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

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ABSTRACT

Aim: To evaluate the cytotoxicity and cell viability of Vinflunine ditartrate, a third-generation vinca alkaloid, in comparison to the standard drug Vinblastine, for the treatment of transitional cell carcinoma (TCC). **Objective:** To analyze the efficacy of Vinflunine ditartrate using various in vitro assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH, and determine its potential as an alternative therapeutic option. **Research:** Vinflunine ditartrate was tested against kidney cancer cell lines using multiple assays to assess cell viability, metabolic activity, and cytotoxicity. Each assay provided quantitative insights into the effect of various concentrations (1, 5, and 10 µM) of Vinflunine ditartrate on cell survival. Results were compared to the control treatment with Vinblastine to establish relative efficacy. **Conclusion:** Vinflunine ditartrate demonstrated a dose-dependent reduction in cell viability and increased cytotoxicity across all assays. The compound showed promising anticancer properties, indicating its potential role as a second-line therapeutic agent for TCC treatment.

KEYWORDS: Vinflunine ditartrate, Transitional cell carcinoma (TCC), Cell viability assay.

INTRODUCTION

Transitional cell carcinoma (TCC), a common type of bladder cancer, is primarily treated with a combination of chemotherapeutic agents. While Vinblastine remains the standard treatment, its efficacy in certain cases is limited, necessitating the exploration of alternative agents. Vinflunine ditartrate, a third-generation vinca alkaloid, has been identified as a potential candidate for second-line therapy in TCC. Structurally similar to Vinblastine, Vinflunine exhibits improved efficacy and tolerability. This study aims to evaluate the cytotoxic and antiproliferative effects of Vinflunine ditartrate on kidney cancer cell lines using multiple in vitro assays, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH cytotoxicity assays. By comparing the outcomes with Vinblastine, this study provides insights into the therapeutic potential of Vinflunine for TCC management.

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.

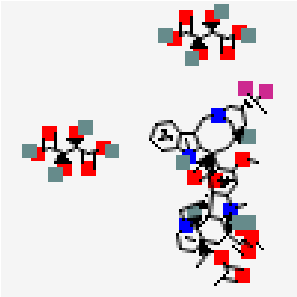
Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol.Record the optical density (OD) or fluorescence intensity for each well.

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. **Vinflunine ditartrate** - A third-generation vinca alkaloid, effective as second-line therapy for TCC.



