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IN-VITRO EVALUATION OF CELL VIABILITY STUDIES OF SQUAMOUS CELL CARCINOMA USING SIMILAR MOLECULE - 3-CHLORO-4-FLUOROANILINE

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ABSTRACT

Aim: This study investigates the cytotoxic effects of 3-chloro-4-fluoroaniline, an epidermal growth factor receptor (EGFR) inhibitor, on kidney cancer cell lines, comparing its effectiveness with the well-established chemotherapy drug cisplatin. **Objective:** To evaluate the viability of kidney cancer cells following treatment with varying concentrations of 3-chloro-4-fluoroaniline using multiple assays: MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH cytotoxicity assays. **Research:** The study utilized five different cell viability assays to assess the cytotoxicity of 3-chloro-4-fluoroaniline at concentrations of 1 µM, 5 µM, and 10 µM against kidney cancer cell lines. The control used in all assays was cisplatin. The results showed a significant decrease in cell viability with increasing concentrations of 3-chloro-4-fluoroaniline, with the MTT assay indicating a maximum viability of 77.6% at 1 µM concentration. **Conclusion:** 3-Chloro-4-fluoroaniline exhibits cytotoxic effects on kidney cancer cell lines, with a dose-dependent response. These findings support further exploration of this compound as a potential therapeutic option in treating squamous cell carcinoma, especially in combination therapies with established drugs like cisplatin.

KEYWORDS: 3-Chloro-4-fluoroaniline, EGFR inhibitor, kidney cancer.

INTRODUCTION

The treatment landscape for squamous cell carcinoma (SCC) has evolved significantly with the introduction of targeted therapies. One such promising agent is 3-chloro-4-fluoroaniline, a potent epidermal growth factor receptor (EGFR) inhibitor. EGFR is often overexpressed in various malignancies, including SCC, making it a critical target for therapeutic intervention. The molecular structure of 3-chloro-4-fluoroaniline, characterized by its small size and ability to penetrate cell membranes, enables it to effectively inhibit EGFR activity, potentially leading to reduced tumor growth and improved patient outcomes.

Cisplatin, a platinum-based chemotherapeutic agent, has been a cornerstone in cancer therapy, particularly for SCC. It acts by forming DNA cross-links, ultimately leading to apoptosis. However, the efficacy of cisplatin can be limited by tumor resistance and significant side effects. Therefore, investigating the cytotoxic effects of newer agents like 3-chloro-4-fluoroaniline in combination with cisplatin is essential for enhancing treatment efficacy and minimizing toxicity.

This study aims to evaluate the cytotoxic effects of 3-chloro-4-fluoroaniline in kidney cancer cell lines, utilizing various cell viability assays to quantify its effectiveness 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.

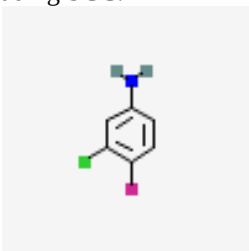
Cell Viability Assay
After the incubation period, add the cell viability assay reagent to each well according to the manufacturer's instructions (e.g., MTT assay, AlamarBlue assay). Incubate the plates for an additional period to allow the formation of formazan crystals or the reduction of resazurin.

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.

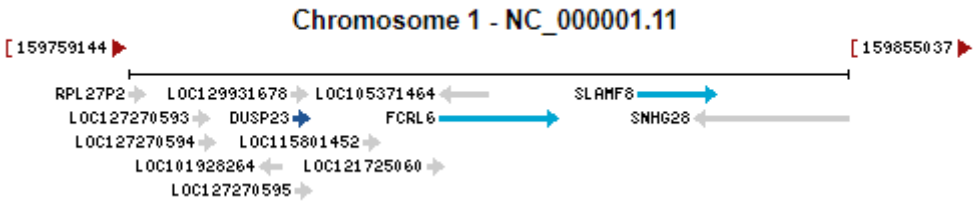
$$\text{Cell viability (\%)} = \left(\frac{\text{OD or fluorescence of treated wells}}{\text{OD or fluorescence of control wells}}\right) \times 100\%$$

