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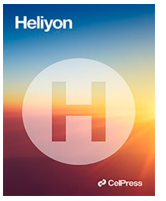

Research article

Cyclin dependent kinase 9 inhibition reduced programmed death-ligand 1 expression and improved treatment efficacy in hepatocellular carcinoma

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ABSTRACT

The anti-programmed death-ligand 1 (PD-L1) antibody is a standard therapy for advanced hepatocellular carcinoma (HCC). Tumor expression of PD-L1 can be induced upon stimulus. Because cyclin-dependent kinase 9 (CDK9) inhibition reduces the expression of inducible proteins, we explored the influence of CDK9 inhibition on PD-L1 expression in HCC cells. We found that PD-L1 expression was low in HCC cells; however, IFN-γ treatment increased this expression. CDK9 inhibitors AZD4573 and atuveciclib reduced the IFN-γ induced PD-L1 expression in a dose-dependent manner. CDK9 knockdown yielded similar results, but CDK9 overexpression reversed the influence of the CDK9 inhibitors. In the orthotopic mouse model, mice treated with a CDK9 inhibitor and an anti-PD-L1 antibody had significantly smaller tumors and exhibited longer survival than mice treated with either agent. In conclusion, CDK9 inhibition could reduce the expression of PD-L1 in HCC cells. Using both CDK9 inhibitors and anti-PD-L1 antibodies is more effective than using either agent alone.

1. Introduction

Programmed cell death protein-1 (PD-1) blockade is a standard treatment for advanced hepatocellular carcinoma (HCC) [1,2]. The survival benefits of using single-agent PD-1 blockade as first-line therapy are noninferior to those of using sorafenib, a multikinase inhibitor [3,4]. When combined with either antiangiogenic therapy or anti-cytotoxic T lymphocyte antigen-4 antibody, PD-1 blockade provides greater survival benefits than sorafenib provides [4–6]. However, not all patients benefit from these aforementioned treatments. Even patients with initial tumor response may develop resistance. The prognosis after the failure of first-line PD-1 blockade

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therapy remains poor [7]. Novel therapies that can enhance the efficacy of PD-1 inhibitors or prevent the resistance to such agents are required.

Based on their functions, cyclin-dependent kinases (CDKs) are divided into cell cycle-dependent CDKs and cell cycle-independent CDKs. Cell cycle–dependent CDKs regulate the progression of cell cycle [8,9]. Inhibitors of cell cycle–dependent CDKs, such as CDKs 4 and 6, are currently in clinical use for breast cancer [10]. However, such inhibitors may not be suitable as HCC treatment because they frequently induce bone marrow suppression, which may exacerbate the preexisting cytopenia among patients with HCC.

By contrast, cell cycle–independent CDKs, such as CDK5 and CDK9, participate in diverse cellular activities, such as transcription, mRNA processing, and the differentiation of neuronal cells [8,9]. Specific inhibitors of such cell cycle–independent CDKs had not been successful until several specific inhibitors of CDK5 or CDK9 were discovered [11–15]. CDK9 is essential for the transcription function of RNA polymerase II (RNAPII) because of its phosphorylation of the C-terminal domain [16,17]. CDK9 inhibition may reduce the expression of many inducible proteins, such as the downstream molecule of c-Myc and several antiapoptotic proteins [18–20]. In our previous study, we discovered the potential of CDK9 inhibition in HCC treatment [20].

Cancer cells regulate the expression of programmed death-ligand 1 (PD-L1) to escape antitumor immunity [21,22], and PD-L1 expression can be induced in response to immune system activity [22,23]. Because CDK9 regulates various inducible proteins, we hypothesized that CDK9 inhibition would decrease PD-L1 expression in tumor cells upon cytokine stimulus and even improve the treatment efficacy of anti-PD-L1 antibodies. We thus planned this study to explore the influence of CDK9 inhibition on HCC PD-L1 expression and the efficacy of PD-1 blockade as HCC treatment.

2. Materials and methods

2.1. Cell lines

We purchased the human HCC cell line HuH7 (RRID: CVCL_0336) from the Japanese Collection of Research Bioresources Cell Bank and the mouse HCC cell line BNL 1 ME A.7R.1 (RRID: CVCL_6371) from American Type Culture Collection. The human HCC cell line Hep3B (RRID: CVCL_0326) was also used and is routinely maintained in our laboratory [24]. All cell lines were maintained in Dulbecco Modified Eagle Medium with 10 % fetal bovine serum, L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (25 ng/mL) at 37 °C in a humidified incubator with 5 % CO_2.

