COMBINED EFFECT OF DECITABINE AND DOXORUBICIN ON PROLIFERATION AND DEGREE OF INVASION OF LEUKEMIC HL-60 CELLS

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ABSTRACT

Purpose: *To investigate the combined effect of decitabine and doxorubicin on the proliferation and degree of invasion of leukemia HL-60 cells.*

Methods: *Human acute myeloid leukemia (HL-60) cells were used for this study. They were randomly divided into four groups: control group, decitabine group, doxorubicin group, decitabine + doxorubicin (DD) group. The cells were cultured in 10 % fetal bovine serum-supplemented RPMI 1640 culture medium. In the decitabine group, decitabin (5.0 μmol/L) was added to the culture medium, while doxorubicin (1.0 μmol/L) was added to the culture medium in the doxorubicin group. Decitabin (5.0 μmol/L) and doxorubicin (1.0 μmol/L) were added to the culture medium in the DD group. The extent of inhibition of cell proliferation was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay kit. Apoptosis and cell cycle distribution of HL-60 cells were measured using a flow cytometer, while Transwell assay was used to determine the degree of invasion of the cells.*

Results: *Inhibition of cell proliferation were significantly higher in the decitabine and doxorubicin groups than in control group, and was significantly higher in DD group than in decitabine and doxorubicin groups ($p<0.05$). The inhibition increased with time across the groups ($p<0.05$). The extent of apoptosis was also significantly higher in the DD group than in decitabine and doxorubicin groups, and apoptosis increased with time across the groups ($p < 0.05$). There were more G0/G1 phase cells in the decitabine and doxorubicin groups than in control group, and G0/G1 phase cells were significantly higher in DD group than in decitabine and doxorubicin groups ($p<0.05$). There were fewer S phase cells in the decitabine and doxorubicin groups, and they were significantly lower in the DD group than in decitabine and doxorubicin groups ($p<0.05$). However, there were no significant differences in the distribution of cells in G2/M phase among the groups ($p>0.05$). Cells in the treatment groups showed less invasiveness, and the number of transmembrane cells were significantly reduced with time ($p<0.05$).*

Conclusion: *These results indicate that combination of DAC with doxorubicin produces synergistic and time-dependent inhibition on the proliferation and invasion of HL-60 cells, thereby increasing the sensitivity of the cells to doxorubicin, arresting the cells at G0/G1 phase, and promoting apoptosis.*

Keywords: *Decitabine, doxorubicin, leukemia HL-60 cells, proliferation, invasion.*

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Introduction

Leukemia is a disease caused by the malignant cloning of hematopoietic stem cells. The cloned cells are deposited in bone marrow and other hematopoietic tissues, where they inhibit hematopoietic functions of normal cells, and the infiltration of other hematopoietic tissues and organs due to their rapid proliferation, differentiation, and resistance to apoptosis. This disease is characterized by symptoms such as anemia, abnormal bleeding, infection, long-term fever, hepato-splenic lymphoid tissue enlargement, and bone pain[1-2]. Doxorubicin, also known as doxorubicin hydrochloride, is an anthracycline antibiotic with a broad spectrum anti-tumor activity suitable for the treatment of acute leukemia, malignant lymphoma, bronchial lung cancer, gastric cancer and other malignant tumors[3-4]. The toxic and side effects associated with doxorubicin lead to the elimination of hemopoietic function of bone marrow and reduction of the number of circulatory platelets and white blood cells (WBCs). Doxorubicin has limited uses

in clinical practice because it can cause heart failure and damage to liver function, and it is associated with drug resistance and long term recurrence[5-6]. Decitabine (DAC) is an adenosine analogue, which inhibits DNA methyltransferase, thereby inhibiting the proliferation of tumor cells and the generation of drug resistance[7]. It exerts some curative effects on recurrent and drug-resistant leukemia. However, its use is characterized by undesirable clinical effects, easy recurrence of tumor, and survival time lower than 5 years[8]. The aim of this study was to investigate the combined effect of decitabine and doxorubicin on the proliferation and degree of invasion of leukemia HL-60 cells.

