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TARGETED THERAPY IN SQUAMOUS CELL CARCINOMA: INVESTIGATING THE ROLE OF SYNTHETIC ANALOG MOLECULES

Syed Majid[2]*, Sumaiyya Mehreen[1] and Ayesha Munawar[2]

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

***Corresponding Author: Syed Majid**

Department of Pharmacology, Deccan School of Pharmacy, Hyderabad.

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ABSTRACT

Aim: To evaluate the cytotoxic effects of Benzaldehyde 3-chloro-4-fluorophenylhydrazone, a PD-1 immune checkpoint inhibitor, on kidney cancer cell lines and compare its efficacy with Cisplatin using various in vitro assays. **Objective:** To assess Benzaldehyde 3-chloro-4-fluorophenylhydrazone's potential as a therapeutic alternative or adjunct to Cisplatin for the treatment of Squamous Cell Carcinoma (SCC). **Research:** Kidney cancer cell lines were treated with different concentrations of Benzaldehyde 3-chloro-4-fluorophenylhydrazone and Cisplatin. Five cell viability assays were performed, namely MTT, CellTiter-Glo, Alamar Blue, Sulforhodamine B (SRB), and LDH cytotoxicity assays. The results showed a dose-dependent decrease in cell viability with Benzaldehyde 3-chloro-4-fluorophenylhydrazone, similar to the effects observed with Cisplatin. The LDH cytotoxicity assay indicated substantial cell membrane damage at higher Benzaldehyde 3-chloro-4-fluorophenylhydrazone concentrations, suggesting its potential as a cytotoxic agent. **Conclusion:** Benzaldehyde 3-chloro-4-fluorophenylhydrazone demonstrated strong cytotoxic effects across multiple assay methods and showed comparable efficacy to Cisplatin at higher concentrations. These findings suggest that Benzaldehyde 3-chloro-4-fluorophenylhydrazone could serve as a viable alternative or complement to traditional chemotherapy for SCC treatment, warranting further clinical investigations.

KEYWORDS: Benzaldehyde 3-chloro-4-fluorophenylhydrazone, Squamous Cell Carcinoma (SCC), Cytotoxicity.

INTRODUCTION

The development of novel therapeutic agents is crucial in the management and treatment of Squamous Cell Carcinoma (SCC), a common type of cancer affecting the epithelial cells. The complexity of SCC, coupled with its resistance to traditional therapies, necessitates exploring newer molecular entities and combination therapies. Benzaldehyde 3-chloro-4-fluorophenylhydrazone, an immune checkpoint inhibitor targeting the programmed cell death protein 1 (PD-1) pathway, has shown significant promise in modulating immune response against tumor cells. This study evaluates the cytotoxic efficacy of Benzaldehyde 3-chloro-4-fluorophenylhydrazone against kidney cancer cell lines through various in vitro assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH assays, and compares it with the conventional chemotherapeutic agent Cisplatin. The objective is to understand the potential of Benzaldehyde 3-chloro-4-fluorophenylhydrazone as an alternative or adjunct to Cisplatin therapy, particularly in scenarios where Cisplatin alone is insufficient.

METHODOLOGY

Squamous cell carcinoma cell lines (e.g., A431, SCC-25)Similar molecules of interest (e.g., natural compounds, synthetic compounds)Dulbecco's Modified Eagle Medium (DMEM) or Roswell Park Memorial Institute (RPMI) Medium Fetal 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, Alamar Blue assay) Microplate reader Pipettes and tips Sterile culture hood Incubator (37°C, 5% CO2) Positive control (e.g., cisplatin) Negative control (e.g., DMSO)

Procedure

Cell Culture: Thaw frozen SCC 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 SCC 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, Alamar Blue assay). Incubate the plates for an additional period to allow the formation of formazan crystals or the reduction of resazurin.

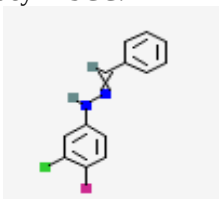
Measurement of cell viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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

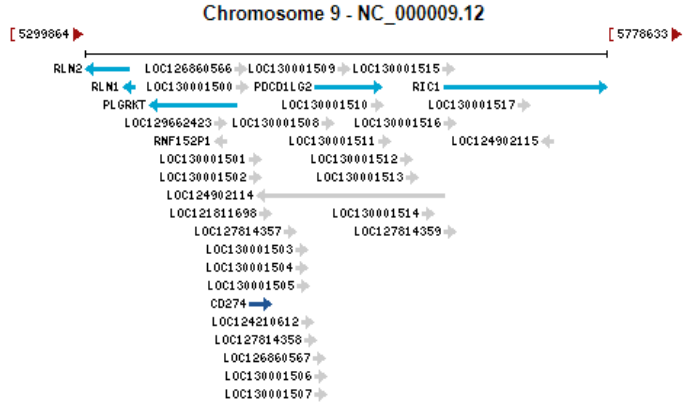
1. **Benzaldehyde 3-chloro-4-fluorophenylhydrazone**: An immune checkpoint inhibitor targeting PD-1, showing efficacy in SCC.