2.2. Compounds

Both human and mouse IFN-γ were purchased from PeproTech (Rehovot, Israel). AZD4573 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Atuveciclib and abemaciclib were acquired for free from Bayer (Leverkusen, Germany) and Eli Lilly (Indianapolis, IN, USA), respectively. Sorafenib and dinaciclib were purchased from LC Laboratories (Woburn, MA, USA), and Selleckchem (Houston, TX, USA), respectively.

2.3. Western blot analysis

We performed Western blot analyses in accordance with the standard protocol. Specific antibodies for CDK9 (RRID: AB_1523330) and mouse PD-L1 (RRID: AB_2773715) were purchased from Abcam (Cambridge, UK). Antibodies against total and phosphorylated signal transducer and activator of transcription 1 (STAT1, RRID: AB_397521, RRID: AB_399503) were purchased from Becton, Dickinson and Company (BD, Franklin Lakes, NJ, USA). Antibodies for phosphorylated RNAPII (RRID: AB_519341) and IRF-1 (RRID: AB_631838) were purchased from Bethyl Laboratories (Montgomery, TX, USA) and Santa Cruz Biotechnology (Dallas, TX, USA), respectively. We purchased the remaining antibodies for Western blotting, including anti-PD-L1 (an E1L3N clone, RRID: AB_2687655) from Cell Signaling Technology (Beverly, MA, USA).

2.4. Flow cytometry

We treated HCC cells with IFN-γ and CDK9 inhibitors at the indicated concentrations for 24 h and subsequently harvested and stained with PE Mouse Anti-Human PD-L1 antibody (RRID: AB_647198) or PE Mouse IgG1, κ Isotype Control (RRID: AB_396091) (BD). Cells staining was quantified using flow cytometry.

2.5. Quantitative reverse transcription PCR analysis

We performed quantitative reverse transcription PCR (RT-qPCR) using qPCRBIO SyGreen Blue Mix Hi-ROX (PCR Biosystems, London, UK) in accordance with the standard protocol. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin served as the internal control in the experiments involving the human and mouse cells, respectively. The primer sequences were 5′-TGCTGAACGCATTTACTGTC (forward) and 5′- ATCTGAAGTGCAGCATTTCC (reverse) for human PD-L1, 5′- AAGCGGCTGTACTG-CAAAAAC (forward) and 5′- TTGATGTGAGGGTCGCTCTTC (reverse) for human GAPDH, 5′- TCGCCTGCAGATAGTTCC (forward) and 5′- GAAGTTGCTGTGCTGAGG (reverse) for mouse PD-L1, and 5′- GTGTGATGGTGGGAATGG (forward) and 5′- GGTCTCAAA-CATGATCTGGG (reverse) for mouse β-actin.

2.6. Orthotopic tumor studies

We used BALB/c male mice aged approximately 5 weeks for experiments. After anesthesia, the liver was exposed by making a small incision on the upper abdomen. Subsequently, we injected 3×10^5 BNL 1 ME A.7R.1 cells into the subcapsular area of the liver. After 1 week, randomization was performed for the mice to receive a vehicle (DMSO and an immunoglobulin G control, RRID: AB_1107780), atuveciclib, an anti-PD-L1 antibody (10F.9G2, Bio X Cell, Lebanon, NH, USA, RRID: AB_10949073), or both atuveciclib and the anti-PD-L1 antibody for 2 weeks. Mouse weight were recorded 3 times per week.

For tumor sizes analysis, the mice were sacrificed 2 weeks after the treatment. The tumors were photographed and measured. In the survival analysis, the mice were not sacrificed unless they met the criteria of animal euthanasia, such as rapid or progressive weight loss, inability to obtain food or water, and moribund status.

The study was approved by National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee, approval number 20210286. The animal study protocols complied with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

2.7. Overexpression and knockdown of CDK9

Vectors overexpressing CDK9 were purchased from OriGene (Rockville, MD, USA). We performed transfection with Maestrofectin transfection reagent (OmicsBio, New Taipei City, Taiwan) after seeding 2×10^5 HuH7 cells in 6-well plates. The cells were allowed to incubate for 48 h afterwards.

