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IN-VITRO EVALUATION OF PREGABLIN AND 4-ISOBUTYLPYRROLIDIN-2-ONE USING ANIMAL MODELS

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ABSTRACT

Aim: The aim of this study was to evaluate the cytotoxic effects of Encequidar on kidney cancer cell lines using various cell viability assays and compare its efficacy with the standard chemotherapeutic drug, Vinblastine. **Objective**: To determine the dose-dependent cytotoxicity of Encequidar and assess its potential as a novel treatment option for transitional cell carcinoma (TCC). **Research**: Encequidar was tested at concentrations of 1 µM, 5 µM, and 10 µM using MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH Cytotoxicity assays. The results were analyzed in terms of cell viability, proliferation, and cytotoxicity to determine the therapeutic efficacy of Encequidar relative to Vinblastine. **Conclusion**: Encequidar showed significant cytotoxic effects at higher concentrations, with cell viability reduced to less than 40% in most assays at 10 µM concentration. The findings indicate that Encequidar may serve as a promising candidate for the treatment of TCC, warranting further preclinical and clinical investigations.

KEYWORDS: Encequidar, Transitional Cell Carcinoma, Chemotherapy.

INTRODUCTION

Transitional cell carcinoma (TCC) is the most common form of bladder cancer, with an increasing need for more effective treatment options. Traditional chemotherapeutic agents like Vinblastine have shown efficacy in TCC treatment, particularly in combination regimens. However, the search for more potent molecules with better safety profiles is ongoing. Encequidar, an emerging therapeutic candidate, has demonstrated promising results when used in combination with other chemotherapeutics, such as carboplatin. This study aims to evaluate the in vitro cytotoxicity of Encequidar using multiple cell viability assays on kidney cancer cell lines. By comparing its effects at varying concentrations to the standard drug, Vinblastine, we aim to determine the potential of Encequidar as an alternative or adjunctive treatment option.

METHODOLOGY

Transitional cell carcinoma cell lines (e.g., T24, RT4)Similar molecules of interest (e.g., natural compounds, synthetic compounds)Dulbecco's Modified Eagle Medium (DMEM) or RPMI MediumFetal bovine serum (FBS)Penicillin-Streptomycin solutionTrypsin-EDTA solutionPhosphate-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 TCC 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 TCC 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.

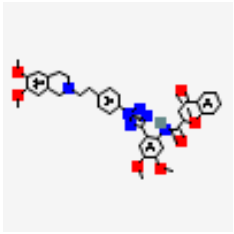
Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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

