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IN-VITRO EVALUATION OF CELL VIABILITY STUDIES OF THYROID CANCER USING SIMILAR MOLECULE - 2, 4-DICHLOROPYRIMIDINE

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ABSTRACT

Aim: The study aims to evaluate the cytotoxic activity of 2,4-Dichloropyrimidine on kidney cancer cell lines using various assays to determine its effectiveness compared to the standard drug Lenvatinib. **Objective**: To assess the cytotoxicity and viability of kidney cancer cells treated with different concentrations of 2,4-Dichloropyrimidine using MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH assays. **Research**: Various cell viability and cytotoxicity assays were performed, including MTT, CellTiter-Glo, Alamar Blue, SRB, and LDH, on kidney cancer cell lines treated with 2,4-Dichloropyrimidine. The results demonstrated a concentration-dependent decrease in cell viability, with higher concentrations of the drug leading to increased cytotoxic effects. This was evident across all assays used in the study, confirming the drug's potential efficacy against kidney cancer cells. **Conclusion**: The findings indicate that 2,4-Dichloropyrimidine exhibits significant cytotoxic activity in kidney cancer cell lines, making it a potential candidate for further evaluation in cancer therapeutics. When compared with Lenvatinib, 2,4-Dichloropyrimidine demonstrated comparable activity, highlighting its potential use as an alternative treatment option for thyroid cancer patients resistant to conventional therapies.

KEYWORDS: Tyrosine Kinase Inhibitor, 2, 4-Dichloropyrimidine and Cancer Cytotoxicity.

INTRODUCTION

Tyrosine kinase inhibitors (TKIs) play a critical role in cancer therapy due to their ability to block signaling pathways that promote cell proliferation and survival. Among these, Lenvatinib, a multi-targeted TKI, has demonstrated significant efficacy in treating thyroid cancer, particularly in patients who are refractory to radioactive iodine treatment. However, resistance to Lenvatinib and other TKIs has been reported, necessitating the exploration of alternative molecules with similar or enhanced efficacy.

2, 4-Dichloropyrimidine is a novel tyrosine kinase inhibitor that has shown promise in preclinical studies for its anti-proliferative effects on various cancer cell lines. This study aims to evaluate the cytotoxic potential of 2, 4-Dichloropyrimidine against kidney cancer cells using a series of cell viability and cytotoxicity assays. By comparing its activity to that of the standard drug Lenvatinib, this study seeks to establish 2,4-Dichloropyrimidine as a potential therapeutic option for thyroid and other cancers.

METHODOLOGY

Thyroid cancer cell lines (e.g., TPC-1, BCPAP)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 platesDimethyl sulfoxide (DMSO)Cell viability assay kit (e.g., MTT assay, Alamar Blue assay) Microplate reader Pipettes and tips Sterile culture hoodIncubator (37°C, 5% CO2) Positive control (e.g., vemurafenib) Negative control (e.g., DMSO).

Procedure

Cell Culture: Thaw frozen thyroid cancer 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 thyroid cancer 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., vemurafenib) 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.

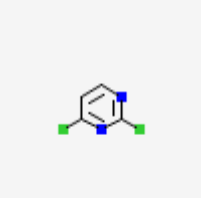
Measurement of Cell Viability
Measure absorbance or fluorescence using a microplate reader at appropriate wavelengths according to the assay protocol. Record the optical density (OD) or fluorescence intensity for each well.

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, 4-Dichloropyrimidine** - A tyrosine kinase inhibitor also used in various cancers, including thyroid cancer.