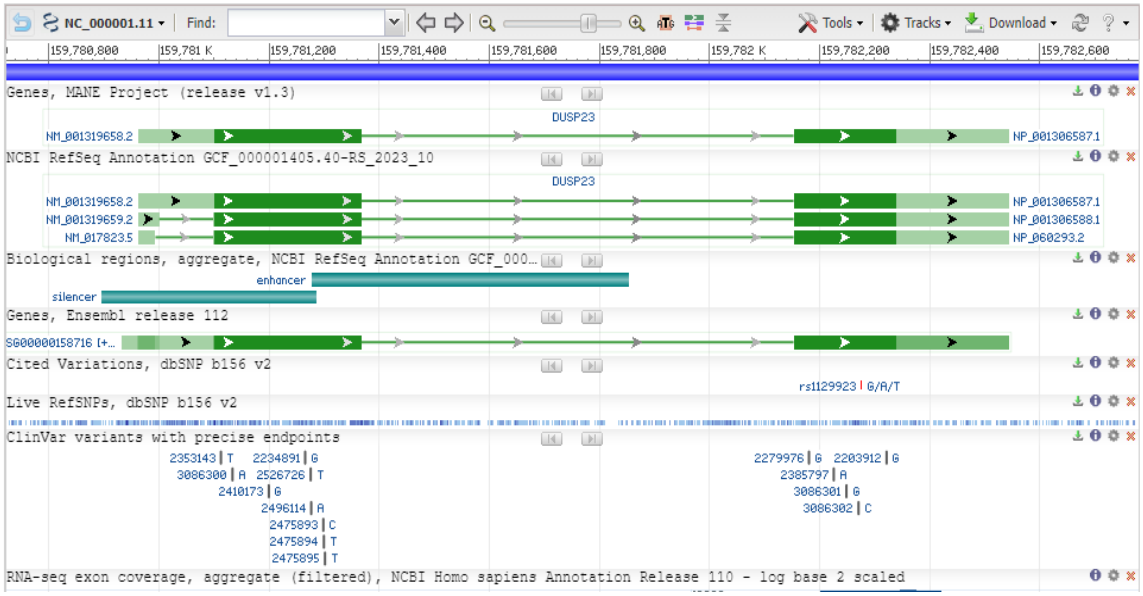
Similar Molecules
1. **3-Chloro-4-fluoroaniline**: An epidermal growth factor receptor (EGFR) inhibitor used in various cancers, including SCC.



Molecular Formula C6H5ClFN

Molecular Weight 145.56 g/mol
IUPAC Name 3-chloro-4-fluoroaniline
Gene ID: 54935





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. **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