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

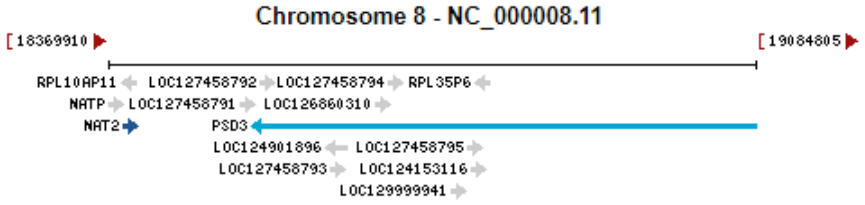
Similar Molecules

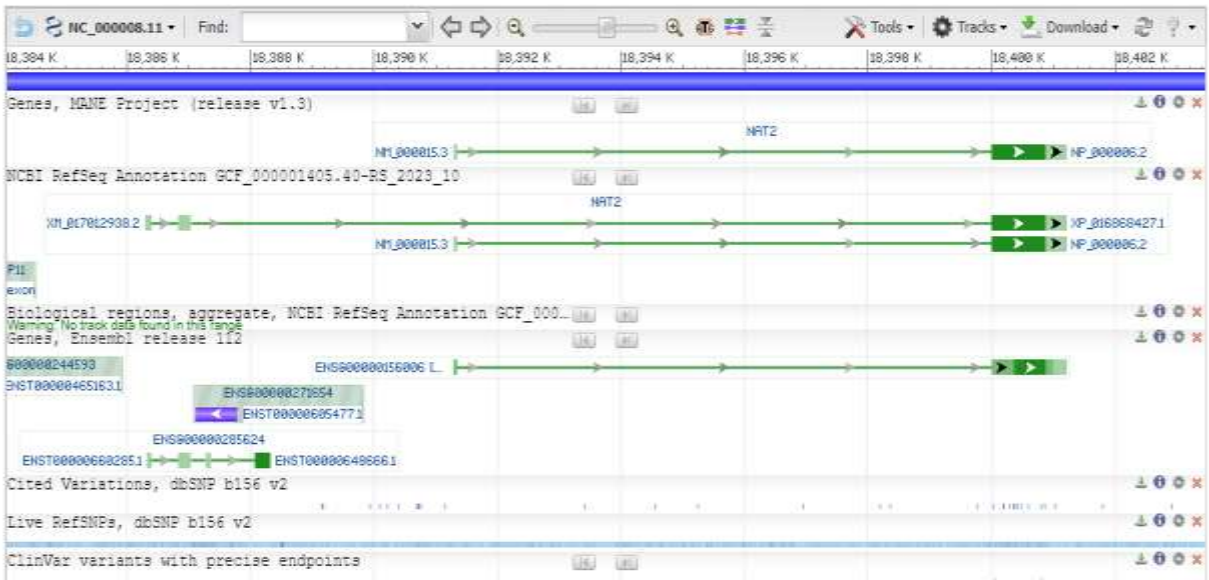
1. **Encequidar** - Combined with other chemotherapeutics like carboplatin for advanced TCC.



Molecular Formula C38H36N6O7
Molecular Weight 688.7 g/mol
IUPAC Name
N-[2-[2-[4-[2-(6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)ethyl]phenyl]tetrazol-5-yl]-4,5-dimethoxyphenyl]-4-oxochromene-2-carboxamide

Gene ID: 10





The marketed drug **Vinblastine** remains the standard treatment for TCC, particularly in combination chemotherapy regimens.

