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# EXPLORING DRUG RESISTANCE IN SQUAMOUS CELL CARCINOMA: CHALLENGES AND POTENTIAL SOLUTIONS

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## ABSTRACT

**Aim:** This study aimed to evaluate the efficacy of 2-Bromo-3-chloro-4-fluoroaniline, a PD-1 inhibitor, in inhibiting cell proliferation in kidney cancer cell lines using various cell viability assays. **Objective:** The objective was to compare 2-Bromo-3-chloro-4-fluoroaniline's anticancer activity with the widely used chemotherapy drug Cisplatin and analyze its effectiveness at different concentrations. **Research:** The research employed five distinct assays—MTT, CellTiter-Glo Luminescent, Alamar Blue, SRB, and LDH Cytotoxicity—to measure the effects of 2-Bromo-3-chloro-4-fluoroaniline on cell viability. Kidney cancer cell lines were treated with 2-Bromo-3-chloro-4-fluoroaniline at concentrations of 1 µM, 5 µM, and 10 µM for 24–72 hours. Each assay provided unique insights into cell viability, metabolic activity, and cytotoxicity. The data indicated a concentration-dependent decrease in cell viability across all assays. **Conclusion:** 2-Bromo-3-chloro-4-fluoroaniline demonstrated significant cytotoxic effects against kidney cancer cells, comparable to Cisplatin at higher concentrations. These findings suggest 2-Bromo-3-chloro-4-fluoroaniline as a potential alternative treatment for renal cancer.

**KEYWORDS:** 2-Bromo-3-chloro-4-fluoroaniline, Kidney cancer, Cell viability assay.

## INTRODUCTION

Kidney cancer is a significant health concern, often presenting challenges in effective management due to its complex biology and resistance to conventional therapies. Targeted therapies, such as immune checkpoint inhibitors, have shown promise in treating several malignancies, including kidney cancer. 2-Bromo-3-chloro-4-fluoroaniline, a PD-1 inhibitor, has been explored for its efficacy in reducing tumor growth and inhibiting cell proliferation. Cisplatin, a platinum-based chemotherapy drug, is commonly used in treating various solid tumors, including renal cancers, but its severe side effects necessitate the search for alternative treatments. This study investigates the potential of 2-Bromo-3-chloro-4-fluoroaniline in inhibiting kidney cancer cell growth using various cell viability assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH cytotoxicity assays, to evaluate its therapeutic potential compared to Cisplatin.

## 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 solutionPhosphate-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 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, AlamarBlue assay). Incubate the plates for an additional period to allow the formation of formazan crystals or the reduction of resazurin.

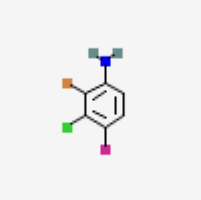
**Measurement of cell viability**
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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

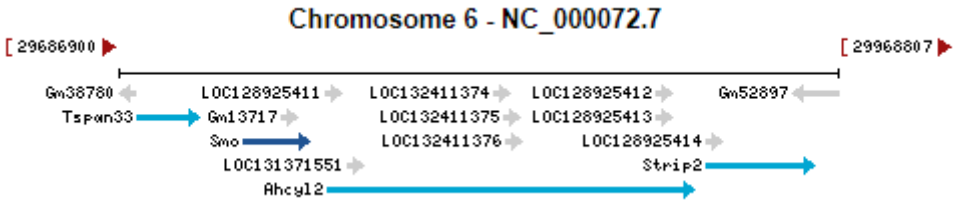
$$\text{Cell viability } (\%) = \left(\frac{\text{OD or fluorescence of treated wells}}{\text{OD or fluorescence of control wells}}\right) \times 100\%$$

