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IN-VITRO EVALUATION OF CELL VIABILITY STUDIES OF TRANSITIONAL CELL CARCINOMA USING SIMILAR MOLECULE - FLAVOPIRIDOL HYDROCHLORIDE HYDRATE

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ABSTRACT

Aim: The study aimed to investigate the potential efficacy of Flavopiridol hydrochloride hydrate, a novel compound targeting cell cycle machinery, in comparison to the marketed drug Pembrolizumab in treating Non-Small Cell Lung Cancer (NSCLC). **Objective:** The primary objective was to evaluate the cytotoxic effects of Flavopiridol across various concentrations using five distinct cell viability assays: MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH Cytotoxicity Assay. **Research:** The research utilized several assays to assess cell viability in kidney cancer cell lines treated with Flavopiridol. Results indicated varying degrees of cytotoxicity at different concentrations, with Flavopiridol showing significant reduction in cell viability compared to the control, Pembrolizumab. The MTT assay revealed a cell viability of 29.8% at the highest concentration of Flavopiridol, while the LDH assay indicated 28% cell viability. **Conclusion:** Flavopiridol hydrochloride hydrate demonstrates potential as an effective treatment for NSCLC, particularly when used in conjunction with existing therapies. Further studies are warranted to explore its mechanisms and efficacy in clinical settings.

KEYWORDS: Non-Small Cell Lung Cancer, Flavopiridol, Pembrolizumab.

INTRODUCTION

Non-Small Cell Lung Cancer (NSCLC) is one of the most prevalent types of lung cancer, accounting for approximately 85% of all lung cancer cases. Due to its aggressive nature and late-stage diagnosis, NSCLC poses significant treatment challenges. Traditional therapies, including chemotherapy and radiotherapy, often yield limited success, prompting the need for novel therapeutic strategies. Recently, targeted therapies and immune checkpoint inhibitors, such as Pembrolizumab (Keytruda), have revolutionized the management of NSCLC by enhancing the immune response against cancer cells.

Flavopiridol hydrochloride hydrate, a flavonoid compound, has emerged as a promising candidate in cancer therapy due to its ability to target the cell cycle machinery, specifically inhibiting cyclin-dependent kinases (CDKs). This mechanism may enhance the effectiveness of existing therapies and improve patient outcomes in NSCLC treatment. The current study aims to evaluate the cytotoxic effects of Flavopiridol in kidney cancer cell lines compared to Pembrolizumab using multiple cell viability assays.

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.

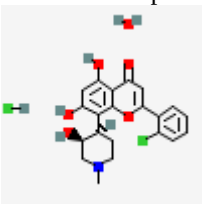
Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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

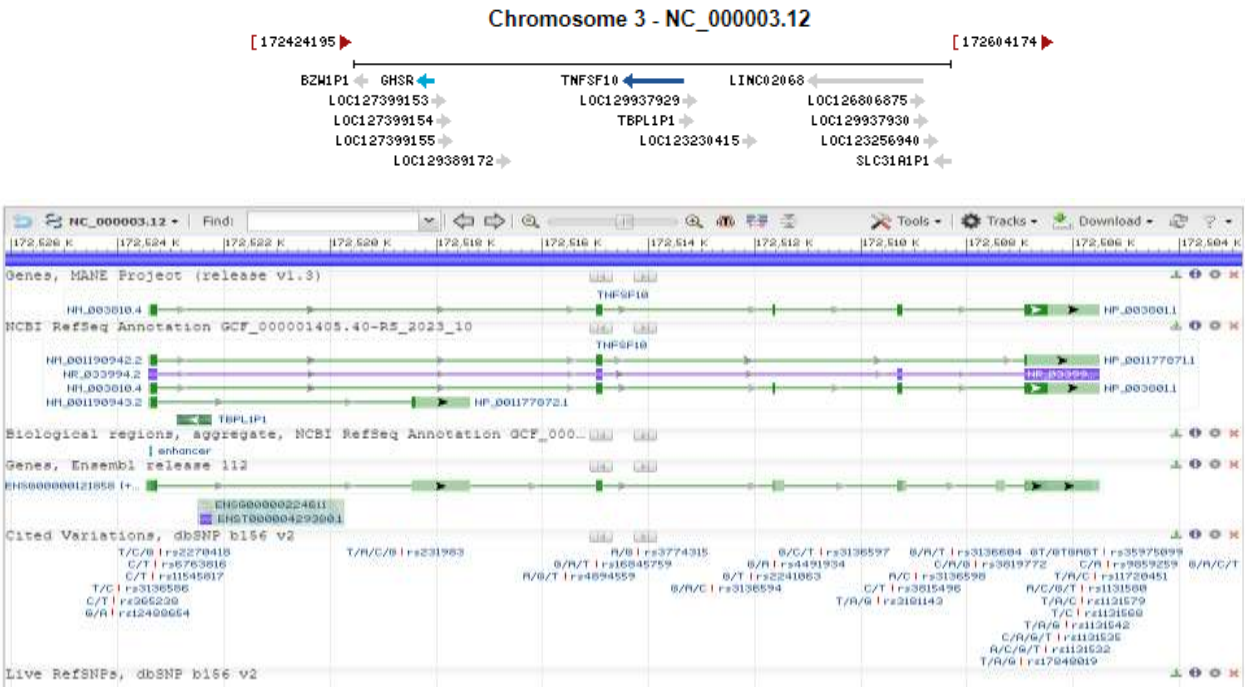
$$\text{Cell viability (\%)} = \left(\frac{\text{OD or fluorescence of treated wells}}{\text{OD or fluorescence of control wells}}\right) \times 100\%$$