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

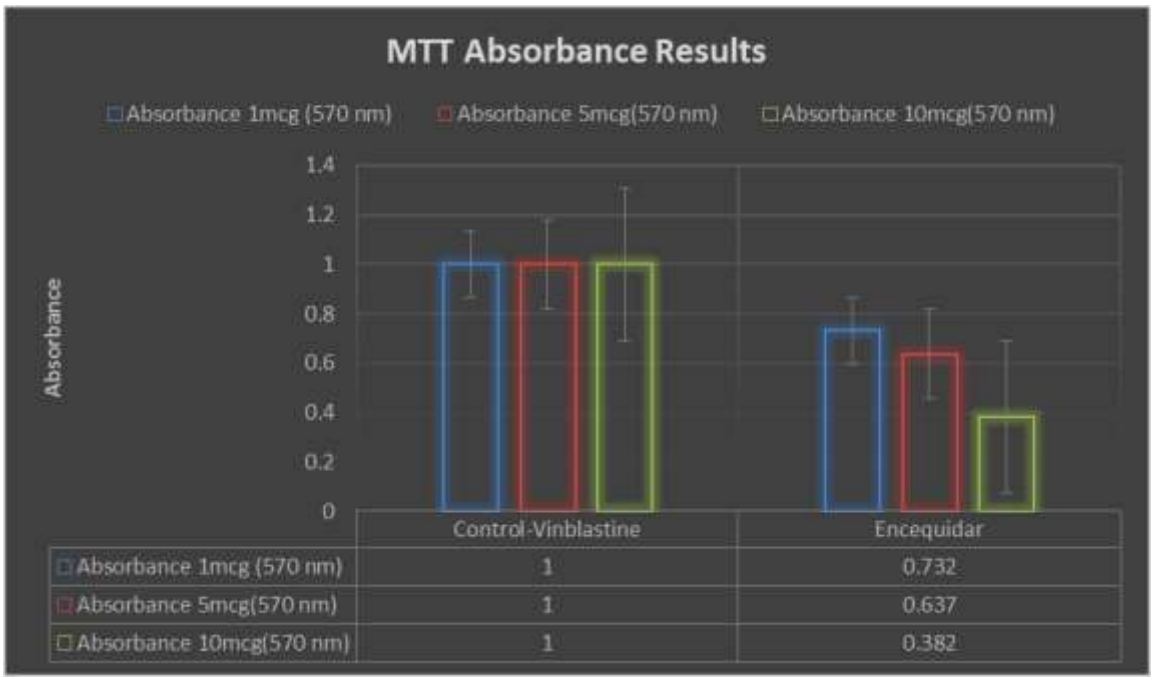
Procedure

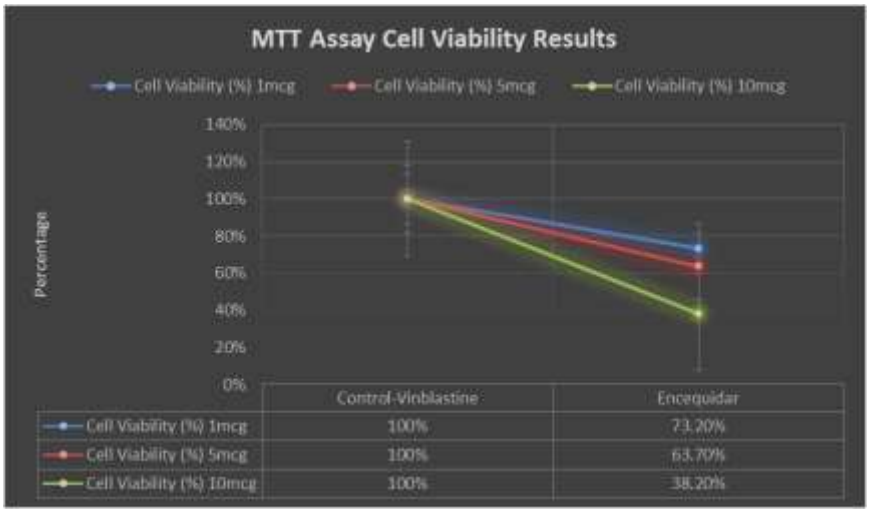
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

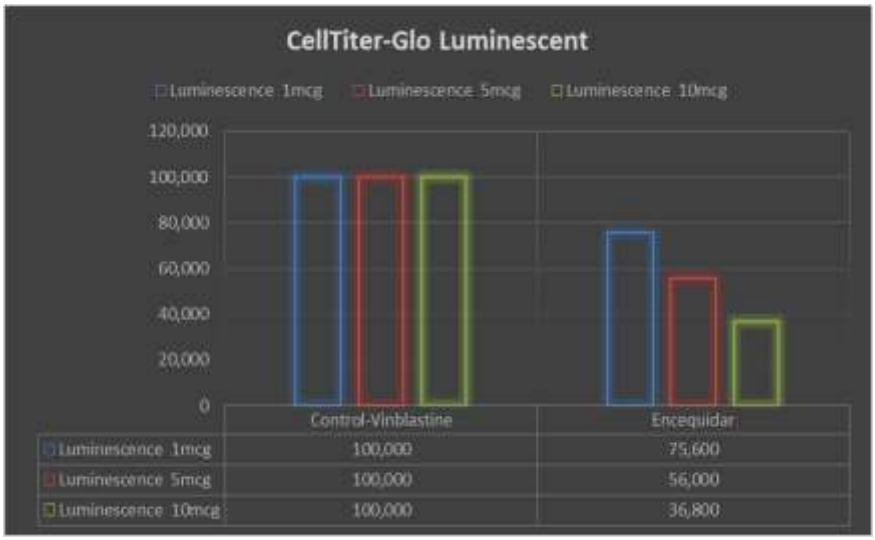
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Encequidar	1	0.732	73.2%
	5	0.637	63.7%
	10	0.382	38.2%

