EXPERIMENTAL STUDY ON THE EFFECT OF INTRACELLULAR ROS LEVEL IN NON-SMALL CELL LUNG CANCER ON RESPONSE TO ABT-263 THERAPY

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ABSTRACT

Objective*: To investigate the effect of reactive oxygen species (ROS) level in non-small cell lung cancer (NSCLC) on response to ABT-263 therapy.*

Methods: *In this study, NSCLC-related cell lines were selected, the responses of different NSCLC cell lines to ABT-263 therapy were analyzed, and the effect of ABT-263 on the apoptosis, MCL-1 ex-pression and ROS level of different cell lines were evaluated.*

Results: *Compared with DMSO-treated cells, the positive percentage of annexin V and/or PI in Calu-1, Calu-3 and BID007 cells treated with ABT-263 increased significantly (P<0.05); PARP deg-radation products were observed in Calu-3 and BID007 cells treated with ABT-263, but not in A549 cells; Western blot analysis indicated that the expression levels of MCL-1 in BID007, Calu-1, Calu-3 and A549 cells were similar. MCL-1 gene silencing can improve the responses of PC-9 and A549 cells to ABT-263 therapy; MCL-1 gene silencing didn't affect the responses of Calu-3 and BID007 cells to ABT-263 therapy; Flow cytometry analysis showed that after ABT-263 treatment, the ROS levels in Calu-1 and Calu-3 cells decreased significantly (P<0.05). Immunofluorescence assay suggested that the ROS levels in Calu-1 and Calu-3 cells were higher than those of H3122 and A549 (P<0.05); The results of MTS test showed that after NAC treatment, the responses of Calu-3 and BID007 cells to ABT-263 therapy decreased significantly (P<0.05). Flow cytometry analysis showed that the ROS level in ABT-263 treated-cells fell significantly when MCL1 gene was silenced (P<0.05).*

Conclusion: *ROS level is closely related to the response of NSCLC cell lines to ABT-263 therapy and ROS can effectively improve the sensitivity to ABT-263 therapy.*

Keywords: *Non-small cell lung cancer, reactive oxygen species, ABT-263, therapeutic response.*

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Introduction

In recent years, studies have con-firmed that abnormal apoptosis plays a key role in the occurrence of cancer[1, 2]. Tumor cells often have overexpression of pro-proliferative BCL-2 family proteins, such as BCL-2, BCL-xL and MCL-1 and inhibit cell apoptosis. BCL-2, BCL-xL, BCL-w and MCL-1 can lead to abnormal apoptosis by binding to and inhibiting the pro-apoptotic BAX/BAK that trigger apoptosis[3, 4]. BAX and BAK share the protein-protein interaction domain, i.e., BH3 motif, and BH3 domain analog can affect the interaction with other BCL-2 family proteins[5]. Based on the above anti-apoptosis mechanism, a variety of apoptosis inducers including BH3 analog have been developed[6]. BH3 analogs induce apoptosis mainly by interfering with the function of BCL-2 family pro-teins. ABT-263 belongs to oral BH3 an-alog, whose inhibition constants for BCL-2, BCL-xL, BCL-2 and BCL-2 are less than 1 nmol/L. Its monotherapy has good anti-tumor effect on xenograft mouse model with small cell lung cancer (SCLC). On the other hand, in preclinical studies of B-cell lymphoma, multiple myeloma and some solid tumors, it has also been proved to improve the an-titumor effect of other chemotherapy drugs[7-9].

The low expression level of MCL-1, a member of BCL-2 family, in SCLC is closely related

to the response of ABT-737, an ABT-263-related com-pound[10]. Other studies suggest that the BIM/MCL-1 ratio can predict the sensitivity of SCLC to ABT-263[11]. However, up to now, the anti-tumor activity of ABT-26 for NSCLC is still controversial, and whether the expression level of MCL-1 or BIM/MCL-1 ratio can predict the anti-tumor activity of ABT-263 for NSCLC or not is also uncertain.

