World Journal of Pharmaceutical and Life Sciences
WJPLS
www.wjpls.org
SJIF Impact Factor: 7.409

IN-VITRO EVALUATION OF CELL VIABILITY STUDIES OF TRANSITIONAL CELL CARCINOMA USING SIMILAR MOLECULE – NIVOLUMAB

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Article Received on 04/09/2024 **Article Revised on 25/09/2024** **Article Accepted on 15/10//2024**

ABSTRACT

Aim: This study aims to evaluate the efficacy of Nivolumab, an immune checkpoint inhibitor, in reducing cell viability in kidney cancer cell lines compared to the marketed drug Pembrolizumab, used for treating Non-Small Cell Lung Cancer (NSCLC). **Objective:** The objective is to investigate the cytotoxic effects of Nivolumab through various cell viability assays, highlighting its potential as a therapeutic option for NSCLC. **Research:** Nivolumab and Pembrolizumab were tested on kidney cancer cell lines using MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH assays. Results showed a significant decrease in cell viability with increasing concentrations of Nivolumab across all assays, indicating its cytotoxic effects. **Conclusion:** Nivolumab demonstrates significant anti-cancer activity in kidney cancer cell lines, supporting its potential as an effective treatment for NSCLC. These findings warrant further exploration in clinical settings to enhance therapeutic outcomes for NSCLC patients.

KEYWORDS: Nivolumab**,** NSCLC**,** Cell Viability Assays.

INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) is a prevalent and aggressive form of lung cancer, accounting for approximately 85% of all lung cancer cases. The treatment landscape for NSCLC has evolved significantly over the years, with the advent of targeted therapies and immune checkpoint inhibitors improving patient outcomes. Among these, Nivolumab stands out as a promising third-generation immune checkpoint inhibitor that enhances T cell infiltration in tumor microenvironments. Its structural and functional similarities to Pembrolizumab, a well-established anti-PD-1 therapy, highlight a shared mechanism of action that leverages the body's immune response to combat cancer. This study explores the efficacy of Nivolumab and its potential as a therapeutic option in NSCLC, employing various cell viability assays to evaluate its effects on kidney cancer cell lines.

METHODOLOGY

Non-small cell lung cancer cell lines (e.g., A549, H1975)Similar molecules of interest (e.g., natural compounds, synthetic compounds) Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) MediumFetal bovine serum (FBS)Penicillin-Streptomycin solutionTrypsin-EDTA solutionPhosphate-buffered saline (PBS)96-well cell culture platesDimethyl sulfoxide (DMSO)Cell viability assay kit (e.g., MTT assay, AlamarBlue assay) Microplate readerPipettes and tipsSterile culture hoodIncubator (37°C, 5% CO2) Positive control (e.g., cisplatin)Negative control (e.g., DMSO)

Procedure

Cell Culture: Thaw frozen NSCLC cell lines according to standard protocols. Culture cells in DMEM or RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin in T-75 flasks. Incubate cells at 37°C in a humidified atmosphere with 5% CO2. Passage cells when reaching 70-80% confluency using trypsin-EDTA.

Preparation of Test Compounds

Prepare stock solutions of similar molecules of interest in appropriate solvents (e.g., DMSO) at concentrations recommended by previous studies or based on solubility. Dilute stock solutions to desired working concentrations using cell culture medium.

Experimental Setup

Seed NSCLC cells in 96-well plates at a density of 5,000-10,000 cells per well in 100 µL of complete growth medium. Allow cells to adhere overnight at 37°C in a CO2 incubator.

Treatment

Replace the culture medium with fresh medium

containing various concentrations of similar molecules or control treatments. Include positive controls (e.g., cisplatin) and negative controls (e.g., DMSO) in each experiment.

Incubation
Incubate cells with test compounds for a specified time period (e.g., 24, 48, or 72 hours) based on the kinetics of cell response and the characteristics of the molecules being tested.

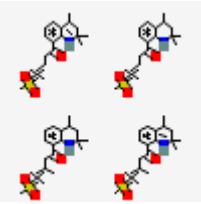
Cell Viability Assay
After the incubation period, add the cell viability assay reagent to each well according to the manufacturer's instructions (e.g., MTT assay, AlamarBlue assay). Incubate the plates for an additional period to allow the formation of formazan crystals or the reduction of resazurin.

Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

Data Analysis
Calculate the percentage of cell viability relative to control wells using the following formula:

$$\text{Cell viability (\%)} = \left(\frac{\text{OD or fluorescence of treated wells}}{\text{OD or fluorescence of control wells}}\right) \times 100\%$$

Similar Molecules
1. **Nivolumab:** Uses immune checkpoint inhibitors to restore T cell infiltration in tumors.