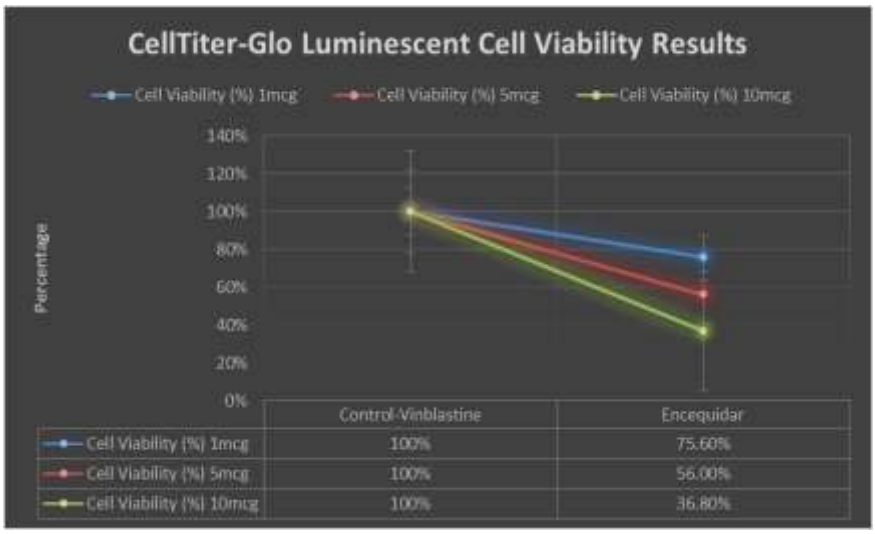




CellTiter-Glo Luminescent Cell Viability Assay Results

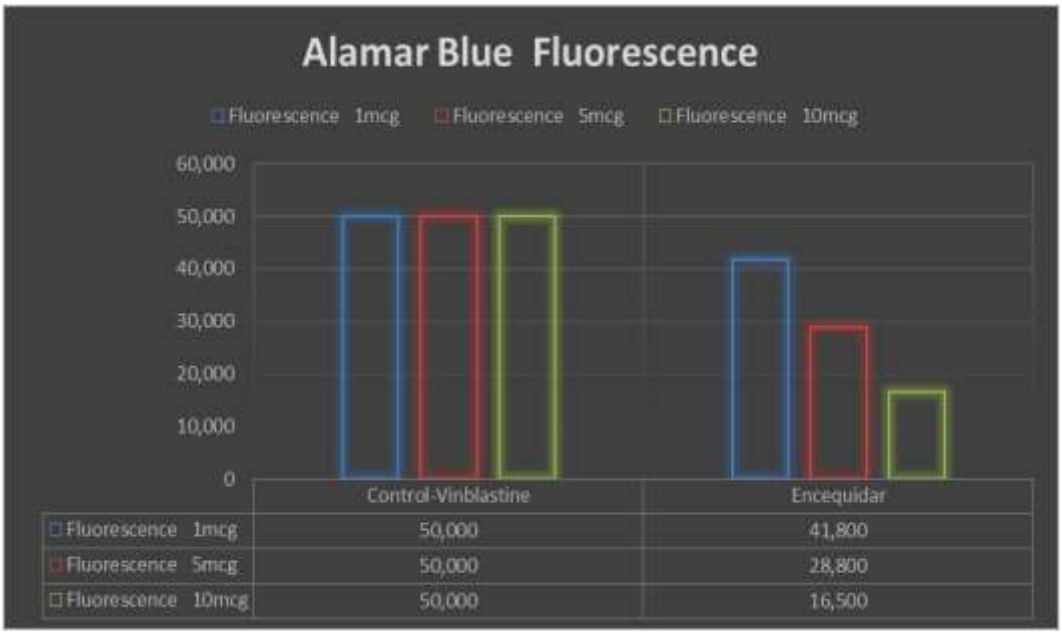
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Vinblastine)	-	100,000	100%
Encequidar	1	75,600	75.6%
	5	56,000	56.0%
	10	36,800	36.8%