An siRNA (siRNA#1) for CDK9 was purchased from Dharmacon (Cambridge, UK). Another siRNA for CDK9 (siRNA#2) and nontarget siRNAs were purchased from Thermo Fisher Scientific (Waltham, MA, USA). We used DharmaFECT 4 Transfection Reagent (Thermo Fisher) to transfect 2×10^5 HuH7 cells with the indicated siRNA (100 nM) in 6-well plates and allowed the cells to incubate

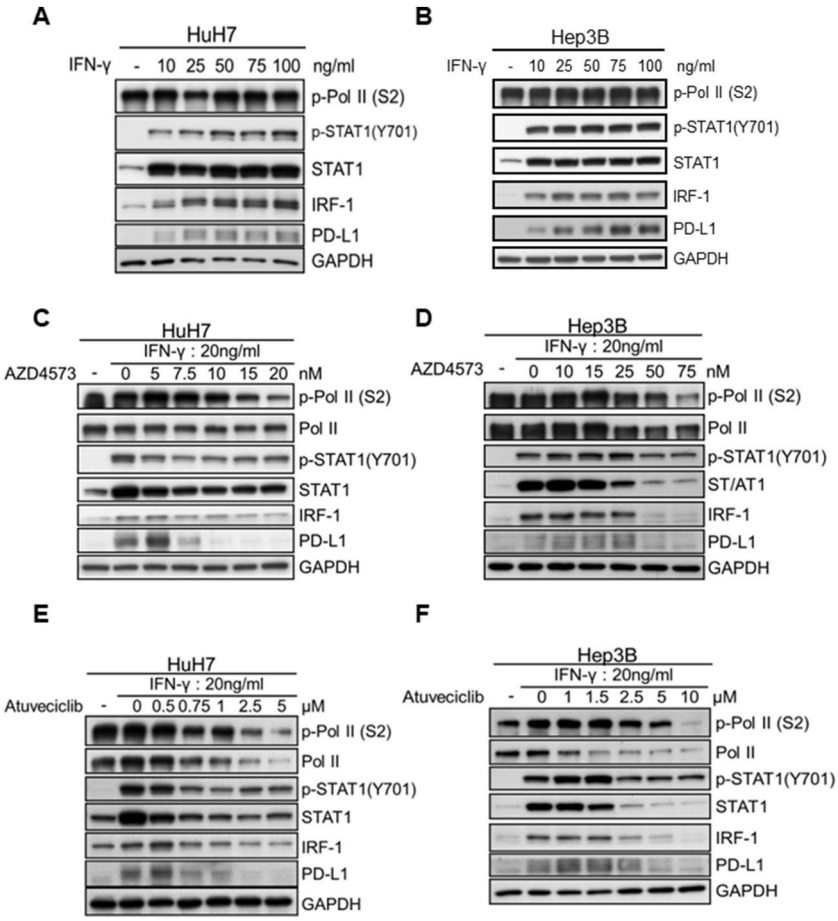

Fig. 1. Western blotting demonstrating PD-L1 expression in HCC cells after treatment with IFN-γ and CDK9 inhibitors. (A–B) Approximately 16 h after seeding, HuH7 (A) and Hep3B (B) cells were incubated with IFN-γ at the indicated concentrations for 24 h. (C–D) Approximately 16 h after seeding, AZD4573 at the indicated concentrations was added to the HuH7 (C) and Hep3B (D) cells together with 20 ng/mL IFN-γ. The cells were harvested after 24 h. (E–F) Experiments similar to those in Fig. 1C and D were performed but atuveciclib was used.

for 48 h.

2.8. Statistical analysis

We used SAS (9.4, SAS Institute, Cary, NC, USA) for statistical analyses and considered a 2-sided p value of <0.05 statistically significant. We used an independent t-test to compare continuous variables, such as expression levels and tumor weights, between the groups. Mouse survival was calculated from the day of treatment initiation and estimated using the Kaplan-Meier method. We used the log-rank test for trend to compare group survival.

3. Results

3.1. CDK9 inhibitors reduced HCC PD-L1 expression

We examined multiple HCC cell lines, and most of them exhibited low PD-L1 expression without stimulus, similar to what was found in most clinical tumor samples from the patients with HCC (Fig. S1). Among them, interferon-γ (IFN-γ) increased PD-L1 expression in HuH7 and Hep3B cells (Fig. 1A and B). We added the specific CDK9 inhibitors AZD4573 and atuveciclib along with IFN-γ. In the HuH7 cells, both AZD4573 and atuveciclib reduced IFN-γ induced PD-L1 expression in a dose-dependent manner (Fig. 1C and E). The downstream signal of IFN-γ, interferon regulatory factor 1 (IRF-1), was reduced by these CDK9 inhibitors; moreover, the phosphorylation of RNAPII, a CDK9 target, was reduced. Similar results were observed in the Hep3B cells (Fig. 1D and F). By contrast, sorafenib, a multikinase inhibitor frequently used in HCC treatment, and abemaciclib, a CDK4/6 inhibitor, failed to influence the IFN-γ induced PD-L1 expression in the HuH7 cells (Figs. S2A and S2B). Dinaciclib, a composite inhibitor of CDKs 1, 2, 5, and 9, reduced PD-