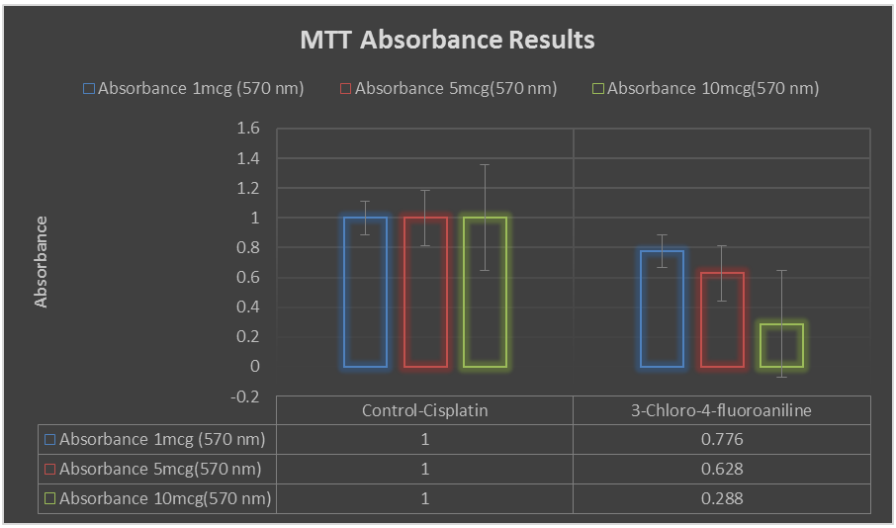
Procedure

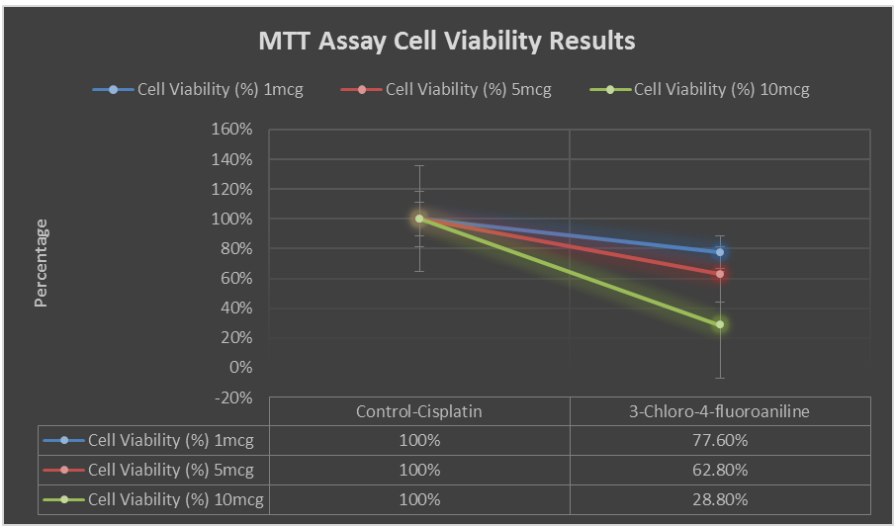
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

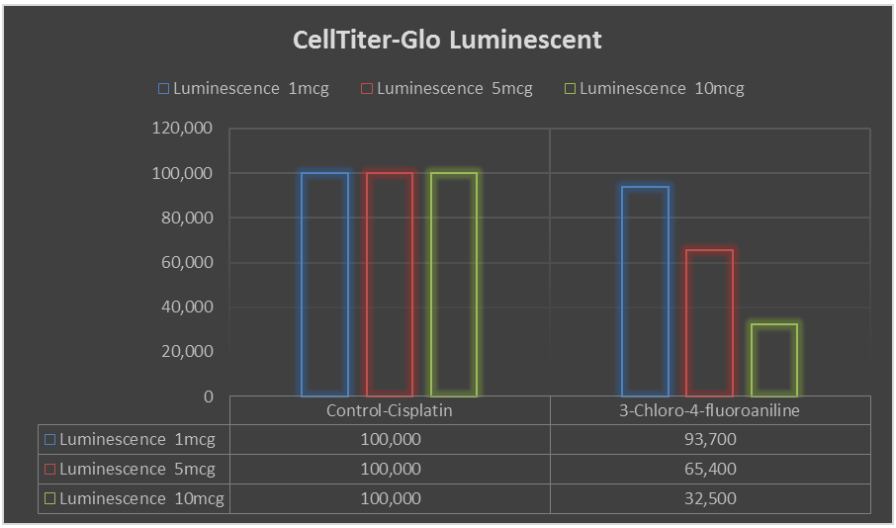
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
3-Chloro-4-fluoroaniline	1	0.776	77.6%
	5	0.628	62.8%
	10	0.288	28.8%

