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1

Evaluation of 5-fluorouracil-treated lung cancer cells by atomic force microscopy

Xiaolin Jiang, Ke Ma,* Cuihua Hu,* Mingyan Gao, Jiashuo Zhang, Ying Wang, Yujuan Chen, Zhengxun Song and Zuobin Wang*

Atomic force microscopy (AFM) can be used to obtain the physical information of single live cancer cells; however, the physical changes in live cells with time based on AFM remain to be studied, which play a key role in the evaluation of the efficacy and side effects of drugs.

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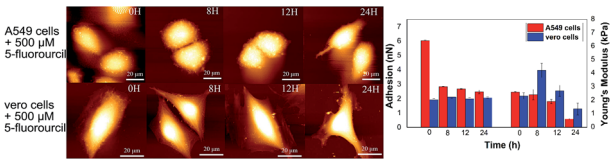

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Analytical Methods

PAPER

Evaluation of 5-fluorouracil-treated lung cancer cells by atomic force microscopy†

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Xiaolin Jiang,[a] Ke Ma,*[a] Cuihua Hu,*[a] Mingyan Gao,[a] Jiashuo Zhang,[b] Ying Wang,[a] Yujuan Chen,[c] Zhengxun Song[a] and Zuobin Wang*[ad]

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Atomic force microscopy (AFM) can be used to obtain the physical information of single live cancer cells; however, the physical changes in live cells with time based on AFM remain to be studied, which play a key role in the evaluation of the efficacy and side effects of drugs. Herein, the treatment of the A549 cell line with the anticarcinogen 5-fluorouracil has been discussed based on the AFM analysis of their continuous physical changes, including their surface morphology, height, adhesion and Young's modulus, with time. In comparison, the African green monkey kidney (Vero) cell line was tested as normal cells to determine the side effects of 5-fluorouracil. The results show that the optimal concentration of 5-fluorouracil is about 500 μM, which presents the best anticancer effect and mild side effects.

1. Introduction

A World Health Organization (WHO) investigation in 2018 revealed that lung cancer ranks the third among serious human diseases for both incidence and mortality with the rates of 22.5% and 18.4%, respectively.[1] Lung cancer occupies a leading status among cancer-related deaths, and non-small cell lung cancer (NSCLC) shares are attributed to 80% of the total lung cancer cases, of which the 5 year survival rate is only 20%.[2–4] Most lung cancer patients are identified at an advanced stage, and only radiation or chemotherapy treatment can work. Chemotherapy treatment is the main method to treat lung cancer, which is based on drugs and medicine.[5,6] However, these drugs always cause extreme side effects to normal cells. Therefore, it is important to evaluate their efficacy and side effects with high accuracy.

5-Fluorouracil (5-FU) is a commonly used chemotherapeutic drug for human cancers, and its effectiveness for cancer treatment has already been verified in clinic and experiment.[7,8] After penetrating the cancer cells, 5-FU is transformed into two major active metabolites: FdUMP and FUTP. FdUMP interacts with thymidylate synthase (TS) and 5,10-CH_2-FH_4 to form a covalent ternary complex, thereby inhibiting the activity of TS and DNA synthesis. Moreover, FUTP can interfere with RNA synthesis.[9] Thus, by inhibiting the synthesis of DNA and RNA, 5-FU exhibits a great value for cancer treatment. However, most of the drug evaluation methods are at the tissue and individual levels. Thus, the nanomechanical property during single-cell apoptosis is still unclear. Therefore, ultrahigh resolution microscopic methods are needed for the detection of nanoscale physical changes.

An atomic force microscope (AFM) has a sub-micrometric resolution and is used in the biological field to provide physical information of biological molecules[10,11] and live cells.[12–14] Thus, it provides a novel physical determination approach with high accuracy. It can provide physical information, including their surface morphology, height, adhesion and Young's modulus, of single cells for quantitative analysis. The Young's modulus is actually the result of complex fitting and several hypotheses of contact regime and tip properties, which lead to significant experimental uncertainty.[15] However, the relative modulus values show a close correlation with the true sample properties. Thus, comprehensive and detailed data can be obtained for single cell analysis.[16–18] Moreover, the AFM images can present the morphological changes of live cells affected by drugs, and the study on the cell membrane ultrastructure can provide a better understanding of the growth and reproduction of live cells.[19–21] Hence, AFM is a suitable and accurate approach to evaluate the efficacy and side effects of drugs in live cells.