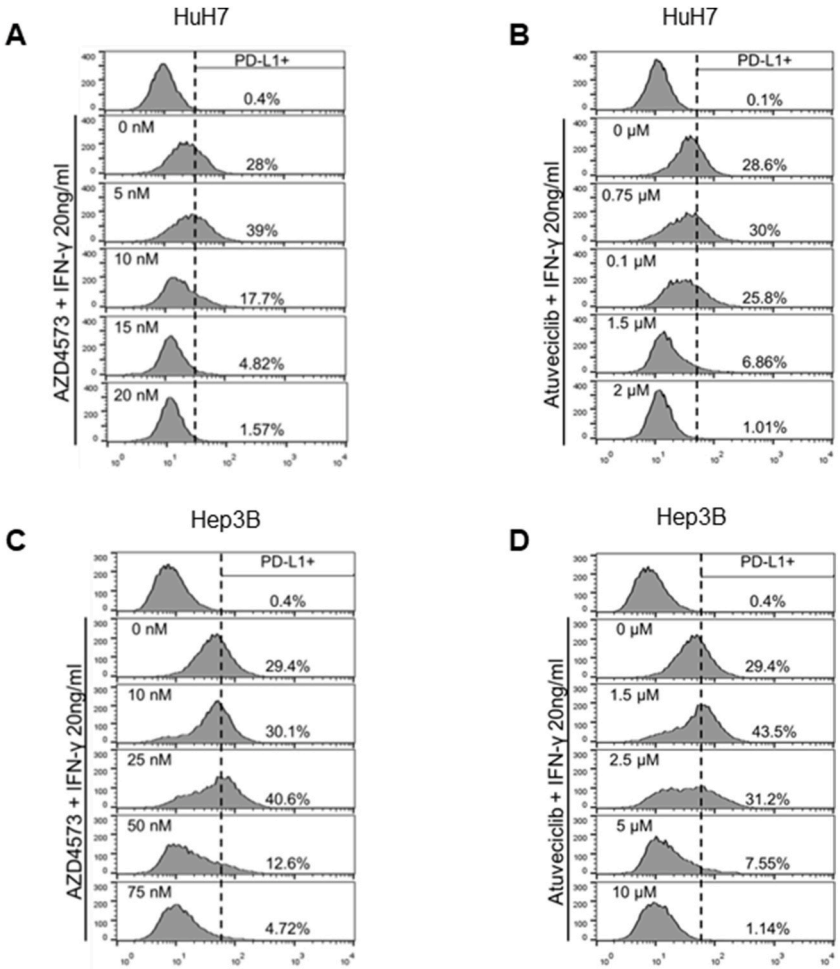

Fig. 2. (A–D) Flow cytometry results revealing membranous PD-L1 expression after CDK9 inhibitor treatment. HuH7 cells were treated with AZD4573 (A) or atuveciclib (B) at the indicated concentration together with 20 ng/mL IFN-γ. After 24 h, the cells were harvested and stained with anti-PD-L1 antibodies or isotype controls. (C–D) Experiments similar to Fig. 2A and B were performed for the Hep3B cells.

L1 expression (Fig. S2C).

3.2. CDK9 inhibitors also decreased PD-L1 membrane and mRNA expression

Flow cytometry revealed that membranous PD-L1 was decreased after AZD4573 and atuveciclib treatment in both the HuH7 (Fig. 2A and B) and Hep3B (Fig. 2C and D) cells. Moreover, the mRNA expression of *CD274* (the gene encoding PD-L1) was significantly reduced after AZD4573 and atuveciclib treatment in both the HuH7 (Fig. 3A and B) and Hep3B (Fig. 3C and D) cells.

In the mouse HCC cell line BNL 1 ME A.7R.1 (BNL), AZD4573 and atuveciclib also reduced IFN-γ induced PD-L1 expression, at both the protein (Fig. 4A and B) and mRNA levels (Fig. 4C and D).

3.3. Influence of CDK9 knockdown and overexpression on HCC PD-L1 expression

CDK9 knockdown mediated by siRNAs reduced the IFN-γ induced PD-L1 expression in the HuH7 (Fig. 5A) and Hep3B (Fig. 5B) cells, a result similar to those obtained by both specific CDK9 inhibitors. Moreover, this knockdown reduced RNAPII phosphorylation and IRF-1 expression. By contrast, the reduction of PD-L1 expression due to the CDK9 inhibitors was reversed by CDK9 overexpress ion (Fig. 5C and D, and Fig. S3).

