Experiment time: 2025-03-22, 18:00 - 2025-03-30, 21:00

Growth Curve Measurement (II)

I. Objective of the experiment

The proliferative ability of T24 cell lines and low, medium and high drug-resistant T24-RC48 cell lines under the treatment of 200ug/ml RC48 was dynamically monitored using the CCK-8 assay. The absorbance values were continuously measured for 5 days (0h,24h,48h,72h,96h,120h), and the growth curve was plotted to evaluate the long-term inhibitory effects of the drug on cell proliferation.

II. Experimental content

2.1 Experimental design

Cell types: T24 wild-type cell line, low/medium/high drug resistance T24-RC48 cell line.

Drug treatment: Vedotin (RC48) concentration gradient (0, 50,100,200,500μg/ml).

Time: 0h (D0),24h (D1),48h (D2),72h (D3),96h (D4),120h (D5).

Number of replicates: 6 wells per group.

2.2 Measurement principle

WST-8 in CCK-8 reagent was reduced to an orange-colored, water-soluble formazan dye by mitochondrial dehydrogenase of living cells, and the absorbance (OD450nm) was positively correlated with the number of living cells.

III. Materials and reagents

3.1 Materials

The 1000μl and 100μl pipette tips and 15ml centrifuge tubes were purchased from Axygen

96-well plates (Corning Inc.) × 6

Micropipettes: Eppendorf

T25 culture flasks were purchased from Corning

Human bladder transitional cell carcinoma cell line (T24)

Low-, medium-, and high-resistance T24-RC48 cell lines

3.2 Reagents

PBS and trypsin were purchased from Gibco, USA

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

Fetal bovine serum (FBS, Gibco)

Disitamab vedotin (RC48, Rongchang Biopharmaceutical Co., Ltd., China)

CCK-8 kit

IV. Experimental instruments

Clean bench: Suzhou purification equipment factory

Benchtop low-speed centrifuge at room temperature: Eppendorf

Microplate reader (Thermo Fisher Scientific)

37℃,5% CO_2 cell culture chamber (Thermo Fisher Scientific)

V. Experimental steps

5.1 T24 and low/medium/high drug-resistant T24-RC48 cell lines in the logarithmic growth phase were harvested, trypsinized, centrifuged, and resuspended. The cell density was adjusted to 3×10^4 cells/mL.

5.2 The cell suspension was inoculated into six 96-well plates according to the experimental design, with each plate containing 12 wells per cell type. Each well received 100μL of culture medium (containing 3×10^3 cells), and edge wells were covered with PBS to prevent evaporation. The plates were then cultured in a 37°C incubator maintained at 5% CO_2 concentration.

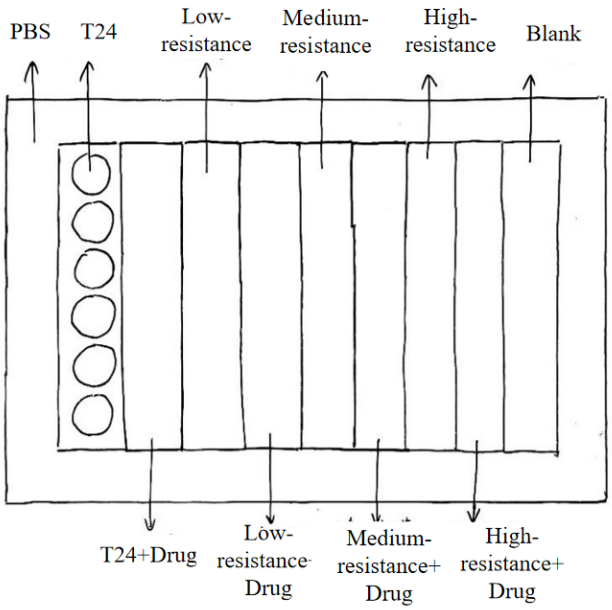

Figure 1 Distribution of cell suspensions in each group in the 96-well plate

5.3 After overnight cell seeding, the culture medium was carefully aspirated from the wells and replace with fresh medium in five 96-well plates (each plate contains six wells of fresh medium supplemented with 200μg/ml RC48). Set time 0 as the reference point and incubate at 37°C with 5% CO_2. For the remaining plate (Plate 1), add the chromogenic solution to the culture wells according to the CCK-8 kit instructions. Incubate for 1 hour and measure the absorbance at 450nm using a microplate reader.

5.4 At each time point (24, 48, 72, 96, and 120 hours), the corresponding 96-well plate was removed from the incubator and processed according to the CCK-8 kit protocol. The absorbance at 450 nm was measured.

5.5 The time of treatment with RC48 was taken as the horizontal coordinate, and the OD value of the experimental group at 450nm was taken as the vertical coordinate with the OD value of the blank control hole as the vertical coordinate to draw the time-OD value curve, namely the cell proliferation curve.