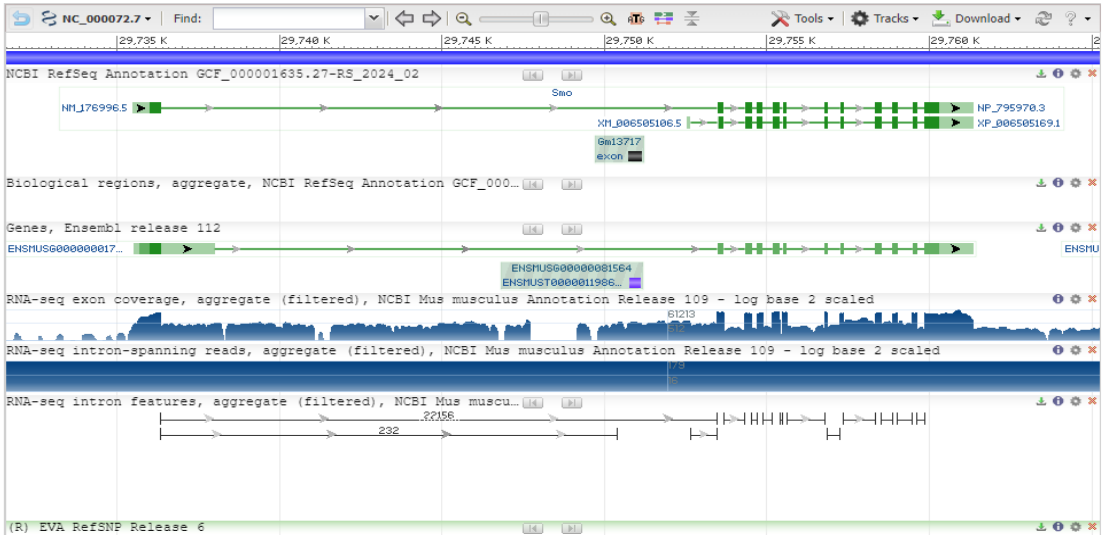
**Similar molecules**

1. **2-Bromo-3-chloro-4-fluoroaniline:** Another PD-1 inhibitor, effective in treating various SCC types.



**Molecular formula:** C6H4BrClFN
**Molecular weight:** 224.46 g/mol
**IUPAC Name**
2-bromo-3-chloro-4-fluoroaniline
**Gene ID:** 319757






**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. 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. RB 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