Herein, the A549 cell line was used for the continuous analysis of the physical changes with time before and after treatment with drugs based on AFM. 5-FU acts as an anticarcinogen,[22,23] which was used to treat the A549 cells with different concentrations. In addition, African green monkey kidney cells,

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known as Vero cells, were tested as normal cells to determine the side effects of 5-FU in under the same conditions. Accordingly, the efficacy and side effects of the drug were determined by analyzing the AFM data, such as surface morphology, height, adhesion and Young's modulus.

2. Experimental

2.1 Cell culture

A549 (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) and African green monkey kidney cells, known as Vero cells (The Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China), were cultured in a T25 cm^2 culture flask using Roswell Park Memorial Institute (RPMI)-1640 media (HyClone Co., USA) with 10% fetal bovine serum (FBS, Gibco Co., USA) and 1% antibiotics (penicillin–streptomycin solution, ThermoFisher Co., USA). The culture flasks were incubated in an incubator (Sanyo, Japan) with 5% CO_2 at 37 °C. The cells were subcultured when they reached 85% confluence, and were washed twice using PBS without calcium and magnesium followed by detachment from the flasks using 1 mL trypsin for 2 min at 37 °C in the cell culture incubator. Then 2 mL fresh culture medium was added to terminate trypsin, and the mixture was transferred to a centrifuge tube and centrifuged for 5 min at 1000 rpm. The supernatant was discarded, and the cells pellets were diluted using fresh media. The diluted cells were dispersed on the a 38 mm culture dish and then cultured for 24 h before use.

2.2 Preparation of 5-FU treatment solution

Commercial 5-FU powder (Aladdin Co., China) was dissolved in RPMI-1640 media at a concentration of 5000 μM, which was used as the 5-FU treatment solution and stored at 4 °C. It was then diluted with RPMI-1640 media (10% FBS) to prepare the 5-FU treatment solution with different concentrations (0, 5, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 μM), which was used for the drug treatment on A549 and Vero cells.

2.3 Sample preparation

A549 and Vero cells were plated in different culture dishes. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) twice, and then different concentrations (0, 500 and 1000 μM) of 5-FU treatment solution (2 mL) were added for further incubation. Next, the cells were washed with PBS three times to remove the dead cells, and 2 mL of RPMI-1640 medium was added before AFM measurements.

2.4 Cell viability assay

A549 and Vero cells in the logarithmic growth phase were plated (5.0×10^3 cells per well) in a 96-well plate (Kangning Co.) and cultured in an incubator at 37 °C, 5% CO_2 for 24 h. After washing with PBS three times, the 5-FU treatment solution (200 μL) with different concentrations (0, 5, 10, 25, 50, 75, 100, 250, 500, 750 and 1000 μM) was then added for another 48 h incubation. Then 20 μL of MTT (Sigma-Aldrich, America) was added to each well for another 4 h culture. The supernatant was removed and 150 μL of DMSO was added to each well, and the OD value at 492 nm was detected using a microplate spectrophotometer (Epoch 2, BioTek, USA).

2.5 AFM analysis

A JPK system (NanoWizard®3, Germany) with an MLCT probe (15 ± 5 kHz resonance frequency, 0.03 ± 0.02 N m^{-1} spring constant, 20 nm tip curvature radius, 15° ± 2.5° tip front angle, 25° ± 2.5° tip back angle, and 17.5° ± 2.5° tip side angle, Bruker, Germany) was used for the measurement of the A549 and Vero cells. The probe was used to scan the cells with 128 × 128 pixels. The 5-FU dosages of 0 μM, 500 μM and 1000 μM were used to treat the cells with different incubation time periods (0, 4, 8, 12, 24 and 48 h), and then the cells were measured by AFM (QI mode with 1.2 nN setpoint) to analyze the surface morphology, height, adhesion and Young's modulus of the cells.

3. Results and discussion

The MTT experimental data shown in Fig. 1 reflects the effect of the dosage of 5-fluorouracil (5-FU) on the A549 and Vero cells after 48 h cell culture. The cell viabilities of the both cell lines decreased with an increase in the concentration of 5-FU from 0 μM to 1000 μM. Additionally, the results indicate that when the concentration of 5-FU reached 500 μM, the A549 cells were almost dead, and their survival rate wA below 2%, which is less than that of the Vero cells (10%). Thus, the concentrations of 0 μM, 250 μM, 500 μM and 1000 μM were selected for the following tests.

