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Modulation of PD-L1 Expression by Cisplatin and Gemcitabine in Bladder Cancer Cells

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Abstract

Bladder cancer (BC) incidence is rising globally, despite available treatment options, including tumor resection, systemic chemotherapy, and immune checkpoint inhibitors targeting PD-1/PD-L1. Gemcitabine and cisplatin are commonly combined as first-line systemic therapy, yet their effects on *PD-L1* expression remain unclear. This study evaluated the effects of cisplatin and gemcitabine, alone and combined, on *PD-L1* expression in a BC cell line, RT-4, and a normal epithelial cell line, ARPE-19. Cells were treated with IC20 doses of each drug, and *PD-L1* expression was quantified by qRT-PCR after 48 hours. In ARPE-19 cells, cisplatin treatment induced a dose-dependent *PD-L1* increase, peaking at a 59.6-fold elevation at higher doses ($p < 0.001$), while gemcitabine alone showed no effect. The combination of low-dose cisplatin and gemcitabine further elevated *PD-L1* expression (25.2-fold, $p < 0.05$). In contrast, in RT-4 cells, low-dose cisplatin downregulated *PD-L1*, but higher doses resulted in a 61.3-fold increase ($p < 0.05$). Gemcitabine alone downregulated *PD-L1* in RT-4 cells; however, the combined treatment produced a 99.4-fold upregulation ($p < 0.001$). These findings highlight dose- and cell type-specific effects of cisplatin and gemcitabine on *PD-L1* regulation, with combination therapy notably upregulating *PD-L1* in BC cells. This underscores the potential for integrating chemotherapy with immune checkpoint inhibitors to counteract chemotherapy-induced immune evasion in BC. Differential responses between normal and cancer cells emphasize the need for tumor-specific therapeutic approaches and further research on PD-L1 regulation to optimize immune checkpoint therapy.

Keywords: Bladder cancer, PD-L1, cisplatin, gemcitabine, combination therapy

1. Introduction

Bladder cancer (BC) is among the most prevalent cancers of the urinary tract, and its incidence is increasing worldwide [1]. Chemotherapy is a commonly used treatment strategy aimed at inhibiting the proliferation of cancer cells and inducing apoptosis [2]. In anticancer therapy, the combination of gemcitabine and cisplatin (GC) chemotherapeutic drugs is used as the first-line [3,4].

In recent years, inhibition of the PD-1/PD-L1 pathway is a critical point in cancer treatment. This pathway is considered a resistance mechanism against cancer immunity, and cancers that show high levels of *PD-L1* expression are important candidates for immunotherapies [5]. PD-L1 is one of the immune checkpoint proteins targeted as a predictive biomarker for the effectiveness of immune checkpoint inhibitors (ICI), suggesting that tumors with high levels of PD-L1 expression are more prone to respond favorably to immunotherapies [6]. Inhibiting PD-L1 is expected to enhance anti-cancer immunity; however, limited information is available regarding the predictive factors influencing its efficacy [7].

BC represents a group of malignancies characterized by a high incidence of metastasis and unfavorable prognosis. The role of PD-L1 on cancer cells stands out as a mechanism that impedes the cancer immunity cycle. Recent clinical trial findings have shown promising results for the treatment of muscle-invasive BC and other malignancies, including advanced biliary tract cancer [8,9]. The effects of combined cisplatin and gemcitabine therapies on the modulation of *PD-L1* gene expression remain unclear.

The primary aim of this study is to examine the impact of gemcitabine and cisplatin treatment, both individually and in combination, on *PD-L1* expression in the RT4, BC cell line and ARPE-19, normal epithelial cell line, to elucidate the impact of combined gemcitabine and cisplatin treatment on *PD-L1* expression and its potential implications for modulating the tumor immune response. By investigating these effects, we aim to enhance the understanding of the molecular impact of combinatory therapeutic approaches for BC, highlighting the pivotal role of PD-L1 as an immunotherapeutic target.

