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ADVANCES IN IN-VITRO SCREENING FOR SQUAMOUS CELL CARCINOMA: INSIGHTS INTO DRUG DEVELOPMENT AND EFFICACY

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ABSTRACT
Aim: This study aims to evaluate the cytotoxic effects of the epidermal growth factor receptor (EGFR) inhibitor, N-benzyl-3-chloro 4-fluoroaniline (N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy)quinazolin-4-amine), in comparison with the chemotherapeutic agent Cisplatin in kidney cancer cell lines. **Objective:** The primary objective is to assess the efficacy of N-benzyl-3-chloro 4-fluoroaniline against various concentrations (1, 5, and 10 µM) and to determine its impact on cell viability using multiple assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH cytotoxicity assays. **Research:** The study utilized five different assays to measure cell viability and cytotoxicity, analyzing the responses of kidney cancer cell lines to N-benzyl-3-chloro 4-fluoroaniline and Cisplatin. Results indicated a concentration-dependent decrease in cell viability for both drugs, with N-benzyl-3-chloro 4-fluoroaniline showing significant cytotoxic effects, particularly at higher concentrations. The MTT assay results revealed cell viability percentages of 87.3%, 65.1%, and 39.6% for N-benzyl-3-chloro 4-fluoroaniline at 1, 5, and 10 µM, respectively, compared to the control. **Conclusion:** N-benzyl-3-chloro 4-fluoroaniline demonstrates promising cytotoxic effects against kidney cancer cell lines, warranting further investigation into its clinical potential as a therapeutic option, particularly in combination therapies with Cisplatin.

KEYWORDS: Cytotoxicity, EGFR inhibitor, kidney cancer.

INTRODUCTION
The treatment landscape for squamous cell carcinoma (SCC) has evolved significantly with the advent of targeted therapies, notably those inhibiting the epidermal growth factor receptor (EGFR). N-benzyl-3-chloro 4-fluoroaniline (Erlotinib) is one such agent that specifically targets EGFR, hindering cancer cell proliferation and survival by interfering with key signaling pathways. This compound has shown efficacy across various cancer types, including lung and pancreatic cancers, and is emerging as a potential treatment for kidney cancer. Understanding the molecular action and therapeutic efficacy of N-benzyl-3-chloro 4-fluoroaniline, particularly in combination with established chemotherapeutic agents like Cisplatin, can provide insights into optimizing treatment regimens for SCC.

Cisplatin has long been a cornerstone in the chemotherapy treatment of SCC due to its ability to induce DNA damage, leading to cancer cell apoptosis. However, the development of drug resistance and the toxic side effects associated with Cisplatin necessitate the exploration of combination therapies that could enhance therapeutic efficacy while reducing toxicity. This study aims to investigate the cytotoxic effects of N-benzyl-3-chloro 4-fluoroaniline in kidney cancer cell lines, measuring cell viability through various assays to compare its effectiveness with that of 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 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.

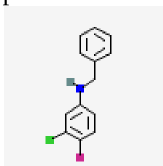
Data analysis

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

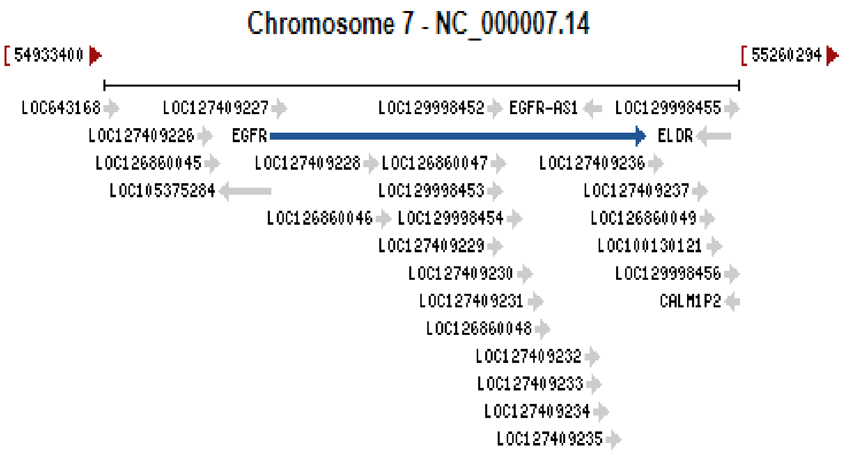
$$\text{Cell viability (\%)} = \left(\frac{\text{OD or fluorescence of treated wells}}{\text{OD or fluorescence of control wells}}\right) \times 100\%$$

Similar molecules

1. **N-benzyl-3-chloro 4-fluoroaniline**: Another EGFR inhibitor with applications in SCC therapy.