The AFM images shown in Fig. 2 reflect the continuous time (from 0 h to 48 h) surface morphology changes in the A549 cells and Vero cells treated with different concentrations of 5-FU (0, 250, 500 and 1000 μM). Here, more than ten cells were measured at a time to obtain the cell morphology. As shown in Fig. 2 and S1,† the surface morphology of the A549 cells and

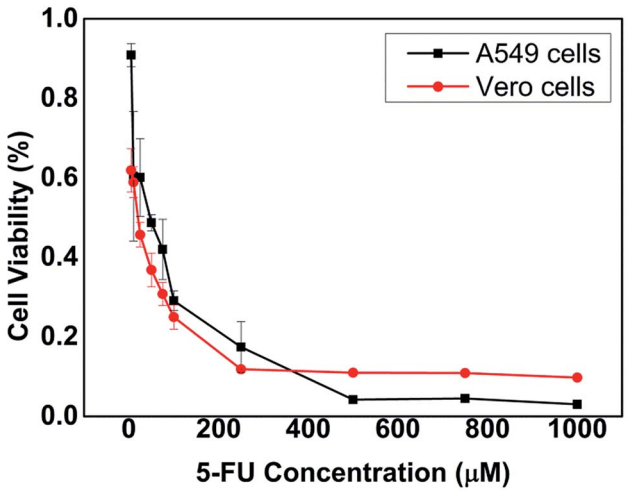

Fig. 1 Cell viabilities of A549 cells and Vero cells after 48 h treatment with increasing concentrations of 5-fluorouracil.

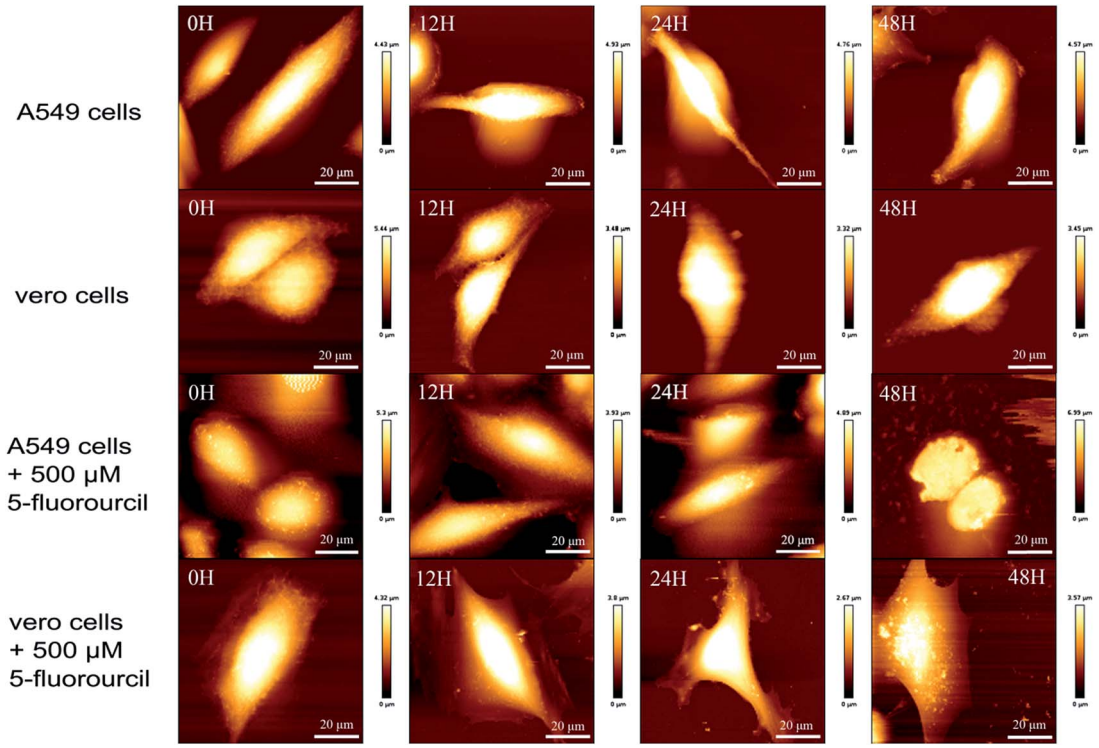

Fig. 2 Morphology of A549 cells and Vero cells treated with different concentrations of 5-fluorouracil (0 and 500 μM) after different incubation times (0, 12, 24 and 48 h); scale bars: 20 μm.