Molecular Formula: C53H66F2N4O20
Molecular Weight: 1117.1 g/mol

IUPAC Name
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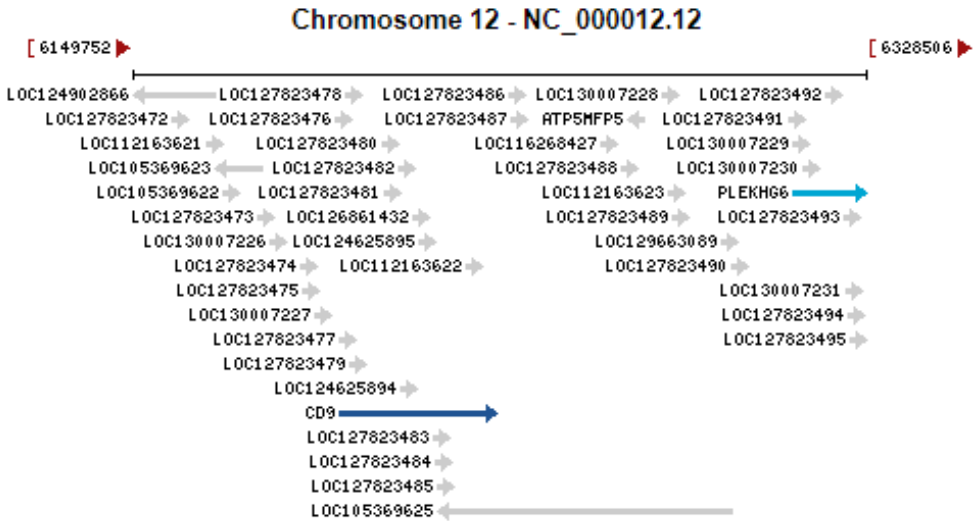
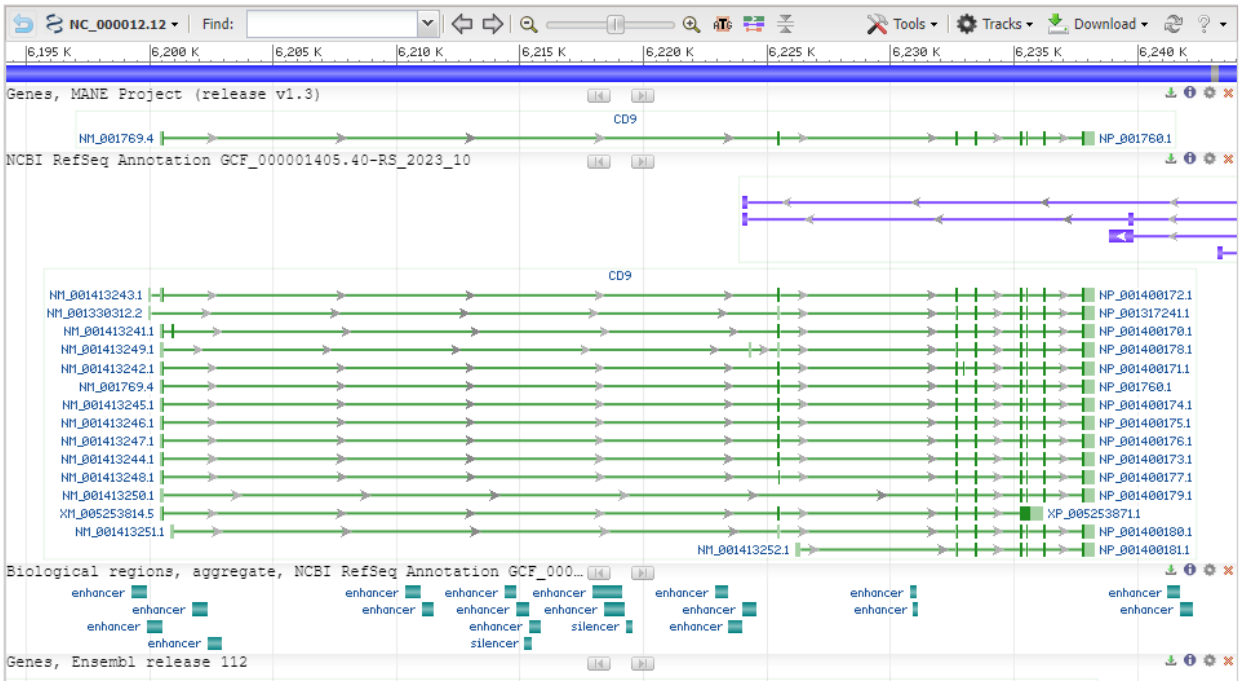
Gene ID: 928





