

## Cationic lipids enhanced cellular uptake and activity of *bcl-2* antisense oligodeoxynucleotide G3139 in HL-60 cells

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**KEY WORDS** antisense oligonucleotides; lipids; HL-60 cells; *bcl-2* genes

### ABSTRACT

**AIM:** To explore the effect of cationic lipid 1, 3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER) on cellular uptake and activity of *bcl-2* antisense oligodeoxynucleotide G3139 in HL-60 cells. **METHODS:** The cell-associated mean fluorescence intensity and the percentage of cells with positive staining for Bcl-2 were measured by flow cytometry. The subcellular distribution of fluorescein isothiocyanate (FITC)-labeled G3139 was observed by fluorescence microscope and the *bcl-2* mRNA level was detected by reverse transcription polymerase chain reaction (RT-PCR). **RESULTS:** ① DOSPER increased cellular uptake of G3139 into HL-60 cells greatly. When DOSPER/G3139 ( $\mu\text{g}:\mu\text{g}$ ) was 2:1, the uptake of G3139 reached top after treatment for 2 h and increased about 20 times compared with application of G3139 alone. In the presence of DOSPER, G3139 was localized in nucleus and cytosol with a bright spotted fluorescence staining. However, G3139 was localized in cytoplasm with faint fluorescence in the absence of DOSPER. ② Cell-associated G3139 could be effluxed out of cells. After treated with G3139 in the presence of DOSPER for 4 h, the cell-associated G3139 could be fitted by  $C(t) = 68.2e^{-0.60t} + 31.8e^{-0.02t}$  (% of initial value), with a half-life of approximately 1.1 h. In the absence of DOSPER, the cell-associated G3139 could be fitted by  $C(t) = 64.8e^{-2.27t} + 35.2e^{-0.04t}$ , with a half-life of about 18 min. ③ In the presence of DOSPER, G3139  $1.0 \mu\text{mol}\cdot\text{L}^{-1}$  specially reduced *bcl-2*

mRNA level, and Bcl-2 protein decreased from  $97\% \pm 4\%$  to  $70.6\% \pm 2.1\%$ . **CONCLUSION:** DOSPER enhanced the activity of G3139 and it might be attributed to increase of the cellular uptake and change of the subcellular distribution of G3139.

### INTRODUCTION

Antisense oligodeoxynucleotides (ODN) were capable of down regulating gene expression and were used for the assessment of gene function and for therapeutic purpose<sup>[1,2]</sup>. Several human clinical trials were ongoing with antisense ODN for the treatment of hematologic malignancies, such as Non-Hodgkin lymphoma<sup>[3]</sup>. However, functional efficacy of ODN required not only the selection of an appropriate target sequence, but also a sufficient intracellular concentration. The latter one depended on the degree of cellular uptake, the subcellular distribution, and the rate of degradation of ODN by cytoplasmic nucleases<sup>[1]</sup>. Cationic lipids have been developed for efficient delivery of ODN into cells for a decade. Data from cell culture experiments suggest that enhancement of cellular uptake and nuclear distribution of ODN by cationic lipids play an important role in increasing the potency of their biologic action<sup>[4]</sup>.

G3139 was an 18-mer full phosphorothioate antisense ODN targeted to the first six codons of the open reading frame of *bcl-2*<sup>[5]</sup>, which has been used to treat Non-Hodgkin lymphoma in II<sub>A</sub> phase of clinical trials in USA. Previous studies have shown that *bcl-2* expression could be inhibited by *bcl-2* antisense ODN<sup>[6]</sup>. The present study was to assess the effect of cationic lipid 1, 3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid (DOSPER) on cellular uptake and activity of G3139 in HL-60 cells, and provide a basis for protocols in delivery of antisense ODN into cultured cells.

### MATERIALS AND METHODS

**Cells and reagents** HL-60 cells were obtained

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from Fujian Institute of Medical Science (Fujian, China); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum from Gibco BRL (Grand Island, NY, USA); Ribonuclease A and propidium iodide (PI) from Sigma (St Louis, MO, USA); Cationic lipid DOSPER (molecular weight 1089) from Boehringer (Mannheim, German); Reverse transcription polymerase chain reaction (RT-PCR) kit from Promega (Madison, WI, USA); Bcl-2 monoclonal antibody from PharMingen (San Diego, CA, USA).