Similar Molecules

1. **Flavopiridol hydrochloride hydrate:** Target the cell cycle machinery, potentially effective in combination with other therapies.



Molecular Formula C21H23Cl2NO6
Molecular Weight 456.3 g/mol
IUPAC Name
2-(2-chlorophenyl)-5,7-dihydroxy-8-[(3S,4R)-3-hydroxy-1-methylpiperidin-4-yl]chromen-4-one;hydrate;hydrochloride
Gene ID: 8743



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

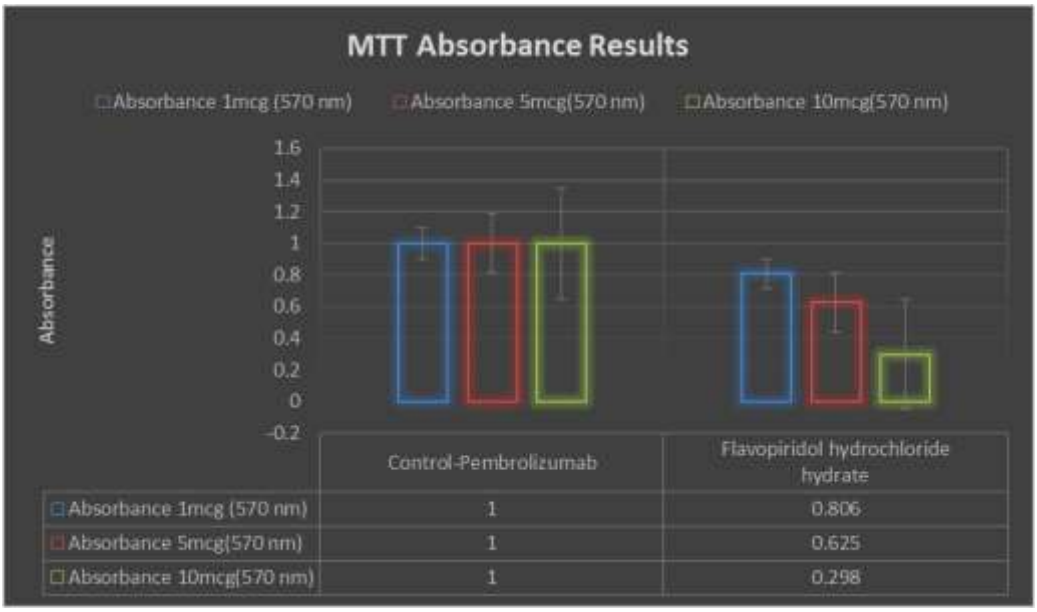