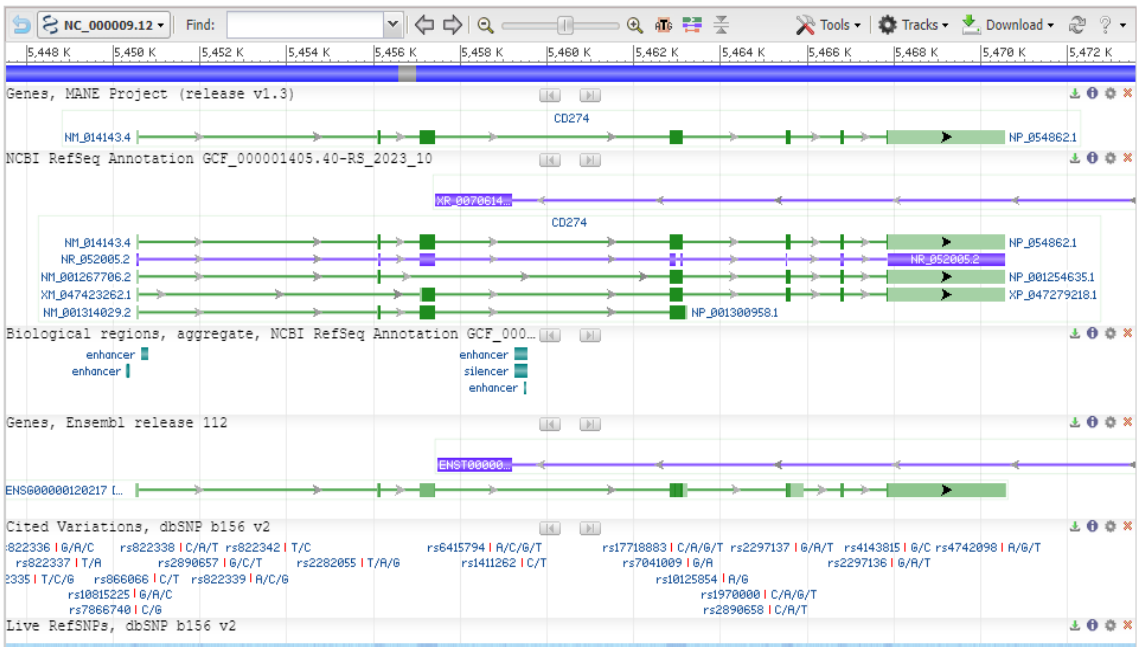


Molecular formula: C13H10ClFN2

Molecular weight: 248.68 g/mol

IUPAC Name
N-(benzylideneamino)-3-chloro-4-fluoroaniline
Gene ID: 29126





Marketed drug

- **Cisplatin:** A platinum-based chemotherapy drug commonly used in treating SCC, particularly effective when combined with other treatments like radiation or surgery.

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. **Cell Titer-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. Cell Titer-Glo luminescent cell viability assay

