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IN-VITRO EVALUATION OF CELL VIABILITY STUDIES OF NON-SMALL CELL LUNG CANCER USING SIMILAR MOLECULE - N-HYDROXY-OSIMERTINIB

Raheem Unnisa Shaik*[1], Dr. Syed Ahmed Hussain[1], Maimuna Fatima, Umaima Batool Osmani[1], Arshiya Tarannum[1], Faheem Unnisa[1] and Nazneen[1]

[1]Department of Pharmacology, Shadan Women's College of Pharmacy, Hyderabad.

***Corresponding Author: Raheem Unnisa Shaik**
Department of Pharmacology, Shadan Women's College of Pharmacy, Hyderabad.

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ABSTRACT

Aim: The study aims to investigate the cytotoxic effects of N-Hydroxy-osimertinib, a third-generation EGFR-TKI, on kidney cancer cells using various in vitro assays. **Objective:** The primary objective is to evaluate the efficacy of N-Hydroxy-osimertinib in reducing cell viability at different concentrations and compare its activity to Pembrolizumab, an established anti-PD-1 therapy for NSCLC. **Research:** Kidney cancer cells were treated with N-Hydroxy-osimertinib at concentrations of 1 µM, 5 µM, and 10 µM. The cytotoxic effects were assessed using MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH Cytotoxicity assays. The results demonstrated a significant, dose-dependent decrease in cell viability across most assays, with cell viability dropping to 31.3% at 10 µM in the MTT assay. However, the LDH Cytotoxicity assay showed an increase in cell viability at 10 µM, suggesting potential alterations in cell membrane integrity or necrotic pathways at higher doses. **Conclusion:** N-Hydroxy-osimertinib exhibited potent anti-proliferative effects on kidney cancer cells, similar to or exceeding those of Pembrolizumab. While the compound shows promise as a potential therapeutic agent for NSCLC, further studies are needed to elucidate its mechanisms of action and validate its efficacy in preclinical and clinical settings.

KEYWORDS: N-Hydroxy-osimertinib, NSCLC, cytotoxicity assays.

INTRODUCTION

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 solution Trypsin-EDTA solution Phosphate-buffered saline (PBS)96-well cell culture plates Dimethyl 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).

METHODOLOGY

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.

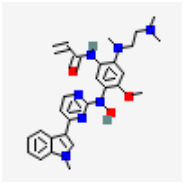
Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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
Five similar molecules and one marketed drug for the treatment of Non-Small Cell Lung Cancer (NSCLC):

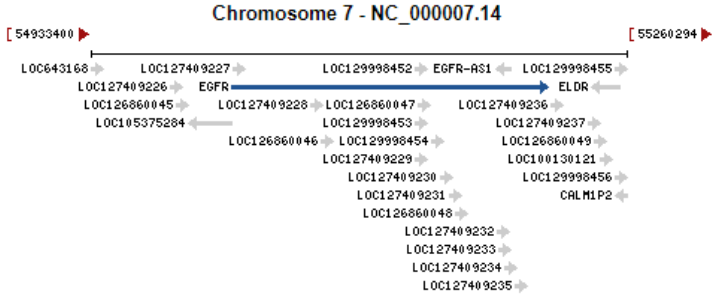
Similar Molecules
1. **N-Hydroxy-osimertinib:** A third-generation EGFR-TKI used as a first-line therapy in advanced NSCLC with EGFR mutations.



Molecular Formula C28H33N7O3
Molecular Weight 515.6 g/mol

IUPAC Name
N-[2-[2-(dimethylamino)ethyl-methylamino]-5-[hydroxy-[4-(1-methylindol-3-yl)pyrimidin-2-yl]amino]-4-methoxyphenyl]prop-2-enamide