2. Materials and Methods

2.1.1. Cell Culture and Application of the Drugs

The RT-4, a human bladder cancer cell line derived from transitional cell carcinoma of the bladder, and the ARPE-19, a human normal retinal pigment epithelial cell line, were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in a 5% CO_2 incubator. Upon reaching 80% confluence, the cells were detached using Trypsin/EDTA and then centrifuged to collect the cell pellets. Cell viability was assessed using trypan blue staining. The cells were seeded into the wells of 6-well plates at a density of $3x10^5$ cells per well and allowed to attach overnight. The cells were then treated with IC20 concentrations of cisplatin, gemcitabine, or their combination.

The concentrations required to achieve a 20% reduction in the viability (IC20) of RT-4 cells were identified as 42 nM for gemcitabine and 2.13 µM for cisplatin at 48h in a prior study conducted in our laboratory [10]. These values represent the doses at which the compounds induce a modest yet measurable decrease in bladder cancer cell growth. These concentrations of the drugs were applied in the current study to examine their impact on *PD-L1* gene expression.

2.1.2. RNA Extraction

The media was removed from the cells treated with the drugs and their combination following 48 hours of incubation. Then, the cells were washed three times with cold PBS. The cells were lysed in the reagent to extract RNA using ONE STEP-RNA Reagent (Cat# BS410A, Bio-Basic, Ontario, Canada) according to the respective company's protocol. Chloroform was then added before being centrifuged to separate the solution into the organic phase and the aqueous phase containing RNA. The aqueous phase was carefully separated, and RNA was precipitated by the addition of isopropyl alcohol, followed by centrifugation to collect the RNA into a pellet. The pellet was then washed with 75% ethanol, centrifuged, air-dried, and subsequently re-dissolved in RNase-free water for future applications.

2.1.3. cDNA Conversion and Real-Time qRT-PCR

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. qRT-PCR was conducted in Roche LightCycler 480 II, employing the LightCycler 480 Probes Master (Roche, Mannheim, Germany). The study utilized TaqMan *PD-L1* (Hs01125301_m1, Thermo Fisher) and the housekeeping gene *GAPDH* for hydrolysis probes. The *GAPDH* primers and YAK probe were sourced from TIB MOLBIOL Syntheselabor (Berlin, Germany). *GAPDH* expression level was used as an internal reference to normalize the mRNA expression of target gene, and the relative expression level of the gene was determined using the $2^{-\Delta\Delta Ct}$ method [11]. For each experimental condition, total RNA was isolated and used to perform three independent qRT-PCR experiments. Each qRT-PCR experiment was carried out in triplicate wells to ensure technical reproducibility. Data presented represent the average of three independent technical replicates.

2.1.4. Statistical analysis

Statistical analysis was performed using the 'QuickCalcs t-test Calculator' from GraphPad Software, available at: https://www.graphpad.com/quickcalcs/ttest1.cfm, with a p-value < 0.05 considered statistically significant. An unpaired, two-tailed t-test was used to assess statistical significance between groups. Gene expression level was determined using the ΔCt method, normalized to the *GAPDH* reference gene. Subsequently, the ΔΔCt method was employed to compare expression levels relative to the ARPE-19 DMSO control group. Statistical comparisons were made between each treatment group and their respective DMSO controls. Results were shown as the mean ± standard deviation (SD) obtained from three independent experiments.

3. Results and Discussion

3.1. Cisplatin-Induced Upregulation and Enhanced Effects with Gemcitabine on *PD-L1* Expression in ARPE-19 Cells

The effect of cisplatin and gemcitabine, both individually and in combination, on *PD-L1* gene expression was evaluated in ARPE-19 and RT-4 cell lines. Cisplatin treatment in ARPE-19 cells led to a dose-dependent increase in *PD-L1* expression. Exposure to a low concentration of cisplatin (2.13 µM) resulted in an 18.7-fold increase in *PD-L1* expression level compared to the untreated cell group ($p < 0.05$) (Figure 1.a). When the cisplatin concentration was increased to 7.5 µM, a dramatic upregulation was observed, with *PD-L1* expression increasing to 59.6-fold above the baseline ($p < 0.001$). This substantial increase showed that higher

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doses of cisplatin can significantly stimulate *PD-L1* gene expression in normal epithelial cells.