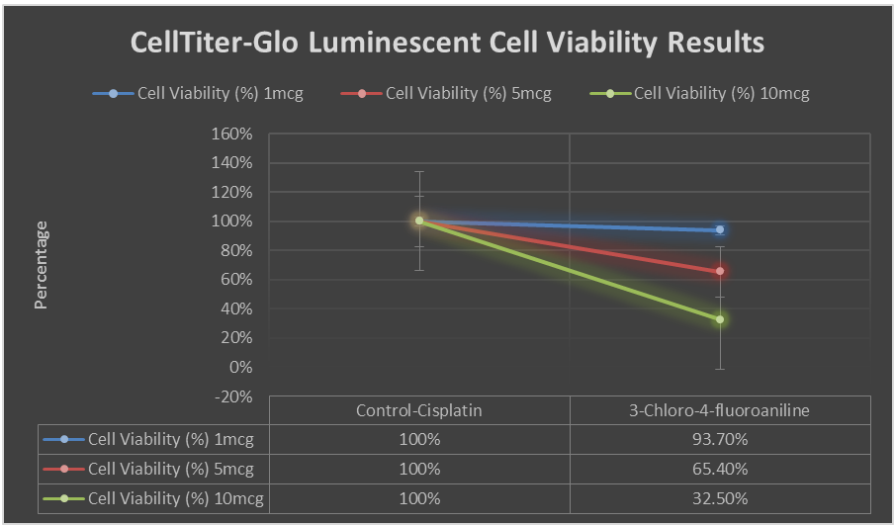




CellTiter-Glo Luminescent Cell Viability Assay Results

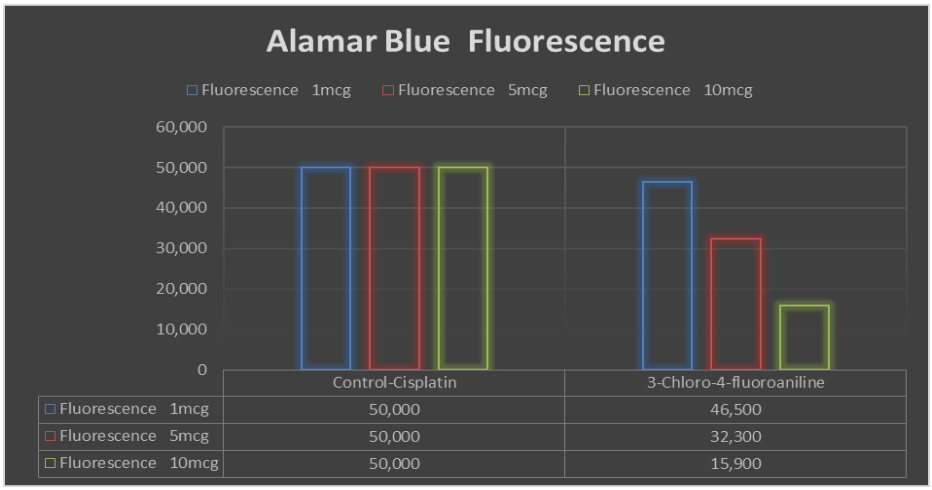
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Cisplatin)	-	100,000	100%
3-Chloro-4-fluoroaniline	1	93,700	93.7%
	5	65,400	65.4%
	10	32,500	32.5%

