Experiment time: 2025-04-21, 19:00 - 2025-04-25, 17:00

Flow Cytometric Analysis of Apoptosis (I)

I. Objective of the experiment

To assess and compare the apoptotic levels of T24 wild-type and low-, medium-, and high-drug-resistant T24-RC48 cell lines, both untreated and following treatment, using an Annexin V-FITC/PI apoptosis detection kit. This analysis aims to elucidate the apoptotic characteristics of the resistant variants and provide an experimental basis for understanding ADC resistance mechanisms in bladder cancer.

II. Experimental content

2.1 Experimental design

Sample grouping: Blank control group (untreated T24 wild-type cell lines and low, medium and high drug-resistant T24-RC48 cell lines);

Drug treatment group (T24 wild-type cell lines and low, medium and high drug resistant T24-RC48 cell lines were treated with videstizumab (RC48) for 36 hours respectively)

Number of repetitions: 3 samples per group.

2.2 Measurement principles

Annexin V-FITC is a fluorescently labeled membrane-associated protein that specifically binds to phosphatidylserine (PS) exposed on the outer leaflet of the plasma membrane in apoptotic cells. Propidium iodide (PI), a nucleic acid dye, penetrates the cell membranes of late-stage apoptotic and necrotic cells, staining the nucleus red. Flow cytometry analysis of both Annexin V-FITC and PI fluorescence signals enables precise differentiation between viable cells, early apoptotic cells, and late apoptotic/necrotic cells.

III. Materials and reagents

3.1 Materials

Human bladder transitional cell carcinoma cell line (T24)

Low, medium, and high drug-resistant cell lines T24-RC48

T25 cell culture flasks (Corning)

6-well plate (Corning Inc.)

15ml centrifuge tube (Axygen Company)

Micropipettes (Eppendorf)

Pipette tips (Axygen company)

3.2 Reagents

Annexin V-FITC/PI cell apoptosis kit (Lianke Biology, AT101)

McCoy's 5A medium (Shanghai Yuanpei Biotechnology Co., Ltd., China)

Fetal bovine serum (FBS, Gibco)

Trypsin (Gibco)

PBS buffer (Gibco)

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Flow cytometer (BD, not the C6 model)

V. Experimental steps

5.1 Seed T24 wild-type and low-, medium-, and high-resistant T24-RC48 cell lines into 6-well plates, with two wells per cell line at a density of 2×10^5 cells per well.

5.2 After 24 hours, for each cell line, replace the medium in one well with fresh complete medium (untreated control), and the medium in the duplicate well with fresh complete medium containing 200μg/mL Disitamab vedotin (RC48). Treat the cells for 36 hours.

5.3 Discard the culture medium and wash the cells with PBS for 3 times.

5.4 Add Accutase solution to digest the cells and gently tap the culture bottle to detach the cells.

5.5 Collect the cell suspension per well and centrifuge at 1000rpm for 5 minutes. Discard the supernatant.

5.6 Wash cells twice with pre-colded PBS and discard the supernatant.

5.7 Dilute 5× Binding Buffer with double distilled water to 1× working buffer. Take 500μl 1× Binding Buffer to resuspend the cells. Add 5μl Annexin V-FITC and 10μl PI to each tube. Vortex

gently and incubate at room temperature in the dark for 5 minutes.

5.8 Harvest 1×10^6 untreated wild-type cells. Resuspend the cell pellet in 500μL of Apoptosis Positive Control Solution and incubate on ice for 30 minutes.

5.9 Centrifuge the cells, discard the supernatant, and resuspend in a small volume of ice-cold 1× Binding Buffer. Mix these induced apoptotic cells with an equal number of untreated, viable wild-type cells.

5.10 Adjust the total volume to 1.5 mL with ice-cold 1× Binding Buffer. Split this mixture equally into three tubes: one unstained control tube, one tube for Annexin V-FITC single stain (add 5μL), and one tube for PI single stain (add 10μL). Incubate all tubes at room temperature in the dark for 5 minutes.

5.11 Analyze the samples using a flow cytometer.

5.12 Use FlowJo software to analyze the flow cytometry data, calculate the apoptosis rate of each group of cells, and generate bar graphs representing the apoptosis rates to compare the apoptosis difference between different drug-resistant groups and wild-type cell lines.