Gemcitabine alone did not significantly alter the expression level of *PD-L1* in ARPE-19 cells, as indicated by a fold change of 1.5 for the lower dose (42 nM) and 5.7 for the higher dose (165 nM), both of which were statistically non-significant ($p > 0.05$). This indicates that gemcitabine alone may not be sufficient to induce notable changes in *PD-L1* expression in non-cancerous epithelial cells.

However, when gemcitabine (42 nM) was combined with low-dose cisplatin (2.13 µM), a notable synergistic effect was observed, resulting in an increase by 25.2-fold in *PD-L1* expression level ($p < 0.05$). This combination treatment significantly augmented *PD-L1* expression compared to gemcitabine or cisplatin alone, indicating that the concurrent administration of these agents can enhance immune checkpoint-related gene expression, potentially influencing immune evasion mechanisms. The literature supports that the JAK/STAT signaling pathway, primarily activated by cytokines such as IFN-γ and IL-6, plays a central role in regulating *PD-L1* expression in tumor cells and is modulated by various tumor-associated genes, highlighting its importance in immune evasion mechanisms across multiple cancer types, including bladder cancer [12]. In addition, NF-κB plays a critical role in regulating *PD-L1* expression in cancer cells, either directly by binding to specific sequences in the PD-L1 promoter or indirectly through inflammation-related pathways, thereby promoting immune evasion and tumor progression [13]. DNA damage response mechanisms, including ATM/ATR/Chk1-mediated checkpoint signaling and the cGAS/STING pathway, regulate *PD-L1* expression in cancer cells through both direct transcriptional activation (e.g., via STAT1/IRF1) and indirect inflammatory signaling, with the extent of *PD-L1* upregulation being influenced by the type and phase of DNA repair, checkpoint fidelity, micronuclei formation, and cytosolic DNA sensing, ultimately linking genomic instability to immune evasion and highlighting the therapeutic potential of combining DNA-damaging agents with immune checkpoint inhibitors [14].

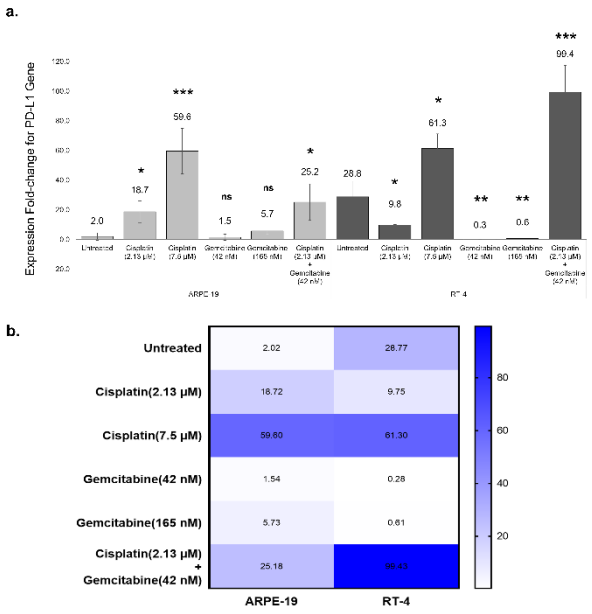

Figure 1. Effects of Cisplatin and Gemcitabine on PD-L1 Gene Expression in ARPE-19 and RT-4 Cells. (a) Bar graphs represent the fold-change in *PD-L1* expression in ARPE-19 and RT-4 cells after 48 hours of treatment with cisplatin concentrations (2.13 µM and 7.5 µM), gemcitabine (42 nM and 165 nM), and their combination (2.13 µM cisplatin + 42 nM gemcitabine). Untreated cells served as the control for each cell line. (b) Heatmap depicting the fold-change in *PD-L1* expression across various treatment conditions for ARPE-19 and RT-4 cells. The color gradient represents the magnitude of expression changes, with darker shades indicating higher levels of upregulation. The data highlight distinct patterns of *PD-L1* modulation between the two cell lines, particularly under combination treatment conditions. Data are presented as the mean ± SD from three qRT-PCR experiments performed using the RNA sample from the same experiment. Each qRT-PCR run was conducted in triplicate wells. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ ns: non-significant.