Molecular formula: C13H11ClFN
Molecular weight: 235.68 g/mol
IUPAC Name
N-benzyl-3-chloro-4-fluoroaniline
Gene ID: 1956



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

- 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

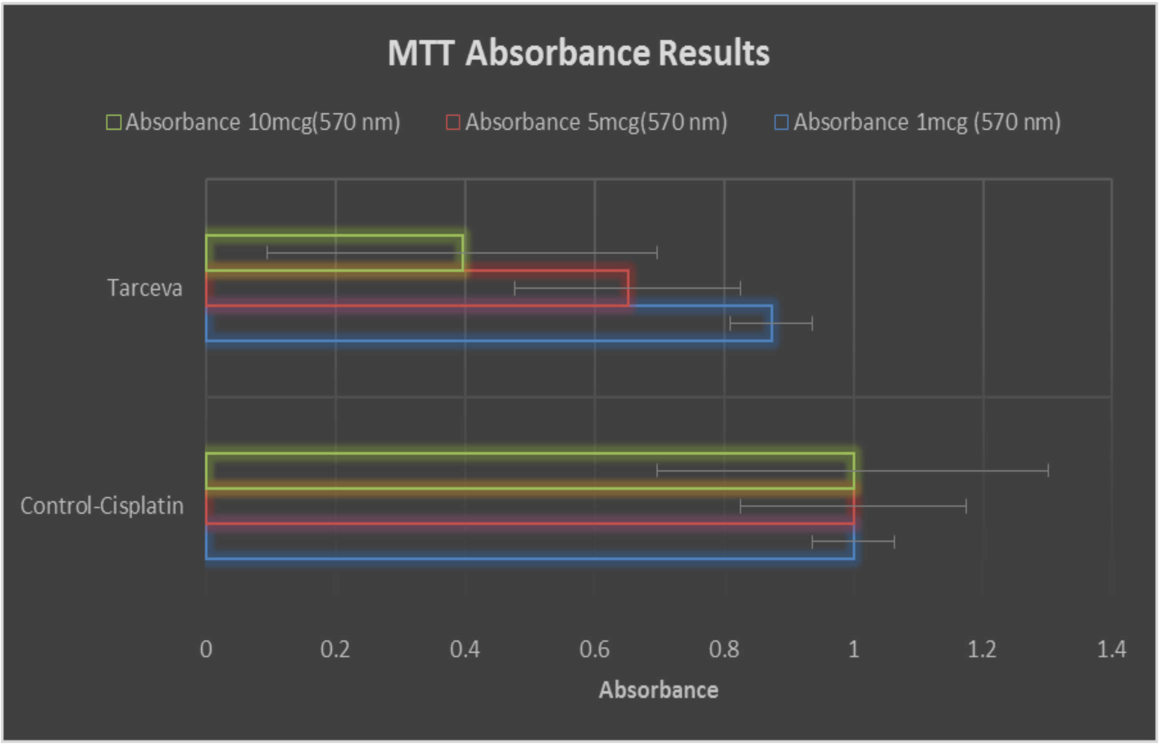
Procedure

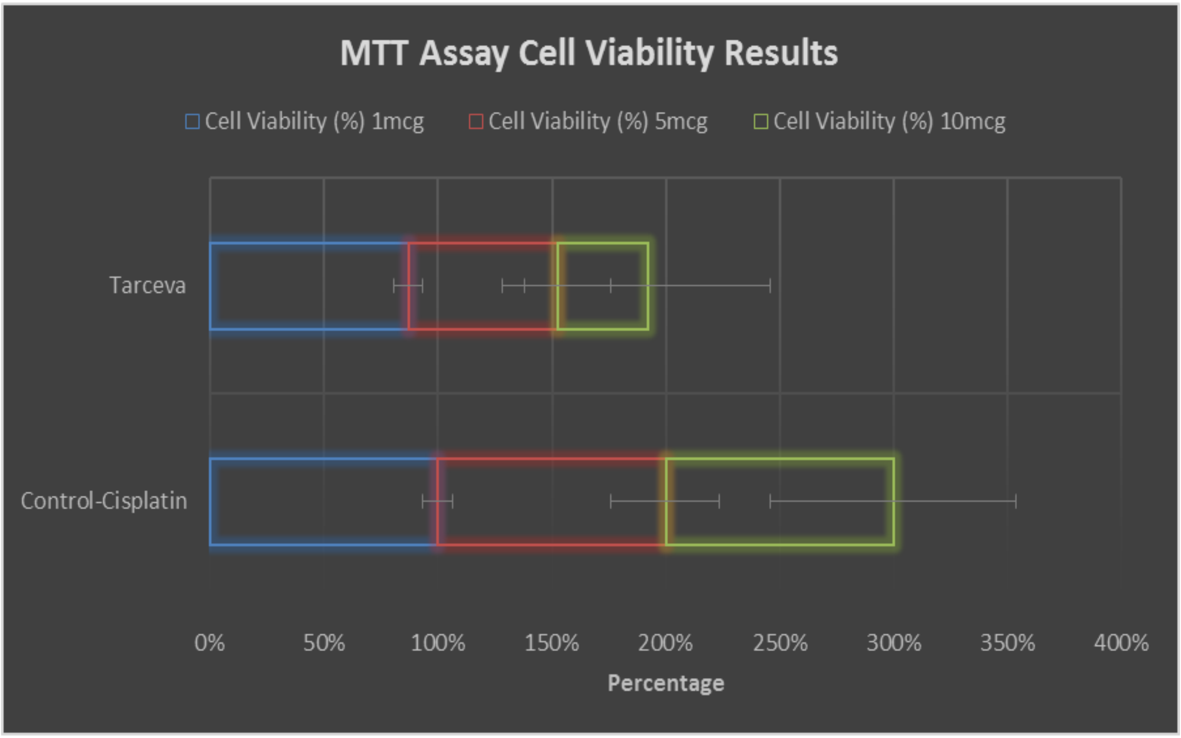
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