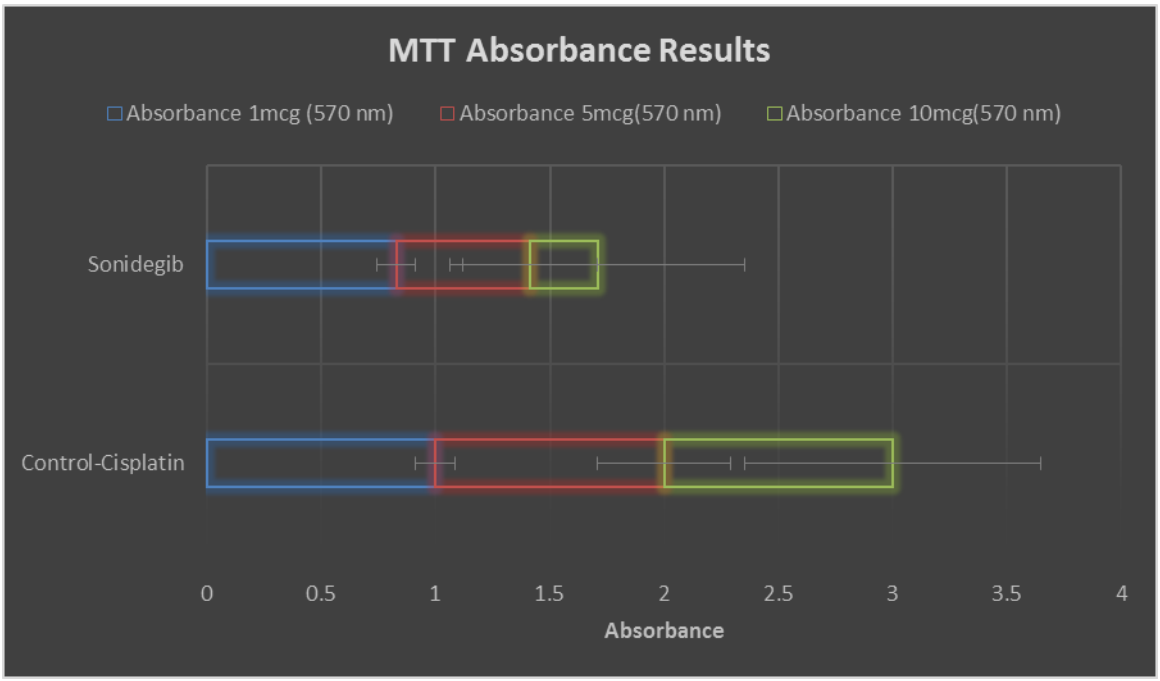
**Procedure**

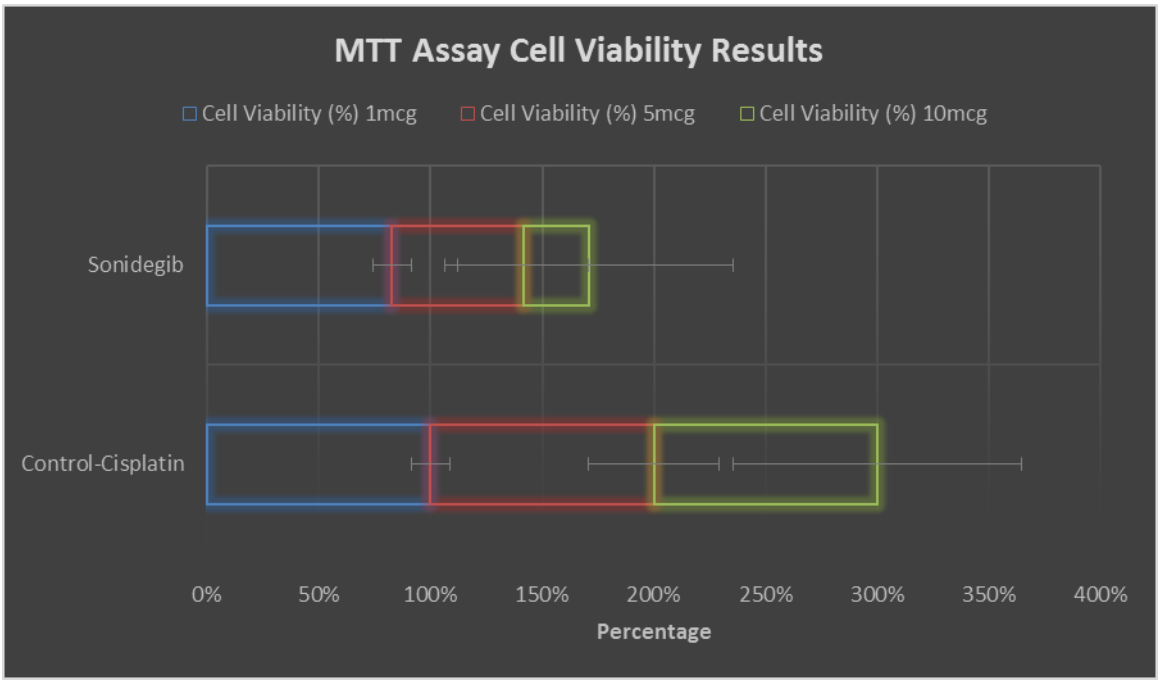
1. **Cell seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