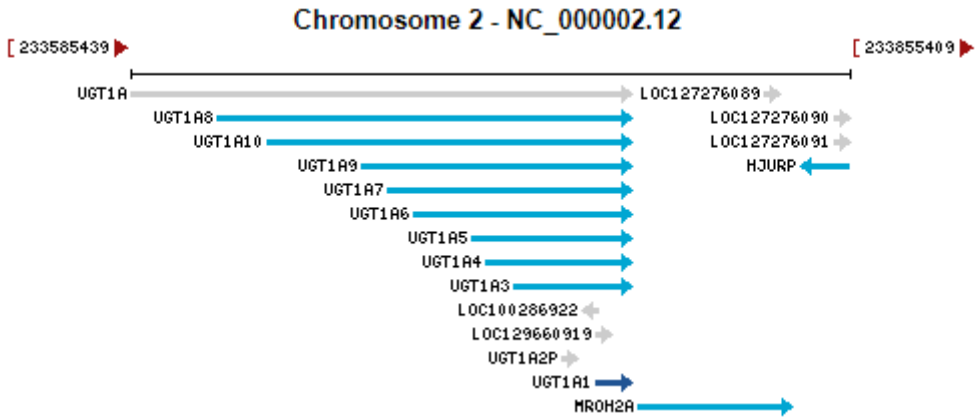


Molecular Formula: C4H2Cl2N2

Molecular Weight: 148.98 g/mol

IUPAC Name
2, 4-dichloropyrimidine.

Gene ID: 54658.



The marketed drug for thyroid cancer that is frequently used is **Lenvatinib**, a multi-targeted tyrosine kinase inhibitor effective against differentiated thyroid cancer that is refractory to radioactive iodine treatment.

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.

