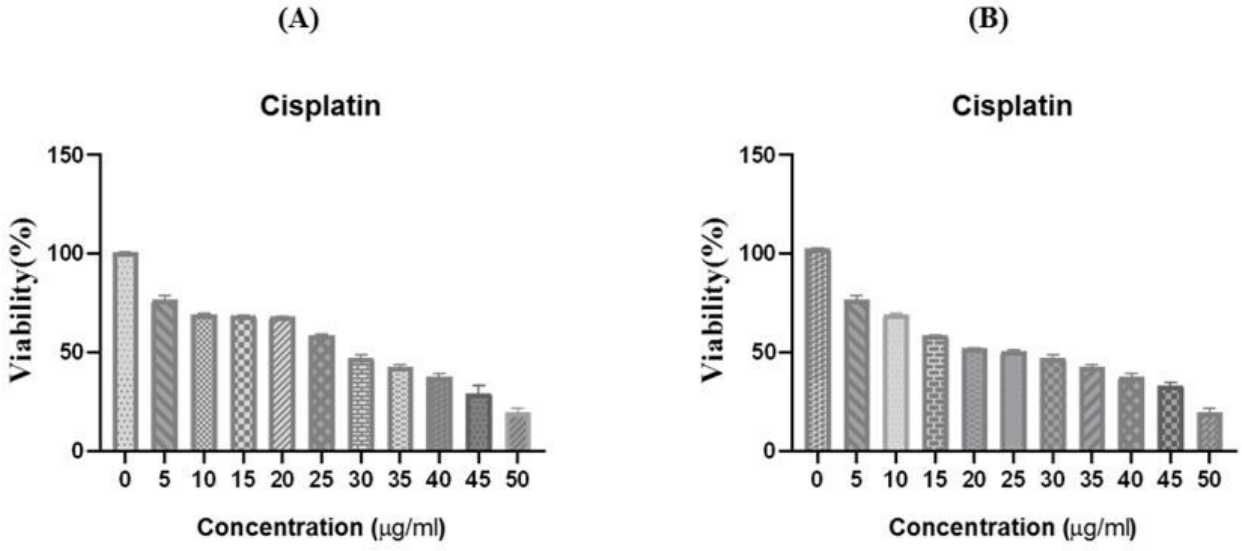

Figure 2

A) The effect of different doses of cisplatin on the viability of A549 cells and its IC50 after 24 hours. B) The effect of different doses of cisplatin on the viability of A549 cells and its IC50 after 48 hours.

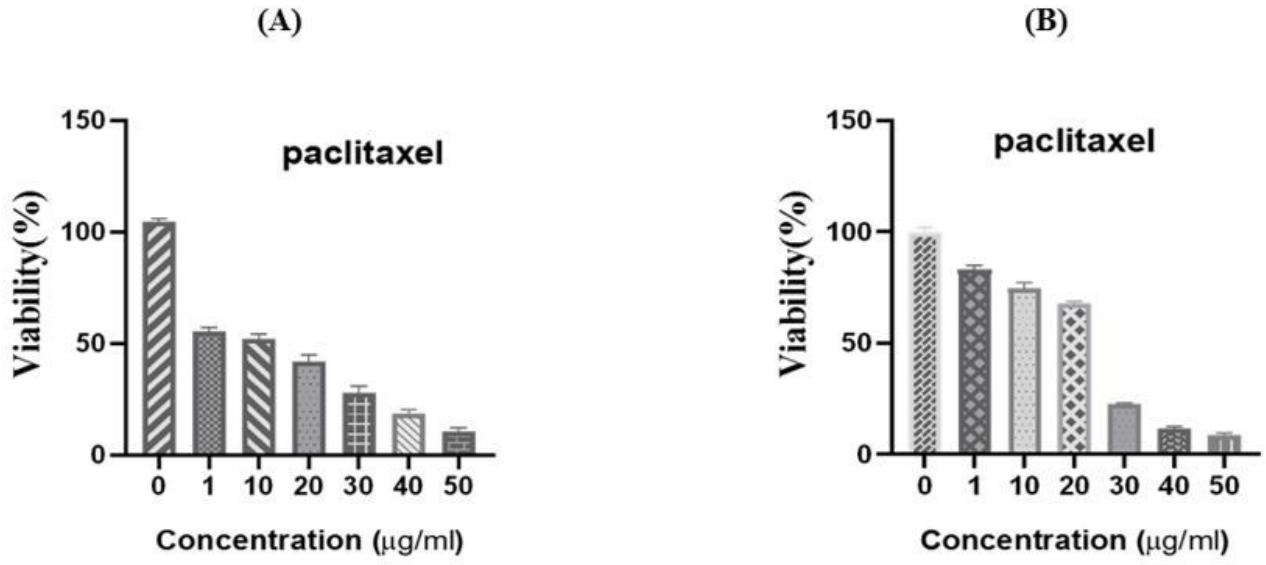

Figure 3

A) The effect of different doses of paclitaxel on the viability of A549 cells and its IC50 after 24 hours. B) The effect of different doses of paclitaxel on the viability of A549 cells and its IC50 after 48 hours.