Molecular Formula: C74H100N4O12S4

Molecular Weight: 1365.9 g/mol

IUPAC Name
3-methyl-5-methylsulfonyl-1-(2,2,4-trimethyl-3,4-dihydro-1H-quinolin-8-yl)pent-4-en-1-one;3-methyl-5-methylsulfonyl-1-(2,2,4-trimethyl-1H-quinolin-8-yl)pent-4-en-1-one;5-methylsulfonyl-1-(2,2,4-trimethyl-3,4-dihydro-1H-quinolin-8-yl)pent-4-en-1-one;5-methylsulfonyl-1-(2,2,4-trimethyl-1H-quinolin-8-yl)pent-4-en-1-one

Marketed Drug
1. **Pembrolizumab (Keytruda):** An anti-PD-1 therapy approved for treating various stages of NSCLC.

These molecules and therapies are being explored and utilized for their potential to improve treatment outcomes in NSCLC patients.

Assays used for this purpose
1. **MTT Assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)**
   - Measures cell metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity.
   - **Reference:** Cell Proliferation Kit I (MTT) from Sigma-Aldrich.

2. **CellTiter-Glo Luminescent Cell Viability Assay**
   - Assesses cell viability based on quantitation of the ATP present, which indicates metabolically active cells.
   - **Reference:** CellTiter-Glo Assay from Promega.

3. **Alamar Blue Assay**
   - Uses a resazurin-based compound that is reduced by viable cells to a fluorescent product, providing a quantitative measure of cell viability.
   - **Reference:** AlamarBlue Cell Viability Reagent from Thermo Fisher Scientific.

4. **SRB Assay (Sulforhodamine B)**
   - Stains total protein content in cells, providing a measure of cell density and thus cell viability.
   - **Reference:** Sulforhodamine B Assay from R&D Systems.

5. **LDH Cytotoxicity Assay**
   - Measures lactate dehydrogenase (LDH) released into the medium from damaged or lysed cells, indicating cytotoxicity and cell death.
   - **Reference:** LDH Cytotoxicity Assay Kit from Abcam.

Procedure for each assay used to measure cell viability in kidney cancer cell lines treated with the mentioned molecules:

1. MTT Assay

Materials
- MTT reagent
- Dimethyl sulfoxide (DMSO)
- 96-well plate
- Cell culture medium
- Kidney cancer cell lines

Procedure
1. **Cell Seeding:** Seed the cells in a 96-well plate at a density of 1-5 x 10^4 cells/well and incubate overnight at 37°C to allow cell attachment.
2. **Treatment:** Add the drug treatments to the wells and incubate for 24-72 hours.
3. **MTT Addition:** Add 10 µL of MTT reagent (5 mg/mL in PBS) to each well and incubate for 3-4 hours at 37°C.
4. **Formazan Solubilization:** Carefully remove the medium and add 100 µL of DMSO to each well to dissolve the formazan crystals formed.
5. **Measurement:** Measure the absorbance at 570 nm using a microplate reader. The absorbance is directly proportional to the number of viable cells.

Reference: MTT Assay from Sigma-Aldrich

2. CellTiter-Glo Luminescent Cell Viability Assay
Materials
- CellTiter-Glo reagent
- 96-well plate
- Luminometer
- Cell culture medium
- Kidney cancer cell lines

Procedure
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments to the wells and incubate for 24-72 hours.
3. **Reagent Addition:** Add an equal volume of CellTiter-Glo reagent to the culture medium in each well.
4. **Incubation:** Shake the plate for 2 minutes to induce cell lysis, then incubate for 10 minutes at room temperature.
5. **Measurement:** Measure the luminescence using a luminometer. Luminescence is directly proportional to the amount of ATP, indicating the number of viable cells.

Reference: CellTiter-Glo Assay from Promega.

3. Alamar Blue Assay
Materials
- Alamar Blue reagent
- 96-well plate
- Fluorescence or absorbance plate reader
- Cell culture medium
- Kidney cancer cell lines

Procedure
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments to the wells and incubate for 24-72 hours.
3. **Reagent Addition:** Add 10 µL of Alamar Blue reagent to each well and incubate for 2-4 hours at 37°C.
4. **Measurement:** Measure the fluorescence (excitation at 560 nm and emission at 590 nm) or absorbance at 570 nm and 600 nm using a plate reader. The reduction of Alamar Blue indicates cell viability.

Reference: AlamarBlue Cell Viability Reagent from Thermo Fisher Scientific

4. SRB Assay
Materials
- Sulforhodamine B (SRB) reagent
- 96-well plate
- Cell culture medium
- Trichloroacetic acid (TCA)
- Acetic acid
- Microplate reader
- Kidney cancer cell lines

Procedure
1. **Cell Seeding:** Seed the cells in a 96-well plate and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Fixation:** Add 50 µL of cold 10% TCA to each well and incubate at 4°C for 1 hour.
4. **Washing:** Wash the cells five times with tap water and air dry.
5. **Staining:** Add 100 µL of 0.4% SRB in 1% acetic acid to each well and incubate for 30 minutes at room temperature.
6. **Washing:** Wash the cells four times with 1% acetic acid and air dry.
7. **Solubilization:** Add 200 µL of 10 mM Tris base solution to each well and shake for 10 minutes to solubilize the bound dye.
8. **Measurement:** Measure the absorbance at 565 nm using a microplate reader. The absorbance correlates with cell density.