Materials and methods

Materials and reagents

Human acute myeloid leukemia cell line (HL-60) was purchased from the Institute of Hematology, Chinese Academy of Medical Sciences. Decitabine was product of JiangSu HaoSen Pharmaceutical Co., Ltd., while doxorubicin was purchased from HaiZheng Pfizer Pharmaceutical Co., Ltd. Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum and annexin v-fitc/iodidine (PI) apoptosis test kit were products of Gibco Co., Ltd. (USA). Transwell chamber and MTT assay kit were purchased from Thermo Fisher Scientific Co., Ltd. CytoFLEX flow cytometer was purchased from Beckman Coulter Co., Ltd.

Cells and groupings

Human acute myeloid leukemia (HL-60) cells were used for this study. They were randomly divided into four groups: control group, decitabine group, doxorubicin group, decitabine + doxorubicin (DD) group. The cells were cultured in 10 % fetal bovine serum-supplemented RPMI 1640 culture medium. In the decitabine group, decitabin (5.0 μmol/L) was added to the culture medium, while doxorubicin (1.0 μmol/L) was added to the culture medium in the doxorubicin group. Decitabin (5.0 μmol/L) and doxorubicin (1.0 μmol/L) were added to the culture medium in the DD group.

Determination of inhibition of cell proliferation (MTT assay)

A concentration of 20 mg/ml MMT was prepared and the HL-60 cells were seeded into 96-well plates containing fetal bovine serum (10%), streptomycin (100 U/mL), penicillin (100 U/mL) and RPMI-1640 (2.0 mmol/L). Incubation was done at 37 oC in an atmosphere containing 5% CO2. The culture medium was changed after 48 h and cells in the exponential phase were selected and used for the determination of cell viability and proliferation. Aliquots of HL-60 cells at the logarithmic phase (1×105 cells/ml) in the four groups were seeded into 96-well plates. Each well was incubated in the dark for 2 h and absorbance was measured at 492 nm using Sunrise enzyme labeling instrument. The inhibition of cell proliferation was determined after 24, 48, and 72 h of incubation. The assay was performed in triplicates and the mean absorbance was calculated. The percentage inhibition was calculated viz:

$$Inhibition\ of\ cell\ proliferation\ (\%) = \frac{(1-Abs)}{Abc \times 100}$$

where Abs=absorbance of sample well; and Abc=absorbance of control well.

Cell invasion ability

This was performed using the Matrigel-coated cell culture insert. Aliquots of HL-60 cells in the logarithmic phase (1 × 106/ml) in the four groups were collected and transferred to new wells containing 1 % fetal bovine serum-supplemented RPMI-1640 medium. After 24, 48, and 72 h of incubation, non-invading cells were identified and removed, while invading cells in the lower chamber media were fixed with methanol and crystal violet dye (1 %). The remaining transmembrane cells were counted using a light microscope. The assay was performed in triplicates.

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using SPSS (19.0). Groups were compared using Student t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Proliferation of cells in the four groups at different time points

Inhibition of cell proliferation were significantly higher in the decitabine and doxorubicin groups than in control group, and was significantly higher in the DD group than in decitabine and doxorubicin groups ($p < 0.05$). The inhibition increased with time across the groups ($p < 0.05$). These results are shown in Table 1.

Group	Time points (h)		
	24	48	72
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Decitabine	30.25 ± 1.45[a]	38.46 ± 1.69[a]	54.39 ± 2.81[a]
Doxorubicin	16.37 ± 1.28[ab]	19.34 ± 1.42[ab]	24.37 ± 1.52[ab]
DD	45.27 ± 2.12[abc]	50.31 ± 2.01[abc]	75.13 ± 2.67[abc]

Table. 1: Cell proliferation in the four groups at different time point (n, %).
[a]p<0.05, when compared to control group; [b]p<0.05, when compared to decitabine group; [c]P<0.05, when compared doxorubicin group.

Apoptosis of cells in the four groups

Table 2 shows that the extent of apoptosis was significantly higher in decitabine and doxorubicin groups than in control group, and was significantly higher in the DD group than in decitabine and doxorubicin groups. Apoptosis increased with time across the groups (p<0.05).