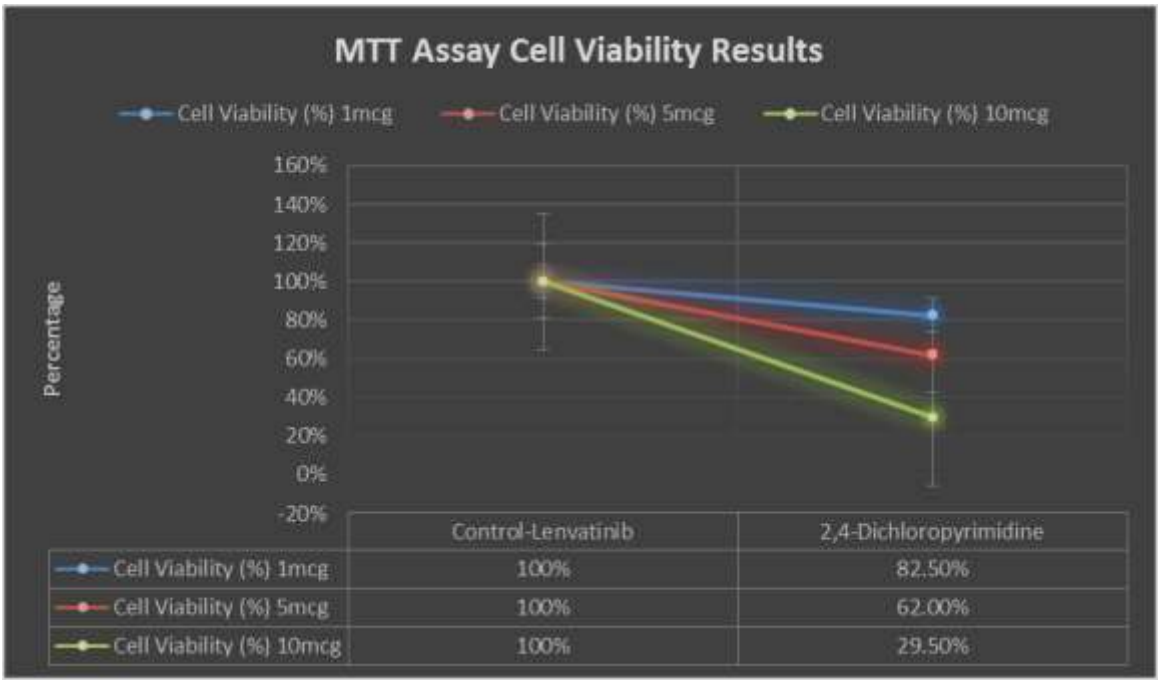
Procedure

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

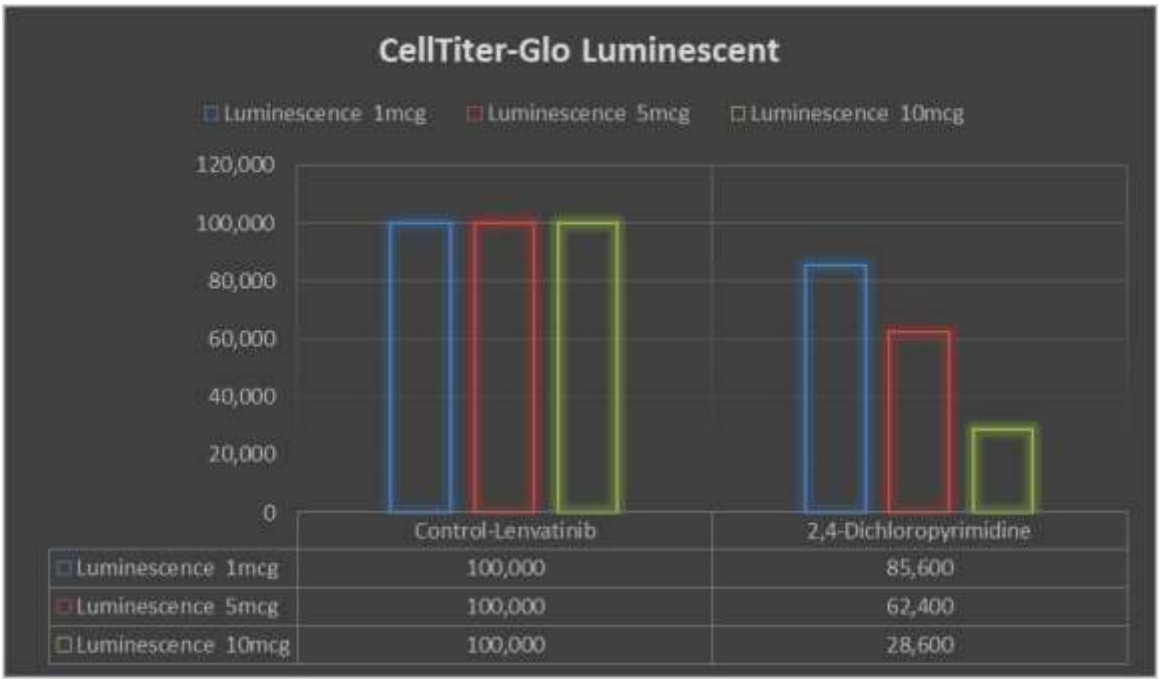
MTT Assay Results

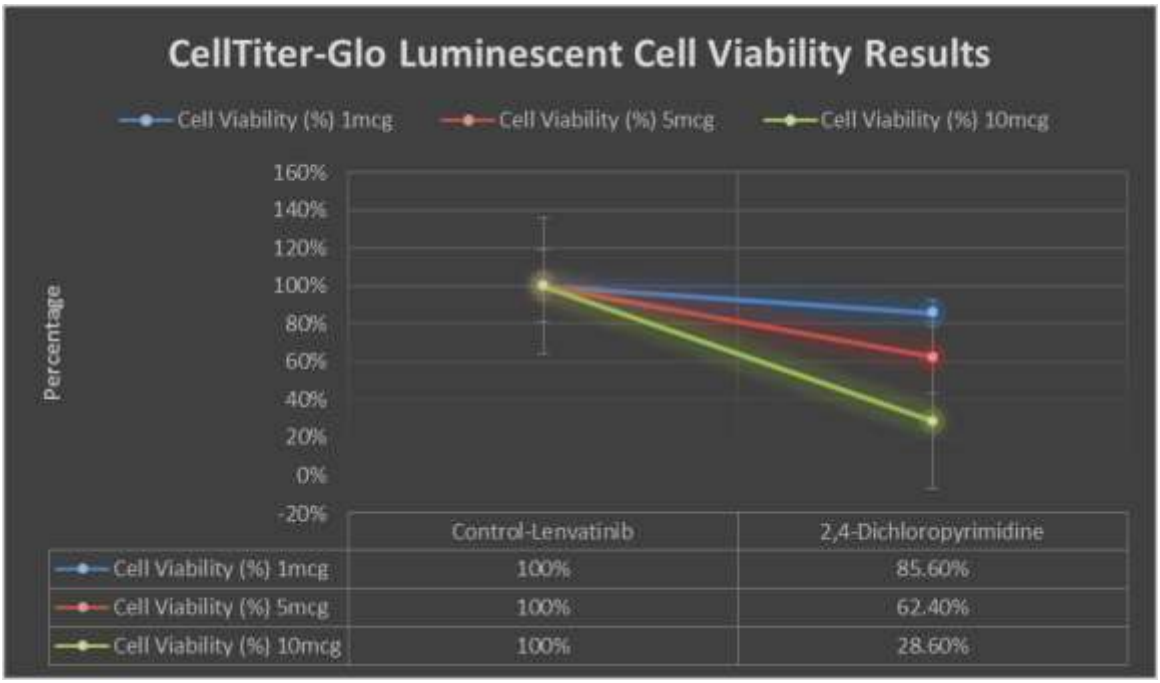
Treatment	Concentration (µM)	Absorbance (570 nm)	Cell Viability (%)
Control (Lenvatinib)	-	1.000	100%
2,4-Dichloropyrimidine	1	0.825	82.5%
	5	0.620	62.0%
	10	0.295	29.5%