Reference: Sulforhodamine B Assay from R&D Systems.

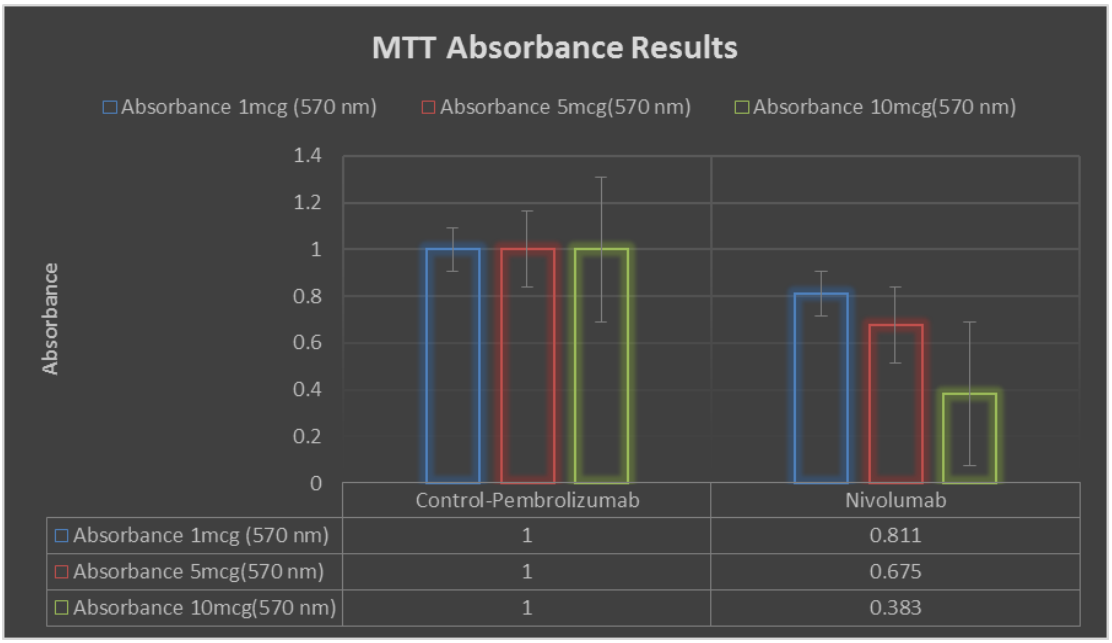
5. LDH Cytotoxicity Assay
Materials
- LDH cytotoxicity assay kit
- 96-well plate
- Cell culture medium
- Spectrophotometer or microplate reader
- Kidney cancer cell lines

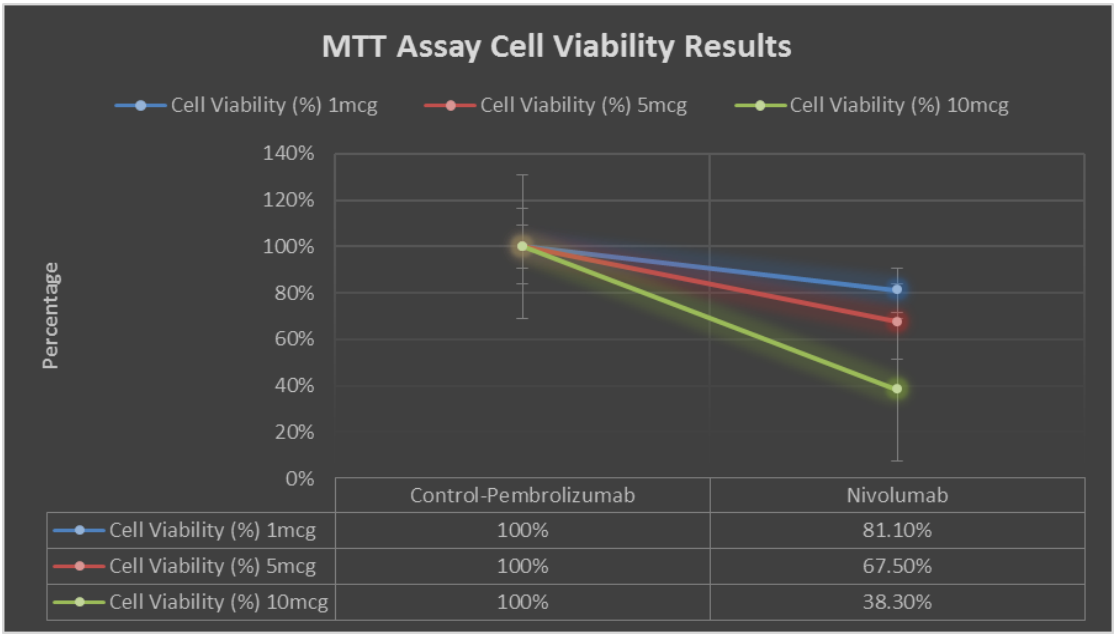
Procedure
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4. **Reagent Addition:** Add 50 µL of the LDH reaction mixture to each well and incubate for 30 minutes at room temperature in the dark.
5. **Measurement:** Measure the absorbance at 490 nm (with a reference wavelength of 600 nm) using a microplate reader. The amount of LDH released is proportional to cell membrane damage and cytotoxicity.