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

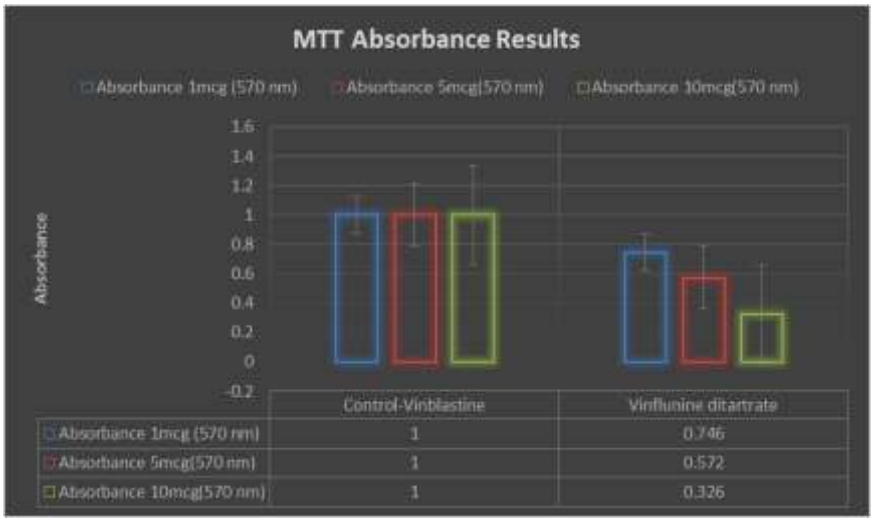
Procedure

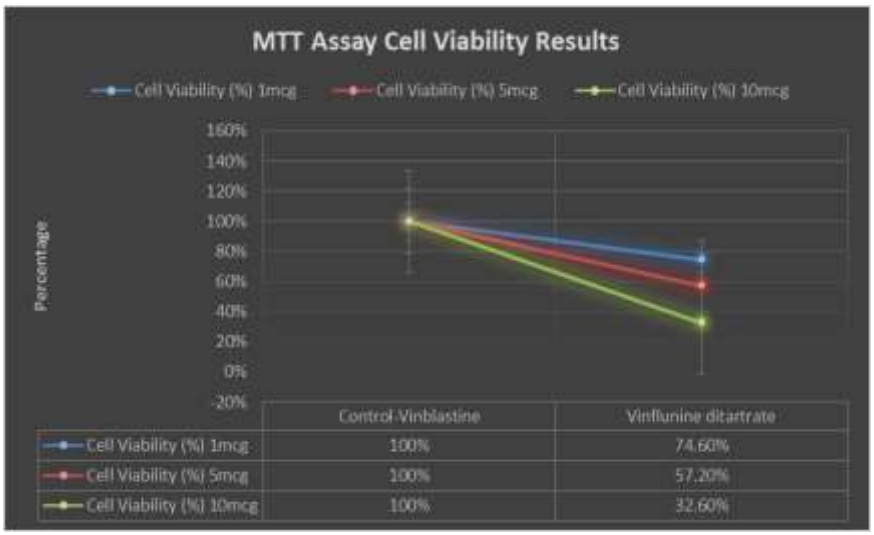
1. **Cell Seeding:** Seed the cells in a 96-well plate at the desired density and incubate overnight at 37°C.
2. **Treatment:** Add the drug treatments and incubate for 24-72 hours.
3. **Supernatant Collection:** Transfer 50 µL of the cell culture supernatant from each well to a new 96-well plate.
4.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

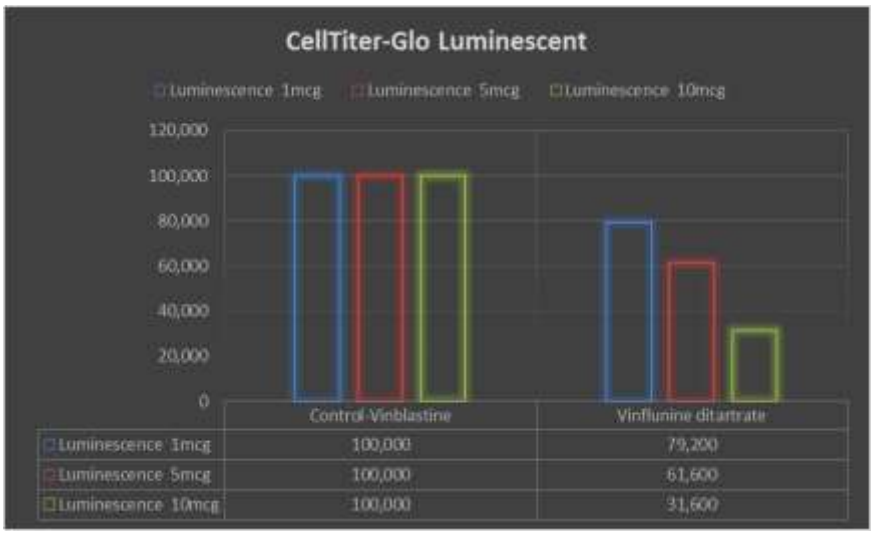
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Vinflunine ditartrate	1	0.746	74.6%
	5	0.572	57.2%
	10	0.326	32.6%