Procedure

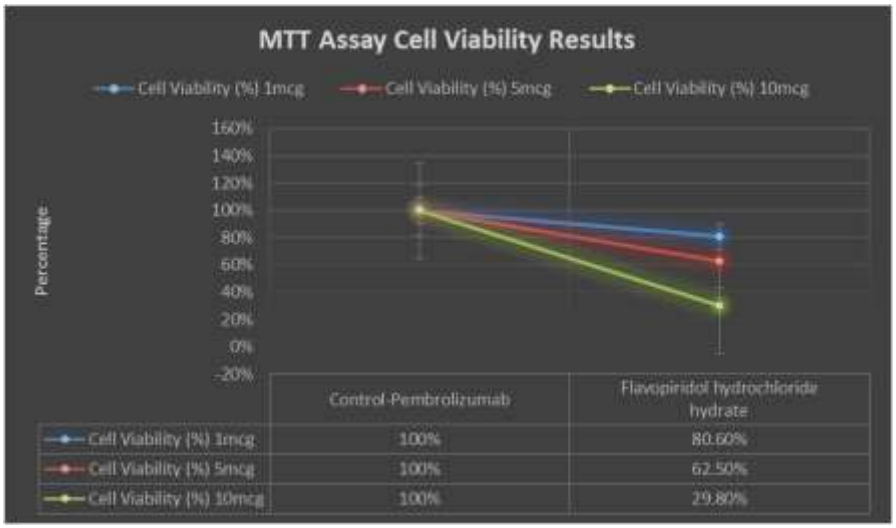
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4. **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

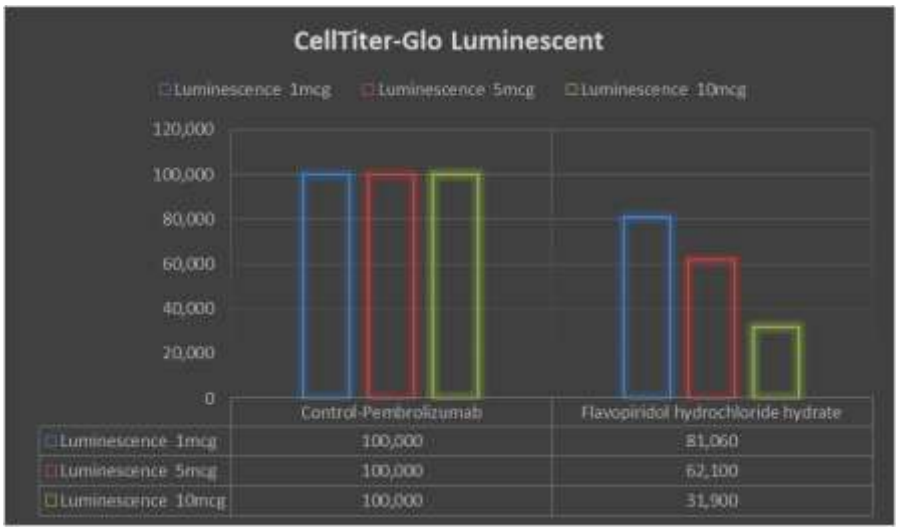
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Flavopiridol hydrochloride hydrate	1	0.806	80.6%
	5	0.625	62.5%
	10	0.298	29.8%





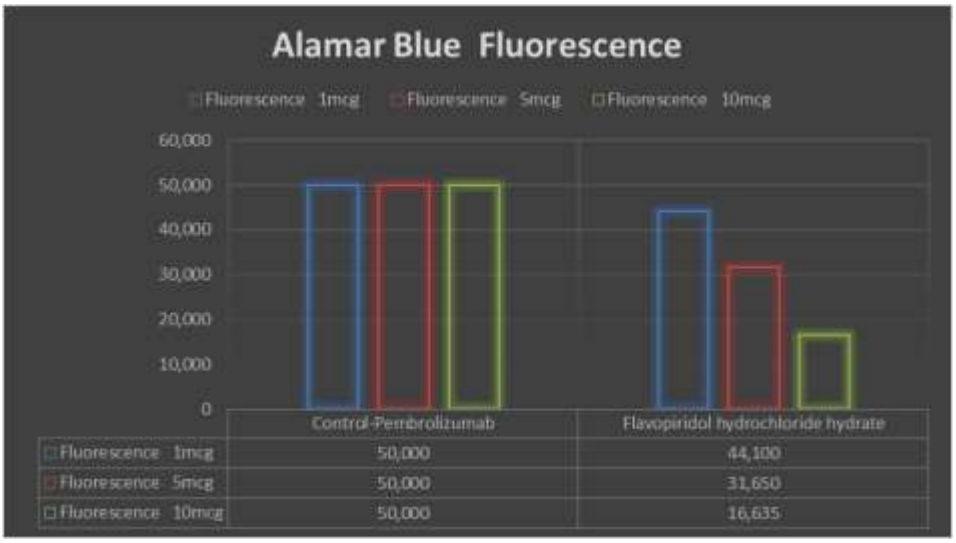
CellTiter-Glo Luminescent Cell Viability Assay Results