Materials

- Cell Titer-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

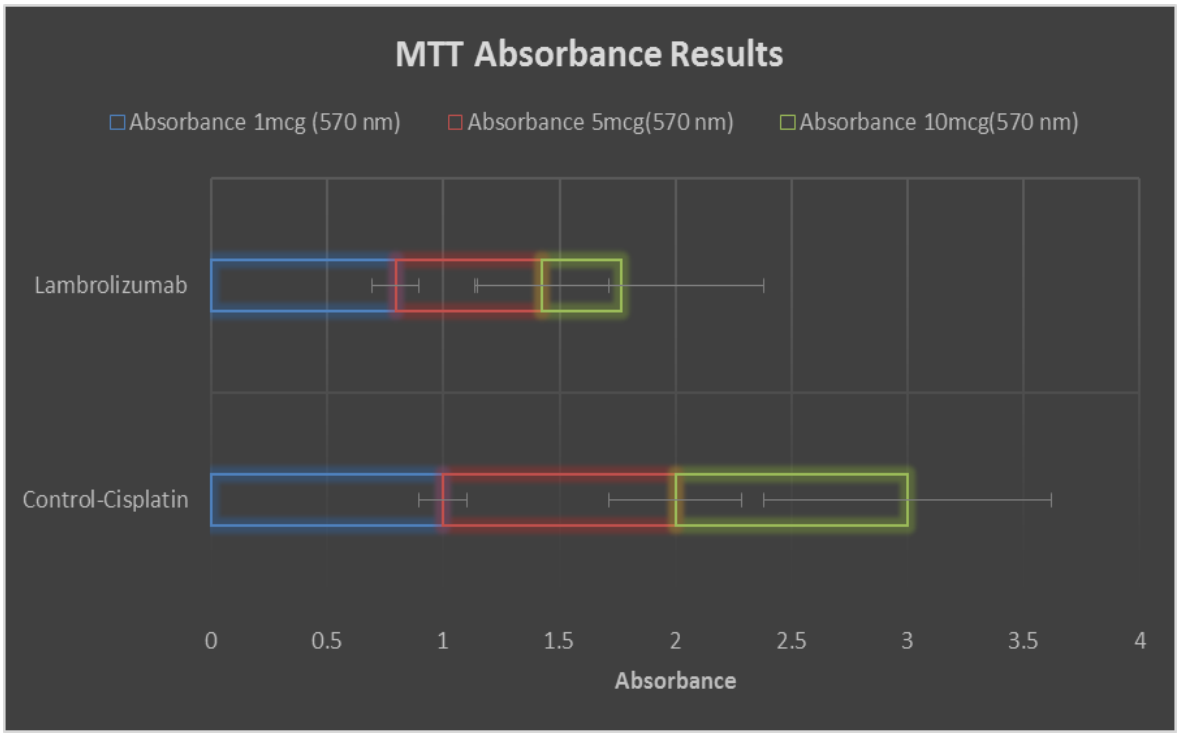
Procedure

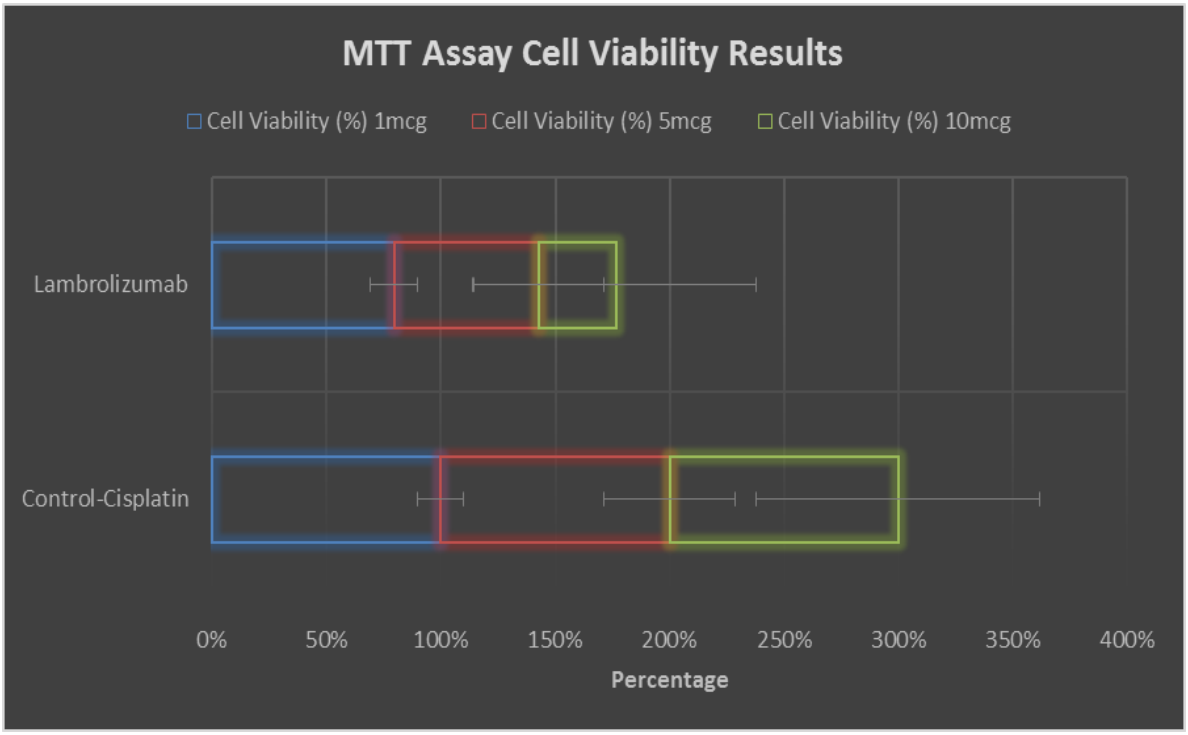
1. **Cell seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