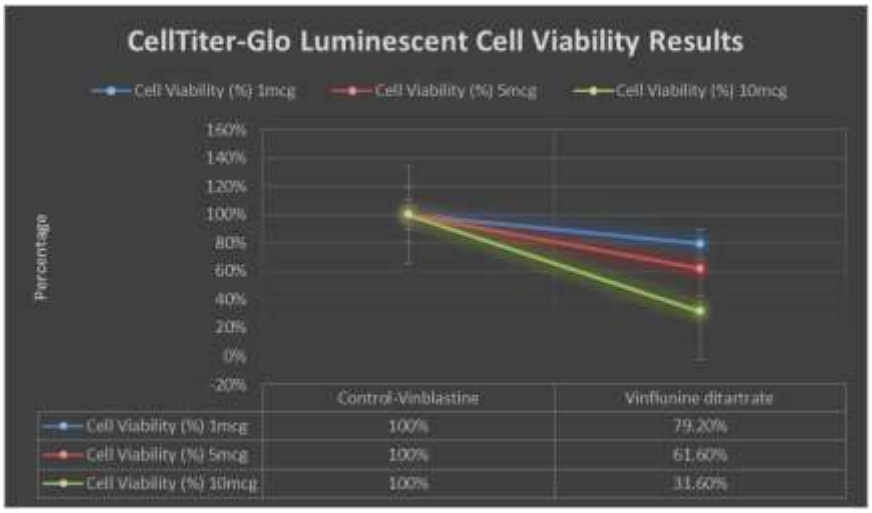




CellTiter-Glo Luminescent Cell Viability Assay Results

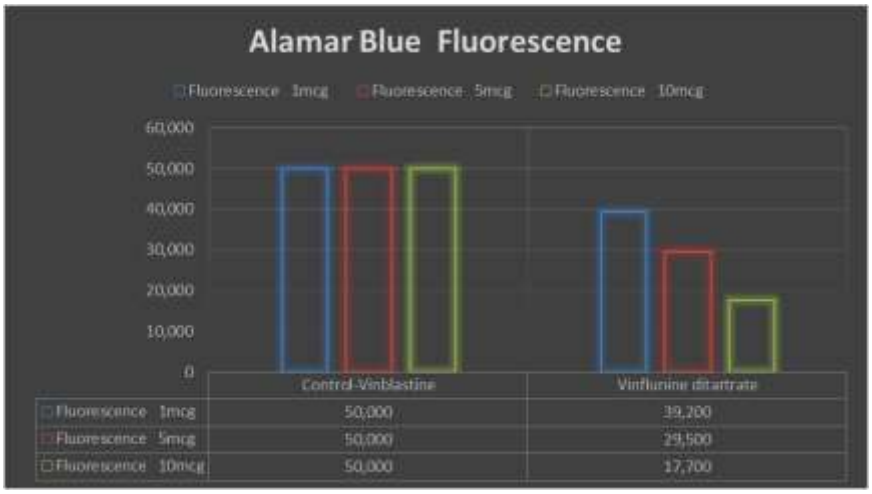
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Vinblastine)	-	100,000	100%
Vinflunine ditartrate	1	79,200	79.2%
	5	61,600	61.6%
	10	31,600	31.6%