Gene ID: 1956



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

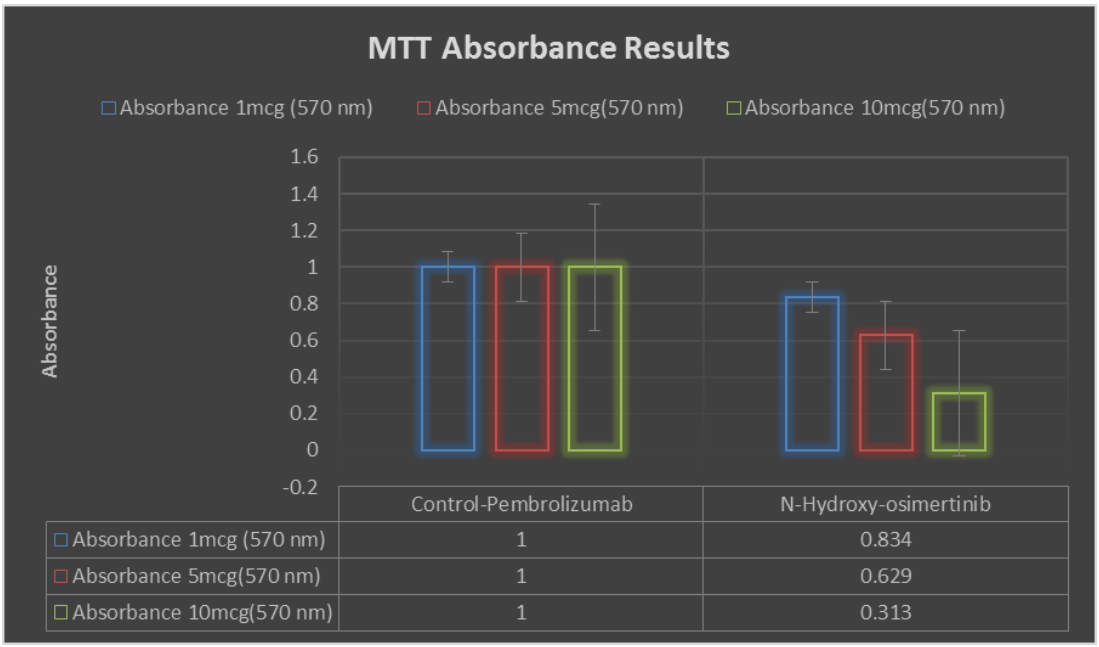
Procedure

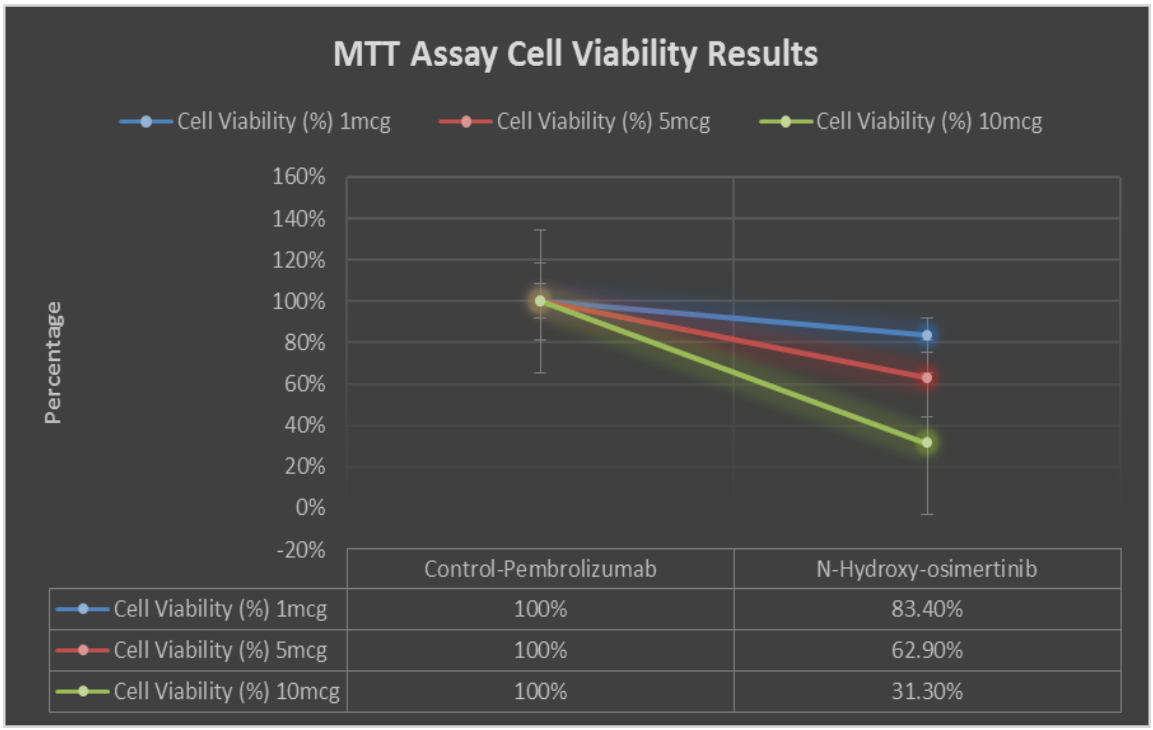
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