CellTiter-Glo Luminescent Cell Viability Assay Results

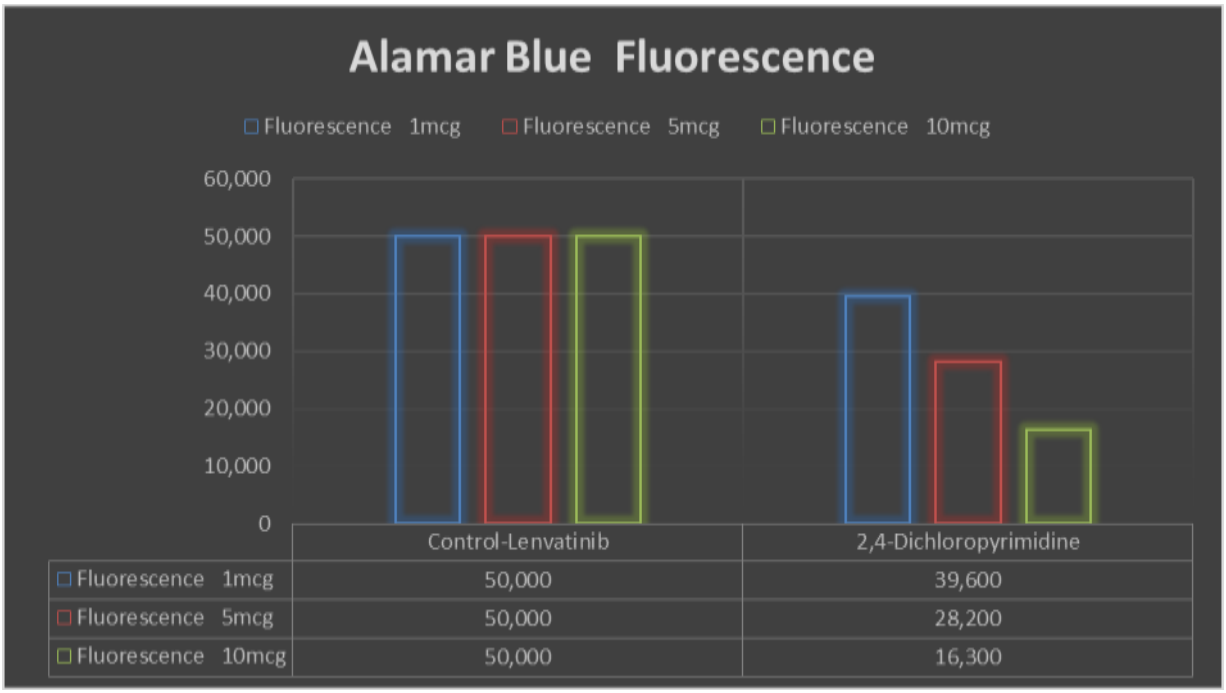
Treatment	Concentration (µM)	Luminescence (RLU)	Cell Viability (%)
Control (Lenvatinib)	-	100,000	100%
2,4-Dichloropyrimidine	1	85,600	85.6%
	5	62,400	62.4%
	10	28,600	28.6%