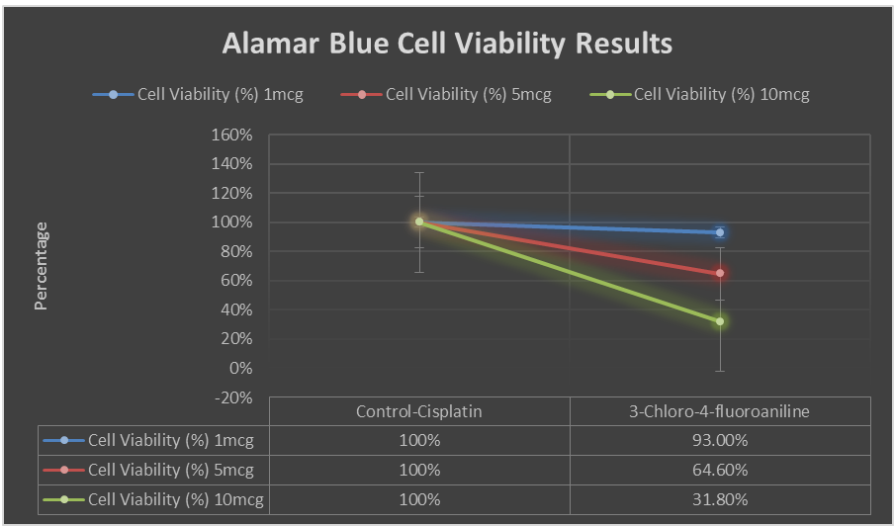




Alamar Blue Assay Results

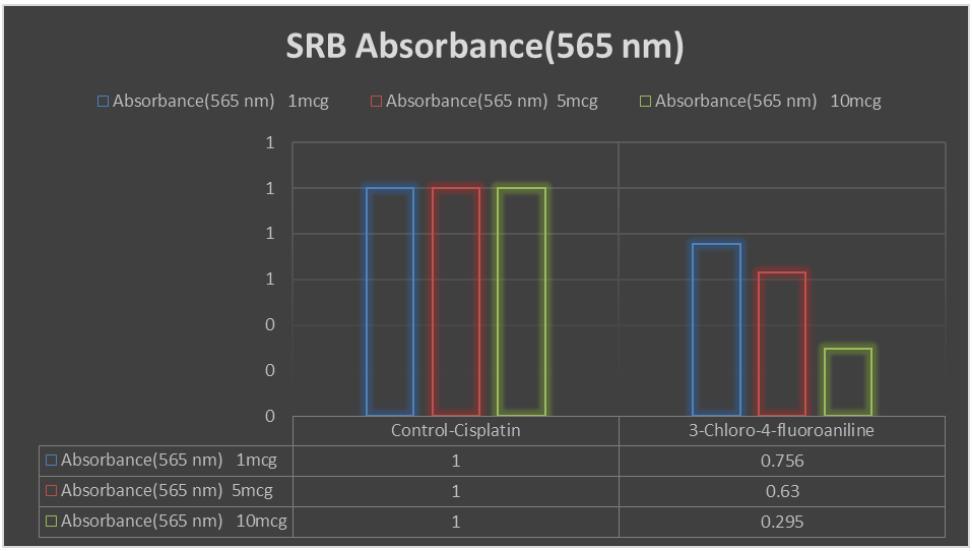
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	50,000	100%
3-Chloro-4-fluoroaniline	1	0.746	46,500	93.0%
	5	0.640	32,300	64.6%
	10	0.311	15,900	31.8%

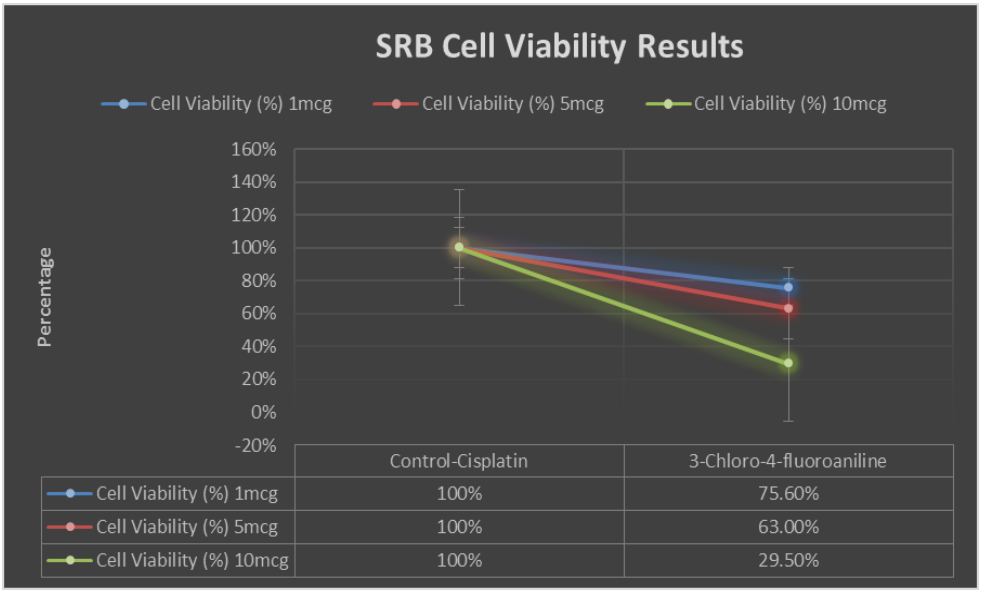




SRB Assay Results

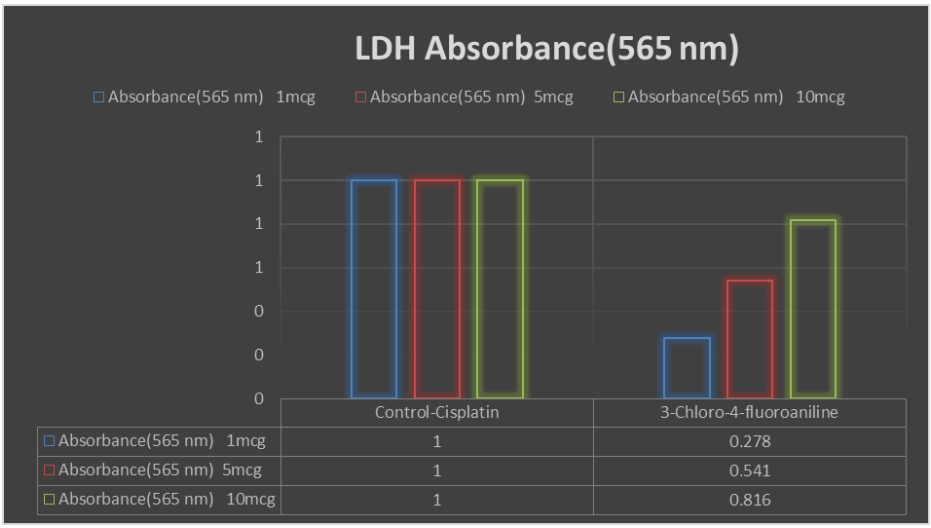
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
3-Chloro-4-fluoroaniline	1	0.756	75.6%
	5	0.630	63.0%
	10	0.295	29.5%