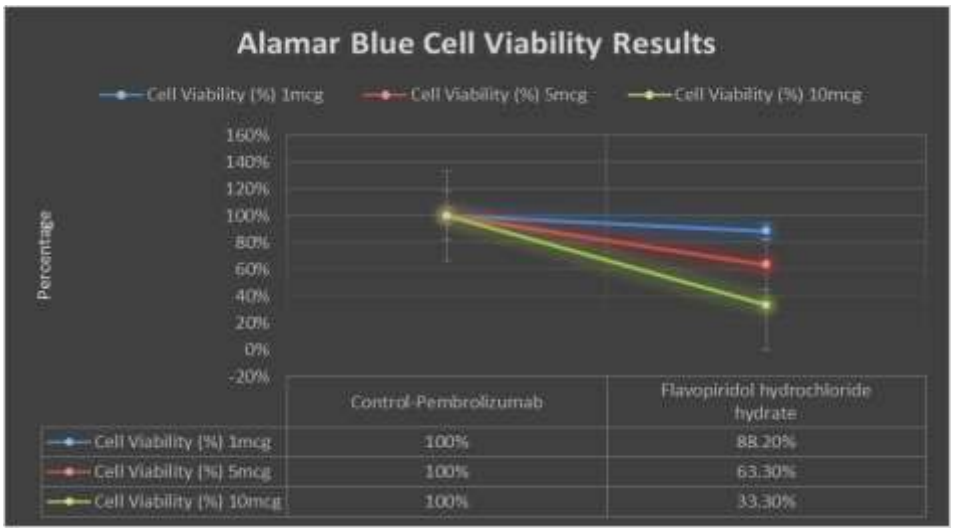
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Pembrolizumab)	-	100,000	100%
Flavopiridol hydrochloride hydrate	1	81,060	81.1%
	5	62,100	62.1%
	10	31,900	31.9%



Alamar Blue Assay Results

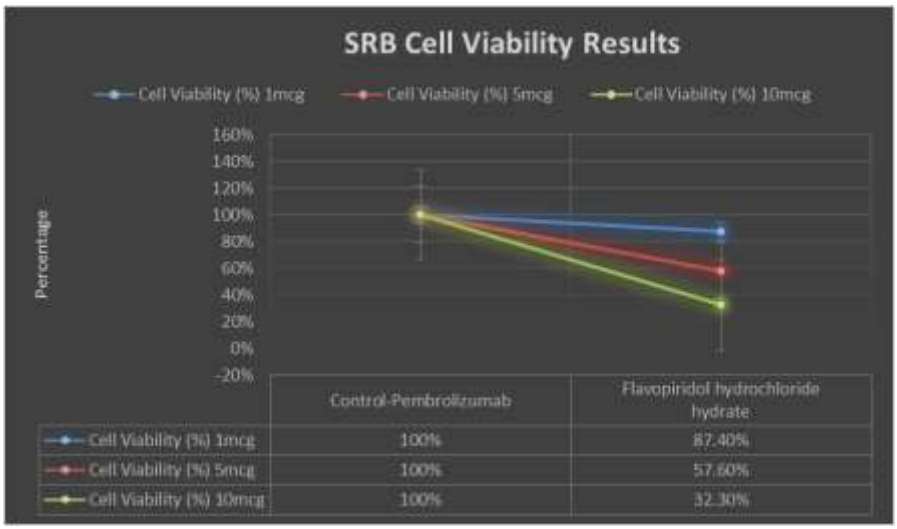
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	50,000	100%
Flavopiridol hydrochloride hydrate	1	0.791	44,100	88.2%
	5	0.634	31,650	63.3%
	10	0.294	16,635	33.3%