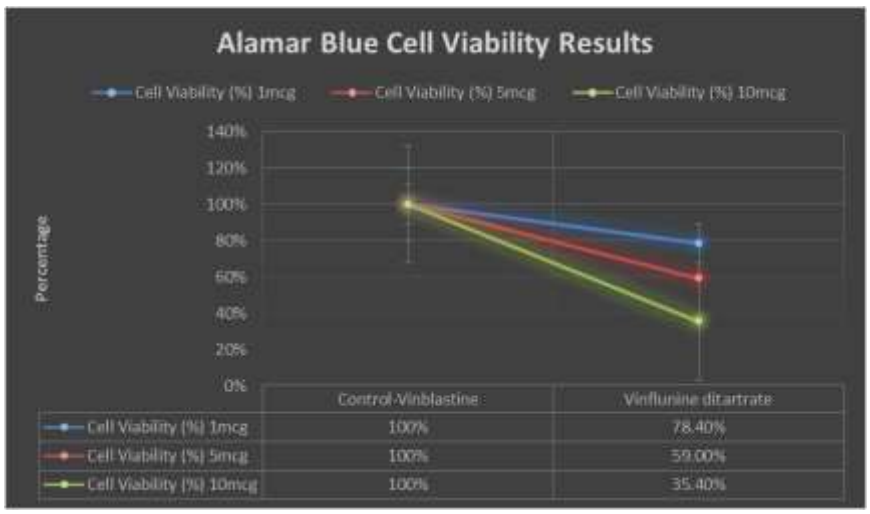




Alamar Blue Assay Results

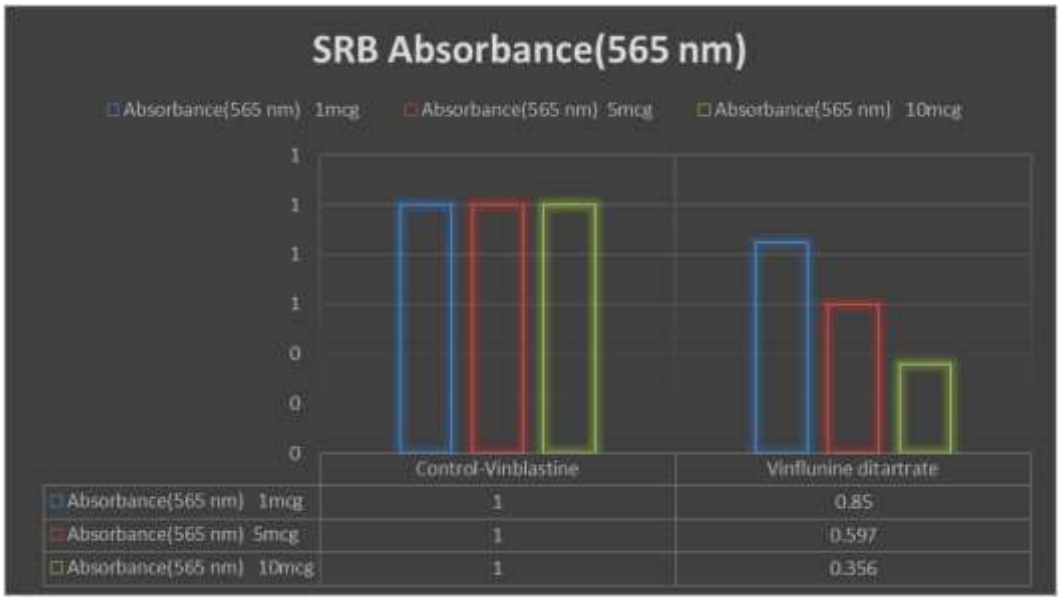
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	50,000	100%
Vinflunine ditartrate	1	0.835	39,200	78.4%
	5	0.636	29,500	59.0%
	10	0.310	17,700	35.4%