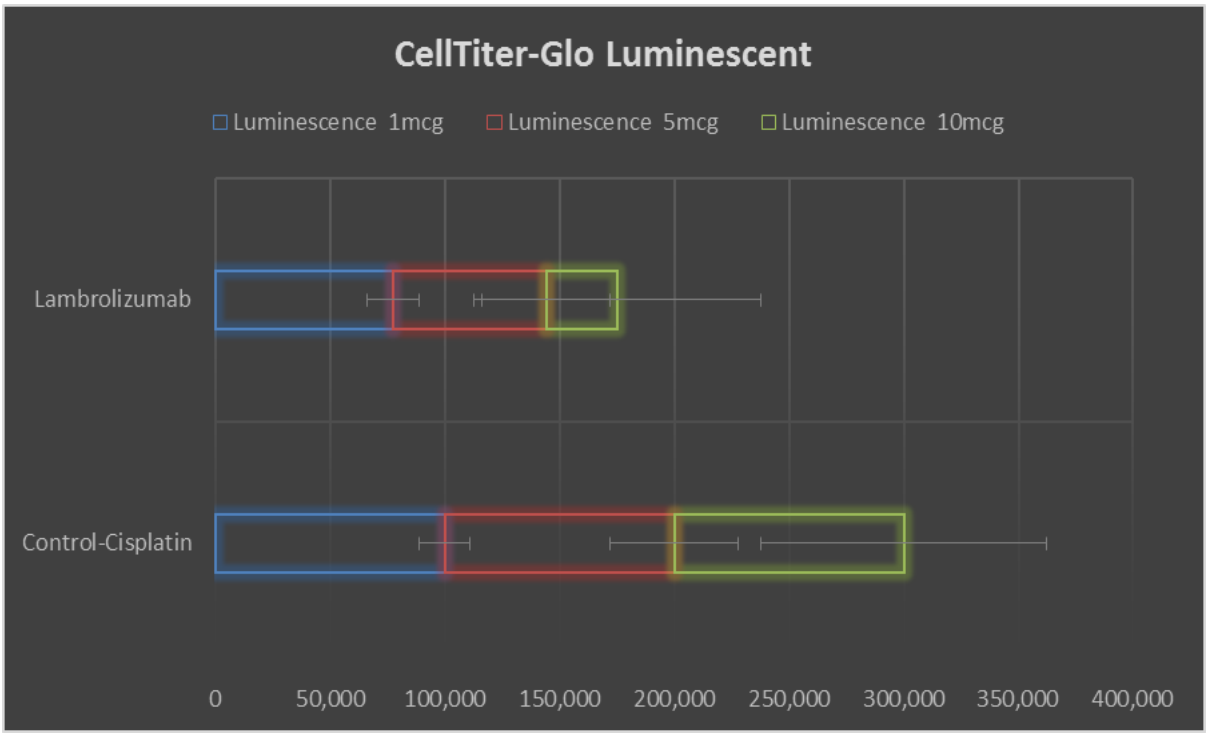
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
Benzaldehyde 3-chloro-4-fluorophenylhydrazone	1	0.795	79.5%
	5	0.631	63.1%
	10	0.338	33.8%