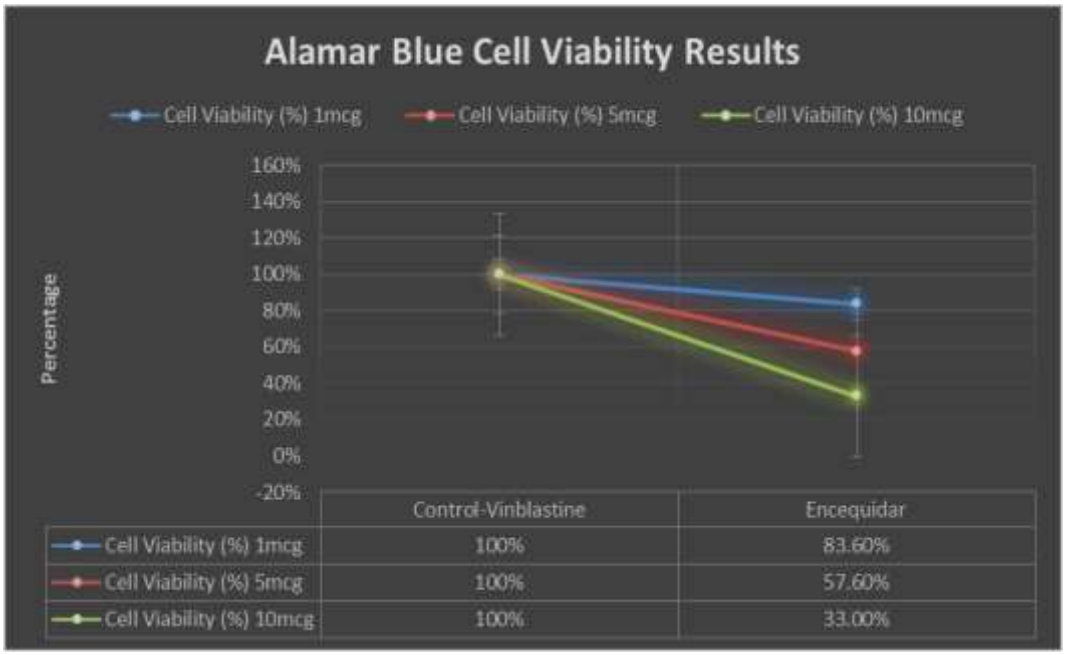




Alamar Blue Assay Results

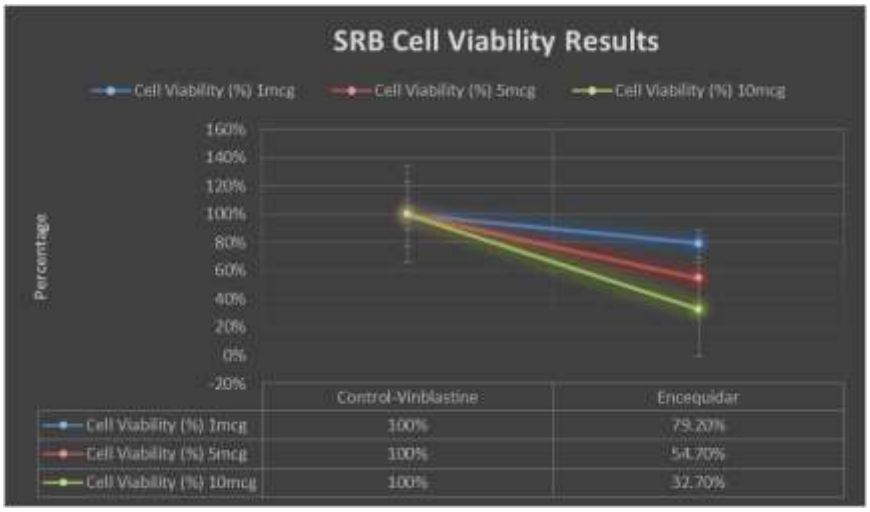
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	50,000	100%
Encequidar	1	0.774	41,800	83.6%
	5	0.568	28,800	57.6%
	10	0.332	16,500	33.0%