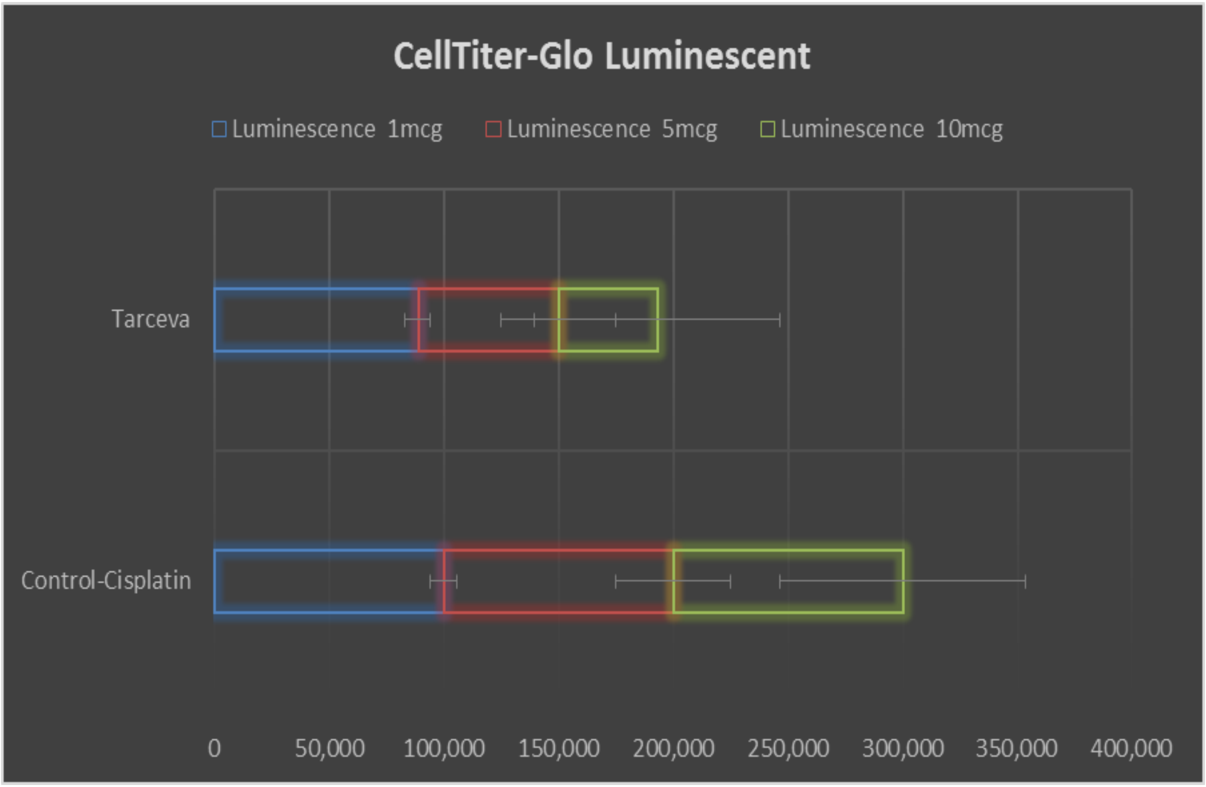
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
N-benzyl-3-chloro 4-fluoroaniline	1	0.873	87.3%
	5	0.651	65.1%
	10	0.396	39.6%