Vero cells had no distinct change during the incubation time in the absence of 5-FU, which reflects that the cells grew in a good condition within 48 h. Similarly, a low content of 5-FU (250 μM) had little influence on the cell growth in 48 h, illustrating that the cells could not be killed by a low concentration of drug (Fig. S2†). After treatment with 500 μM 5-FU, the A549 cells died after 24 h, but the Vero cells were still alive in an unhealthy status after the same period. This indicates that 500 μM 5-FU has ascertainable anticancer efficacy on the A549 cells and mild side effect on the Vero cells within 24 h. As the concentration of 5-FU increased to 1000 μM, the time for apoptosis to occur in the Vero cells shifted to an earlier time of 24 h and 12 h for the A549 cells. This demonstrates that the drug concentration has a serious side effect on the Vero cells in 24 h. Therefore, 500 μM of 5-FU should be the preferred concentration with good anticancer efficacy for NSCLC cells and mild side effects for normal cells.

Besides, the loss of cell volume signals cell death.[24,25] As shown in Fig. S3† corresponding to Fig. 2, cell deformation and membrane collapse appeared in the presence of 5-FU. Also, the cell volume loss deteriorated with an extension in the treatment time. Thus, cell volume loss is a direct character to evaluate drug efficiency.

The cell height variation with time quantitatively corresponds to the cell condition in accordance with the morphology. After drug treatment with 0 μM or 250 μM 5-FU, the A549 cell height decreased at 8–10 h and then recovered (Fig. 3), indicating that A549 cells undergo mitosis in 8 h and then continue growing. For the Vero cells, the cell height declined at 0–2 h and 6–8 h, indicating that the Vero cells experienced two mitosis processes. While, after treatment with 500 μM 5-FU, both the A549 and Vero cells went through one mitosis process at 6–8 h and then continued growing. Moreover, under the treatment of 1000 μM 5-FU, the A549 cell height declined at 10 h, indicating that the A549 cells directly entered the apoptotic phase without undergoing any mitosis process. Whereas, the Vero cells underwent a mitosis process at 2–4 h and then kept growing afterward. In general, the addition of different 5-FU concentrations had an effect on the cell height, where a higher concentration of drug resulted in a lower cell height.

The adhesion map of A549 cells and Vero cells was investigated (Fig. S4†), which represents the adhesion force between the unfunctionalized AFM tip and cell. Without the drug treatment, the adhesion force of the A549 cells showed no change until a sudden reduction appeared at 8 h, indicating that the mitosis process can reduce the cell adhesion force (Fig. 4). The cell adhesion force remained stable after 8 h, indicating that the cell growth has no effect on the adhesion force of A549 cells. In comparison, the adhesion force of the Vero cells remained stable during 48 h, which demonstrates that the cell mitosis and cell growth had no effect on the adhesion ability of the Vero cells. After treatment with 250 μM 5-FU, the adhesion force of both A549 cells and Vero cells exhibited no change compared to that without drug treatment. Similarly, the adhesion force of both A549 cells and Vero cells treated with 500 μM exhibited a consistent trend, illustrating that the concentration of 500 μM has a slight influence on the cell adhesion force. However, in