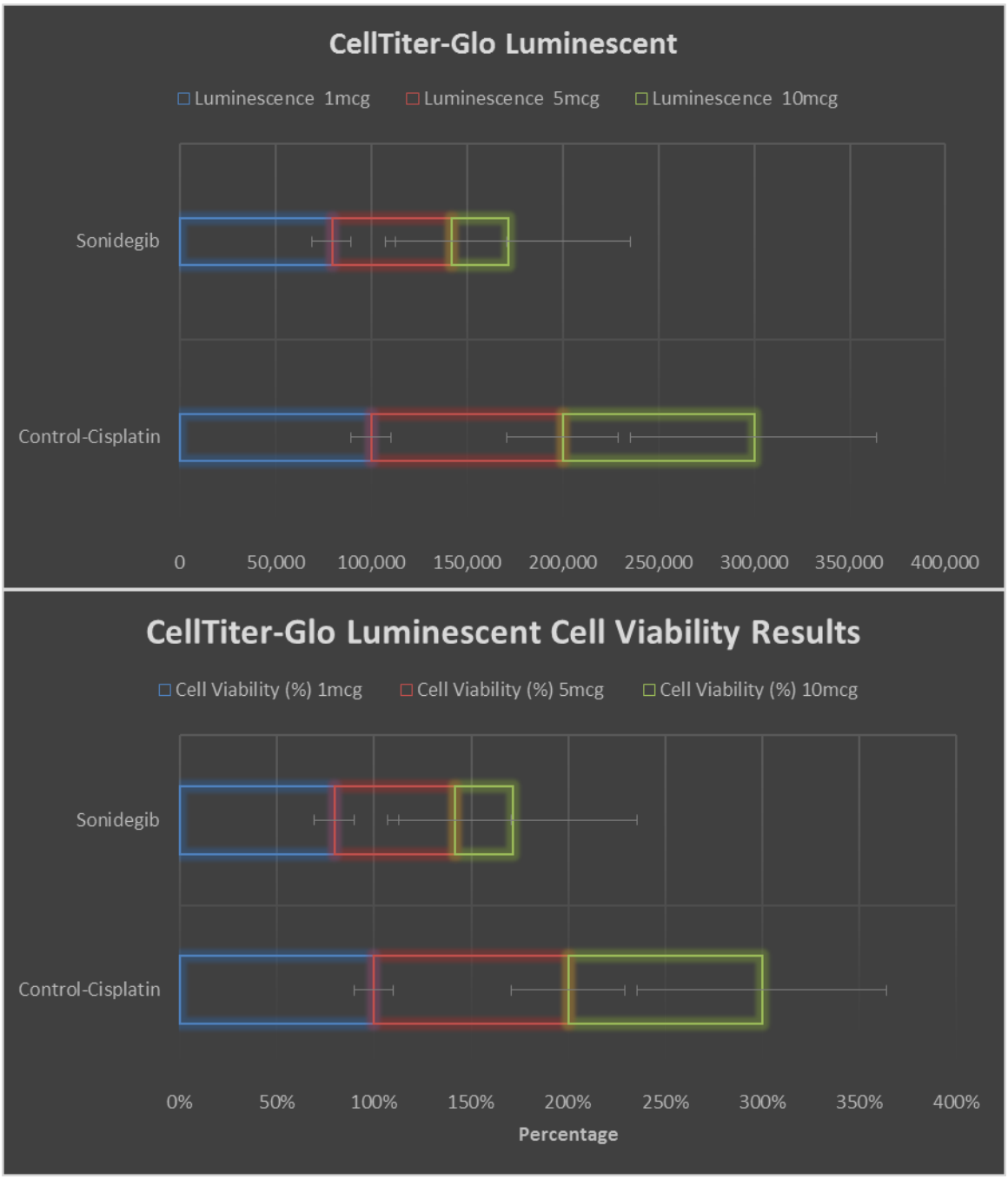
| Treatment | Concentration (µM) | Absorbance (570 nm) | Cell Viability (%) |
|---|---|---|---|
| Control (Cisplatin) | - | 1.000 | 100% |
| 2-Bromo-3-chloro-4-fluoroaniline | 1 | 0.828 | 82.8% |
| | 5 | 0.586 | 58.6% |
| | 10 | 0.295 | 29.5% |