This study aims to analyze the correlation between the intracellular ROS level in NSCLC and response to ABT-263 ther-apy, explore the influence of ROS on the regulation of response to ABT-263 therapy in NSCLC. Below, the process will be reported.

Materials and methods

Cell culture

The selected cell lines included H146[SCLC], Calu-1[KRASG12C], Ca-lu-3[HER2 amplification], BID007 [EGFR exon 20 insertion (A763, Y764ins-FQEA)], H3122[EML4-ALK E13; A20 fusion], A549[KRASG12S], PC-9[EGFR exon 19 deletion, EGFRdelE746-A750], H358[NSCLC], H441[NSCLC] and H460[NSCLC]. A549 and Calu-3 were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and anti-biotics (10 U/mL penicillin and 50μg/mL streptomycin); Other cell lines were cul-tured in RPMI-1640 medium containing 10% FBS and antibiotics (10 U/mL penicillin and 50μg/mL streptomycin). Culture conditions: 5% CO_2, humidified, 37°C.

Reagent

ABT-263 was supplied by Selleck Chemicals; the anti-MCL-1 antibody (5453) and anti-PARP antibody (9532) were provided by CST; the anti-actin an-tibody, NAC and cisplatin were supplied by Sigma Aldrich.

MTS cell proliferation assay

The cells were inoculated in a 96-well plate at a concentration of 3000 cells/well and cultured for 24h. After that, the cells were treated using ABT-263 at different concentrations. The control group was treated with DMSO at the same concentration, and the absorbance at 490 nm was measured after treating for 72h.

Apoptosis test

The cells were inoculated in a 6-well plate at a concentration of 30,000 cells/well. 1mol/L ABT-263 was added to treat for 36h. The control group was treated with DMSO at the same concentration. The percentage of apoptotic cells was calculated by BD FACSCalibur™ flow cytometer.

Western blot analysis

The cells were treated with ABT-263 at a concentration of 0.1~1μmol/L for 24h. The control group was treated using DMSO. The total protein concentration was determined by BCA protein assay. The same amount of protein was added to SDS-PAGE (12.5%) and then transferred to VDF membrane. 5% skim milk powder was added to block.

The mixture was incubated overnight at 4°C after the primary antibody was added, and then incubated for 1h after the secondary anti-body was added. Eventually, LumiGLO reagent and hydrogen peroxide were added. The culture was developed using X-ray imaging and then assessed.

Gene silencing mediated by siRNA

The cells were transfected using MCL1-targeting siRNA and non-MCL1-targeting control siRNA. The transfection reagent was LIP2000. The knockout of MCL1 gene was confirmed by qRTPCR and western blot analysis.

Test of intracellular ROS level

The cells were treated with CM-H2DCFDA (100μmol/L) and cul-tured at 37°C for 15min. The ROS level was tested by Gallios Flow Cytometer from Beckman Coulter and ROS assay kit (Abcam). To begin with, the cells were inoculated in a 96-well plate at a con-centration of 10,000 cells/well, and ABT-263 was added to treat for 24h.

The cells were washed using HBSS buffer, stained with H2DCFDA (20μmol/L) and cultured at 37°C for 40min. After that, the cells were washed with HBSS buffer, and the fluorescence intensity was measured at 485nm (excitation) and 535nm (emission) through Cytation™ 5 Multi-Mode Reader.

Statistical processing

SPSS24.0 software was selected to analyze data. Among them, measurement data were compared with t test and expressed as ($\bar{x}\pm s$). The significance level was $\alpha=0.05$.

Results

Analysis of the responses of different NSCLC cell lines to ABT-263 therapy

H146 was highly sensitive to ABT-263 thera-

py, IC50=0.03μM; Calu-1, Calu-3 and BID007 were sensitive to ABT-263 therapy, but the sensitivity was lower than that of H146 cells, IC50=0.85μM, 1.74μM, 1.94μM. See Table 1.