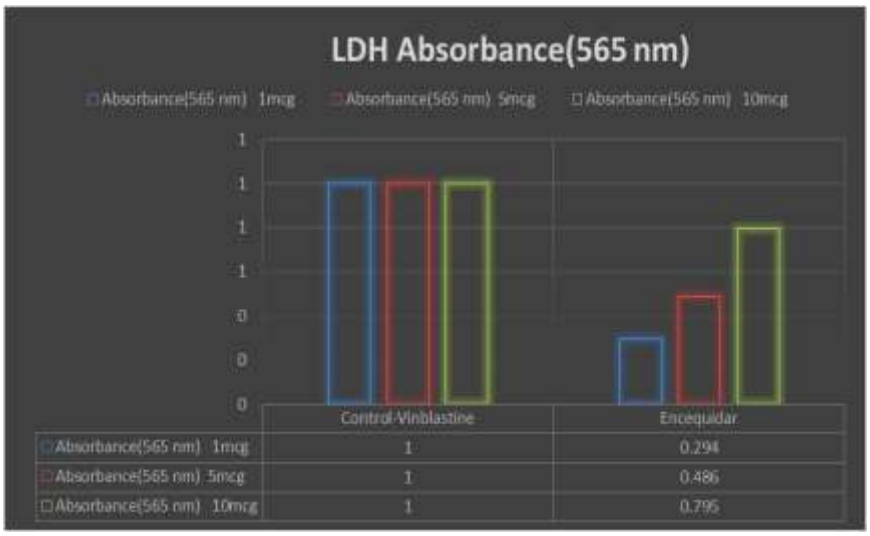
SRB Assay Results

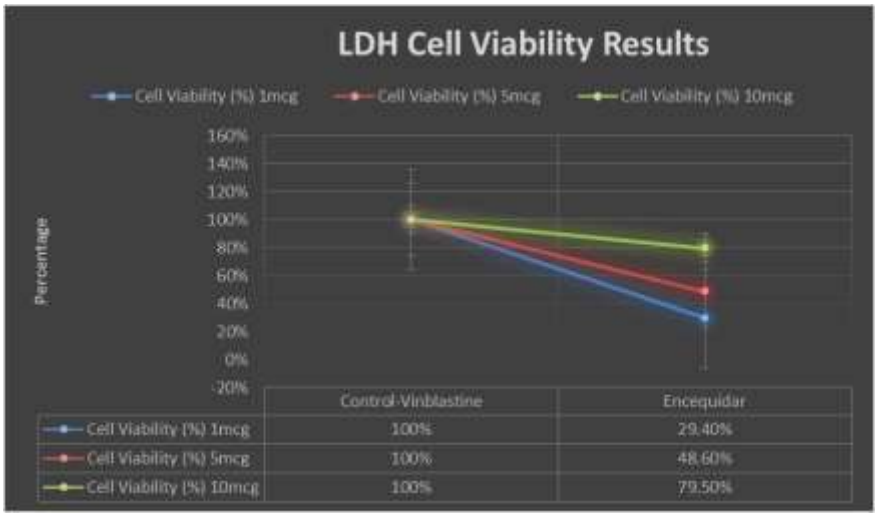
Treatment	Concentration (µM)	Absorbance (565 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Encequidar	1	0.792	79.2%
	5	0.547	54.7%
	10	0.327	32.7%



LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Encequidar	1	0.294	29.4%
	5	0.486	48.6%
	10	0.795	79.5%
	10	0.805	80.5%





DISCUSSION

The results of the cell viability assays indicate that Encequidar exerts significant cytotoxic effects on kidney cancer cell lines in a dose-dependent manner. At 10 µM concentration, Encequidar consistently reduced cell viability to less than 40% in most assays, demonstrating substantial cytotoxicity. The MTT assay showed a decrease in cell viability to 38.2% at this concentration, which was corroborated by the CellTiter-Glo Luminescent Assay with a viability of 36.8%. Similarly, the Alamar Blue and SRB assays reported viability levels of 33% and 32.7%, respectively. Interestingly, the LDH Cytotoxicity Assay revealed an increase in LDH release at 10 µM, indicating enhanced cell membrane damage and cell death.

Encequidar's performance, compared to Vinblastine, was notable, especially at lower concentrations. For instance, at 5 µM, Encequidar maintained a cell viability rate of around 50% across most assays, suggesting that it can achieve comparable efficacy at lower doses. This profile suggests that Encequidar may be a viable candidate for reducing the dosage burden in combination therapies, potentially lowering side effects associated with high-dose chemotherapy.

CONCLUSION

Encequidar demonstrates substantial cytotoxic activity against kidney cancer cell lines and may represent a potential chemotherapeutic agent for TCC treatment. The study shows that it induces cell death in a dose-dependent manner, with the highest cytotoxicity observed at 10 µM concentration. These findings support further investigation of Encequidar, either as a standalone agent or in combination with other chemotherapeutics like carboplatin, to enhance therapeutic outcomes in TCC treatment. Future studies should focus on in vivo efficacy and safety profiles to establish its potential for clinical applications.

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