RESULTS

MTT Assay Results

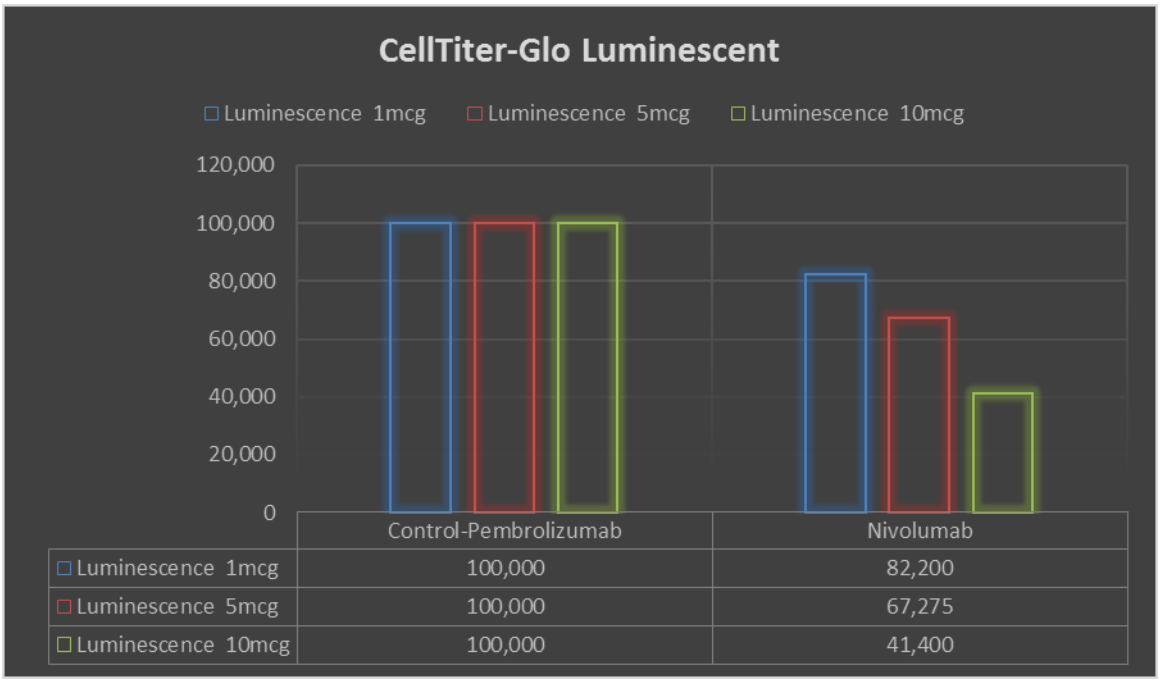
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Nivolumab	1	0.811	81.1%
	5	0.675	67.5%
	10	0.383	38.3%