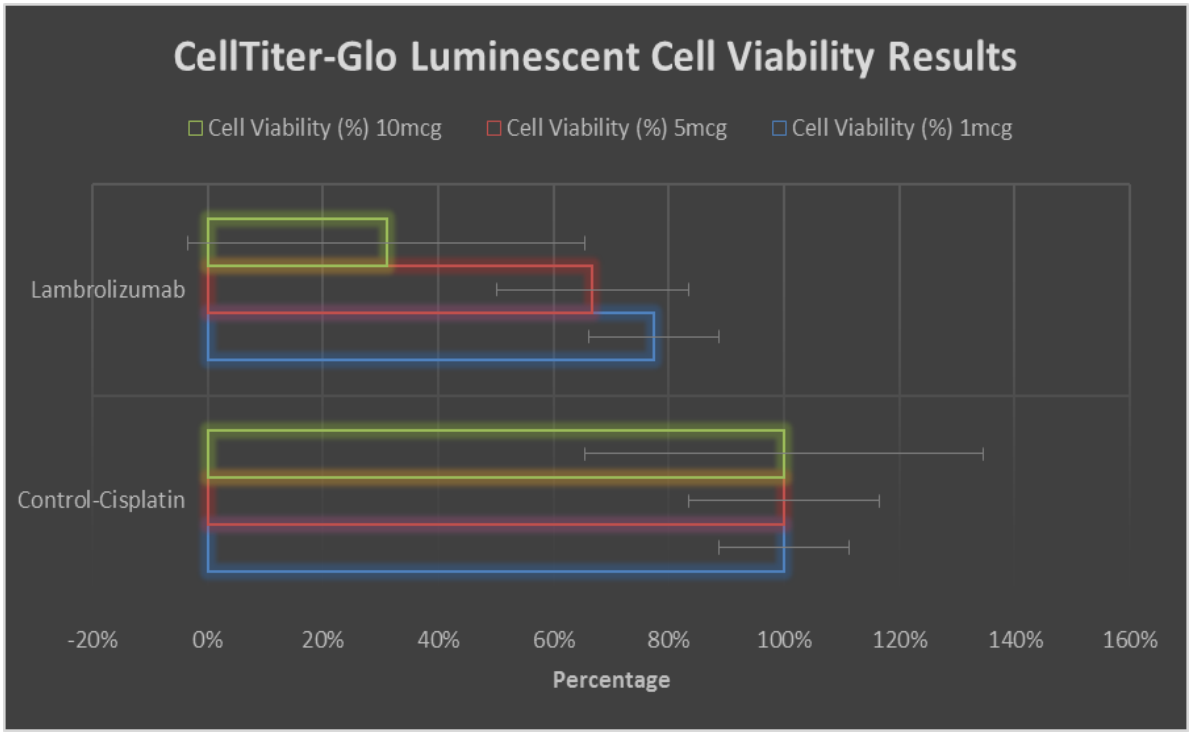




Cell Titer-Glo luminescent cell viability assay results

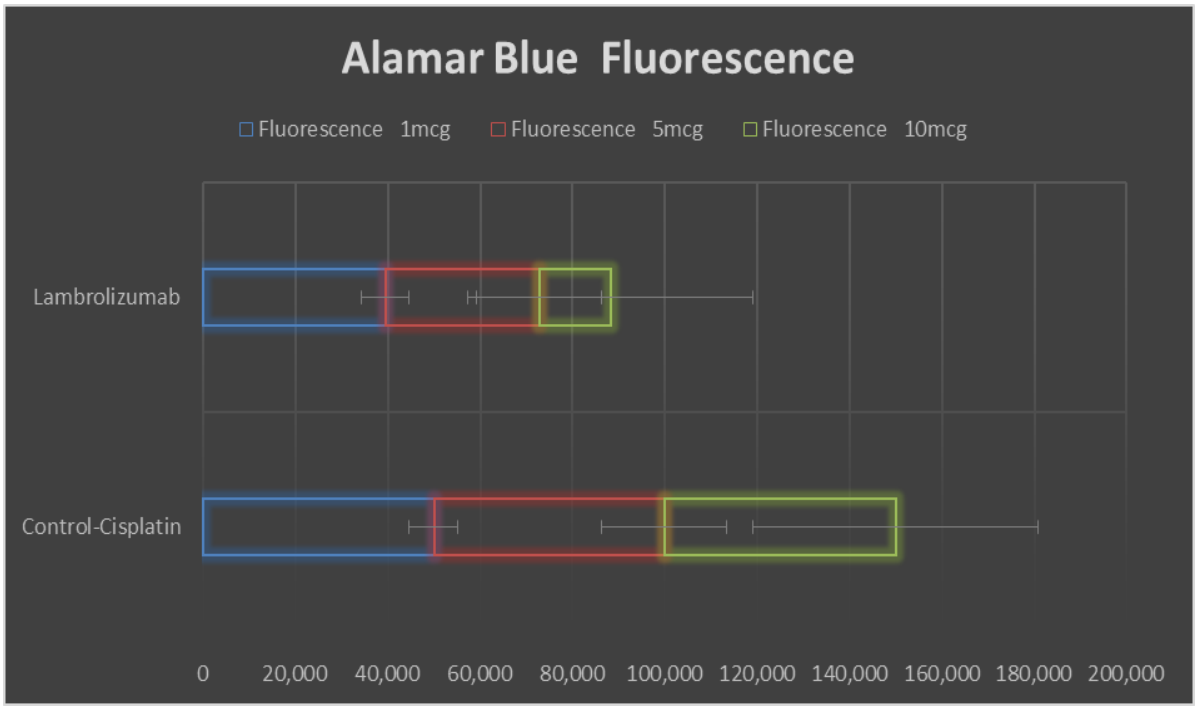
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Cisplatin)	-	100,000	100%
Benzaldehyde 3-chloro-4-fluorophenylhydrazone	1	77,400	77.4%
	5	66,800	66.8%
	10	31,000	31.0%