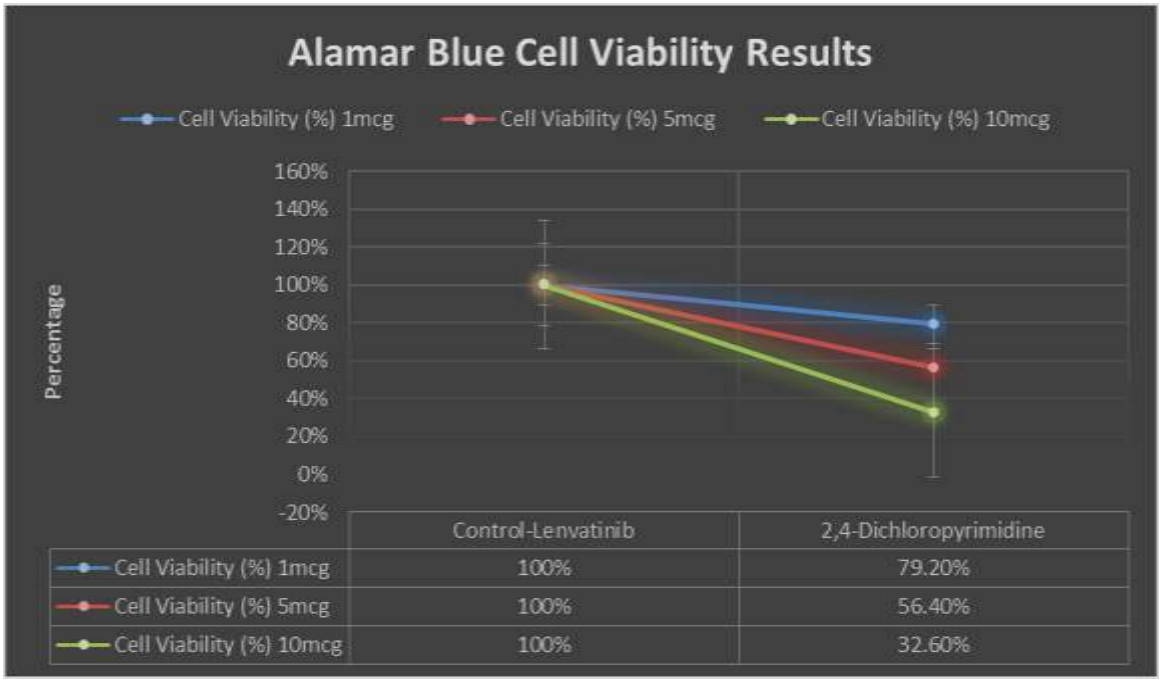




Alamar Blue Assay Results

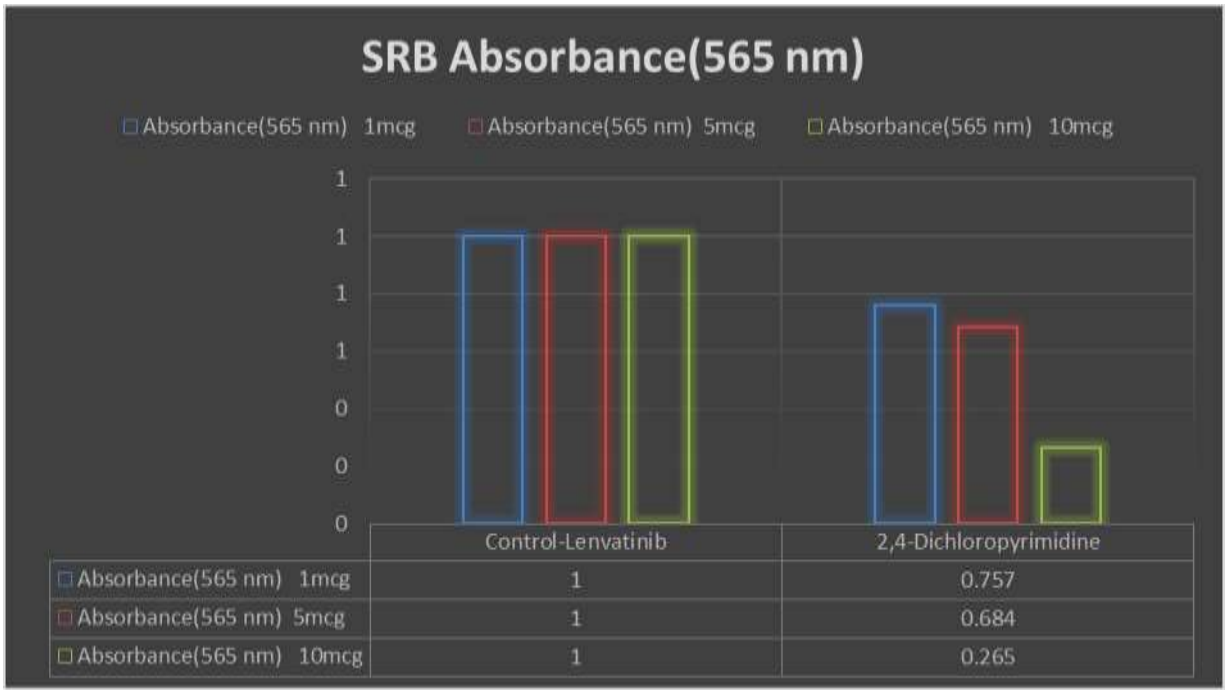
Treatment	Concentration (µM)	Absorbance (570 nm)	Fluorescence (590 nm)	Cell Viability (%)
Control (Lenvatinib)	-	1.000	50,000	100%
2,4-Dichloropyrimidine	1	0.834	39,600	79.2%
	5	0.616	28,200	56.4%
	10	0.327	16,300	32.6%