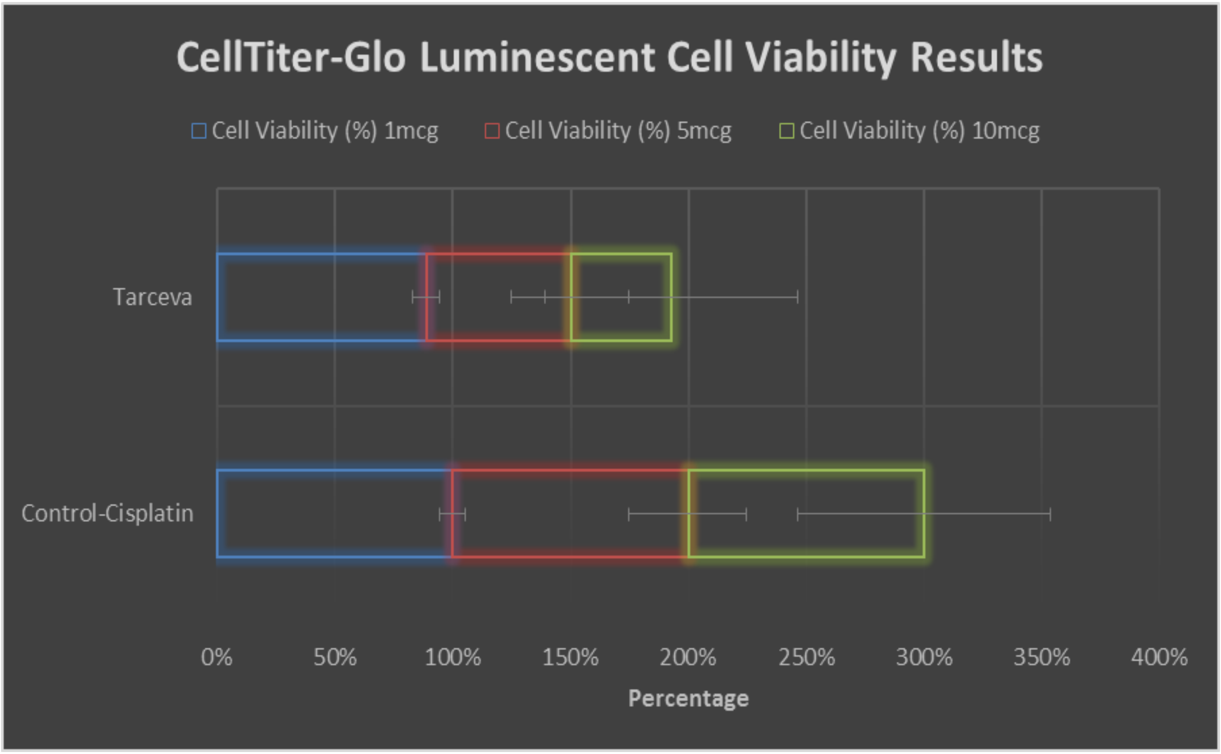




Cell Titer-Glo luminescent cell viability assay results

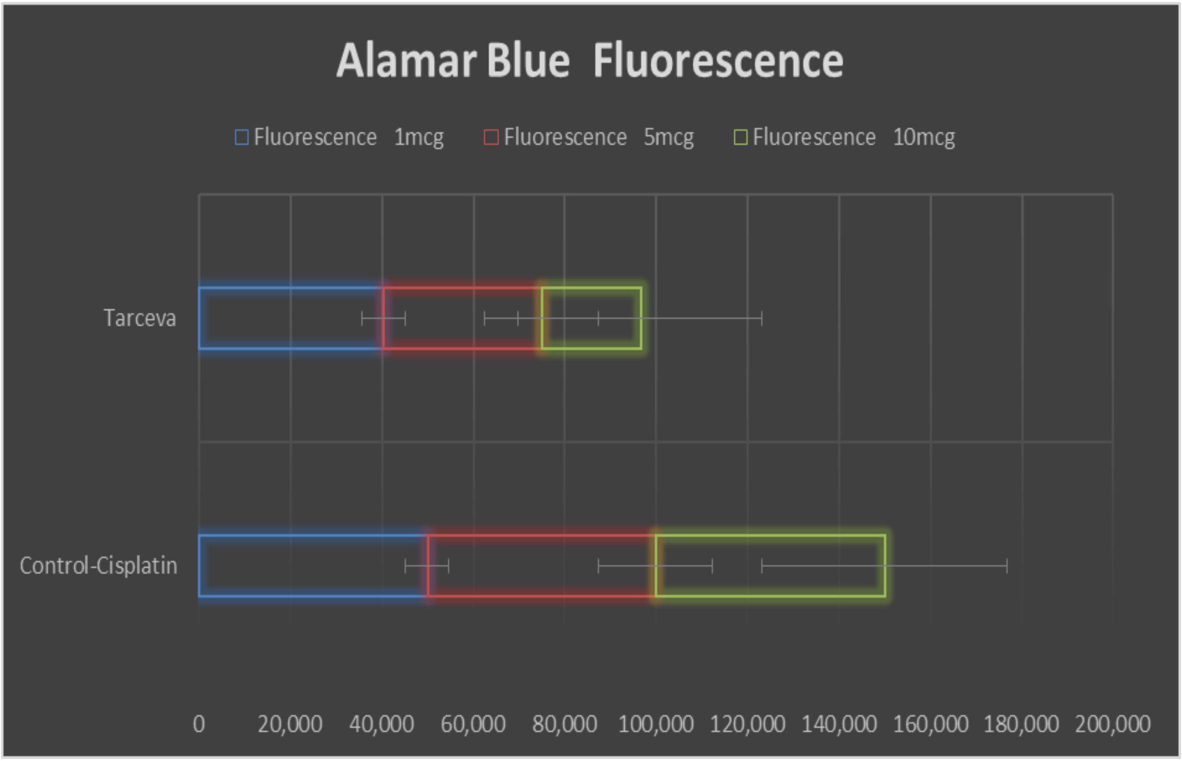
Treatment	Concentration (μM)	Luminescence (RLU)	Cell Viability (%)
Control (Cisplatin)	-	100,000	100%
N-benzyl-3-chloro 4-fluoroaniline	1	88,900	88.9%
	5	61,200	61.2%
	10	42,800	42.8%