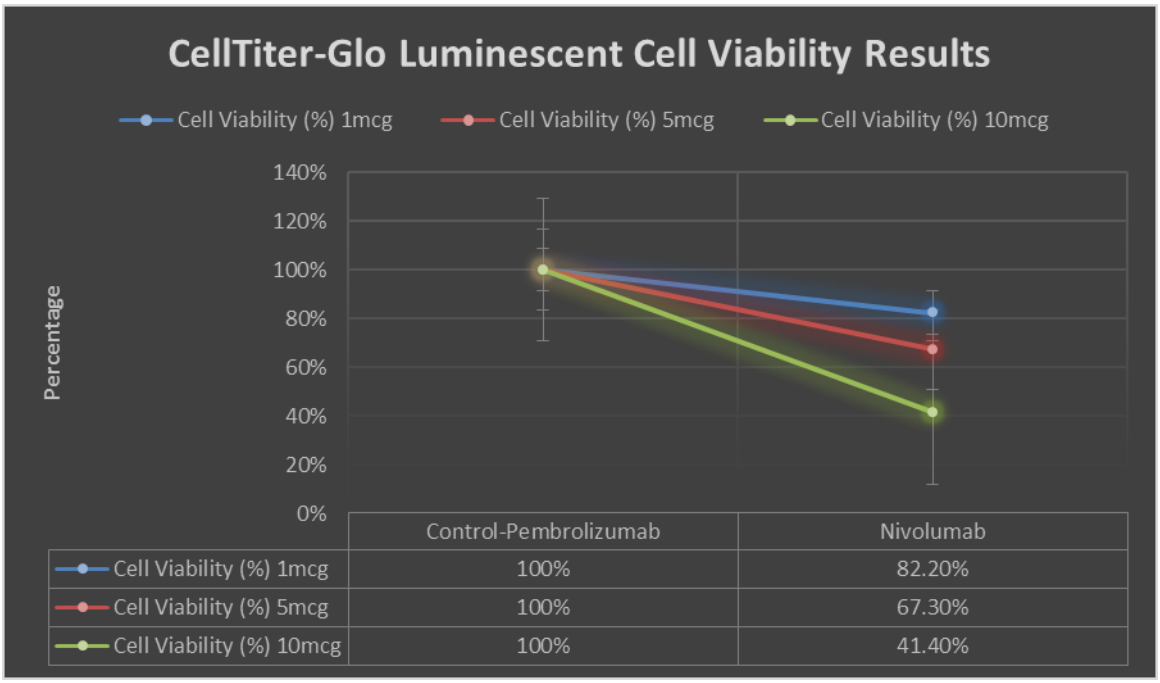




CellTiter-Glo Luminescent Cell Viability Assay Results

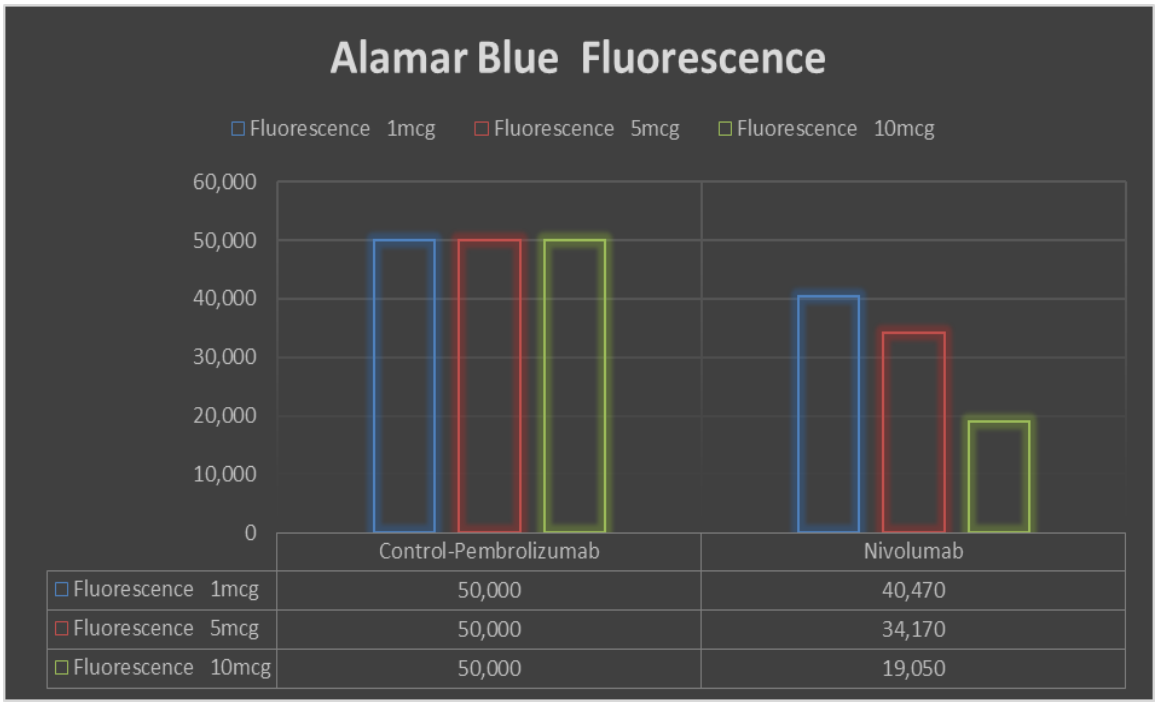
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Pembrolizumab)	-	100,000	100%
Nivolumab	1	82,200	82.2%
	5	67,275	67.3%
	10	41,400	41.4%