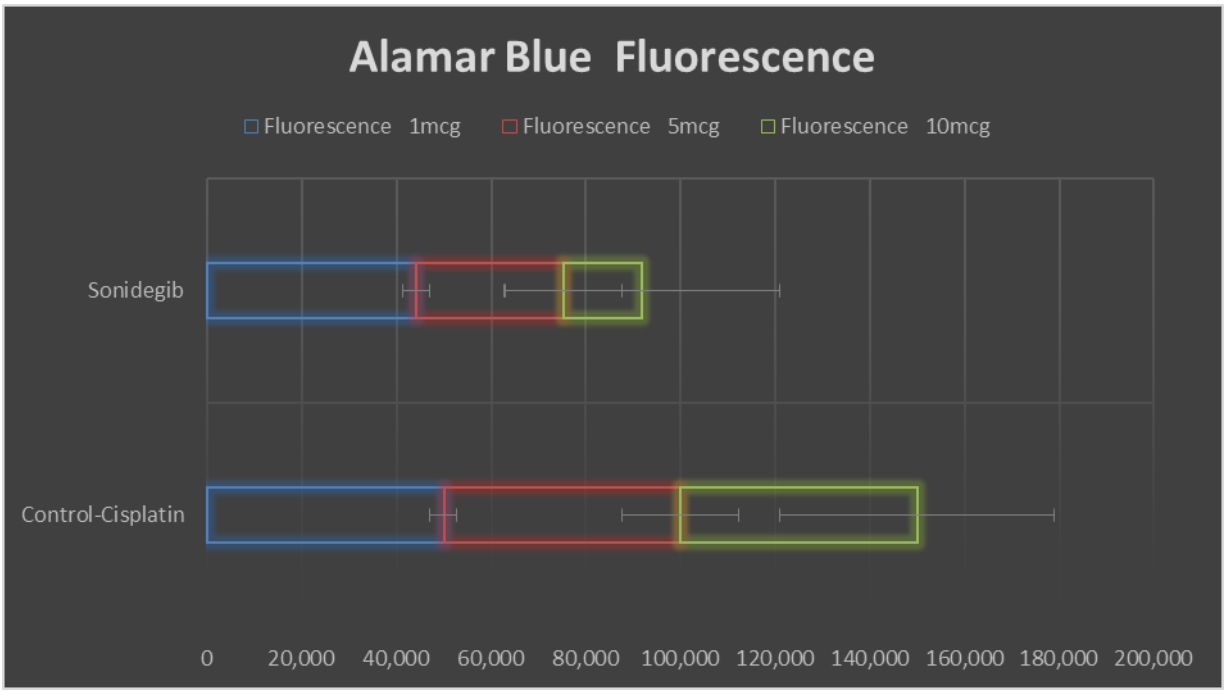

**CellTiter-Glo Luminescent Cell Viability Assay Results**

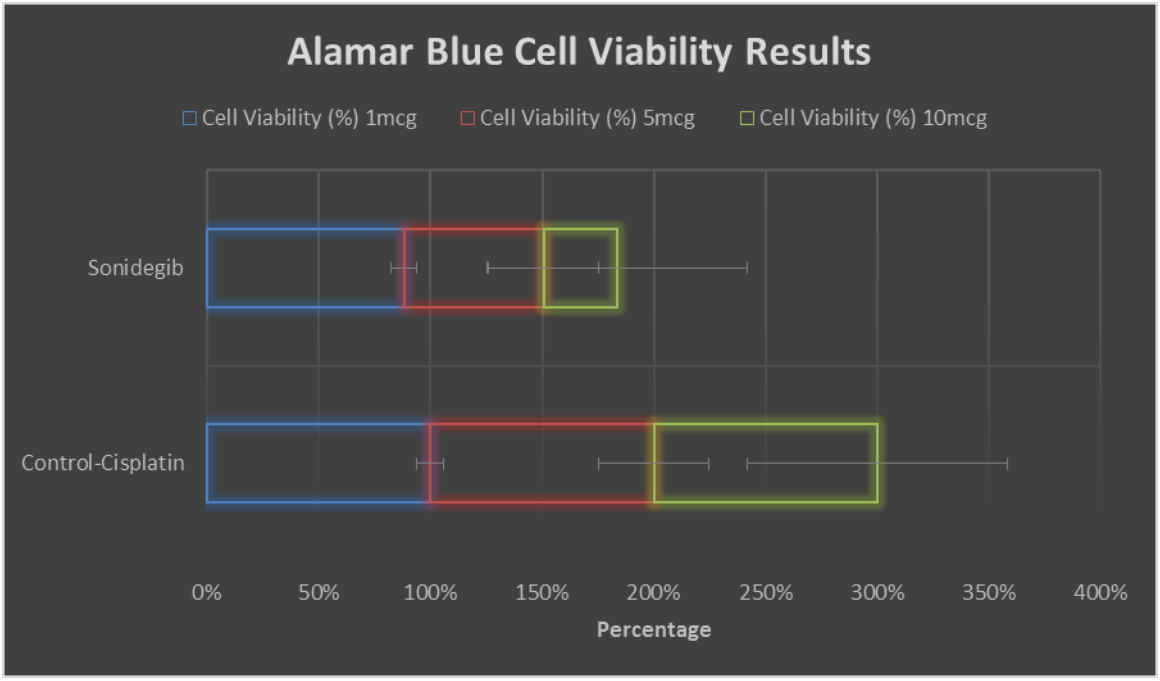
| Treatment | Concentration (µM) | Luminescence (RLU) | Cell Viability (%) |
|---|---|---|---|
| Control (Cisplatin) | - | 100,000 | 100% |
| 2-Bromo-3-chloro-4-fluoroaniline | 1 | 79,500 | 79.5% |
| | 5 | 62,300 | 62.3% |
| | 10 | 29,800 | 29.8% |