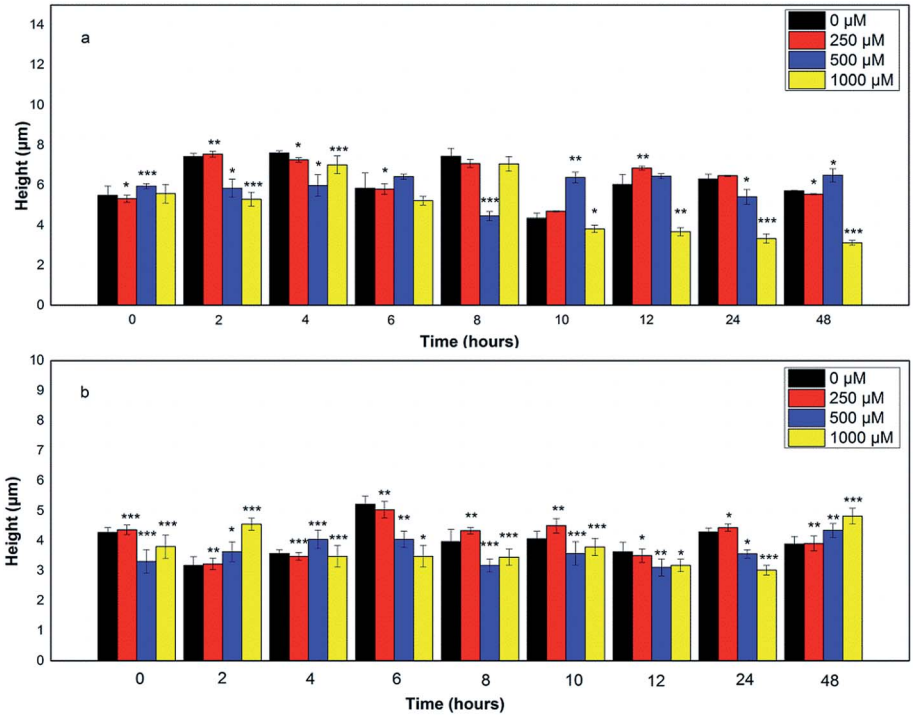

Fig. 3 Cell height of A549 cells (a) and Vero cells (b) after treatment with different concentrations of 5-fluorouracil (0, 250, 500 and 1000 μM) within a constant incubation time (0, 2, 4, 6, 8, 10, 12, 24, and 48 h) (data presented as the mean ± SE. *P < 0.05; **P < 0.01; and ***P < 0.001 compared with the 0 μM group).

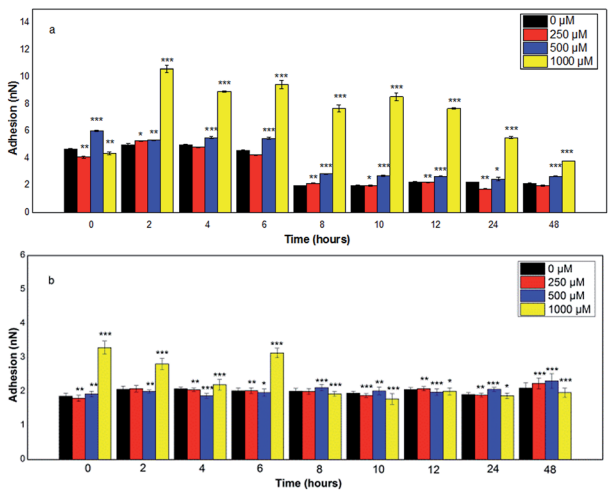

Fig. 4 Adhesion force of A549 cells (a) and Vero cells (b) after treatment with different concentrations of 5-fluorouracil (0, 250, 500 and 1000 μM) within a continuous incubation time (0, 2, 4, 6, 8, 10, 12, 24 and 48 h) (data presented as the mean ± SE. *P < 0.05; **P < 0.01; and ***P < 0.001 compared with the 0 μM group).

the presence of 1000 μM 5-FU, the adhesion force of the A549 cells increased gradually and then decreased rapidly. This indicates that the high concentration of drug increased the cell adhesion force, and then the dead cells led to a decline in cell adhesion. In comparison, the adhesion force of the Vero cells showed a wavy decrease due to the excessive drug intake and excretion during the mitosis process. After the mitosis process, the adhesion force remained unchanged. Therefore, the excessive drug concentration will vastly increase the adhesion force of both A549 and Vero cells within a certain period (2–8 h). As shown in Fig. S5,† the changing tendency of the cell retraction force is similar to that of the cell adhesion force.[26] However, the cell retraction force was lower than cell adhesion force due to van der Waals force and repulsive force.[27]

The Young's modulus explains the elasticity of the cell membrane. The Young's modulus map of the A549 cells and Vero cells is shown in Fig. S6,† and the relative Young's modulus parameters are presented in Fig. 5. The Young's modulus of both A549 cells and Vero cells remained relatively stable in the absence of drug, illustrating that the mitosis process generally does not affect the cell membrane elasticity. Additionally, under the treatment of 250 μM 5-FU, the Young's modulus tendency underwent a slight change. Thus, the low-dose treatment did not affect the Young's modulus of the cells. However, after treatment with 500 μM 5-FU, the Young's modulus of the A549 cells showed a wave-like pattern, changing every two hours due to the drug intake and excretion during the mitosis process. Finally, the Young's modulus reached a minimum value at 24 h due to cell death. On the contrary, the Young's modulus of the drug-treated Vero cells exhibited a moderate decline compared to the cells without the drug treatment. This indicates that the cell surface elasticity of the normal cell membrane can be reduced by drug intake. When the drug concentration increased to 1000 μM, the Young's modulus of the A549 cells exhibited a sharp increase and