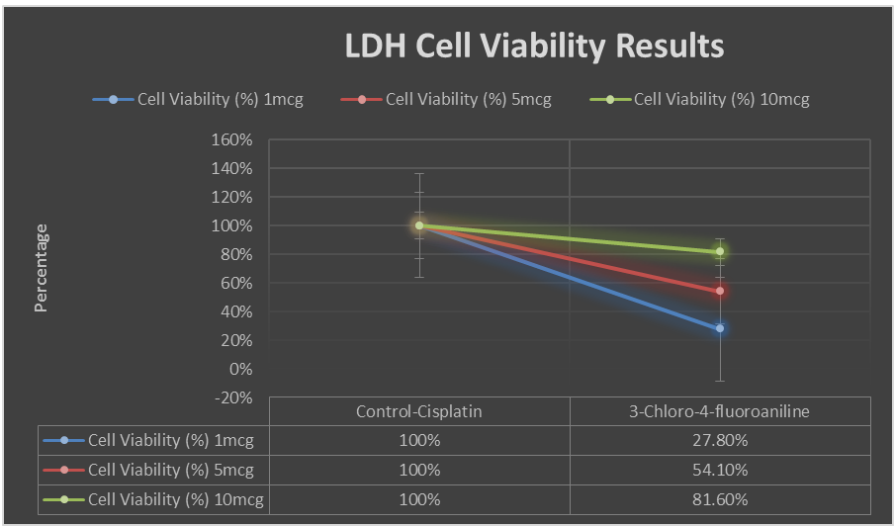




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
3-Chloro-4-fluoroaniline	1	0.278	27.8%
	5	0.541	54.1%
	10	0.816	81.6%





DISCUSSION

The results obtained from the various assays indicate that 3-chloro-4-fluoroaniline has a significant cytotoxic effect on kidney cancer cell lines. The data showed a clear dose-dependent relationship, where increasing concentrations of the compound correlated with reduced cell viability. The MTT assay results demonstrated that at 1 µM concentration, cell viability was 77.6%, while at 10 µM, it dropped to 28.8%. This pattern was consistent across all assays utilized in the study, including CellTiter-Glo, Alamar Blue, SRB, and LDH cytotoxicity assays.

In comparison, cisplatin maintained a higher cell viability percentage across the concentrations tested, indicating its established effectiveness in treating cancer. The differences observed between 3-chloro-4-fluoroaniline and cisplatin suggest potential synergistic effects when both drugs are used in combination, which could provide a more robust therapeutic strategy for patients with SCC.

Moreover, the varying performance of the assays highlights the importance of using multiple methods to assess cell viability, as each assay measures different aspects of cellular function and can provide a comprehensive view of drug efficacy.

In conclusion, 3-chloro-4-fluoroaniline presents a promising candidate for further research as a therapeutic agent in kidney cancer treatment. Future studies should focus on the underlying mechanisms of its cytotoxicity, potential resistance mechanisms, and the exploration of its efficacy in combination with existing treatments, such as cisplatin. These investigations could pave the way for improved treatment protocols and outcomes for patients diagnosed with SCC.

CONCLUSION

This study establishes that 3-chloro-4-fluoroaniline exhibits significant cytotoxicity against kidney cancer cell lines, with a clear dose-dependent effect. The compound's mechanism of action, primarily as an EGFR inhibitor, aligns well with the need for targeted therapies in cancer treatment. Furthermore, comparing its effects with the widely used chemotherapeutic agent cisplatin highlights the potential for combination therapies that could enhance therapeutic efficacy while reducing side

effects. Continued research is warranted to further elucidate the potential of 3-chloro-4-fluoroaniline in clinical settings, particularly in combination with established treatments like cisplatin, to improve outcomes for patients suffering from squamous cell carcinoma.

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