VI. Experimental results

6.1 Cell morphology observation

As can be seen from the inverted phase microscope photo in Figure 2, after 120 hours, all cell lines (T24 wild type and low/medium/high drug-resistant T24-RC48) maintained a relatively good

growth state, with relatively intact cell morphology, close adhesion, clear edges, no obvious apoptotic bodies or fragments, and the cell confluence was about 100%.

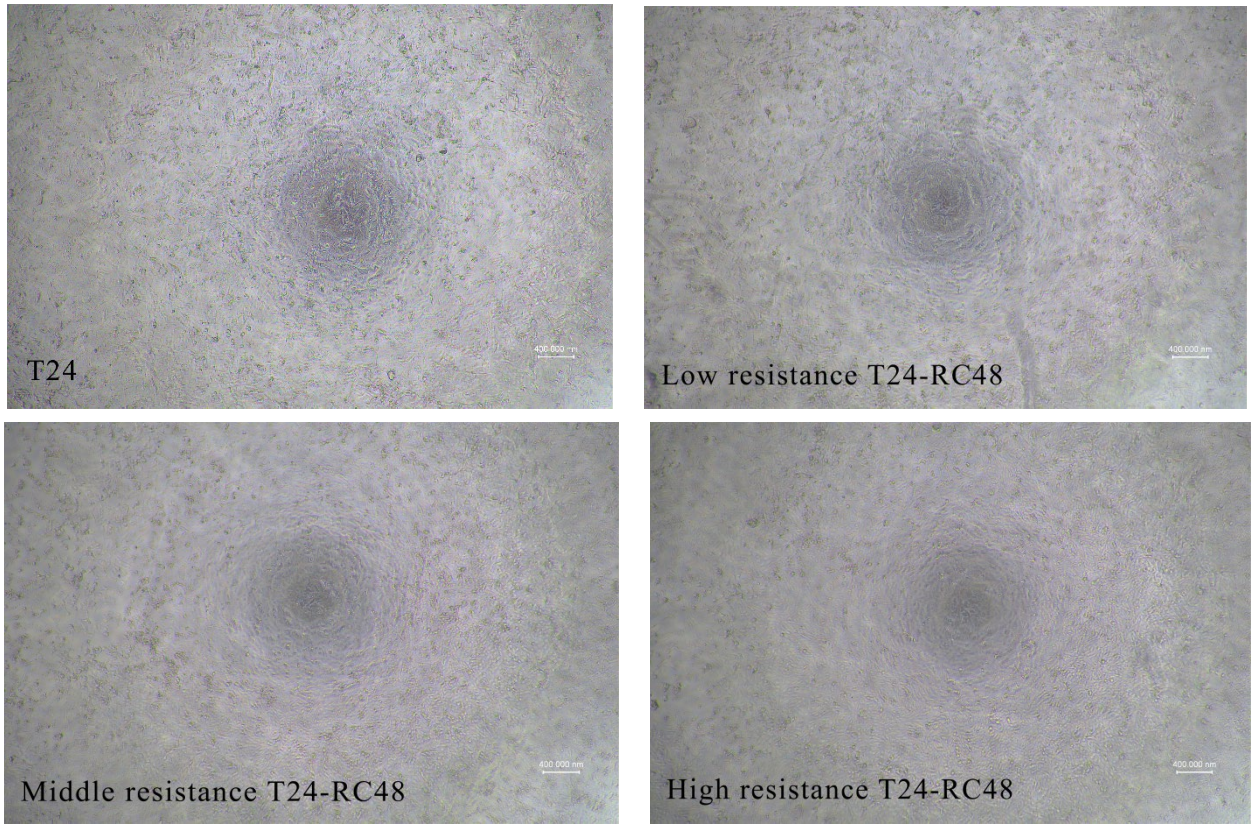

Figure 2 Photos of low/medium/high drug-resistant cell lines T24-RC48 after 120h (no drug)

As shown in the inverted phase contrast microscopy image (Figure 3), after 120 hours of drug treatment, both the T24 cell line and its low/mid/high drug-resistant variants (T24-RC48) exhibited significantly reduced cell densities compared to the untreated group. The T24 wild-type cells demonstrated massive shedding with shrinking residual cells showing typical apoptotic morphology. The T24-RC48 low-resistance variant maintained only slightly higher surface density than the wild-type, while its cells lost normal morphology and their viability was indeterminate. The T24-RC48 medium-resistance variant showed approximately 50% cell density with improved overall morphology compared to the wild-type and low-resistance counterparts, though numerous abnormally shaped cells remained. Notably, the T24-RC48 high-resistance variant demonstrated the highest cell density among all treated groups, exhibiting intact morphology with visible mitotic phases in proliferative areas.