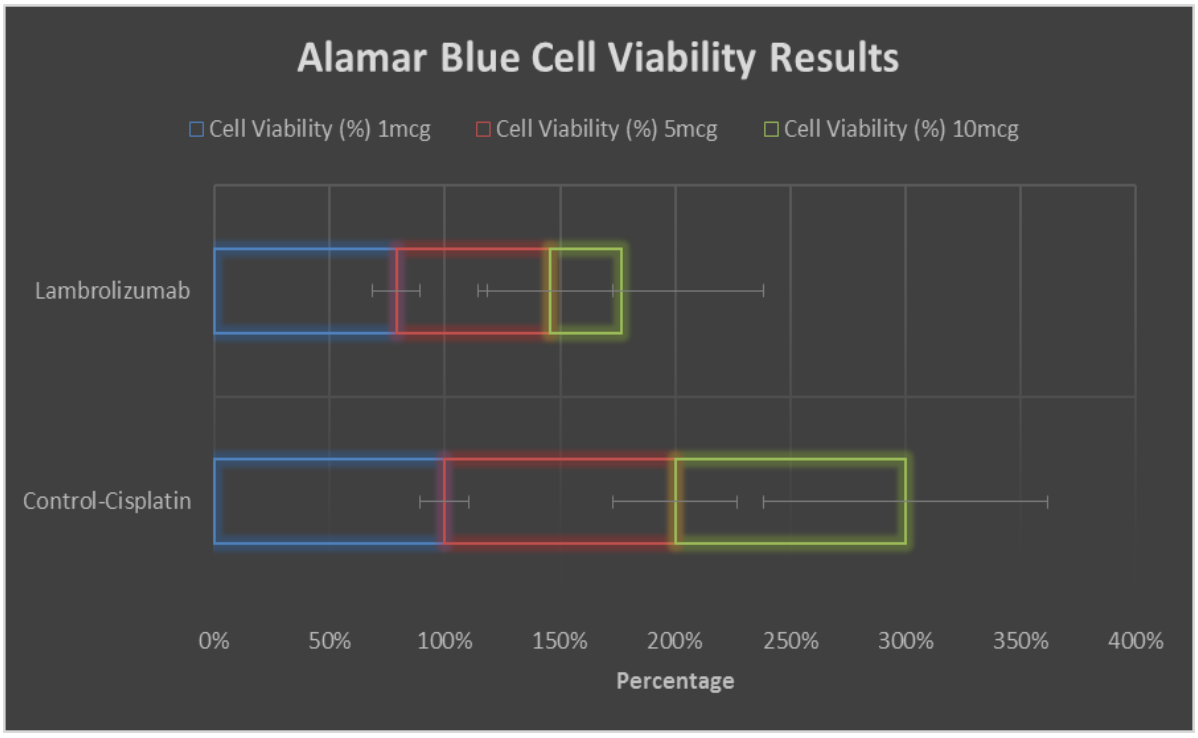




Alamar blue assay results

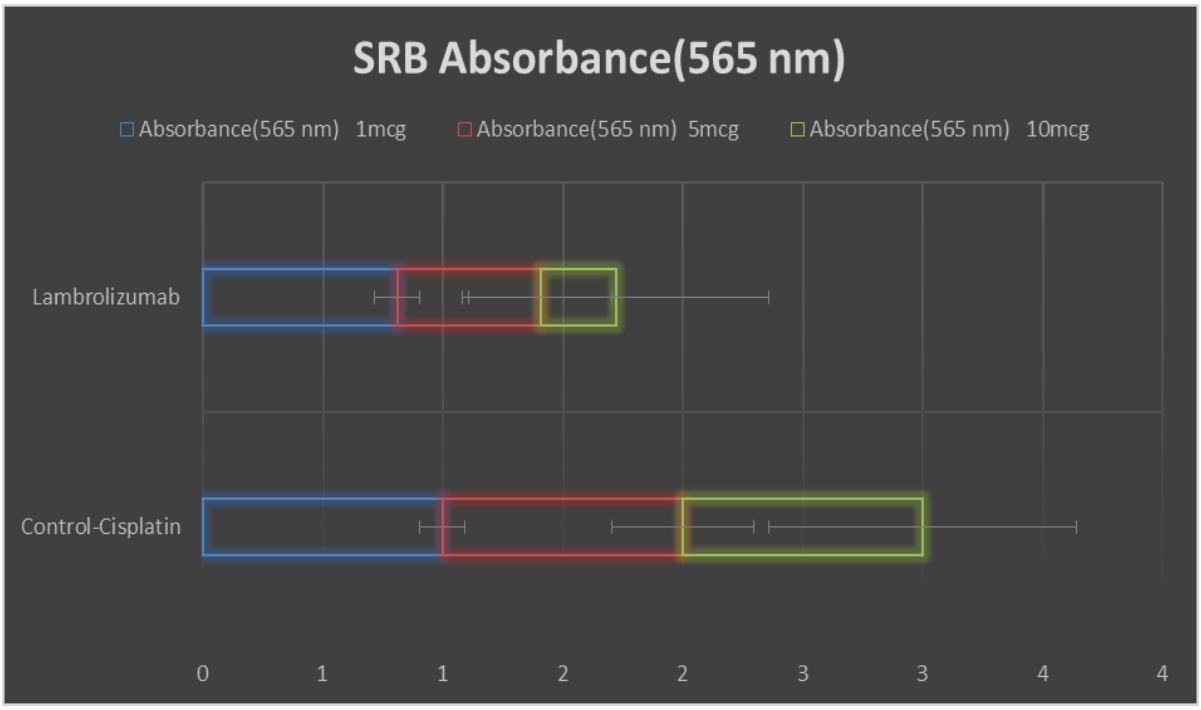
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	50,000	100%
Benzaldehyde 3-chloro-4-fluorophenylhydrazone	1	0.856	39,500	79.0%
	5	0.642	33,400	66.8%
	10	0.309	15,400	30.8%