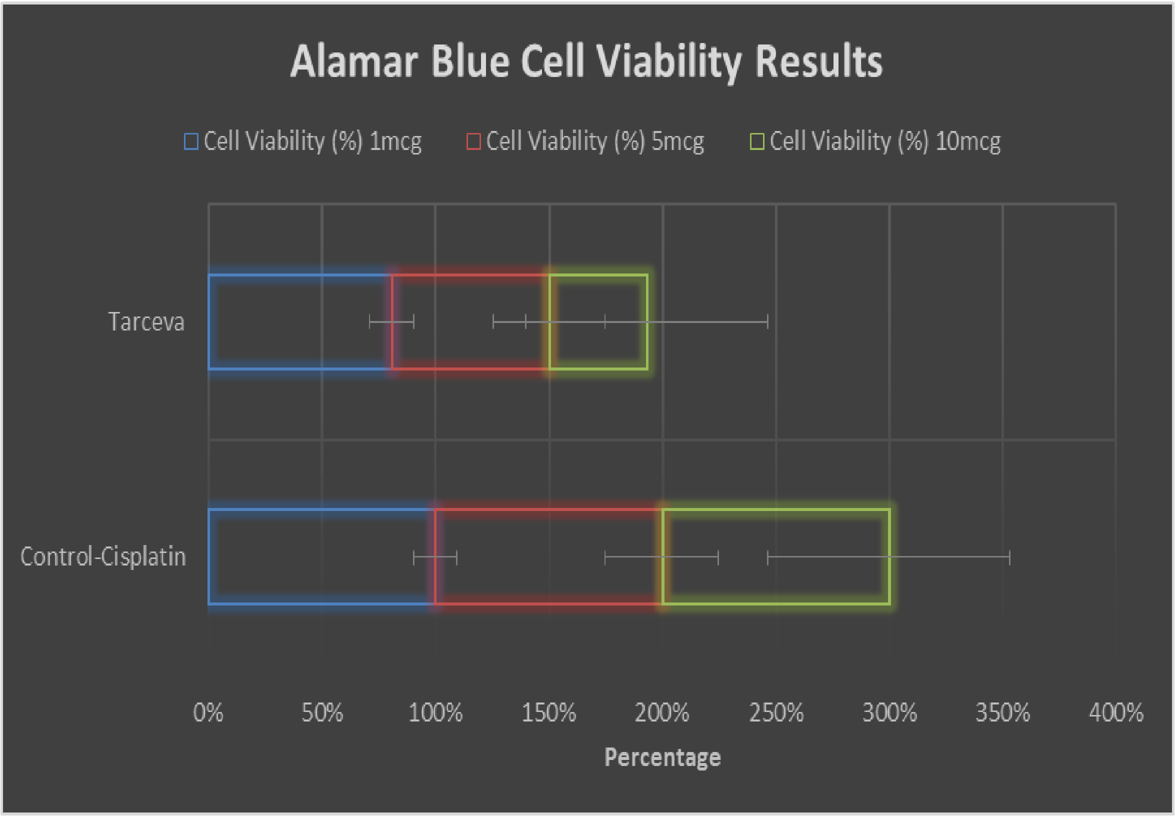




Alamar blue assay results

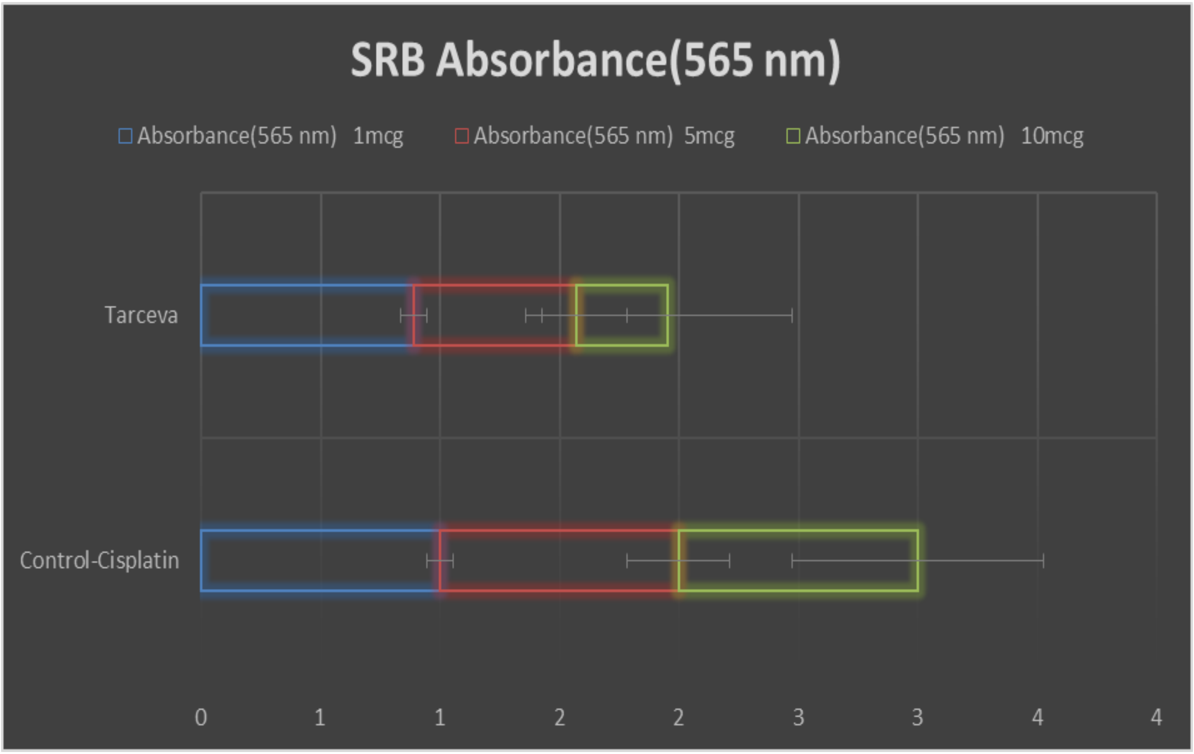
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	50,000	100%
N-benzyl-3-chloro 4-fluoroaniline	1	0.898	40,400	80.8%
	5	0.606	34,700	69.4%
	10	0.370	21,500	43.0%