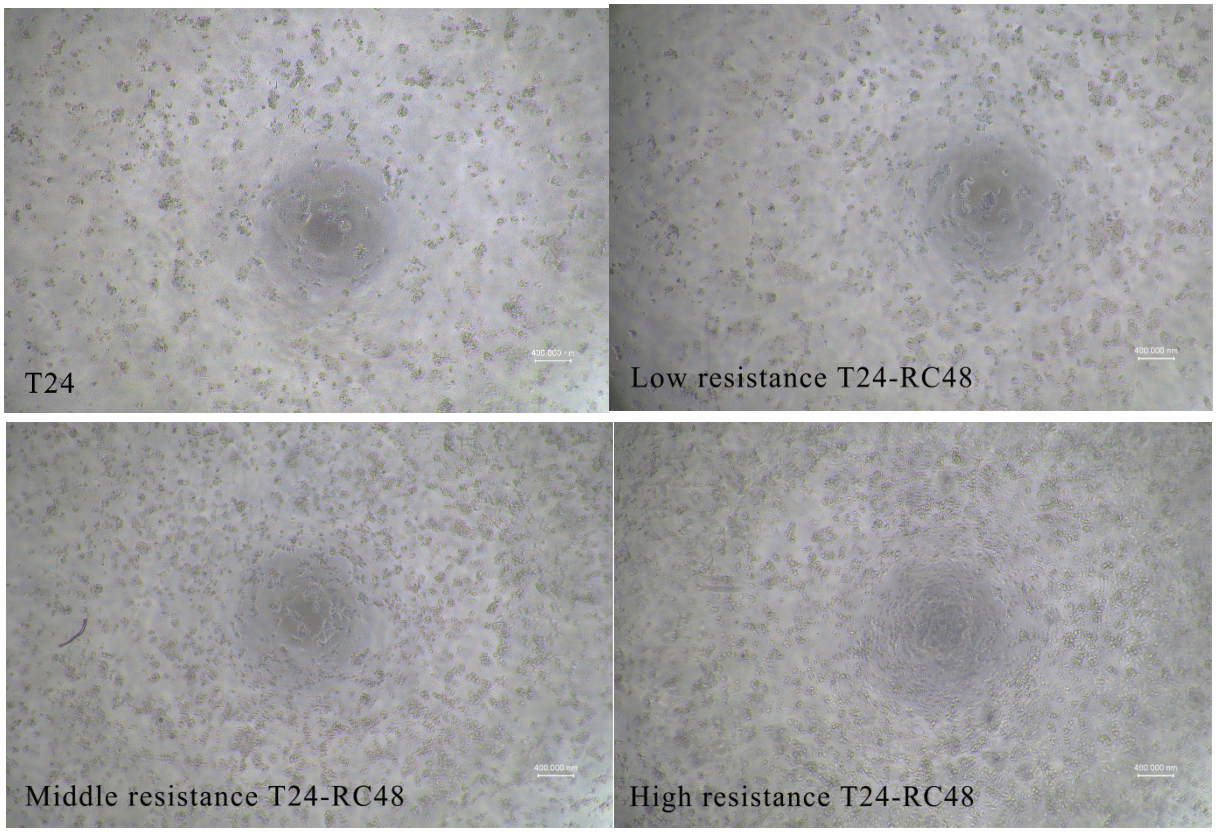

Figure 3 Photos of low/medium/high drug-resistant cell lines T24-RC48 after 120h of drug addition

6.2 Changes in cell proliferation capacity

Under the condition of no addition of RC48 drug treatment (left figure), the four cell lines showed a relatively stable proliferation trend, and their doubling time was not significantly different, indicating that under normal culture conditions, the proliferation of the four cells was similar.

When treated with 200μg/ml of RC48 (right panel), the OD value of wild-type T24 cells decreased sharply from 2.1 to 0.48, corresponding to an inhibition rate of 77.1%. The inhibitory effect on drug-resistant strains gradually diminished with increasing resistance levels: low-resistance strains showed OD=0.55 (72.5% inhibition) on day 5, moderate-resistant strains OD=0.61 (60.5% inhibition), and highly resistant strains OD=1.53 (19.4% inhibition). This demonstrates an inverse correlation between the level of drug resistance and the inhibitory efficacy of RC48. Notably, highly resistant strains maintained near-normal proliferation rates post-treatment, confirming their strong drug-resistant phenotype.