3.4. Combination of CDK9 inhibitors and immunotherapy

In the orthotopic mouse model, liver tumors were significantly smaller in the mice treated with atuveciclib in combination with an anti-PD-L1 antibody than in the mice treated with either agent alone (Fig. 6A and B). The mice tolerated the treatment well without obvious weight differences among the treatment groups (Fig. 6C). In survival analysis, the mice treated with atuveciclib in combination with an anti-PD-L1 antibody exhibited the longest survival compared with the mice treated with either agent alone (Fig. 6D; $p = 0.037$). A mouse treated with the combination survived more than 270 days.

4. Discussion

In this study, CDK9 inhibition reduced IFN-γ induced PD-L1 expression in multiple HCC cell lines. Two specific CDK9 inhibitors, atuveciclib and AZD4573, and siRNA-mediated CDK9 knockdown had similar effects on PD-L1 expression. To exclude the possible off-target effects of small molecule inhibitors, we confirmed the results through genetic manipulation of CDK9 expression. In our animal models, the combination of CDK9 inhibitors and anti-PD-L1 antibodies demonstrated superior treatment efficacy to that of either treatment alone.

CDK9 influences RNAPII function by phosphorylating its C-terminal domain. Therefore, CDK9 inhibition may reduce the

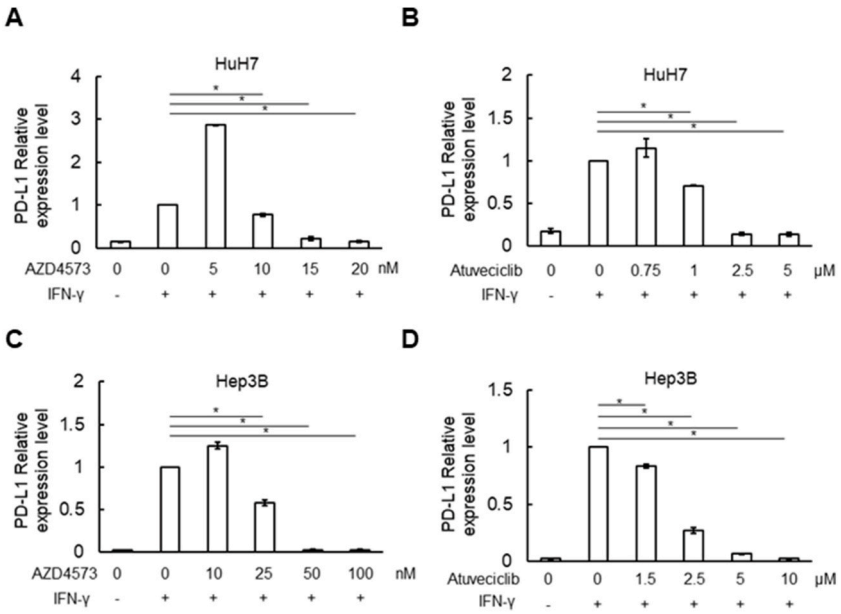

Fig. 3. (A–D) Expression of *CD274* mRNA after CDK9 inhibitor treatment. HuH7 cells were treated with AZD4573 (A) or atuveciclib (B) at the indicated concentration, together with 20 ng/mL IFN-γ. Hep3B cells were treated with AZD4573 (C) or atuveciclib (D) at the indicated concentration, together with 20 ng/mL IFN-γ. The cells were harvested after 24 h. *CD274* mRNA expression was analyzed using RT-qPCR with GAPDH as the internal control. Expression is reported as fold changes relative to cells treated with the vehicle (*denotes $p < 0.05$).