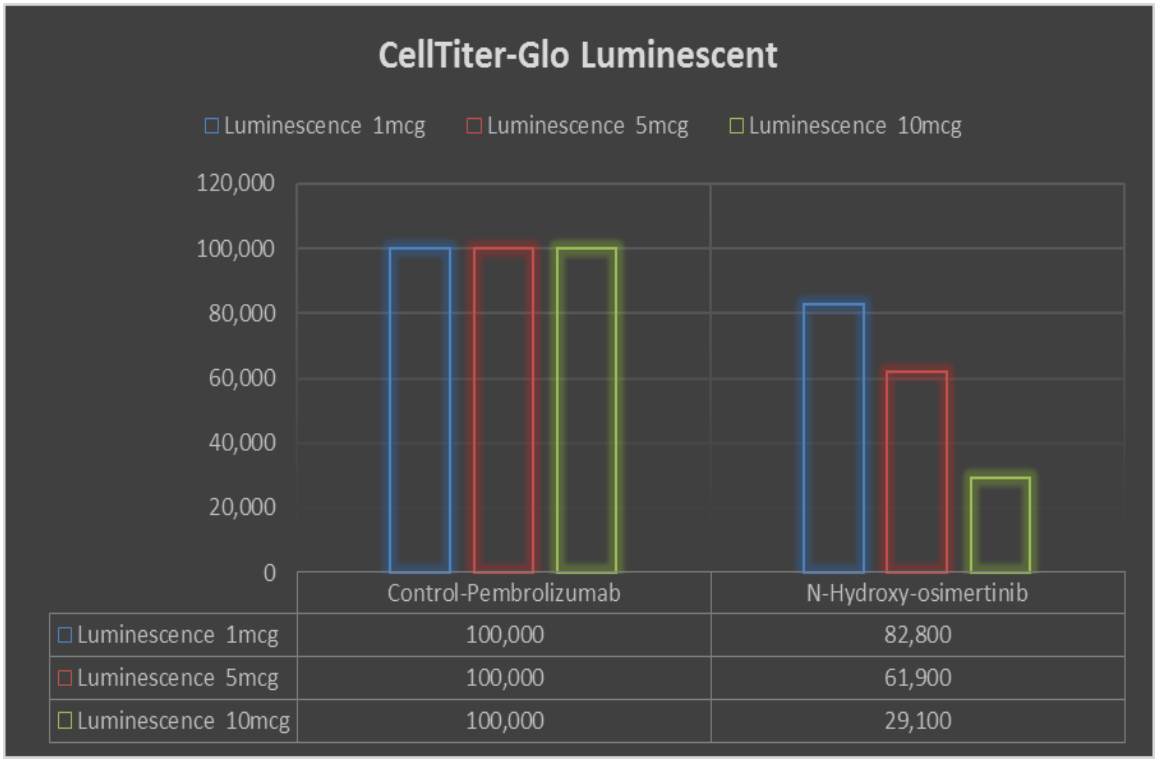
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
N-Hydroxy-osimertinib	1	0.834	83.4%
	5	0.629	62.9%
	10	0.313	31.3%