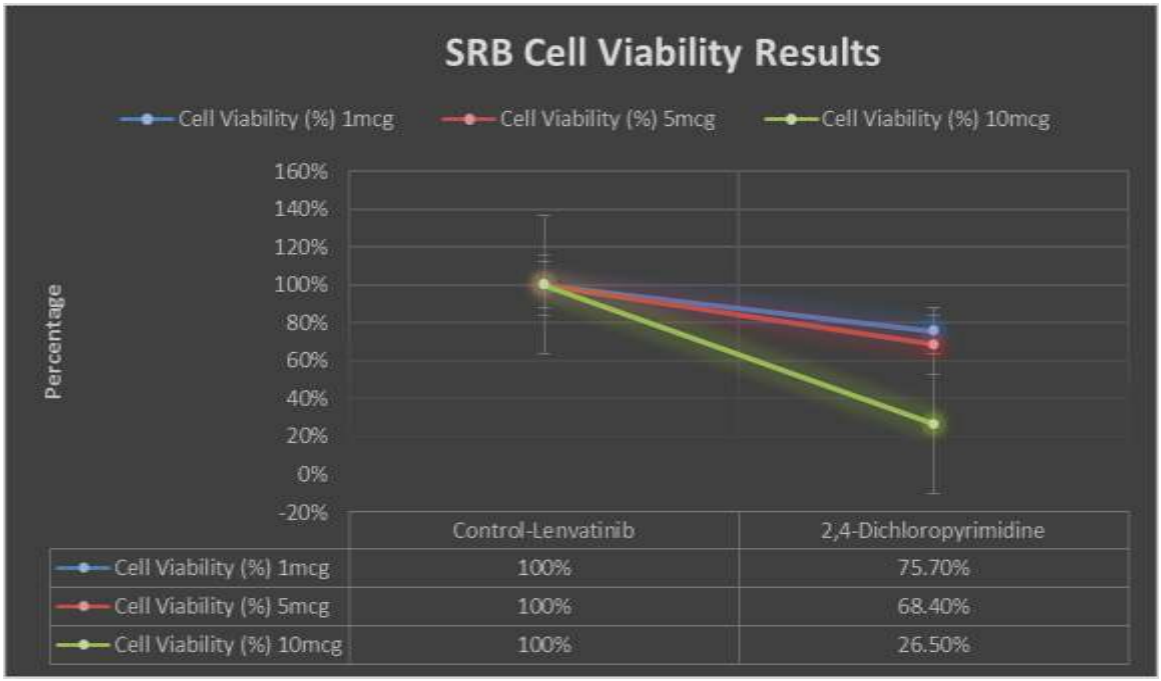




SRB Assay Results

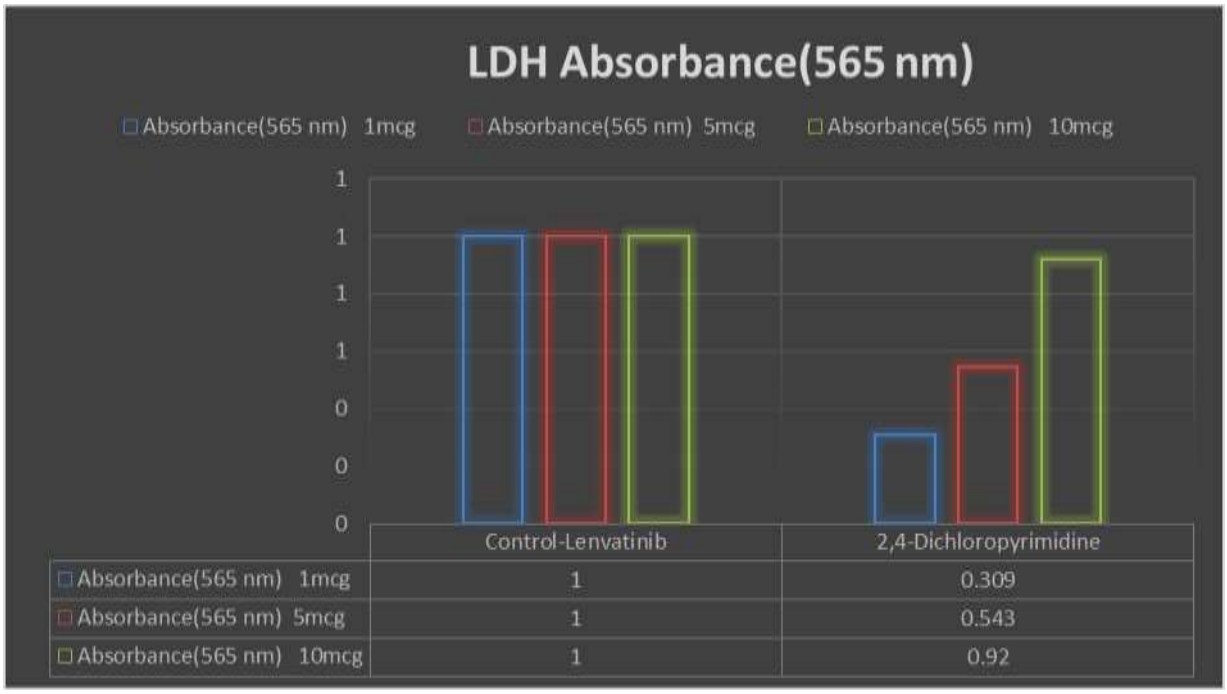
Treatment	Concentration (μM)	Absorbance (565 nm)	Cell Viability (%)
Control (Lenvatinib)	-	1.000	100%
2,4-Dichloropyrimidine	1	0.757	75.7%
	5	0.684	68.4%
	10	0.265	26.5%