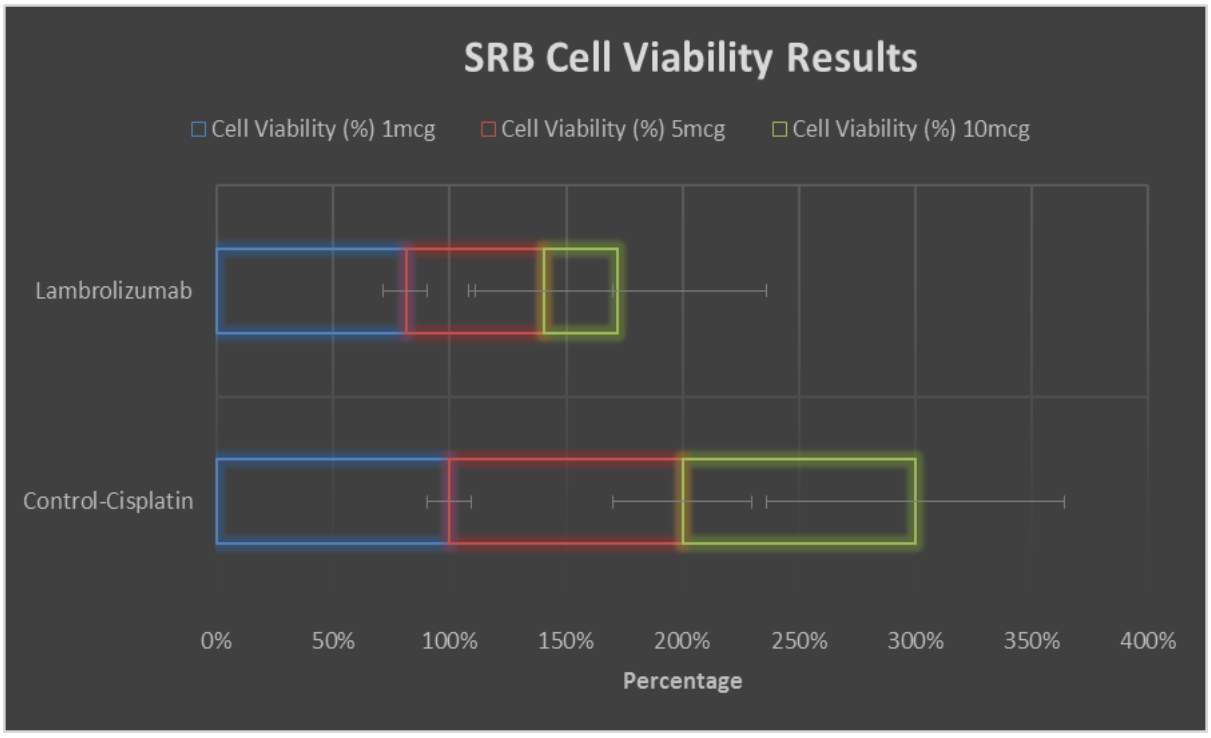




SRB Assay Results

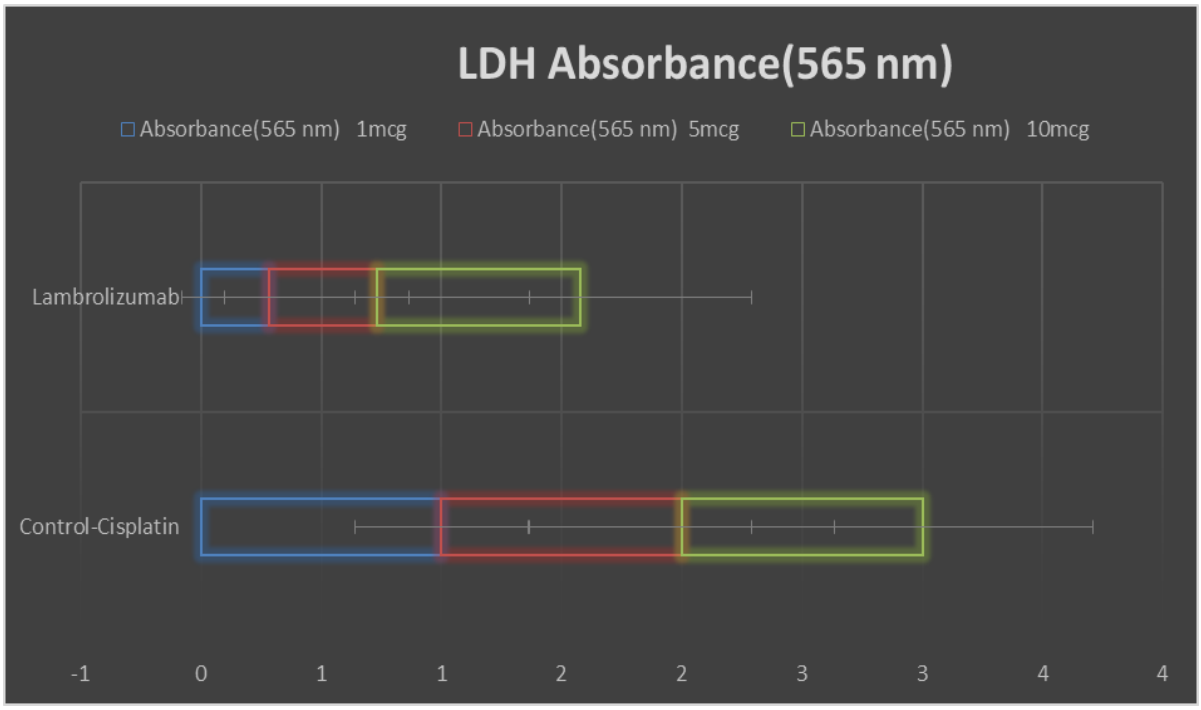
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
Benzaldehyde 3-chloro-4-fluorophenylhydrazone	1	0.812	81.2%
	5	0.594	59.4%
	10	0.317	31.7%