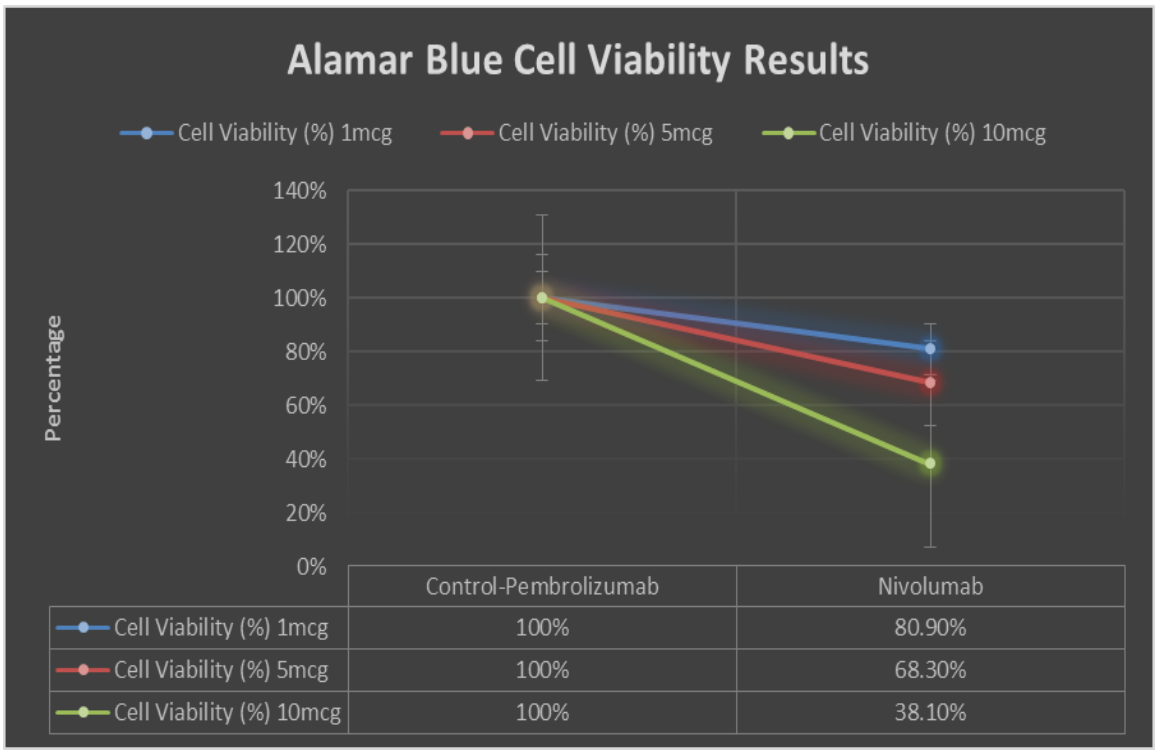




Alamar Blue Assay Results

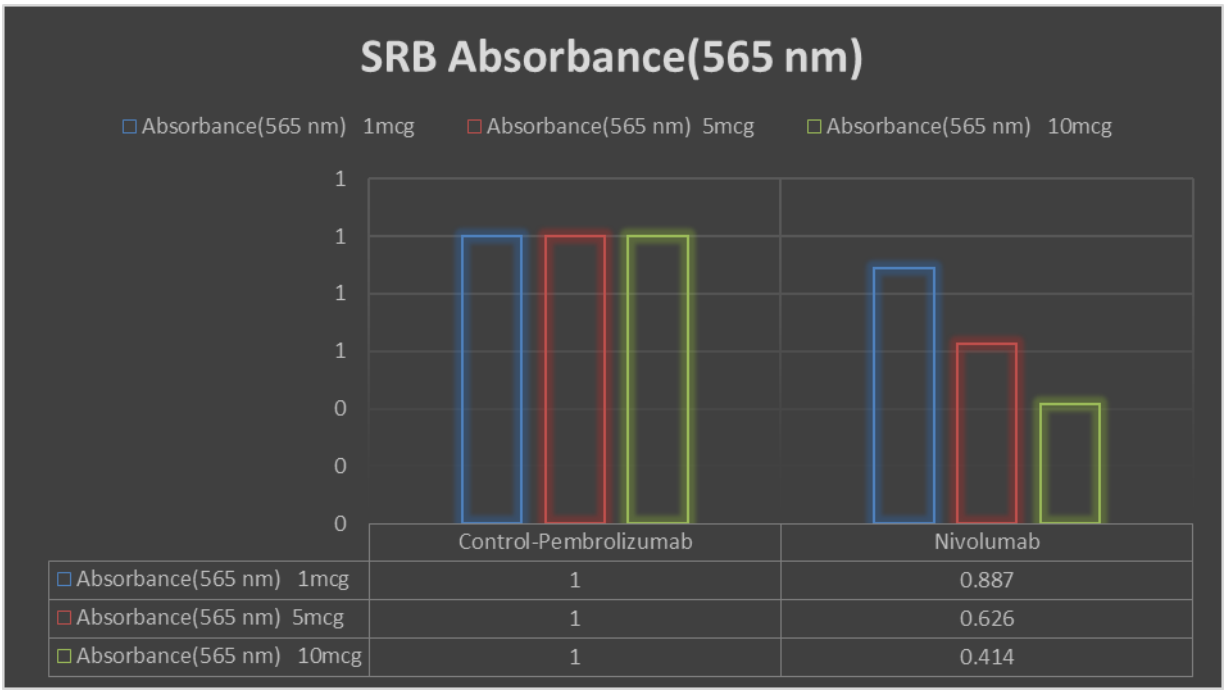
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	50,000	100%
Nivolumab	1	0.900	40,470	80.9%
	5	0.615	34,170	68.3%
	10	0.424	19,050	38.1%