Cell Line Type	IC50 (**μM**)
H146	0.03
Calu-1	0.85
Calu-3	1.74
BID007	1.94
PC9	3.40
H358	3.62
H441	4.59
H460	7.26
A549	15.30
H3122	17.15

Table 1: Comparison of IC50 values of ABT-263 for different NSCLC cell lines.

The effect of ABT-263 on apoptosis of Calu-1, Calu-3 and BID007

Compared with DMSO-treated cells, the positive percentage of annexin V and/or PI in Calu-1, Calu-3 and BID007 cells treated with ABT-263 increased significantly (P<0.05). See Figure 1.

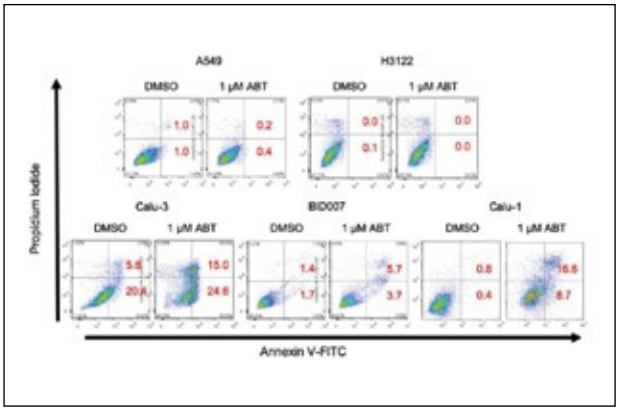

Figure 1: Results of flow cytometry analysis.

MTS cell proliferation assay of MCL-1 Expressions

MCL-1 gene silencing can improve the responses of PC-9 and A549 cells to ABT-263 therapy; MCL-1 gene silencing didn't affect the responses of Calu-3 and BID007 cells to ABT-263 therapy. See Table 2.

Group	0.01μM ABT 263	0.1μM ABT 263	1μM ABT 263
PC-9 cells			
Negative control group	98.43±5.71	97.20±4.64	85.46±5.64
MCL siRNA group	81.33±3.41	45.40±2.47	27.93±1.05
A549 cells			
Negative control group	103.68±4.57	100.52±4.30	99.35±3.67
MCL siRNA group	98.05±3.30	80.20±3.83	39.16±2.37
Calu-3			
Negative control group	99.84±2.21	98.47±2.69	79.38±4.60
MCL siRNA group	98.33±2.69	98.10±2.75	42.06±3.41
BID007 cells			
Negative control group	102.89±4.42	100.92±4.68	83.70±4.31
MCL siRNA group	99.56±3.44	98.20±3.21	62.63±3.10

Table 2: MTS cell proliferation assay of MCL-1 expressions in PC-9, A549, Calu-3 and BID007 cells (%).

The effect of ROS level on response to ABT-263 therapy

Flow cytometry analysis showed that after ABT-263 treatment, the ROS levels in Calu-1 and Calu-3 cells de-creased significantly (P<0.05). See Table 3.

Group	DMSO	1μM ABT 263
A549	17.45±3.09	16.51±1.12
H3122	84.74±10.56	82.67±8.50
H146	95.34±13.70	0.04±0.01
Calu-3	83.34±11.59	12.91±3.46
Calu-1	920.97±105.12	32.25±6.84

Table 3: Flow cytometry analysis of ROS levels.

The effect of NAC treatment and MCL gene silencing on the responses of NSCLC cells to ABT-263 therapy

The results of MTS test showed that after NAC treatment, the responses of Calu-3 and BID007 cells to ABT-263 therapy decreased significantly (P<0.05). See Table 4.