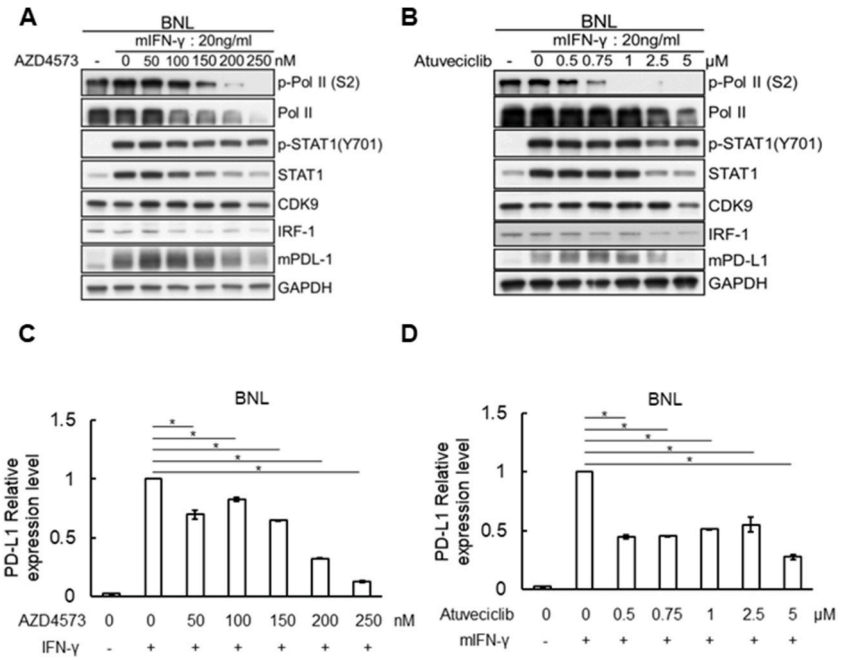

Fig. 4. (A–B) Western blotting demonstrating mouse PD-L1 expression after CDK9 inhibitor treatment. AZD4573 (A) or atuveciclib (B) at the indicated concentration were added to mouse BNL cells after seeding, together with 20 ng/mL mouse IFN-γ. The cells were harvested after 24 h. (C–D) Mouse *CD274* mRNA expression after CDK9 inhibitor treatment. BNL cells treated with AZD4573 (C) or atuveciclib (D) at the indicated concentration, together with 20 ng/mL mouse IFN-γ. The cells were harvested after 24 h. *CD274* mRNA expression was analyzed using RT-qPCR with β-actin as the internal control. Expression is reported as fold changes relative to cells treated with the vehicle (*denotes $p < 0.05$).

transcription of many inducible proteins, such as survivin, Bim_{EL}, Mcl-1, and X-linked inhibitor of apoptosis protein [11,12,20]. In this study, CDK9 inhibition reduced PD-L1 expression at the mRNA level. Such a decrease led to a reduction in PD-L1 expression, particularly that of membranous PD-L1. This effect is crucial because tumors require membranous PD-L1 to bind to the PD-1 receptors of immune cells. Because cancer cells frequently express PD-L1 to counteract antitumor immunity, prevention of such a response may improve the efficacy of immunotherapy.

CDK inhibitors, such as dinaciclib, are typically poorly selective of cell cycle-dependent and cell cycle-independent CDKs [25,26]. Such nonselective inhibitors may have undesirable side effects, such as bone marrow suppression, due to the inhibition of cell cycle-dependent CDKs [27–29]. Specific CDK9 inhibitor, including atuveciclib and AZD4573, which were used in the current study, may exhibit superior safety profile [11–13]. A phase 1 clinical trial of VIP152, another specific CDK9 inhibitor, revealed high tolerability [14], which is a crucial characteristic for a compound to be used in combination therapy.

The findings of reducing PD-L1 expression through CDK9 inhibition increased the efficacy of anti-PDL1 antibodies might sound counterintuitive because some reports demonstrated that positive PD-L1 expression in tumor tissues was associated with a better objective response rate in patients who received PD-1 blockade therapy [30]. However, in phase 3 clinical trials comparing PD-1 blockade with sorafenib as first-line therapy for advanced HCC, PD-L1 expression was not associated with overall survival [4,31]. Such conflicting results implied that the baseline PD-L1 expression might be the most crucial factor for the effectiveness of PD-1 blockade. Instead, reducing PD-L1 expression through CDK9 inhibition may ensure the blockade of the PD-L1/PD-1 signaling even if the anti-PD1 or PD-L1 antibody alone failed to do so.

Combination therapy is key in the application of PD-1 blockade as first-line therapy for advanced HCC. Although PD-1 blockade is an effective treatment for HCC [32,33], the use of PD-1 inhibitors as a single agent does not provide superior survival benefits to those of antiangiogenic targeted therapy, such as sorafenib [3,4,31]. By contrast, the combination of antiangiogenic therapy or other immune checkpoint inhibitors with PD-1 inhibitors has demonstrated superior survival benefits to those of sorafenib [4–6,34]. Therefore, CDK9 inhibitors may constitute a promising candidate for such combination therapies. Because of the mechanism underlying CDK9 function, its inhibition may influence immune checkpoints other than PD-L1 and improve the efficacy of inhibitors of checkpoints other than PD-1. This topic warrants further investigation.