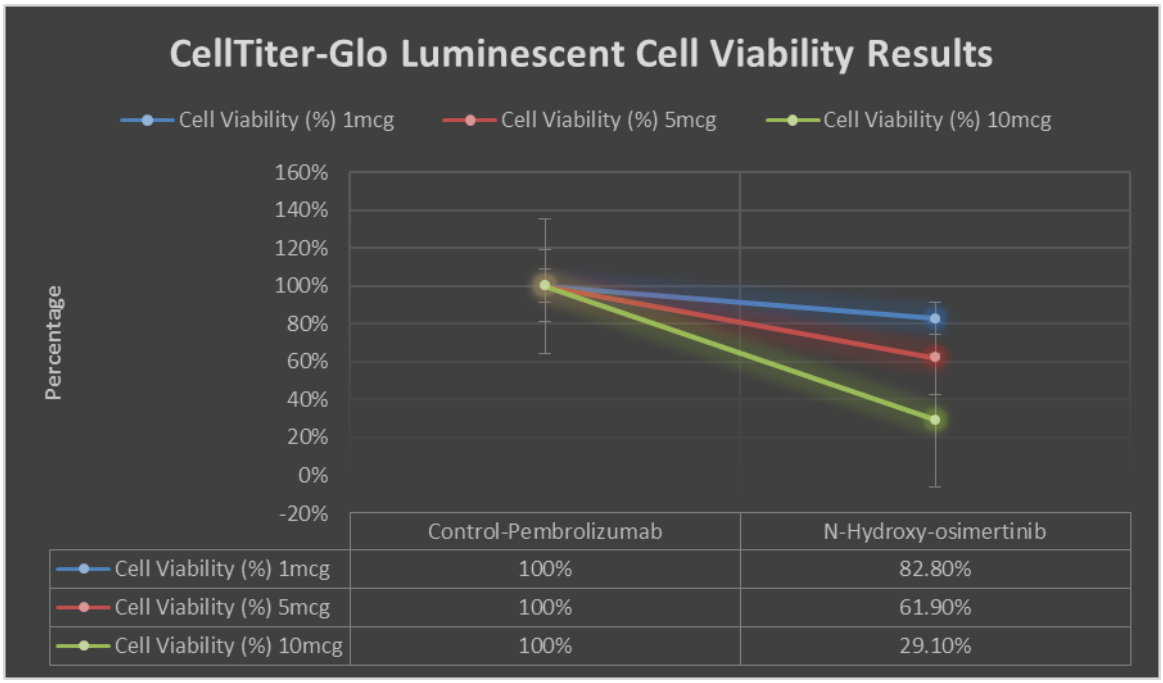




CellTiter-Glo Luminescent Cell Viability Assay Results

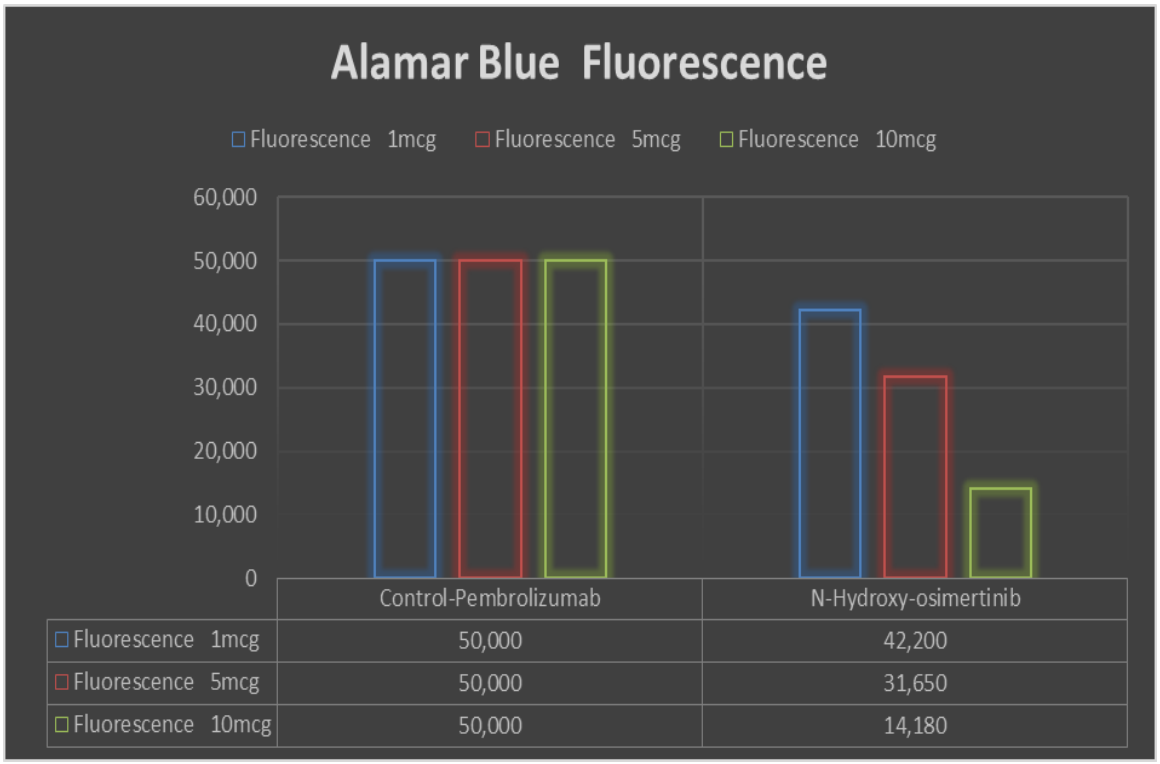
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Pembrolizumab)	-	100,000	100%
N-Hydroxy-osimertinib	1	82,800	82.8%
	5	61,900	61.9%
	10	29,100	29.1%