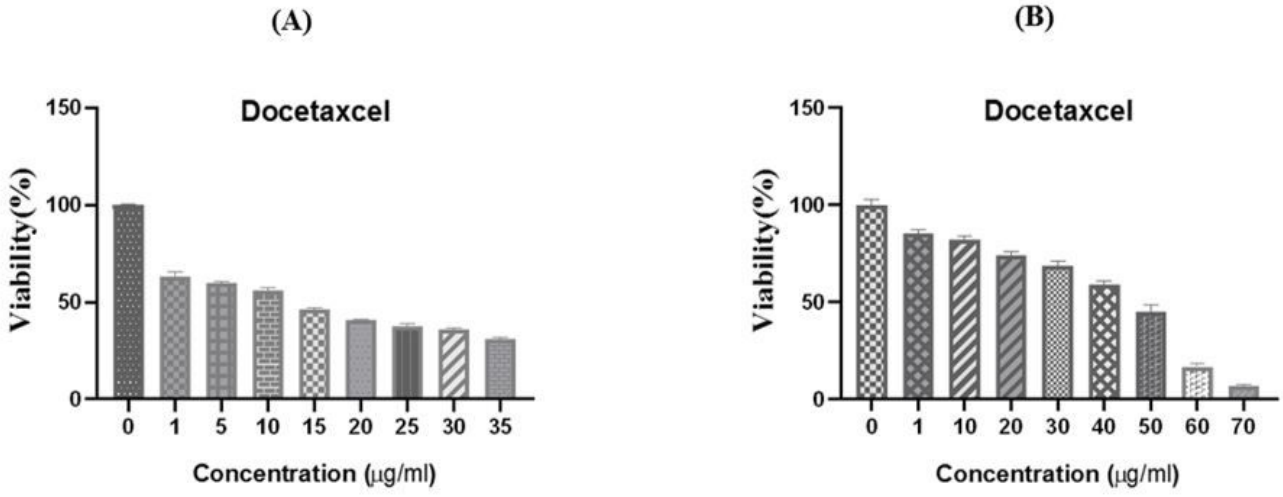

Figure 4

A) The effect of different doses of docetaxel on the viability of A549 cells and its IC50 after 24 hours. B) The effect of different doses of docetaxel on the viability of A549 cell line and its IC50 after 48 hours.

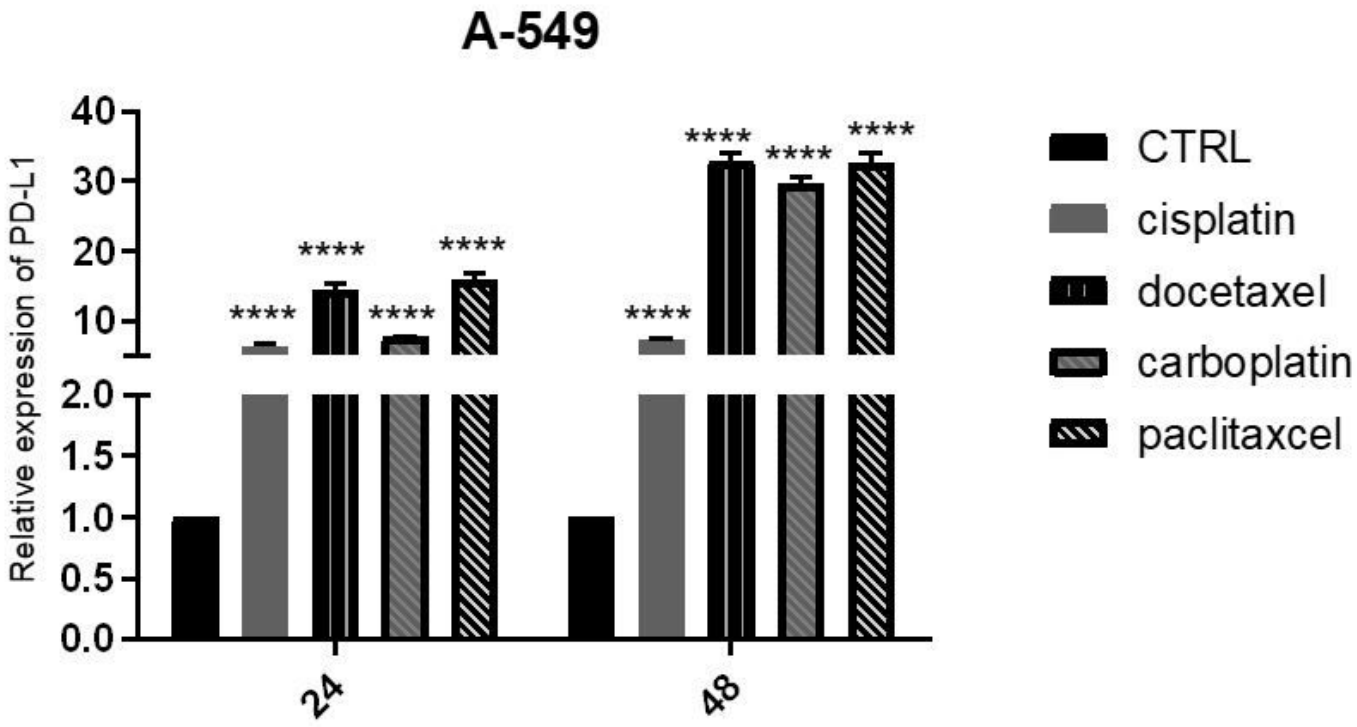

Figure 5

Considering GAPDH as the control, the expression of PD-L1 increased after 24 and 48 hours of treatment with Carboplatin, Docetaxel, cisplatin, and Paclitaxel. Statistical analysis shows that these changes were statistically significant ($p < 0.0001$). These drugs have a greater effect on increasing PD-L1 expression in 48 hours than 24 hours after treatment.