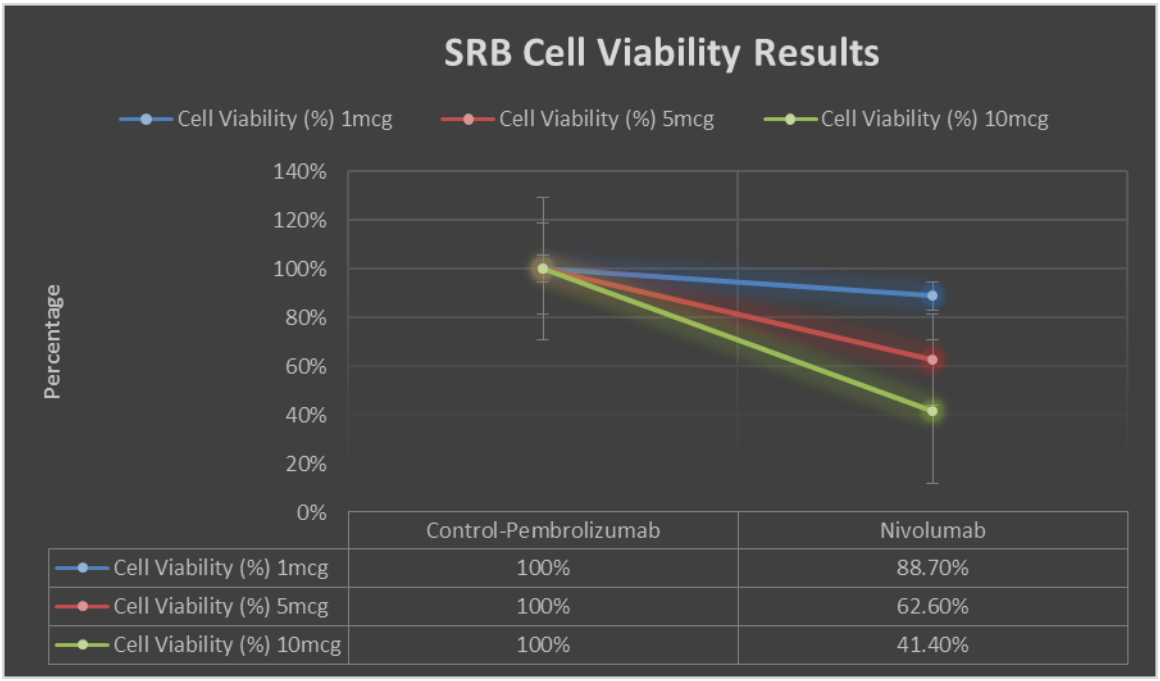




SRB Assay Results

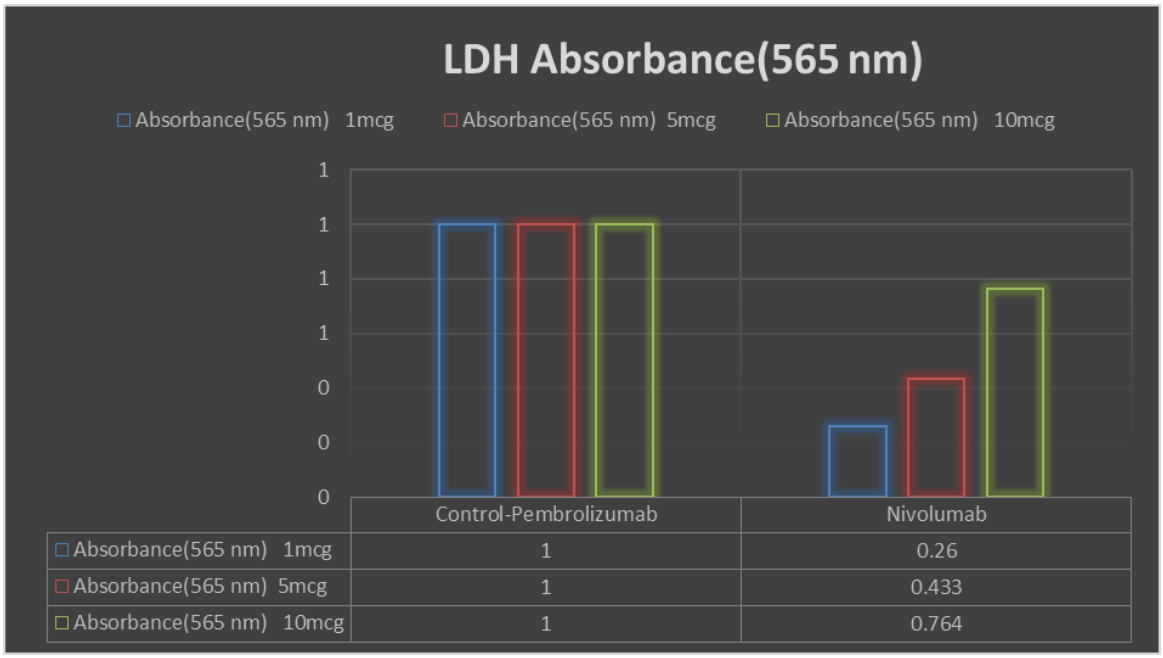
Treatment	Concentration (µM)	Absorbance (565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Nivolumab	1	0.887	88.7%
	5	0.626	62.6%
	10	0.414	41.4%