In conclusion, CDK9 inhibition could reduce the expression of PD-L1 in HCC cells. In addition, the combination of CDK9 inhibitors and anti-PD-L1 antibodies was more effective than either therapy alone.

5. Limitations of the study

1. Our *in vivo* experiments based on mouse models, which might not represent the human conditions adequately.

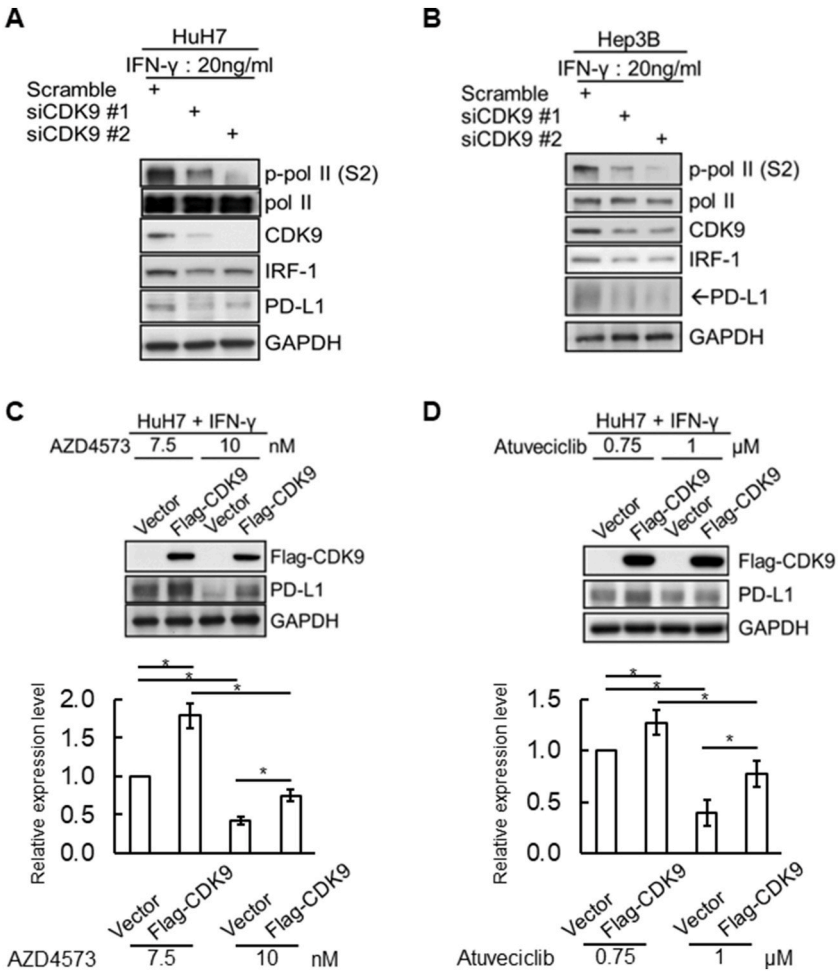

Fig. 5. (A–B) Western blotting for PD-L1 expression after CDK9 knockdown. Approximately 16 h after seeding, CDK9-specific siRNAs were added to HuH7 (A) and Hep3B (B) cells. After 48 h, 20 ng/mL IFN-γ was added, and the cells were incubated for another 24 h. (C–D) Western blotting demonstrating the influence of CDK9 inhibitors on PD-L1 expression in cells overexpressing CDK9. Empty vectors or vectors overexpressing CDK9 were added to the HuH7 cells after seeding. After 48 h, we treated the cells with AZD4573 (C) or atuveciclib (D) at the indicated concentration, together with 20 ng/mL IFN-γ. The cells were harvested after 24 h. PD-L1 protein expression was analyzed with GAPDH as the internal control. Expression is reported as fold changes relative to cells treated with the empty vector and a lower dose of CDK9 inhibitor (*denotes $p < 0.05$).

2. The orthotopic model we used in nude mice relied on injection of established cell lines into the mouse livers, which was different from the carcinogenesis process in patients with HCC.

Data availability

All data generated or analyzed during this study are included in this article and its supplementary material files. Further enquiries can be directed to the corresponding author.