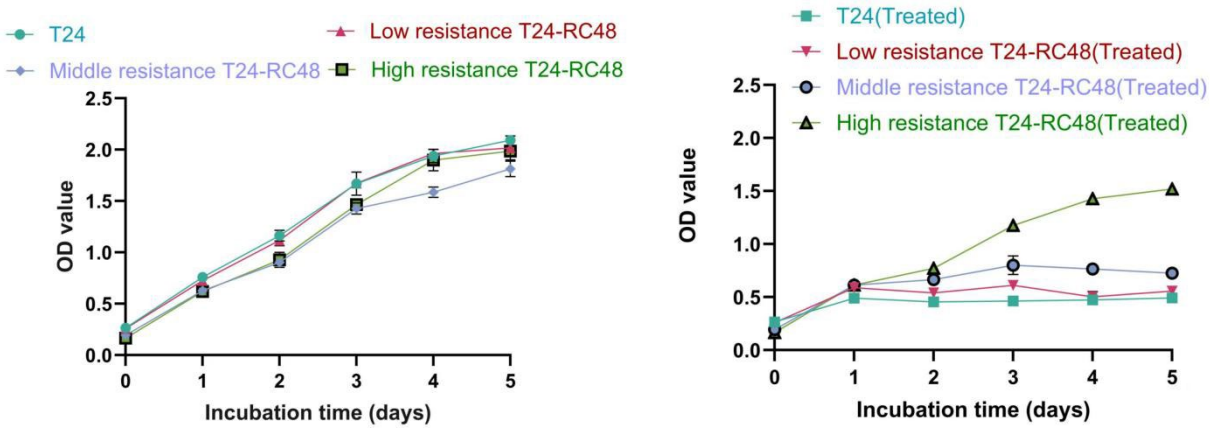

Figure 4 Growth curves of T24 and low/medium/high drug resistant T24-RC48 cell lines without drug (left) and with drug (right)

VII. Results analysis

After 120 hours of culture, the morphology of cells without medication was relatively intact, and the cell density was close to 100%, indicating that the initial cell seeding density and the duration of the experiment were appropriate in this experiment.

After 120 hours of drug treatment, the growth status and morphology of T24 cell lines and their low/medium/high drug resistant strains (T24-RC48) showed significant differences:

1） The cell density of T24 wild-type cell lines was significantly reduced, a large number of cells fell off, and the volume of residual cells was reduced, showing typical apoptosis morphology. This indicates that the drug had the strongest killing effect on wild-type cells, and the cells were highly sensitive to the drug.

2） The cell density of the adherent part of T24-RC48 low-resistant strain was only slightly higher than that of wild-type, and the cell morphology was abnormal, so the survival status could not be determined. This indicated that the drug resistance of low-resistant strain was weak, and the drug could still significantly inhibit its growth.

3） The cell density of the drug-resistant strains in T24-RC48 was about 50%, and the morphology of the drug-resistant strains was improved compared with the wild type and low drug-resistant strains, but there were still a large number of abnormally morphological cells. This indicates that the drug resistance of the drug-resistant strains was enhanced, but it was not completely resistant to the drug action.

4） The T24-RC48 highly drug-resistant strain had the highest cell density, good morphology, and the division phase could be observed in the proliferating area. This indicates that the highly drug-resistant strain has developed strong tolerance to the drug and can maintain

normal proliferation.

In the absence of RC48 treatment, all four cell lines showed a stable proliferation trend with similar doubling time, indicating that there was no significant difference in proliferation capacity of these four cells under normal culture conditions, and there was no significant difference in doubling time between T24-RC48 drug-resistant cell line and parent T24 cell.

Following drug treatment, the doubling time of the T24-RC48 cell lines did not increase significantly and was notably shorter than that of parent T24 cells treated with the drug. This indicates that compared to conventional T24 cell lines, the T24-RC48 cell line cultivated in this study demonstrates stronger drug resistance to RC48. Although drug treatment affects the growth rate of T24-RC48 cells, this impact is substantially less than the inhibitory effect of RC48 on T24 cells.

The data demonstrate a positive correlation between cellular drug resistance and changes in cell density and morphology: higher drug resistance correlates with lower cellular sensitivity, while improved survival rates and proliferative capacity are observed. This suggests that drug resistance may be maintained through enhanced cellular tolerance to medications, thereby sustaining their growth and division capabilities. Such resistance likely stems from adaptive mechanisms gradually developed by T24-RC48 cells during prolonged culture and screening processes, which reduce the inhibitory effects of drugs on cellular proliferation. These findings hold significant implications for investigating the drug resistance mechanisms of T24 cells toward RC48. The results validate T24-RC48 cell lines as suitable models for studying RC48 resistance mechanisms, laying the groundwork for deeper mechanistic investigations. This indicates that future research should focus on exploring specific resistance mechanisms in T24-RC48 cells (such as drug efflux, metabolic alterations, or apoptosis inhibition), providing theoretical foundations for developing more effective anticancer drugs and therapeutic strategies.