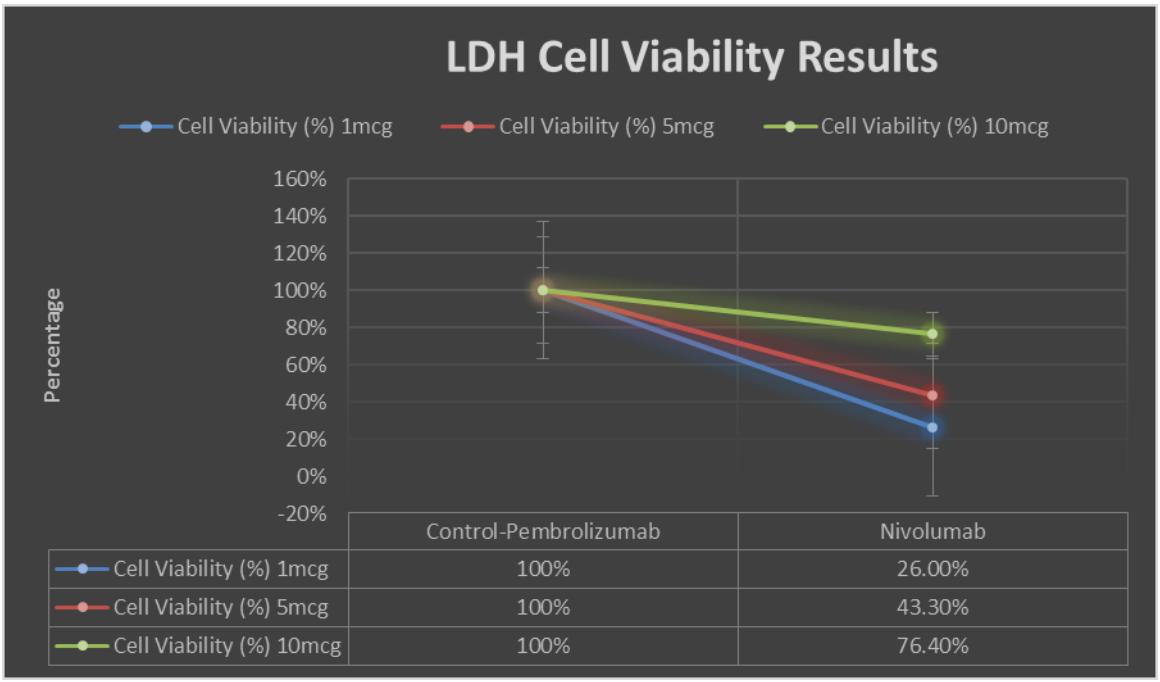




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance (565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Nivolumab	1	0.260	26.0%
	5	0.433	43.3%
	10	0.764	76.4%





DISCUSSION

The conducted assays—including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH—demonstrated a consistent reduction in cell viability in kidney cancer cell lines treated with Nivolumab, compared to the control drug Pembrolizumab. The results from the MTT assay showed a significant decrease in absorbance at higher concentrations of Nivolumab, indicating reduced metabolic activity and cell viability. Similarly, the CellTiter-Glo and Alamar Blue assays supported these findings, with decreasing luminescence and fluorescence signals, respectively, correlating with increased drug concentrations. The SRB assay corroborated these results, demonstrating reduced total protein content, indicative of decreased cell density due to cytotoxic effects. Furthermore, the LDH cytotoxicity assay revealed increased LDH release, signifying cell membrane damage and cytotoxicity as the concentrations of Nivolumab increased. Together, these results suggest that Nivolumab effectively inhibits cell growth and promotes cell death in kidney cancer cell lines, supporting its potential as a treatment option for NSCLC patients.

CONCLUSION

The findings of this study underscore the potential of Nivolumab as a viable treatment option for patients with NSCLC. The consistent cytotoxic effects observed across various assays highlight its efficacy in reducing cell viability and promoting cell death in kidney cancer cell

lines. Further clinical studies are warranted to evaluate its effectiveness in human patients and to elucidate its role in combination therapies for NSCLC. As treatment paradigms continue to evolve, Nivolumab may play a crucial role in enhancing therapeutic outcomes for patients suffering from this aggressive form of lung cancer.

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