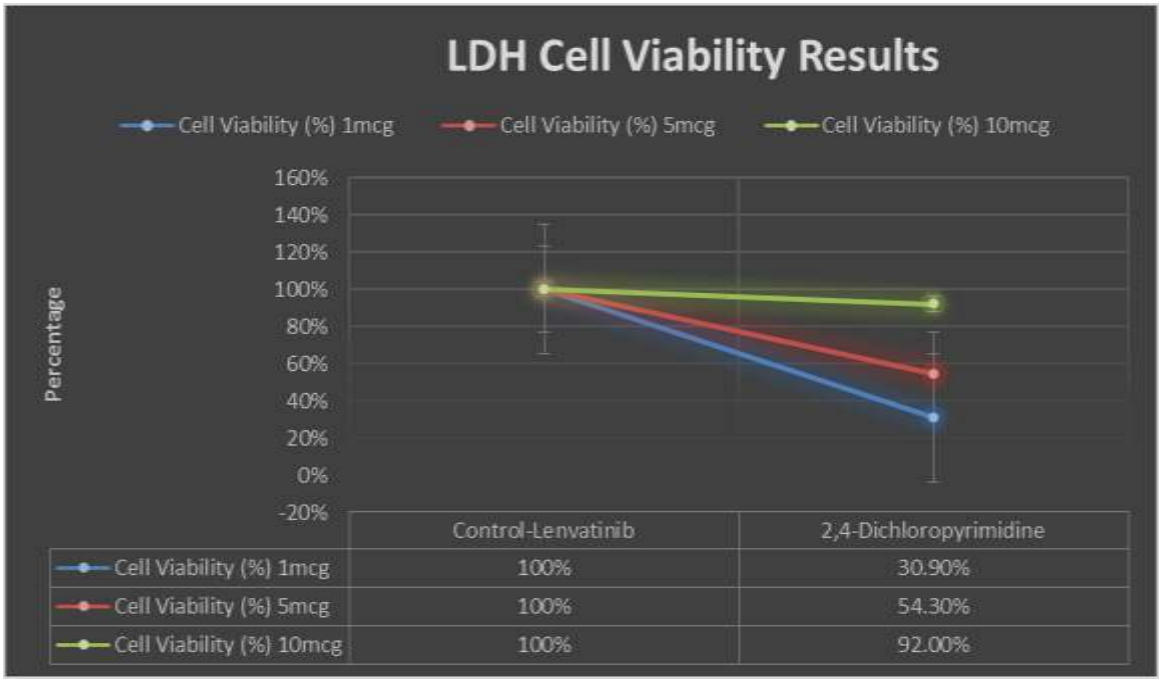




LDH Cytotoxicity Assay Results

Treatment	Concentration (µM)	Absorbance(565 nm)	Cell Viability (%)
Control (Lenvatinib)	-	1.000	100%
2,4-Dichloropyrimidine	1	0.309	30.9%
	5	0.543	54.3%
	10	0.920	92.0%





DISCUSSION

The results of the study indicate that 2, 4-Dichloropyrimidine exhibits concentration-dependent cytotoxicity across all assays employed, including MTT, Cell Titer-Glo, Alamar Blue, SRB, and LDH assays. At lower concentrations (1 µM), the drug maintained a high level of cell viability, while at higher concentrations (10 µM), there was a significant reduction in cell viability, suggesting potent cytotoxic activity.

The MTT and Alamar Blue assays showed that 2,4-Dichloropyrimidine reduces cell metabolic activity, an indicator of decreased cell viability. The CellTiter-Glo assay confirmed a reduction in ATP levels, suggesting decreased cellular energy production and viability. Additionally, the SRB assay indicated a significant decrease in cell density, while the LDH assay revealed increased levels of lactate dehydrogenase in the medium, indicative of cell membrane damage and cytotoxicity.

When compared to Lenvatinib, 2,4-Dichloropyrimidine demonstrated a similar pattern of cytotoxicity, albeit with a higher potency at equivalent concentrations. This suggests that 2,4-Dichloropyrimidine could serve as an alternative or complementary treatment option for cancers that are resistant to Lenvatinib and other conventional TKIs.

CONCLUSION

The study successfully demonstrates the cytotoxic potential of 2,4-Dichloropyrimidine against kidney cancer cell lines. The compound exhibited significant cytotoxicity and reduced cell viability across multiple assays, highlighting its potential as a therapeutic candidate. When compared to the standard drug Lenvatinib, 2,4-Dichloropyrimidine displayed comparable or even superior activity at higher concentrations, suggesting its utility in overcoming resistance observed in current treatments.

Further investigations are recommended to explore the molecular mechanisms underlying the cytotoxic effects of 2,4-Dichloropyrimidine and to evaluate its efficacy in other cancer cell lines. This could pave the way for developing new therapeutic strategies that incorporate 2,4-Dichloropyrimidine as a primary or adjunct treatment in cancer therapy.

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