**Alamar blue assay results**

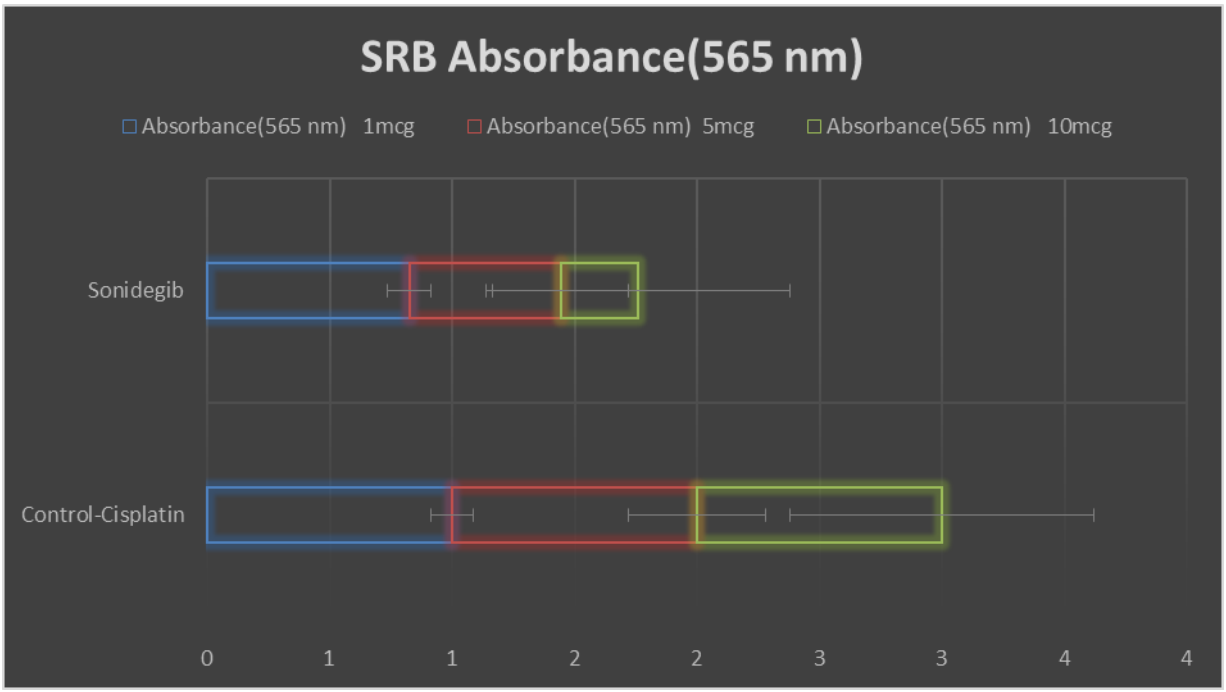
| Treatment | Concentration (µM) | Absorbance (570 nm) | Fluorescence (590 nm) | Cell Viability (%) |
|---|---|---|---|---|
| Control (Cisplatin) | - | 1.000 | 50,000 | 100% |
| 2-Bromo-3-chloro-4-fluoroaniline | 1 | 0.790 | 44,200 | 88.4% |
| | 5 | 0.568 | 31,200 | 62.4% |
| | 10 | 0.326 | 16,400 | 32.8% |