3.2. Combination Treatment with Cisplatin and Gemcitabine Leads to Significant Modulation of *PD-L1* Expression in RT-4 BC Cells

In RT-4 cells, the basal expression level of *PD-L1* was higher compared to untreated ARPE-19 cells, with a fold change of 28.8. Cisplatin treatment at 2.13 µM led to a downregulation of *PD-L1* expression to 9.8-fold ($p < 0.05$), hence, a suppressive effect of low-dose cisplatin on the expression level of *PD-L1* in BC cells was observed. Conversely, higher cisplatin concentration (7.5 µM) significantly increased *PD-L1* expression to 61.3-fold ($p < 0.05$), indicating a dose-dependent biphasic response to cisplatin, where lower concentrations may reduce expression while higher doses can markedly enhance it.

Treatment with gemcitabine at both tested concentrations (42 nM and 165 nM) resulted in a significant downregulation of the expression of *PD-L1*, with by 0.3 and 0.6-fold changes, respectively ($p < 0.01$). This reduction implies that gemcitabine may successfully inhibit RT-4 cell *PD-L1* expression level, which could reduce the capacity of tumor cells to elude immune surveillance.

Interestingly, the combination of low-dose cisplatin (2.13 µM) and gemcitabine (42 nM) resulted in a substantial increase in the expression of *PD-L1* to 99.4-fold ($p < 0.001$). This upregulation in the gemcitabine and cisplatin combination treatment group was significantly higher than the response observed with either agent alone, indicating an additive effect that may increase immune checkpoint expression. This finding is of particular interest as it suggests that the GC might activate compensatory immune evasion mechanisms in BC cells by upregulating *PD-L1*, which could have implications for the design of combination therapies involving ICI.

3.3. Comparison Between BC and Normal Epithelial Cells

Figure 1.b presents a heatmap of *PD-L1* expression fold-changes across all treatment groups for both cell lines. The heatmap clearly shows distinct patterns of gene regulation between ARPE-19 and RT-4 cells. Comparative analysis of the two cell lines revealed distinct patterns of *PD-L1* regulation in chemotherapy response. While both cell lines exhibited dose-dependent increases in the expression level of *PD-L1* with high-dose cisplatin, the combination of low-dose cisplatin with gemcitabine induced a stronger upregulation in RT-4 cells than in ARPE-19 cells. Additionally, gemcitabine alone had an inhibitory effect on *PD-L1* in RT-4 cells but not in ARPE-19 cells. These differential responses highlight the cell line-specific regulation of immune checkpoint molecules in response to chemotherapy, emphasizing the need to consider tumor type and treatment context when designing therapeutic strategies targeting PD-L1.

The data show that chemotherapy can modulate the expression of *PD-L1* in both normal epithelial and cancer cells, with combination treatments potentially exerting synergistic effects on gene regulation. These findings support the rationale for further investigating chemotherapy-induced *PD-L1* upregulation and its implications for the efficacy of ICI in cancer therapy.

Currently, the treatment methods recommended in the guidelines for BC include systemic chemotherapy and radiation therapy [15]. In particular, GC is applied in antitumor therapy as the first treatment option [3,4]. PD-1 and PD-L1 antibodies have recently been employed into the treatment of advanced or metastatic BC treatment [16,17]. In comparison to other urological cancers, such as prostate and kidney cancer, BC patients have fewer treatment options, and more research is needed to understand the development of drug resistance and develop new treatment strategies [18].