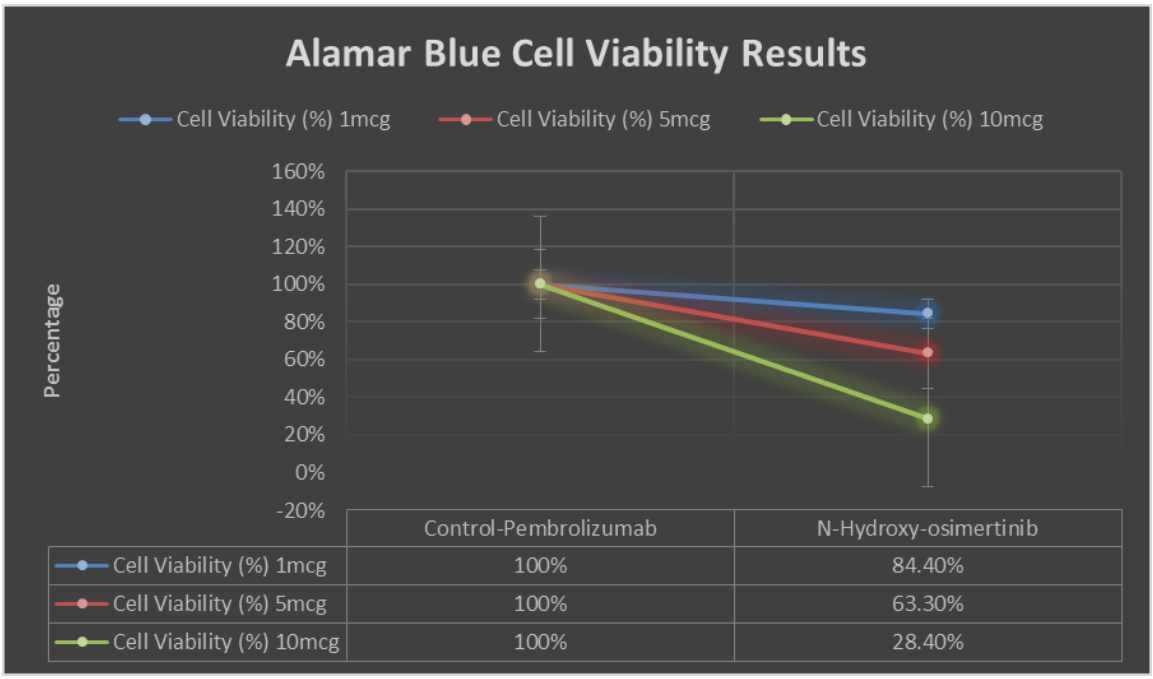




Alamar Blue Assay Results

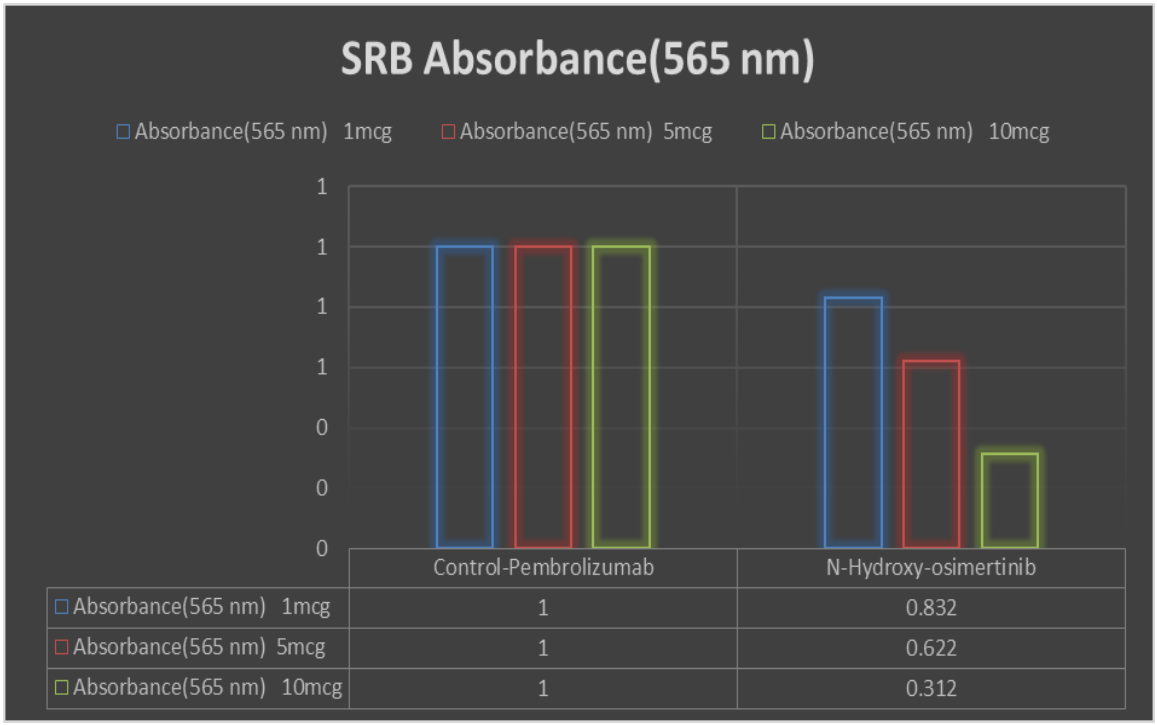
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	50,000	100%
N-Hydroxy-osimertinib	1	0.851	42,200	84.4%
	5	0.564	31,650	63.3%
	10	0.316	14,180	28.4%