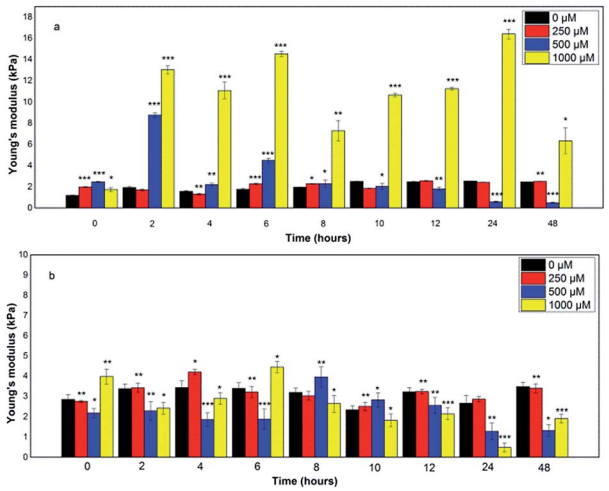

Fig. 5 Young's modulus of A549 cells (a) and Vero cells (b) after treatment with different concentrations of 5-fluorouracil (0, 250, 500 and 1000 μM) within a continuous incubation time (0, 2, 4, 6, 8, 10, 12, 24 and 48 h) (data presented as the mean ± SE. *P < 0.05; **P < 0.01; and ***P < 0.001 compared with the 0 μM group).

showed a similar wavy changing tendency compared with the cells treated with 500 μM 5-FU. However, the Young's modulus of the A549 cells presented a rather high value probably due to the excessive drug intake. After the high concentration of drug intake, the Young's modulus of the cancer cells underwent a rapid increase and then a slight decline. This decline trend was not obvious because a high dose of drug remained inside the dead cells, resulting in the high Young's modulus value compared to the cells treated with a suitable dose of drug. Meanwhile, the Young's modulus of the Vero cells presented a stable variation trend under low-dose treatment (0 μM and 250 μM 5-FU) and the high-dose treatment initially increased their Young's modulus. Contrary to the A549 cells, the Young's modulus of the Vero cells was much lower after the high-dose treatment. This is probably because the Vero cells could not intake the high dose of drug, and a large amount of drug remained in the culture medium. Subsequently, the decline in Young's modulus is probably due to cell mitosis, which efficiently decreased the drug concentration in the cells. After the mitosis process, the cells kept swallowing the drug and the Young's modulus increased again. Finally, the Young's modulus was constantly reduced when the cells enter the apoptosis phase. It should be noted that the Young's modulus was calculated using Hertz/Sneddon model fitting. Additionally, uncertainties are inevitably caused by the probe, such as tip shape, tip size, contact point and Poisson ratio.[15] Therefore, the relative difference between samples is more meaningful for studying the mechanical properties of cells before and after drug treatment.

Actually, cell physical changes significantly contribute to cellular growth.[28,29] This study clearly showed the continuous changes in cell surface morphology, height, adhesion force and Young's modulus with time at different concentrations of 5-fluorouracil. Simultaneously, the drug efficacy and side effects of 5-fluorouracil in live cells were analyzed. The calculated physical change in adhesion (Fig. 4 and S2†) and Young's modulus (Fig. 5 and S3†) can be used as an effective value to evaluate the effects of 5-FU and other biological molecules on cancer cells. Therefore, the results provide new evidence for pharmacodynamic assay.

4. Conclusion

In conclusion, the optimal concentration of 5-FU was 500 μM. At this concentration, the time for apoptosis to occur in A549 cells and Vero cells was 24 h and 48 h, respectively. During this period, the height, adhesion and Young's modulus of the A549 cells and Vero cells varied moderately. This suggests that 500 μM 5-FU was capable of killing A549 cells with mild side effects on the Vero cells in 24 h. However, excessive drug (1000 μM) resulted in the apoptosis of the A549 cells in 12 h and Vero cells in 24 h, which interrupted the metabolism process of the Vero cells with a serious side effect. Therefore, the concentration of 500 μM of 5-FU presented the best anticancer effect with mild side effects.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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