The present study demonstrates that cisplatin and gemcitabine, either individually or in combination, can significantly modulate *PD-L1* gene expression in both ARPE-19 and RT-4, indicating that chemotherapy influences immune checkpoint regulation. The findings of the present study suggest that the complex interaction between dose and cell type may be important for treatment strategies to increase the efficacy of ICI. Cisplatin is a platinum-based chemotherapeutic agent that inhibits DNA replication by binding to DNA [19]. The anticancer efficacy of cisplatin is not limited to the induction of apoptosis through DNA cross-linking and mitosis inhibition; it is also significantly enhanced by the immunomodulatory effects of cisplatin. These immunological effects play a crucial role in combating tumors, thereby increasing the therapeutic effectiveness [20]. A significant increase was observed at higher concentrations (7.5 µM). Higher doses of cisplatin may promote *PD-L1* gene expression in non-cancerous epithelial cells, as seen by the dose-dependent elevation of the expression of *PD-L1* in ARPE-19 cells following cisplatin treatment. In contrast, a biphasic response was observed in RT-4 cells, where low-dose cisplatin (2.13 µM) resulted in downregulation, while high-dose cisplatin (7.5 µM) significantly upregulated *PD-L1* expression. This biphasic effect highlights the differential responses between normal and cancerous cells, possibly reflecting variations in the underlying molecular mechanisms of *PD-L1* regulation. Our findings suggest that high-dose cisplatin may increase *PD-L1* expression in both cell types and promote immune escape in a dose-dependent manner, which may affect the efficacy of immune checkpoint therapies. Furthermore, cisplatin has been previously reported to increase *PD-L1* expression in hepatoma and breast cancer cells [21,22].

Gemcitabine alone had contrasting effects on the two cell lines. Gemcitabine did not significantly alter the expression of *PD-L1* in ARPE-19 cells, while it significantly downregulated *PD-L1* in RT-4 cells at both tested concentrations (42 nM and 165 nM). Gemcitabine may have a tumor-specific inhibitory effect on immune checkpoint expression, which could potentially reduce the ability of BC cells to escape immune surveillance. The ability of gemcitabine to decrease *PD-L1* levels in cancer cells could be advantageous in reducing immune resistance, which might enhance the response to immunotherapy. However, the lack of significant change in *PD-L1* expression in ARPE-19 cells indicates that gemcitabine may not broadly affect immune checkpoint regulation in normal epithelial cells, suggesting a more targeted effect on tumor cells. Compared to gemcitabine alone, the GC provides a significant survival benefit and

exhibits synergistic or additive effects without significantly increasing toxicity [23-25]. The combination of gemcitabine with cisplatin and carboplatin, in the treatment of solid tumors, including breast, ovarian, and non-small cell lung cancer [26-28].

Chemotherapy has the potential to create synergistic effects with immunotherapies that enhance tumor cell immunogenicity [29,30]. The combination of low-dose cisplatin (2.13 µM) and gemcitabine (42 nM) resulted in an additive effect on the upregulation of the expression of *PD-L1*, particularly in RT-4 cells, where a 99.4-fold increase was observed. This substantial increase shows that the combination treatment might cause cancer cells to engage in compensatory processes, which would boost their immune escape potential. The observed synergy between cisplatin and gemcitabine could be attributed to multiple factors, including increased cellular stress and DNA damage, which may trigger upregulation of immune checkpoints as a defense mechanism against the cytotoxic effects of chemotherapy. These results align with earlier research indicating that the expression of *PD-L1* may be elevated by chemotherapeutic drugs as a stress response. It is currently known that the expression of *PD-L1* in cancer cells contributes to immune system resistance [31,32]. Increased expression of *PD-L1* is associated with poor clinical prognosis in renal and gastric carcinomas [33,34], and breast cancer [35].

Gemcitabine and cisplatin are cytotoxic agents that can cause cellular damage and could increase tumor antigen presentation [36-39]. Additionally, many patients with advanced urothelial cancer experience recurrence after receiving cisplatin-based first-line therapy [40].

The findings of this study have significant implications for the design of combination therapies involving chemotherapy and ICI. The significant PD-L1 upregulation observed in the GC group may enhance the efficacy of anti-PD-1/PD-L1 therapies. However, the enhanced expression of *PD-L1* could also lead to increased immune evasion, necessitating the concurrent use of immune checkpoint blockade to counteract this effect. The differential response between normal epithelial and cancer cells also highlights the importance of considering the tumor microenvironment and cellular context when developing therapeutic strategies. A recent phase II, investigator-initiated, multicentre study (HCRN GU 16–257) reported that clinical complete response after gemcitabine, cisplatin, plus nivolumab resulted in bladder preservation [8].