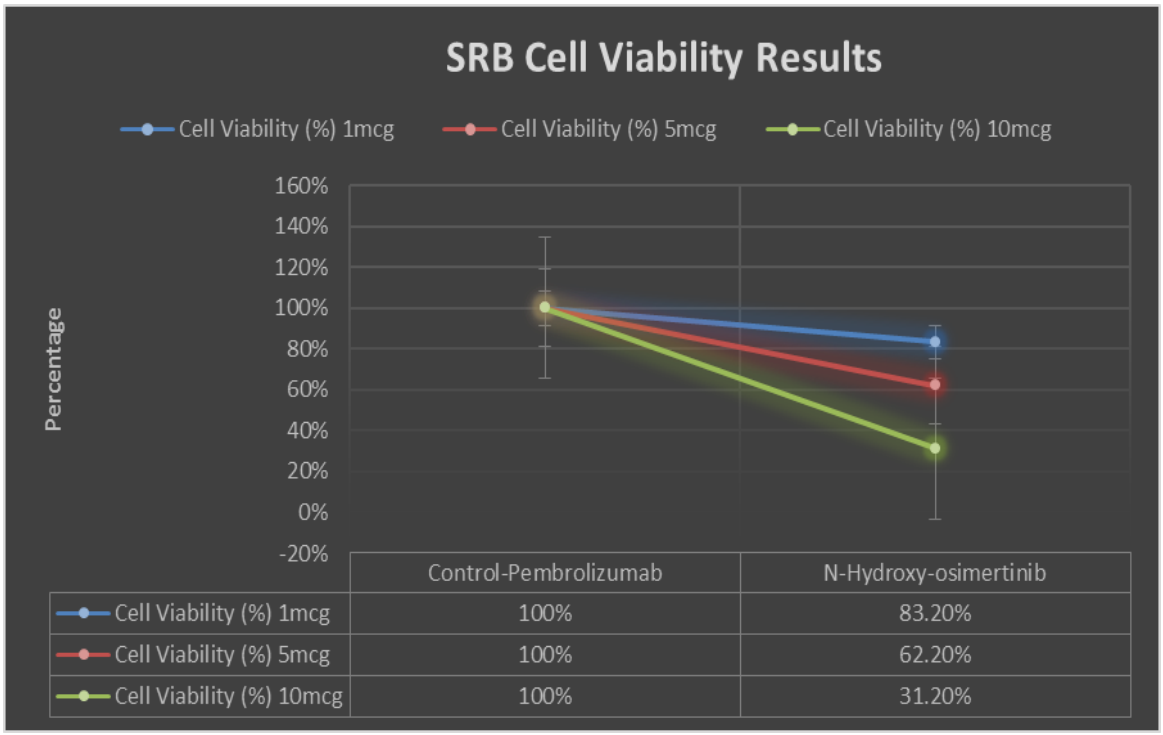




SRB Assay Results

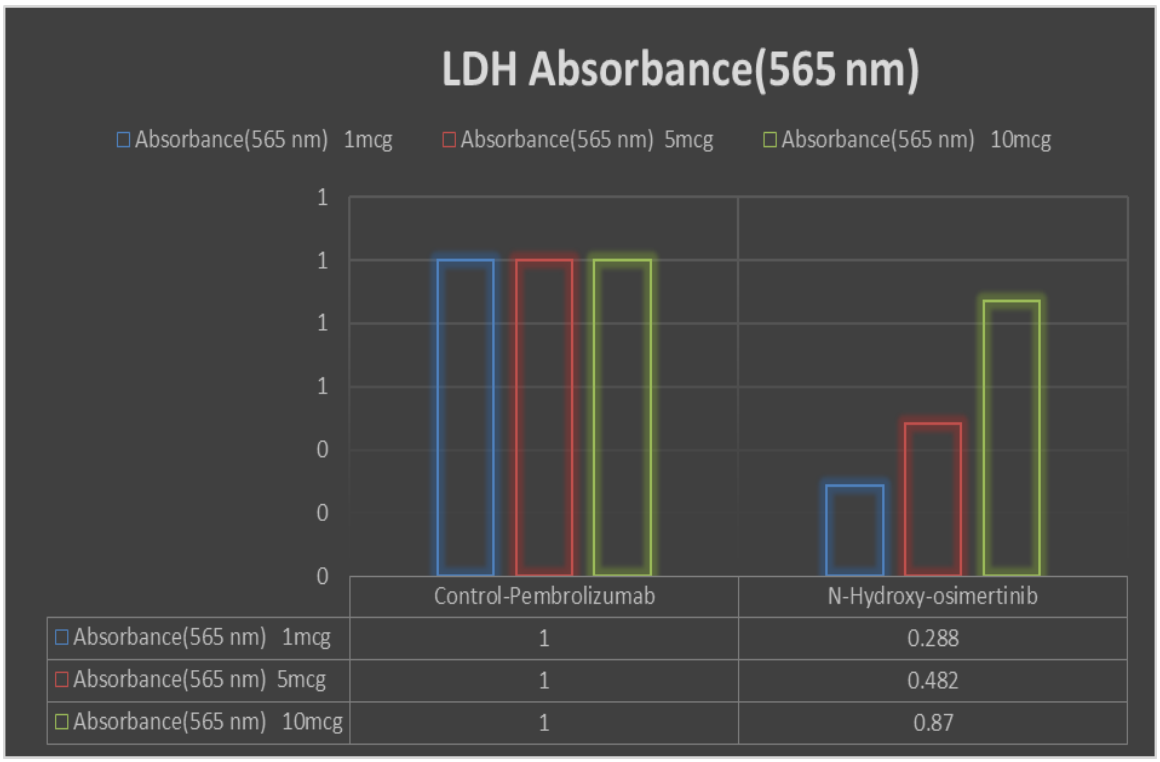
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
N-Hydroxy-osimertinib	1	0.832	83.2%
	5	0.622	62.2%
	10	0.312	31.2%