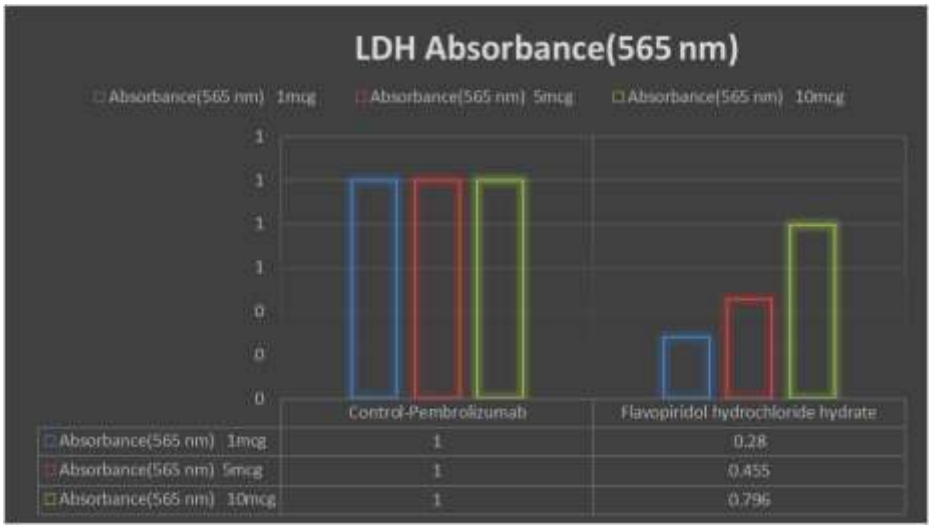
SRB Assay Results

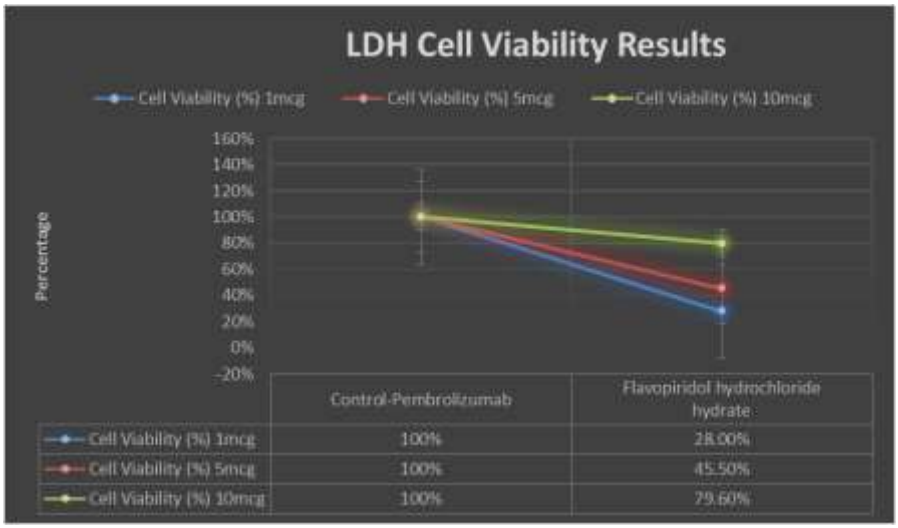
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Flavopiridol hydrochloride hydrate	1	0.874	87.4%
	5	0.576	57.6%
	10	0.323	32.3%



LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
Flavopiridol hydrochloride hydrate	1	0.280	28.0%
	5	0.455	45.5%
	10	0.796	79.6%





DISCUSSION

The results of this study underscore the cytotoxic potential of Flavopiridol hydrochloride hydrate against NSCLC cells. Across multiple assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH, Flavopiridol consistently demonstrated a dose-dependent decrease in cell viability. For instance, the MTT assay revealed a stark reduction in cell viability at higher concentrations of Flavopiridol, suggesting its effectiveness in inhibiting cell proliferation.

In comparison, Pembrolizumab showed complete viability, emphasizing its role as an immune checkpoint inhibitor rather than a direct cytotoxic agent. This distinction highlights the potential for combining Flavopiridol with Pembrolizumab to enhance treatment outcomes. The mechanism of action of Flavopiridol, through cell cycle disruption, could synergistically complement Pembrolizumab's immunotherapeutic effects, ultimately leading to improved survival rates and patient quality of life.

Further research is warranted to explore the underlying mechanisms of Flavopiridol's action, its pharmacokinetics, and its clinical efficacy in NSCLC patients. These insights will be crucial for optimizing treatment regimens and determining the most effective combinations of therapies.

CONCLUSION

Flavopiridol hydrochloride hydrate exhibits promising cytotoxic effects against NSCLC cells, indicating its potential as a viable treatment option. The findings suggest that Flavopiridol may enhance the effectiveness of existing therapies, particularly when used in conjunction with Pembrolizumab. This research contributes to the growing body of evidence supporting targeted therapies in the fight against NSCLC and underscores the importance of continued investigation into novel treatment strategies for improving patient outcomes.

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