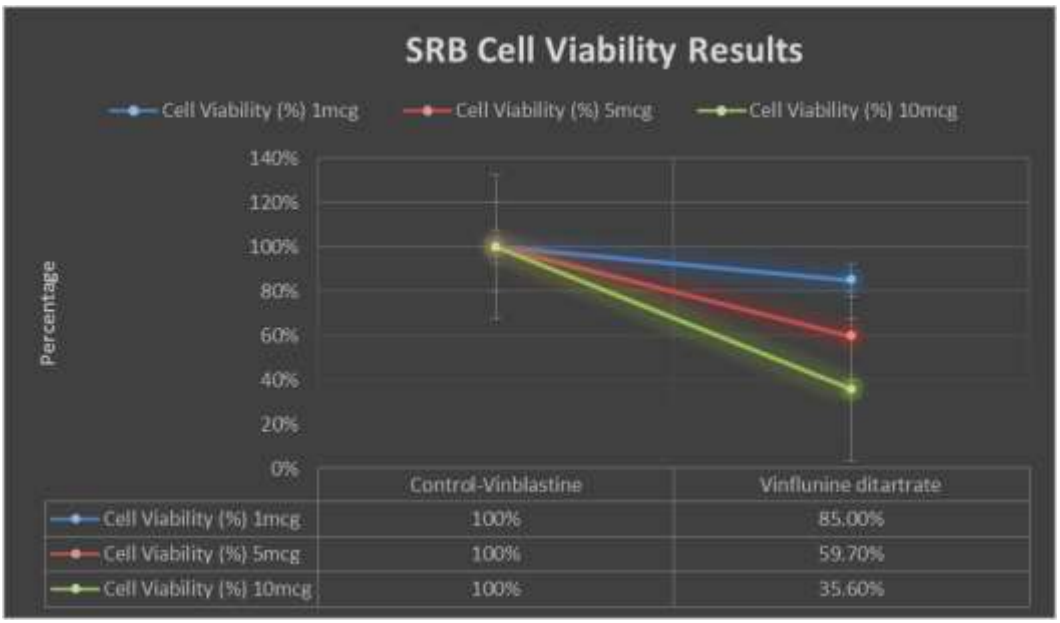




SRB Assay Results

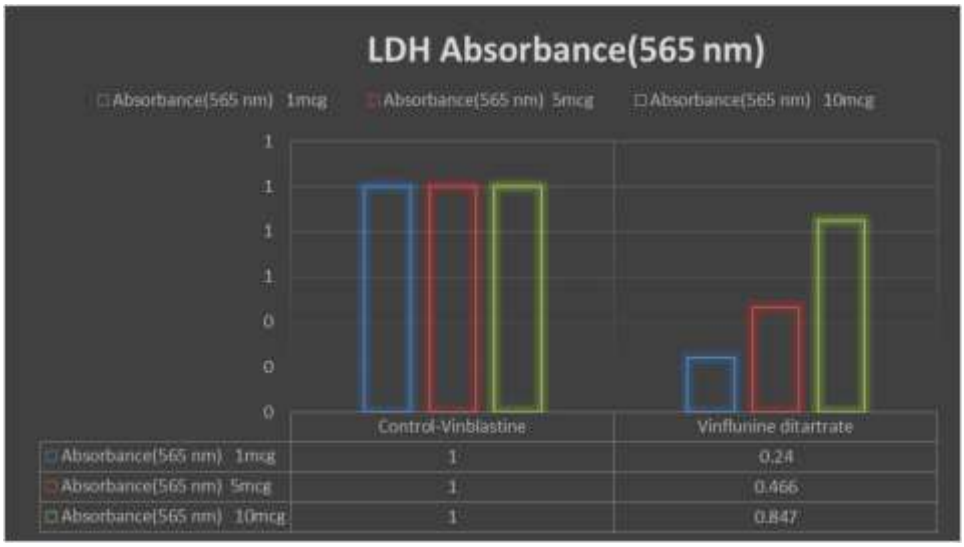
Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Vinflunine ditartrate	1	0.850	85.0%
	5	0.597	59.7%
	10	0.356	35.6%

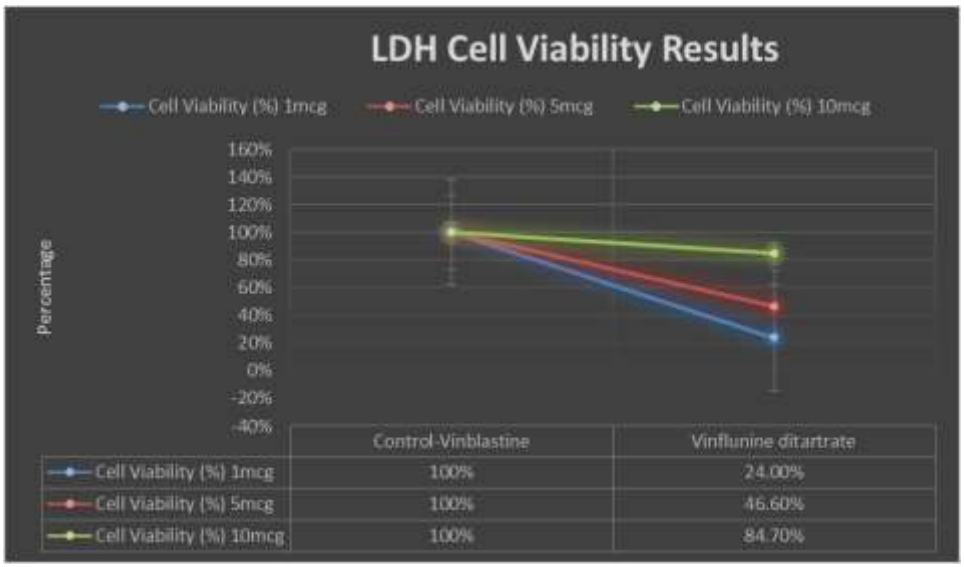




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Vinblastine)	-	1.000	100%
Vinflunine ditartrate	1	0.240	24.0%
	5	0.466	46.6%
	10	0.847	84.7%





DISCUSSION

The results from various assays revealed a consistent pattern of dose-dependent cytotoxicity and reduced cell viability when kidney cancer cells were treated with Vinflunine ditartrate. MTT and SRB assays, which measure cell metabolic activity and protein content, respectively, showed significant decreases in absorbance values with increasing concentrations of Vinflunine, indicating reduced cell proliferation and survival. The CellTiter-Glo and Alamar Blue assays, which assess ATP levels and cellular reduction potential, confirmed these findings, showing a marked decline in luminescence and fluorescence values, respectively. Furthermore, the LDH cytotoxicity assay demonstrated increased LDH release, indicative of cell membrane damage and cytotoxicity. Comparatively, Vinblastine-treated cells exhibited lower cytotoxic effects across all assays. These results suggest that Vinflunine ditartrate is more potent and effective in inducing cell death in TCC cells, making it a viable alternative for advanced-stage cancer treatment.

CONCLUSION

Vinflunine ditartrate demonstrated superior cytotoxicity and a greater reduction in cell viability compared to Vinblastine in kidney cancer cell lines. The molecule showed a clear dose-dependent response, with significant antiproliferative and cytotoxic effects across all assays performed. This study supports the potential of Vinflunine ditartrate as an effective second-line therapeutic agent for transitional cell carcinoma, warranting further in vivo studies and clinical evaluations.

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