VI. Experimental results

Table 1 Apoptosis and Survival Rates of Bladder Cancer Cells with Varying Drug Resistance

	Wild type	Low levels of resistance	Intermediate drug resistance	High levels of resistance	Wild type (medicate)	Low levels of resistance (medicate)	Intermediate drug resistance (medicate)	High levels of resistance (medicate)
Apoptosis rate (%)	4.05	1.8	1.93	1.69	4.34	2.92	2.48	2.08
Viability (%)	95.95	98.2	98.07	98.31	95.66	97.08	97.52	97.92

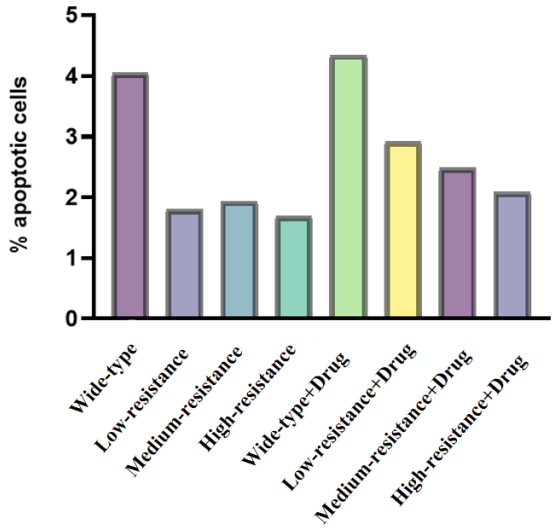

Figure 1 Comparison of Apoptosis Rates of Bladder Cancer Cells with Different Resistance Levels

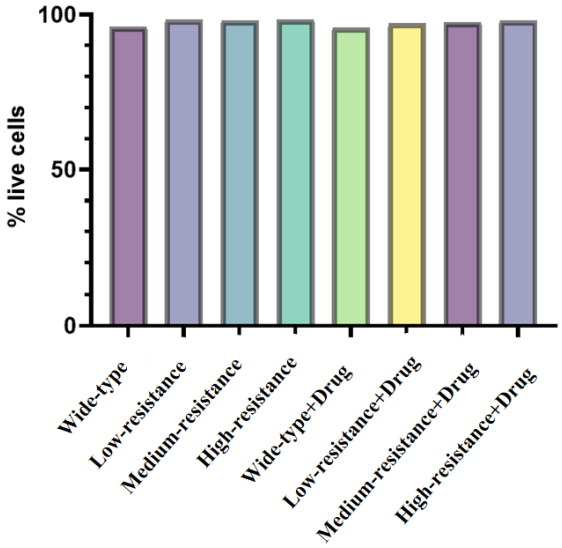

Figure 2 Comparison of Cell Viability of Bladder Cancer Cells with Different Degrees of Drug Resistance

VII. Results analysis

The survival rates of the T24 wild-type cell line and its low-, medium-, and high-drug-resistant variants (T24-RC48) showed minimal overall variation. While apoptosis rates in bladder cancer cells with varying drug resistance levels demonstrate a negative correlation with resistance severity, all cell lines exhibited relatively low apoptosis rates. This phenomenon may be attributed to the following factors:

1) The initial plate density (2×10^5/ hole) is too high, resulting in enhanced inhibition of intercellular contact, reduced drug permeability efficiency, and insufficient induction of apoptosis signal.

2) The 36-hour drug treatment may not have reached the peak of the apoptosis process, and some cells did not enter the apoptosis stage, resulting in a decrease in the efficiency of Annexin V-FITC/PI labeling.

3) Late-stage apoptotic or necrotic cells may have been separated from the culture plate and floated in the culture medium, which were not included in the test samples, resulting in an underestimate of the apoptosis rate data.

To address these potential issues, the following modifications were implemented for the subsequent formal experiment:

1) The cell inoculation density was adjusted to 1×10^5/cell to reduce contact inhibition and improve the interaction efficiency between drugs and cells.

2) The processing time of RC48 was extended to 48 hours to ensure that the apoptosis process was fully completed.

3) Before digesting the adherent cells, collect the suspended cells in the culture medium, combine and centrifuge them for testing to avoid data loss.