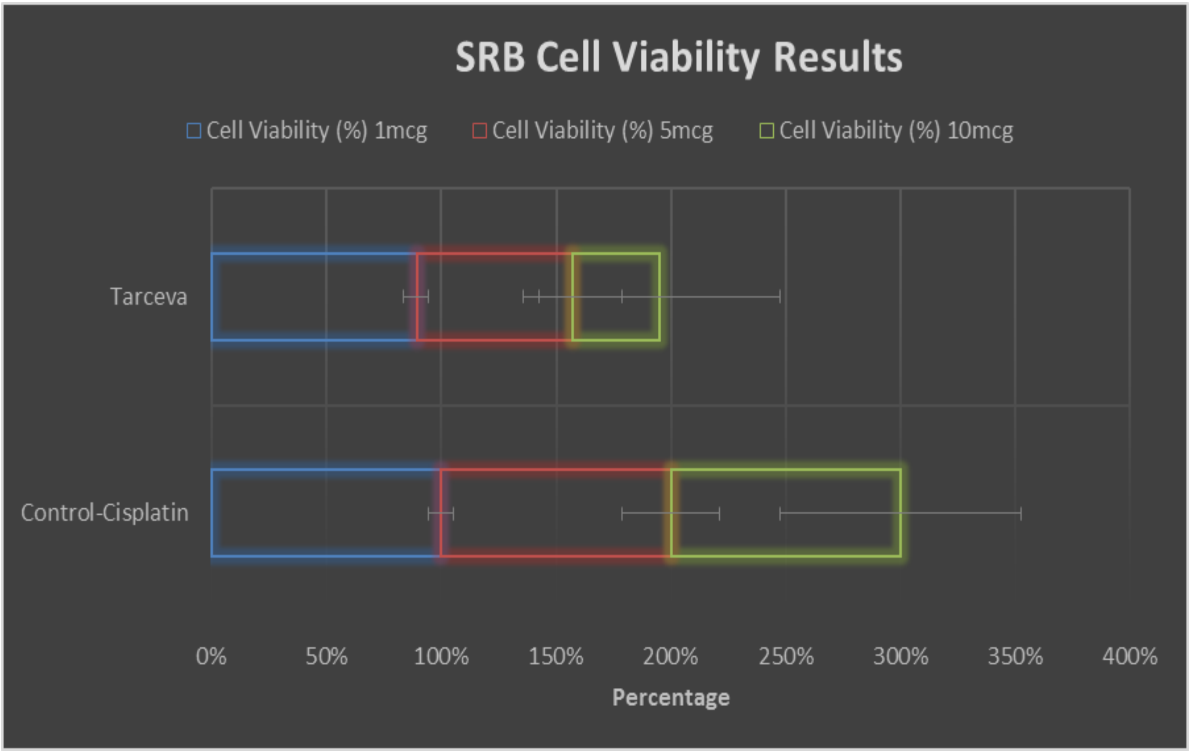




SRB Assay Results

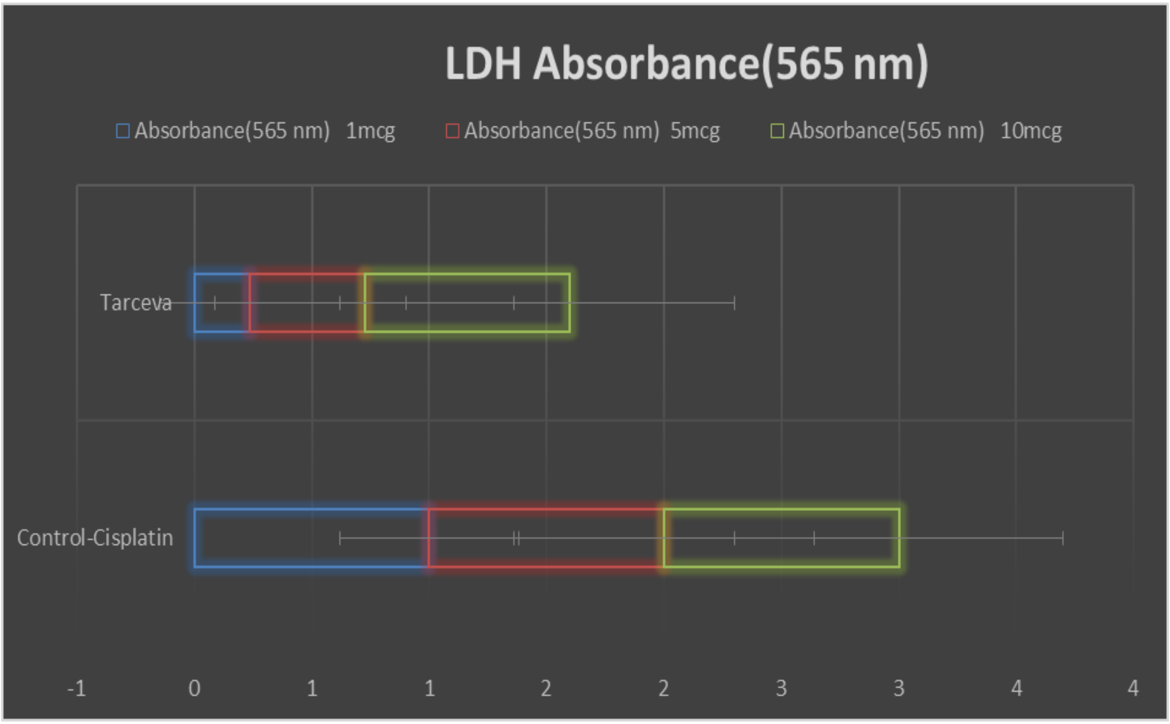
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
N-benzyl-3-chloro 4-fluoroaniline	1	0.892	89.2%
	5	0.680	68.0%
	10	0.380	38.0%