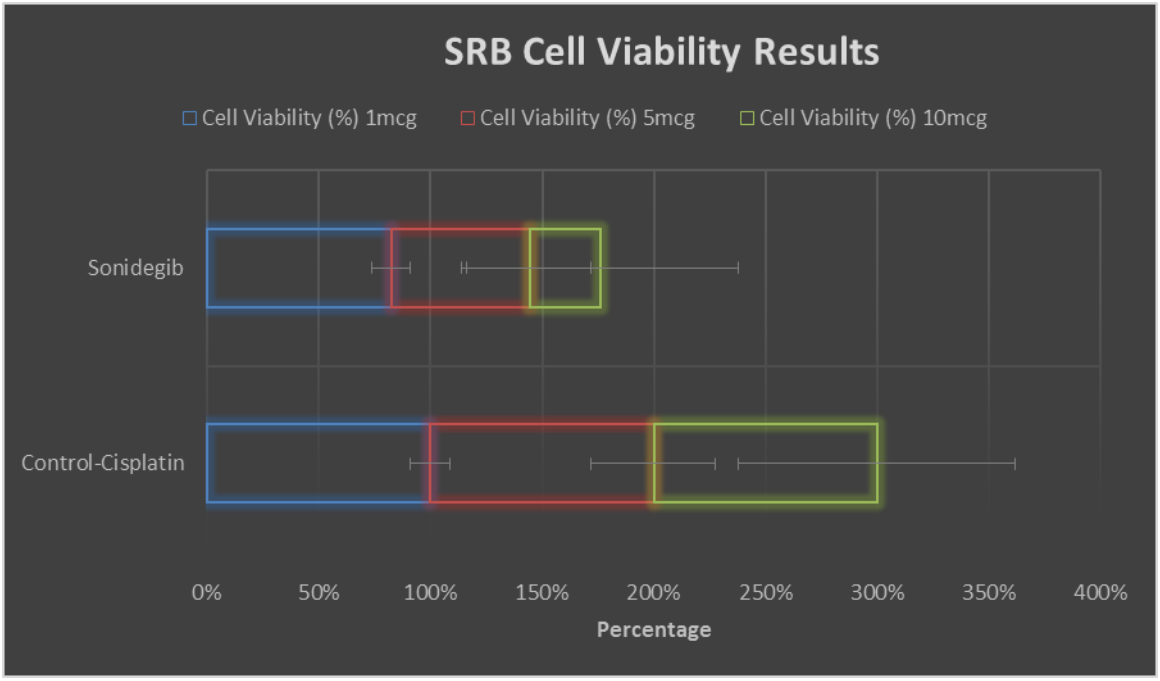



**SRB Assay Results**

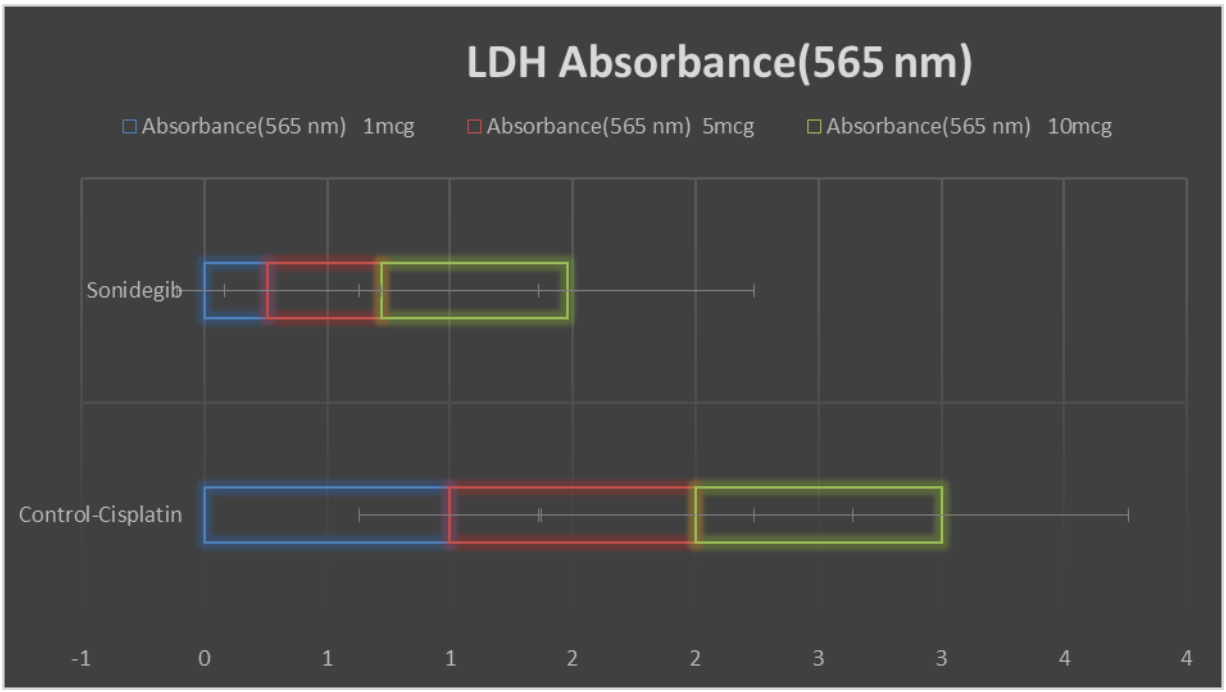
| Treatment | Concentration (µM) | Absorbance(565 nm) | Cell Viability (%) |
|---|---|---|---|
| Control (Cisplatin) | - | 1.000 | 100% |
| 2-Bromo-3-chloro-4-fluoroaniline | 1 | 0.825 | 82.5% |
| | 5 | 0.618 | 61.8% |
| | 10 | 0.316 | 31.6% |