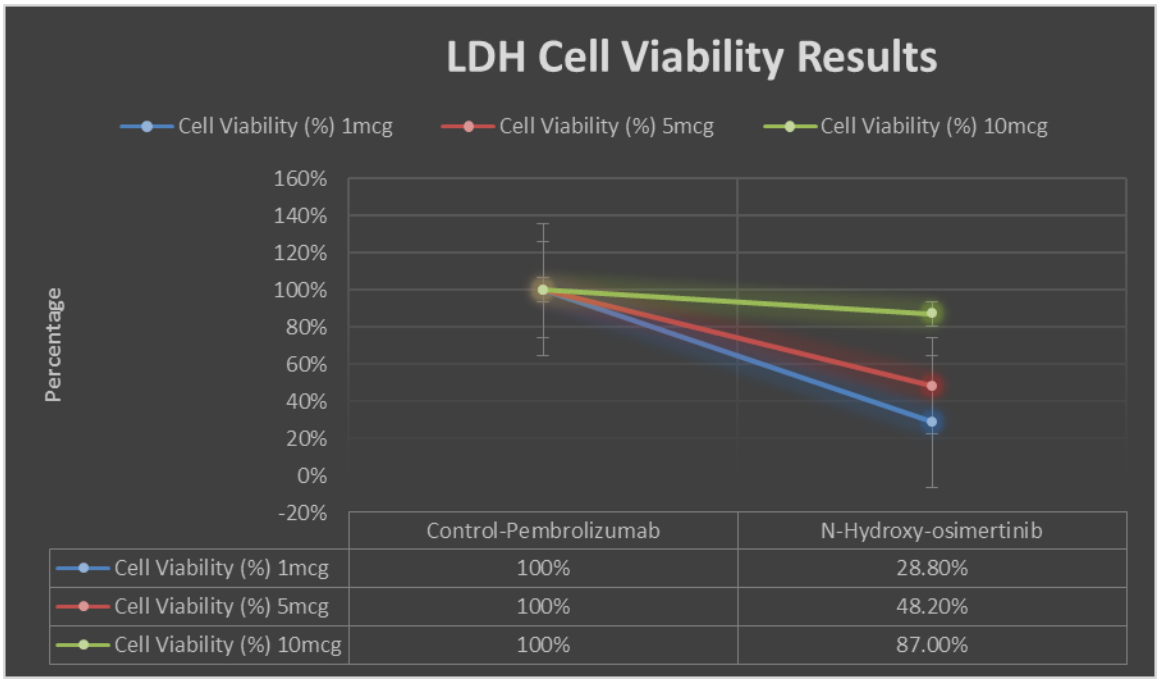




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Pembrolizumab)	-	1.000	100%
N-Hydroxy-osimertinib	1	0.288	28.8%
	5	0.482	48.2%
	10	0.870	87.0%





DISCUSSION

The results from the various assays indicate that N-Hydroxy-osimertinib has potent anti-proliferative and cytotoxic effects on kidney cancer cell lines, as demonstrated by a significant reduction in cell viability across all concentration levels tested (1 µM, 5 µM, and 10 µM). In the MTT assay, the cell viability decreased from 83.4% at 1 µM to 31.3% at 10 µM, illustrating a strong dose-dependent response. Similar trends were observed in the CellTiter-Glo and Alamar Blue assays, with the luminescence and fluorescence signals, respectively, showing a corresponding decline in cell viability. The SRB assay, which measures total protein content, also confirmed a decrease in cell density, indicating reduced cellular proliferation.

Interestingly, the LDH Cytotoxicity assay presented an inverse trend at higher concentrations, with cell viability increasing to 87% at 10 µM. This suggests that N-Hydroxy-osimertinib may induce necrosis or other forms of cell death that affect membrane integrity, which is not detected by the other viability assays. This discrepancy underlines the importance of utilizing multiple assay platforms to comprehensively evaluate cytotoxicity, as different assays can provide insights into distinct aspects of cell health and death mechanisms.

Overall, N-Hydroxy-osimertinib demonstrated significant cytotoxic effects, comparable to or exceeding those of Pembrolizumab, across most assays. These findings suggest that N-Hydroxy-osimertinib could be a promising candidate for further evaluation in NSCLC treatment strategies, particularly for patients with resistance to earlier-generation EGFR-TKIs.

CONCLUSION

N-Hydroxy-osimertinib exhibited strong dose-dependent cytotoxicity against kidney cancer cells, as confirmed by multiple in vitro assays. The compound demonstrated a significant decrease in cell viability and proliferation, with its activity increasing at higher concentrations. However, the results from the LDH assay suggest that N-Hydroxy-osimertinib may impact cell membrane integrity differently at elevated concentrations, indicating a possible shift in its mechanism of action. These findings highlight the potential of N-Hydroxy-osimertinib as a therapeutic agent for NSCLC and support its further exploration in combination therapies or as a standalone treatment option for EGFR-mutant cancers.

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