CRediT authorship contribution statement

Yu-Yun Shao: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Min-Shu Hsieh:** Investigation. **Yi-Hsuan Lee:** Investigation. **Hung-Wei Hsu:** Investigation, Formal analysis. **Rita Robin Wo:** Investigation, Formal analysis. **Han-Yu Wang:** Investigation, Formal analysis. **Ann-Lii Cheng:** Writing – review & editing, Funding acquisition. **Chih-Hung Hsu:** Writing – review & editing, Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

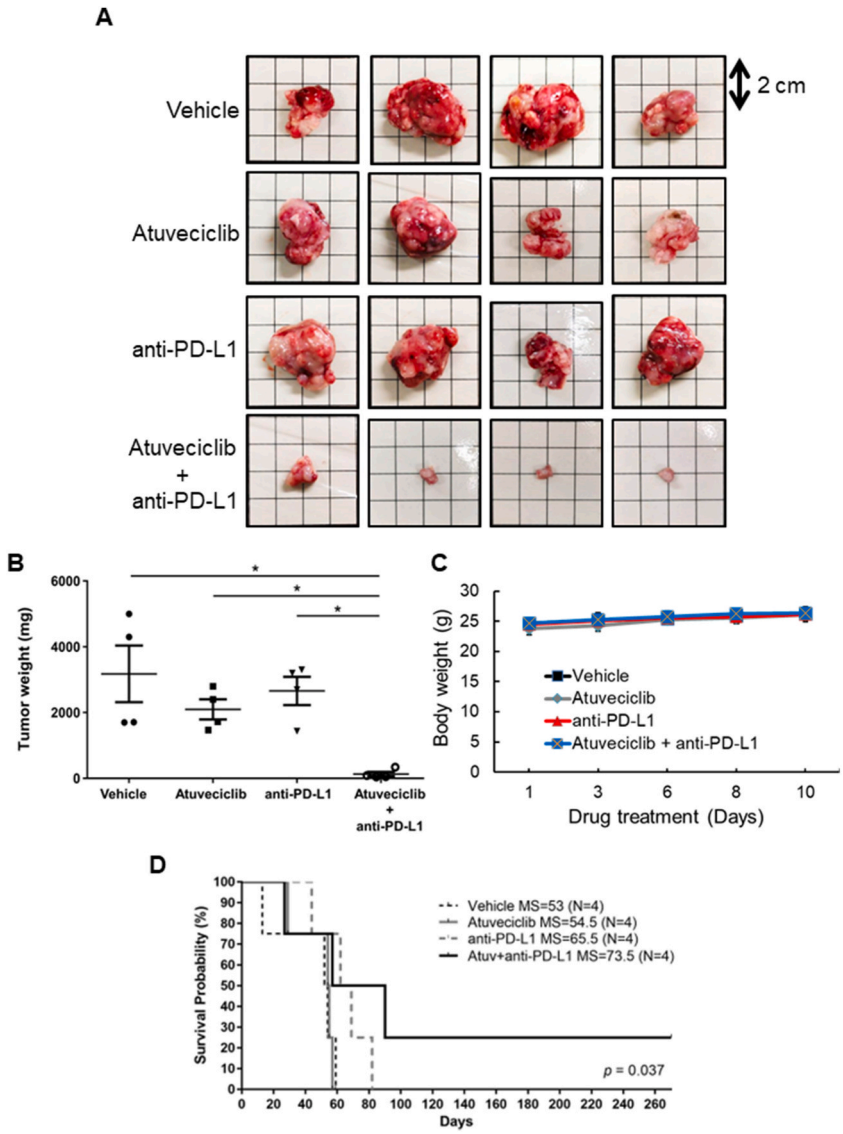

Fig. 6. Orthotopic mouse HCC model. We implanted 3×10^5 BNL cells in the subcapsular area of the liver in BALB/c mice. After 1 week, we started treatment with vehicle (n = 4), atuveciclib (6.25 mg/kg orally, once daily, 5 days per week; n = 4), an anti-PD-L1 antibody (5 mg/kg via intraperitoneal injection 2 times per week; n = 4), or combination treatment (n = 4). (A–C) After 2 weeks, the mice were sacrificed. Tumors were photographed (A) and measured (B) (*denotes $p < 0.05$). Mouse weights were also measured (C). In another separate but similar experiment, the mice were not sacrificed unless they met the animal euthanasia criteria after 2 weeks of the indicated treatment. The Kaplan-Meier method was used to calculate survival from the day of treatment initiation (D). The p value was conducted using the log-rank test for trend.

influence the work reported in this paper.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e34289.

List of Abbreviations

PD-1	programmed cell death protein-1
HCC	hepatocellular carcinoma
CDK	cyclin-dependent kinase
RNAPII	RNA polymerase II
PD-L1	programmed death-ligand 1
IFN-γ	interferon-γ
STAT1	signal transducer and activator of transcription 1
IRF-1	interferon regulatory factor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

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