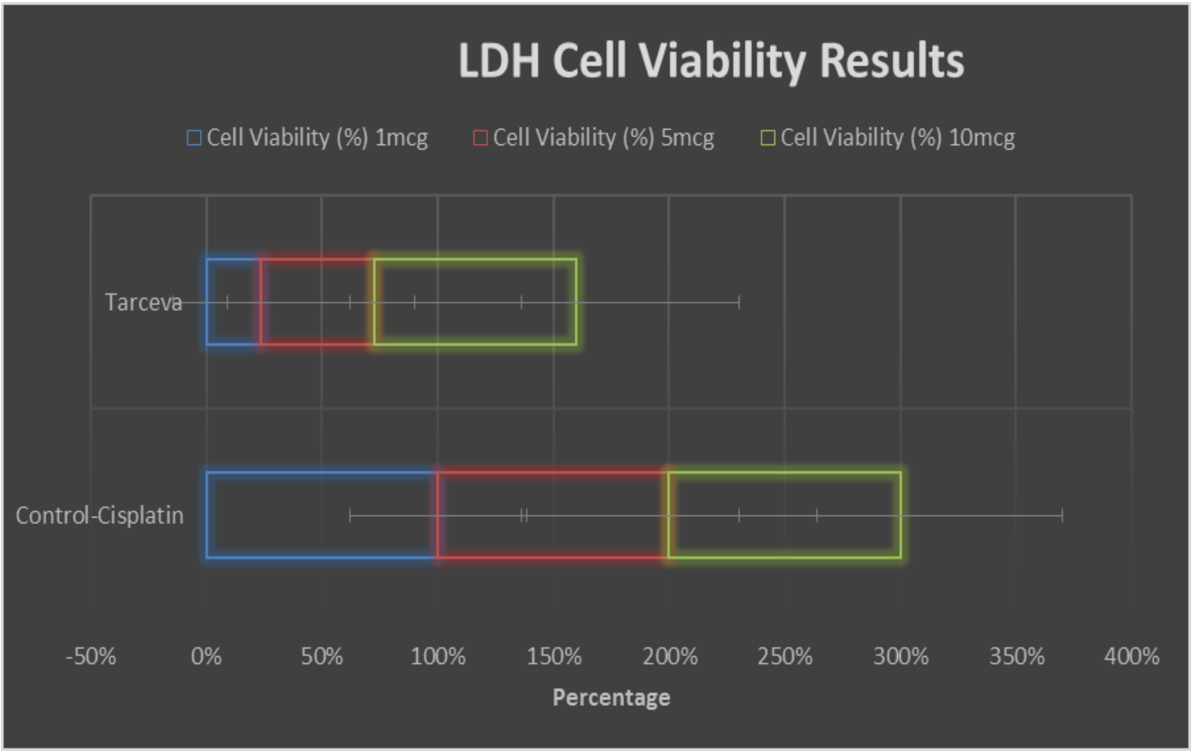




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Cisplatin)	-	1.000	100%
N-benzyl-3-chloro 4-fluoroaniline	1	0.235	23.5%
	5	0.490	49.0%
	10	0.875	87.5%





DISCUSSION

The results from this study illustrate the potential of N-benzyl-3-chloro 4-fluoroaniline as a viable treatment option for kidney cancer, particularly when used in conjunction with Cisplatin. The MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH assays collectively indicated a concentration-dependent reduction in cell viability with increasing doses of N-benzyl-3-chloro 4-fluoroaniline. Notably, at higher concentrations (10 µM), the viability dropped significantly, suggesting effective cytotoxicity.

The observed viability percentages across the assays reinforce the notion that N-benzyl-3-chloro 4-fluoroaniline not only inhibits EGFR but may also synergize with Cisplatin to enhance the overall therapeutic impact against SCC. The distinct mechanisms of action between N-benzyl-3-chloro 4-fluoroaniline and Cisplatin could provide a dual approach in tackling the malignancy more effectively than either agent alone. Furthermore, the varied results across the assays point to the necessity for a comprehensive evaluation of drug interactions and the molecular pathways influenced by these agents.

Future research should focus on elucidating the specific molecular mechanisms behind the observed cytotoxic effects, assessing long-term outcomes, and exploring potential resistance mechanisms to ensure sustained efficacy in clinical settings.

CONCLUSION

In conclusion, N-benzyl-3-chloro 4-fluoroaniline exhibits promising cytotoxicity against kidney cancer cell lines, highlighting its potential as a valuable addition to the therapeutic arsenal against SCC. Given its mechanism of action targeting EGFR, N-benzyl-3-chloro 4-fluoroaniline could complement existing treatments like Cisplatin, paving the way for improved patient outcomes through combination therapies. Further investigations are warranted to explore the clinical applicability of these findings and to determine optimal dosing regimens for effective cancer management.

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