Cisplatin upregulates *PD-L1* in ARPE-19 and has a biphasic effect in RT-4. Gemcitabine downregulates *PD-L1* in RT-4 only (Figure 2). Combined low doses of both drugs lead to a significant *PD-L1* increase in RT-4 cells. Moreover, our findings emphasize the need for personalized approaches in cancer treatment. Given the distinct patterns of *PD-L1* regulation observed in ARPE-19 and RT-4 cells, the type and specific characteristics of tumor type may need to be considered when designing chemotherapy regimens. This could involve tailoring the doses of cisplatin and gemcitabine to modulate *PD-L1* expression in a manner that enhances antitumor immunity while minimizing immune escape.

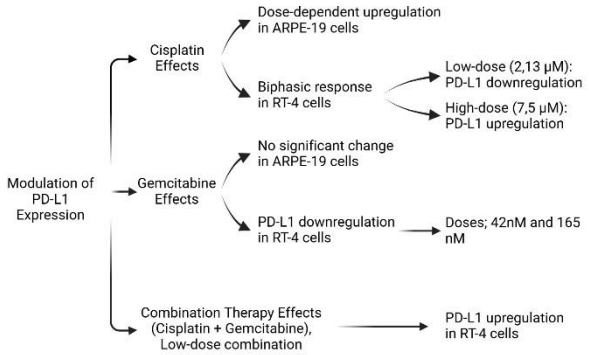

Figure 2. Modulation of the Expression of PD-L1 in ARPE-19 and RT-4 Cells Following Cisplatin, Gemcitabine, and Combination Treatments. This figure was created in BioRender. Nalkıran, İ. (2024) https://BioRender.com/x93v056

This study was conducted using an in vitro model, which, while controlled and reproducible, does not fully replicate the complexity of the tumor microenvironment or immune interactions present in vivo. Specifically, the observed modulation of *PD-L1* expression in response to cisplatin and gemcitabine may differ under the influence of cytokines, stromal components, and immune cell signaling that are absent in our culture system. To strengthen translational relevance, future studies should validate these findings in xenograft or syngeneic bladder cancer models and investigate whether chemotherapy-induced *PD-L1* upregulation correlates with response to immune checkpoint blockade in clinical contexts. Additionally, dissecting the contribution of key signaling pathways such as JAK/STAT, ERK, and DNA damage response mechanisms, using targeted inhibitors may help clarify the molecular basis of *PD-L1* regulation following chemotherapy.

This study supports the rationale for combining chemotherapy with ICI in cancer therapy. By understanding how chemotherapy modulates immune checkpoints, more effective treatment strategies can be developed, potentially leading to improved clinical outcomes for patients with BC and other malignancies.

4. Conclusion

This study demonstrates that cisplatin and gemcitabine modulate *PD-L1* expression in bladder cancer cells, with cisplatin upregulating *PD-L1* in both ARPE-19 and RT-4 cells in a dose-dependent manner, and gemcitabine downregulating *PD-L1* in RT-4 cells. The combination of both drugs significantly increased *PD-L1* expression

in RT-4 cells, suggesting a potential impact on immune evasion. While these effects are currently being tested in clinical trials for cancer therapy, this study also highlights that similar impacts on *PD-L1* regulation were observed in normal cells, emphasizing the complexity the effect of chemotherapy on immune checkpoints. These findings support the rationale for combining chemotherapy with ICI to enhance treatment efficacy. Future research should explore the underlying mechanisms of these effects and validate the results in clinical settings to optimize cancer treatments while considering the impact on both cancerous and normal cells.

Author's Contributions

İhsan Nalkıran: Drafted and wrote the manuscript, performed the experiments, and conducted the data analysis.

Hatice Sevim Nalkıran: Assisted with the interpretation of results and contributed to manuscript preparation.

Ethics

There are no ethical issues after the publication of this manuscript.

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