Group	DMSO	3μM ABT 263	10μM ABT 263
Calu-3 cells			
Control group	97.55±1.31	23.32±1.10	7.40±0.53
NAC 0.5mM group	98.81±1.05	28.49±1.79	5.58±0.40
NAC 2.5mM group	99.03±0.70	42.66±1.70	30.37±1.94
BID007 cells			
Control group	98.24±1.05	90.26±1.37	41.07±2.49
NAC 0.5mM group	97.29±1.80	110.15±2.07	67.63±2.49
NAC 2.5mM group	98.53±1.31	113.38±2.97	105.38±2.12

Table 4: Analysis of MTS test results after NAC treatment (%).

Discussion

In recent years, a variety of new drugs have been widely used in the treatment of lung cancer but it is still the main cause of cancer-related death throughout the world[12]. NSCLC patients account for about 85% of the total number of patients with lung cancer. More than 40% of the patients are in the advanced stage when first diagnosed, and the 5-year OS is less than 20%[13].

In this study, the sensitivity of different NSCLC cell lines to ABT-263 treatment was analyzed. The author discovered that three ABT-263-sensitive cell lines, that is, Calu-1, Calu-3 and BID007, were sensitive to ABT-263 therapy, but their sensitivity was lower than that of H146 cells, IC50=0.85μM, 1.74μM, 1.94μM. In NSCLC cell lines, the low level of MCL-1 was connected with the response to ABT-263 therapy. But according to the results of this work, MCL-1 gene silencing can improve the responses of PC-9 and A549 cells to ABT-263 therapy. MCL-1 gene silencing didn't affect the responses of Calu-3 and BID007 cells to ABT-263 therapy, suggesting that the expression level of MCL-1 cannot be used as a biomarker to predict the response to NSCLC therapy. Recent studies have shown that there is no correlation between the responses of NSCLC cell lines to ABT-263 therapy and the expression level of MCL-1[14, 15]. In our study, however, flow cytometry analysis showed that after ABT-263 treatment, the ROS levels in Calu-1 and Calu-3 cells decreased significantly ($P<0.05$). Immunofluorescence assay suggested that the ROS levels in Calu-1 and Calu-3 cells were higher than those of H3122 and A549 ($P<0.05$). The results of MTS test indicated that after NAC treatment, the responses of Calu-3 and BID007 cells to ABT-263 therapy decreased significantly ($P<0.05$). Flow cytometry analysis demonstrated that the ROS level in ABT-263 treated-cells fell significantly when MCL1 gene was silenced ($P<0.05$), which further suggested that there was a correlation between response to ABT-263 therapy and ROS level in NSCLC cell lines.

In this work, the author found that the sensitivity of some NSCLC cell lines to ABT-263 treatment was just fine, but their therapeutic response was often worse than that of SCLC cell lines (H146). This evidence may be indicative of the need to increase the dose of ABT-263, to improve clinical efficacy. However, it should be noted that ABT-263 treatment showed high toxicity in early studies, so the feasibility of this regimen is still open for debate[16, 17].

Previous studies have evidenced the correlation between ROS, BCL-2 proteins and response to ABT-263 therapy, but the specific mechanism of this relationship has not been clarified yet[18-20]. Some scholars posit that leu-kemia stem cells with low ROS level and high BCL-2 level have relatively low apoptosis level and rate. But other studies argue that high BCL-2 level can induce autophagy and reduce the ROS level in HCC cells[21-23]. Our finding confirms that intracellular ROS level can be used to predict the responses of NSCLC cell lines after ABT-263 treatment. The apoptosis induced by ABT-263 after the treatment of sensitive NSCLC cell lines is often accompanied by a drop in ROS level. ROS has been proved to induce apoptosis, but some tumor cells rely on ROS, and the decrease of ROS level can also lead to apoptosis. Based on the above evidence, the author believes that the overall curative effect of ABT-263 alone on NSCLC patients is average, but there may be synergistic effect if it is combined with platinum-based chemotherapy drugs, radiotherapy and other ROS-inducing therapies.

To sum up, ROS level is closely related to the response of NSCLC cell lines to ABT-263 therapy and ROS can effectively improve the sensitivity to ABT-263 therapy.

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