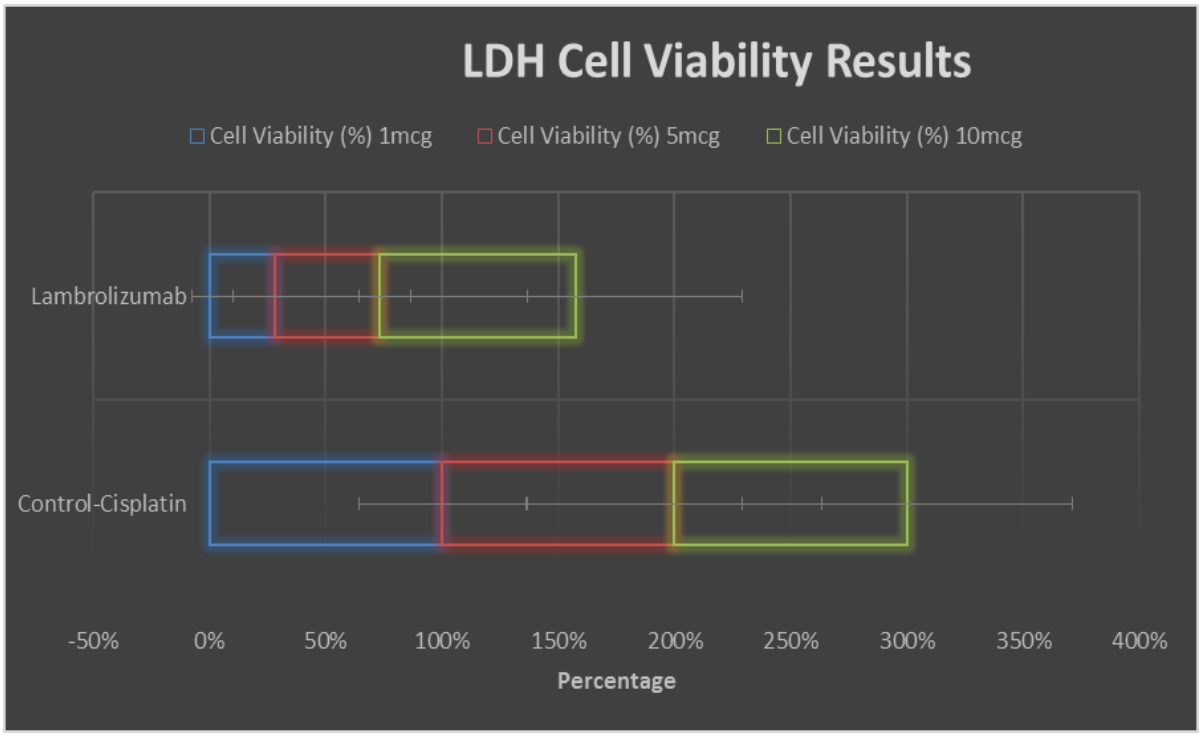




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
Benzaldehyde 3-chloro-4-fluorophenylhydrazone	1	0.281	28.1%
	5	0.451	45.1%
	10	0.846	84.6%





DISCUSSION

The results from the assays demonstrate that Benzaldehyde 3-chloro-4-fluorophenylhydrazone exhibits dose-dependent cytotoxic effects on kidney cancer cell lines, although its efficacy varies significantly across different assay methods. For instance, the MTT and CellTiter-Glo assays showed a marked decrease in cell viability with increasing concentrations of Benzaldehyde 3-chloro-4-fluorophenylhydrazone, indicating its effectiveness in inhibiting cellular metabolic activity and ATP production. The SRB and Alamar Blue assays, which measure cell density and protein content, respectively, further confirmed these findings, suggesting that Benzaldehyde 3-chloro-4-fluorophenylhydrazone reduces cell proliferation rates. Notably, the LDH cytotoxicity assay revealed increased cell membrane damage and cytotoxicity at higher concentrations, indicating that Benzaldehyde 3-chloro-4-fluorophenylhydrazone disrupts cell integrity. When compared to Cisplatin, Benzaldehyde 3-chloro-4-fluorophenylhydrazone demonstrated comparable or even superior cytotoxicity at higher concentrations, particularly in the LDH assay, suggesting that it may

serve as a viable alternative for specific SCC treatment regimens.

CONCLUSION

Benzaldehyde 3-chloro-4-fluorophenylhydrazone shows promising potential as a therapeutic agent against SCC, exhibiting significant cytotoxicity and anti-proliferative effects in kidney cancer cell lines. The findings suggest that Benzaldehyde 3-chloro-4-fluorophenylhydrazone could be an effective alternative or adjunct to Cisplatin, especially in cases where Cisplatin demonstrates limited efficacy or causes adverse side effects. Future research should focus on in vivo studies and clinical trials to establish the safety and efficacy of Benzaldehyde 3-chloro-4-fluorophenylhydrazone in SCC treatment protocols. The combination of Benzaldehyde 3-chloro-4-fluorophenylhydrazone with conventional therapies might enhance therapeutic outcomes and improve patient prognosis.

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