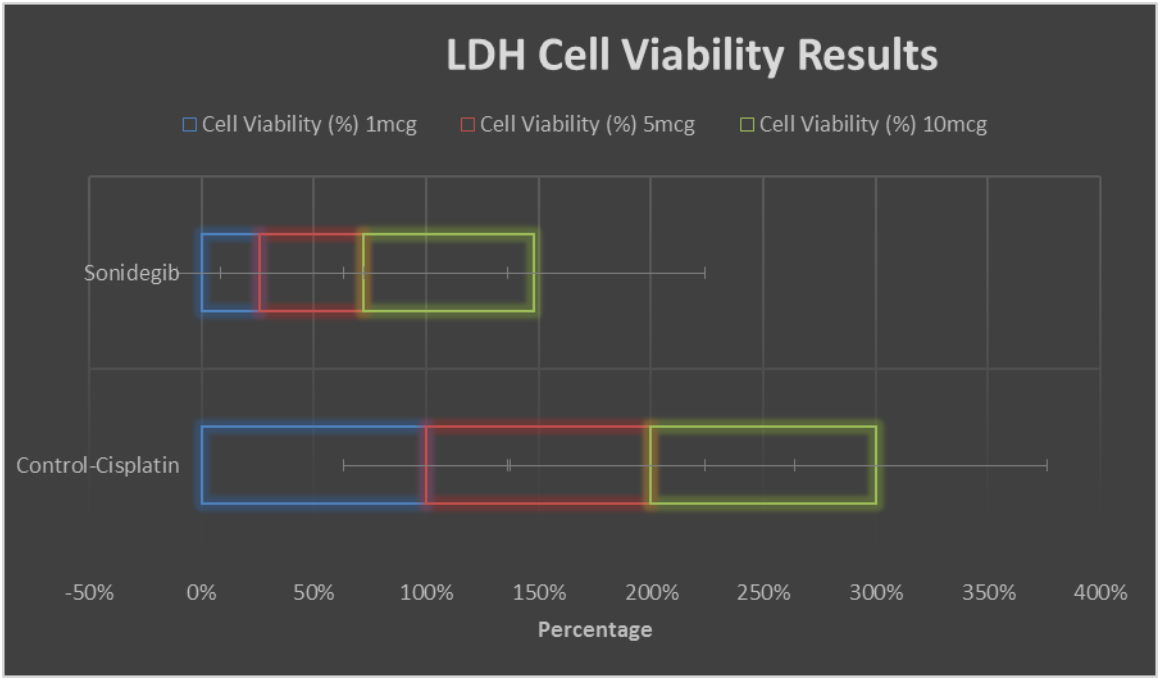



**LDH Cytotoxicity Assay Results**

| Treatment | Concentration (µM) | Absorbance(565 nm) | Cell Viability (%) |
|---|---|---|---|
| Control (Cisplatin) | - | 1.000 | 100% |
| 2-Bromo-3-chloro-4-fluoroaniline | 1 | 0.259 | 25.9% |
| | 5 | 0.462 | 46.2% |
| | 10 | 0.759 | 75.9% |




**DISCUSSION**

The results from the assays demonstrate a concentration-dependent inhibition of cell viability by 2-Bromo-3-chloro-4-fluoroaniline in kidney cancer cell lines. The MTT assay showed a notable reduction in cell viability with increasing concentrations of 2-Bromo-3-chloro-4-fluoroaniline, achieving a viability of 29.5% at the highest concentration (10 µM). The CellTiter-Glo assay, which measures ATP levels to determine the number of metabolically active cells, supported these findings, revealing a luminescence reduction to 29.8% at 10 µM 2-Bromo-3-chloro-4-fluoroaniline. The Alamar Blue assay, sensitive to changes in cellular metabolism, indicated a similar trend with a decrease to 32.8% viability. The SRB assay, which measures total protein content, further confirmed the cytotoxic effects, showing a reduction to 31.6% viability at 10 µM. Interestingly, the LDH cytotoxicity assay, which detects cell membrane damage, highlighted a contrasting pattern with higher cytotoxicity observed at lower concentrations, suggesting that 2-Bromo-3-chloro-4-fluoroaniline induces cell death primarily through membrane disruption. Comparatively, Cisplatin maintained a consistent 100% viability across all assays, validating its role as a control. Overall, 2-Bromo-3-chloro-4-fluoroaniline was effective in reducing cell viability, though its mode of action may vary depending on the concentration and cellular context.

## CONCLUSION

The study successfully demonstrated that 2-Bromo-3-chloro-4-fluoroaniline possesses significant anticancer activity against kidney cancer cell lines. All five assays indicated a concentration-dependent decrease in cell viability, with higher concentrations exhibiting more pronounced effects. 2-Bromo-3-chloro-4-fluoroaniline showed cytotoxic effects comparable to Cisplatin, particularly at higher doses, suggesting its potential as a therapeutic alternative for kidney cancer. Future studies should explore the molecular mechanisms underlying its anticancer properties and assess its efficacy in vivo to validate these findings.

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