Group	Time points (h)		
	24	48	72
Control	2.64 ± 0.65	3.12 ± 0.74	4.21 ± 1.02
Decitabine	18.56 ± 1.22[a]	25.34 ± 1.32[a]	32.45 ± 1.96[a]
Doxorubicin	5.38 ± 0.74[ab]	7.34 ± 0.85[ab]	10.34 ± 1.11[ab]
DD	24.36 ± 1.34[abc]	36.31 ± 1.36[abc]	48.25 ± 2.23[abc]

Table. 2: Comparison of cell apoptosis among the four groups (n, %).
[a]p<0.05, when compared to control group; [b]p<0.05, when compared to decitabine group; [c]p<0.05, when compared doxorubicin group.

Cell cycle distribution in the four groups

There were more G0/G1 phase cells in the decitabine and doxorubicin groups than in control group, and they were significantly higher in DD group than in decitabine and doxorubicin groups (p<0.05). However, there were fewer S phase cells in the decitabine and doxorubicin groups, and they were significantly lower in the DD group than in decitabine and doxorubicin groups (p<0.05). There were no significant differences in the distribution of cells in G2/M phase among the groups (p>0.05). These results are shown in Table 3 and Figure 1.

Group	G_0/G_1	S	G_2/M
Control	33.57 ± 3.36	54.29 ± 4.67	11.24 ± 1.67
Decitabine	49.16 ± 2.58[a]	40.75 ± 3.58[a]	9.24 ± 1.85
Doxorubicin	52.23 ± 3.54[ab]	37.25 ± 2.54[a]	10.34 ± 1.38
DD	66.37 ± 4.31[abc]	22.45 ± 2.51[abc]	9.46 ± 1.79

Table. 3: Effect of the various treatments on cell cycle distribution of HL 60 cells (%).
[a]p<0.05, when compared to control group; [b]p<0.05, when compared to decitabine group; [c]P<0.05, when compared doxorubicin group.

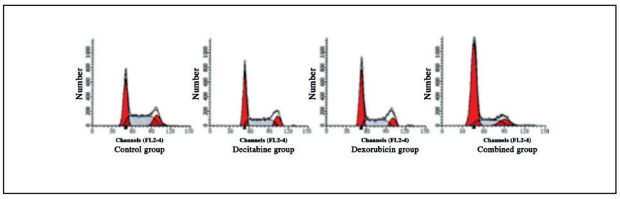

Figure 1: Cell cycle distribution of HL-60 cells after 72 h of treatment.

The degree of invasion of HL-60 cells

The degree of invasion was significantly lower in the treatment groups than in control group, and was significantly lower in DD group than in decitabine and doxorubicin groups (p < 0.05. Cells in the control group showed strong invasiveness, and the number of transmembrane cells were significantly increased with time (p < 0.05). However, cells in the treatment groups showed less invasiveness, and the number of transmembrane cells were significantly reduced with time (p < 0.05). The results are shown in Table 4 and Figure 2.

Group	Time points		
	475.34 ± 26.21	636.26 ± 35.64	785.36 ± 42.18
Control	412.37 ± 24.58[a]	360.67 ± 34.26[a]	321.01 ± 21.45[a]
Decitabine	436.25 ± 25.16[ab]	465.38 ± 26.87[ab]	389.76 ± 26.16[ab]
Doxorubicin	394.37 ± 26.38[abc]	310.37 ± 22.37[abc]	220.13 ± 21.45[abc]
DD	45.27 ± 2.12[abc]	50.31 ± 2.01[abc]	75.13 ± 2.67[abc]

Table. 4: Invasive ability of HL 60 cells (nmol/L) in the various groups.
[a]p<0.05, when compared to control group; [b]p<0.05, when compared to decitabine group; [c]P<0.05, when compared doxorubicin group.

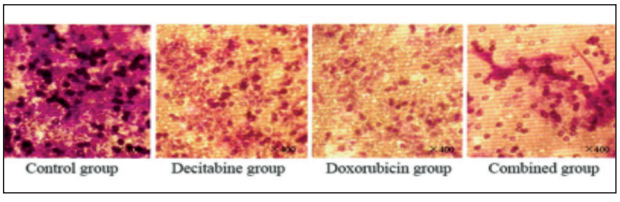

Figure 2: Degree of invasion of cells in the four groups after 72 h of treatment.

Discussion

Doxorubicin is an antitumor antibiotic which inhibits the activity of topoisomerase II and nucleic acid synthesis, thereby promoting abnormal mitosis and chromosomal separation via combination with DNA in tumor cells[9]. However, it is toxic to the heart, and it produces long-term resistance[10]. Decitabine (DAC) is a specific DNA methyltransferase inhibitor. The reduced activity of DNA methyltransferase blocks the methylation of cytosine residues in DNA, and inhibits DNA methylation via the formation of a stable covalent complex with the enzyme after phosphorylation[11-12]. In addition, DAC demethylates methylated DNA, and represses the activity of DNA methyltransferase[13]. Studies have shown that DAC is cytotoxic at high doses and exerts a dose-dependent double inhibition on tumor cells at low doses via demethylation[14-15].

Previous studies have suggested that the mechanism of doxorubicin resistance is linked to its activation of PI3K/Akt/NF signaling pathway, because the inhibition of the PI3K/Akt signaling pathway reduces the expression of drug-resistant genes, while increasing the sensitivity of tumor cells to anti-cancer drugs[16-17]. In a previous study, Zhang et al used LY294002, a PI3K inhibitor to pretreat gastric cancer cells and K562 drug-resistant strains, and found that the sensitivity of the pretreated cells to drugs was significantly increased when compared to single-drug treatment[18]. In addition, the expressions of p-gp and p-akt were significantly reduced, an indication that PI3K/Akt is related to drug resistance[10]. Combined therapy has proven to be effective in inhibiting drug resistance, relative to treatment with doxorubicin alone[19]. Studies have also shown that the combination of DAC with doxorubicin can significantly inhibit the expression of drug-resistant proteins in drug-resistant K562 cells and improve the sensitivity of drug-resistant strains to doxorubicin[20-21]. Therefore, drug combination is more advantageous in overcoming the problem of drug resistance in tumor cells.

In the present study, inhibition of cell proliferation was significantly higher in the decitabine and doxorubicin groups than in control group, and was significantly higher in the DD group than in decitabine and doxorubicin groups. The inhibition increased with time across the groups. These results suggest that the combination of the two drugs may have a synergistic effect, and that DAC may increase the sensitivity of HL-60 cells to doxorubicin. These results are in agreement with those previously reported[22-23].

Previous studies have shown that the combination of DAC with doxorubicin induces apoptosis in HL-60 cells, which obstructs the cell cycle. In this study, the extent of apoptosis was significantly higher in decitabine and doxorubicin groups than in control group, and was significantly higher in the DD group than in decitabine and doxorubicin groups. Apoptosis increased with time across the groups.

There were more G_0/G_1 phase cells in the decitabine and doxorubicin groups than in control group, and were significantly higher in DD group than in decitabine and doxorubicin groups. There were fewer S phase cells in the decitabine and doxorubicin groups, and S phase cells were significantly lower in the DD group than in decitabine and doxorubicin groups. However, there were no significant differences in the distribution of cells in G_2/M phase among the groups. These results appear to suggest that the cell may not have successfully completed their normal cycle, causing resistance to apoptosis in drug combination.

Extramedullary tumor cell infiltration in leukemia is the major reason for its high recurrence and poor clinical effect. Therefore, inhibiting the invasiveness of white blood cells (WBCs) is key in the prevention and control of extramedullary cell infiltration. In this study, after DAC or doxorubicin treatment, the number of transmembrane penetration of HL-60 cells was significantly reduced, and the number of transmembrane penetration was significantly lower in DD group than in single drug group. These results indicate that the combination of DAC and doxorubicin may synergistically inhibit the invasion and metastasis of drug-resistant tumors.

Conclusion

The results of this study have shown that the combination of DAC and doxorubicin has synergistic and time-dependent effects and effectively inhibits the proliferation and invasion of HL-60 cells. Thus, the sensitivity of the cells to doxorubicin is enhanced, the cell cycle is arrested at G0/G1 phase, and apoptosis is increased.

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