**Oligodeoxynucleotide synthesis and labeling** G3139 (molecular weight 5947) with the sequence 5'-TCTCCCAGCGTGCGCCAT-3' and sense ODN (5'-ATGGCGCACGCTGGGAGA-3') were synthesized, and G3139 was labeled with fluorescein isothiocyanate (FITC) from Gened Synthesis Inc (USA). All of the ODN were purified by high performance liquid chromatography (HPLC).

**Mixture of G3139 and DOSPER on agarose gel** Mixture of FITC-labeled G3139 and DOSPER was performed according to the supplier's recommendation. Briefly, DOSPER and G3139 diluted with nuclease-free distilled water were mixed at various mass ratios and kept at room temperature for 15 min. Then the mixture was run on 3% agarose gel. Electrophoresis was performed in a Tris-edetic acid-acetic acid buffer (pH 8.1). The gel was photographed under UV transillumination.

**Treatment of cells with G3139 and DOSPER** HL-60 cells were grown in the DMEM supplemented with 5% heat-inactivated fetal bovine serum, benzylpenicillin  $100 \text{ kU} \cdot \text{L}^{-1}$ , streptomycin sulfate  $100 \text{ mg} \cdot \text{L}^{-1}$ , and 1% glutamine at  $37 \text{ }^\circ\text{C}$ , in a humidified atmosphere of 5%  $\text{CO}_2$ . Cells were seeded on 24-well plate with  $1 \times 10^5$  cells per well in a volume of  $500 \mu\text{L}$ . After 12 h of seeding the cells, mixtures of G3139 and DOSPER at different ratios were added, and incubated for 30 min to 24 h. The concentration of G3139 was from  $0.3$  to  $4.0 \mu\text{mol} \cdot \text{L}^{-1}$ .

**Quantification of cell-associated G3139 by flow cytometry** After treatment with FITC-labeled G3139 in the presence or absence of DOSPER, cells were washed with cold phosphate buffer saline (PBS, containing  $\text{NaCl } 13.7 \text{ mmol} \cdot \text{L}^{-1}$ ,  $\text{KCl } 0.27 \text{ mmol} \cdot \text{L}^{-1}$ ,  $\text{Na}_2\text{HPO}_4 0.43 \text{ mmol} \cdot \text{L}^{-1}$ ,  $\text{KH}_2\text{PO}_4 0.14 \text{ mmol} \cdot \text{L}^{-1}$ , pH 7.3) and with PBS containing 1% bovine serum albumin (BSA), respectively. To remove G3139 bound to the cell membrane, the acid-salt elution method was used<sup>(7)</sup>. Briefly, the cells were suspended in an ice-cold

solution containing  $\text{NaCl } 0.5 \text{ mol} \cdot \text{L}^{-1}$  and acetic acid  $0.2 \text{ mol} \cdot \text{L}^{-1}$  (pH 2.5), incubated at  $4 \text{ }^\circ\text{C}$  for 10 min and centrifuged at  $1000 \times g$  for 5 min. Then the cells were washed twice with PBS/BSA and suspended in  $300 \mu\text{L}$  PBS/BSA containing  $\text{PI } 0.3 \mu\text{mol} \cdot \text{L}^{-1}$ . Relative mean fluorescence intensity (MFI) was determined by flow cytometry (FACScan, Becton Dickinson, USA). Data were analysed with CellQuest™ software (Becton Dickinson, USA). Cell-associated G3139 was expressed as mean fluorescence intensity (MFI).

**Subcellular distribution of G3139** After treatment with FITC-labeled G3139 in the presence or absence of DOSPER for the indicated time, the cells were washed four times with cold PBS. Then the subcellular distribution of G3139 was determined by Olympus fluorescent microscopy.

**Measurement of Bcl-2 protein by flow cytometry** Bcl-2 protein was measured as described by Liu and Zhu<sup>(8)</sup>. Briefly, HL-60 cells were collected by centrifugation and washed with PBS. After fixation with 2% paraformaldehyde for 20 min and permeabilization with 0.5% Triton-X 100, cells were incubated with mouse monoclonal antibody against Bcl-2 for 30 min, followed by incubation with FITC-conjugated rabbit anti-mouse IgG for 30 min at room temperature in the dark. After washing twice with PBS, cells were measured by flow cytometry and the percentage of cells with positive staining for Bcl-2 protein was determined.

**Identification of bcl-2 mRNA by RT-PCR** Total RNA was isolated from HL-60 cells with guanidium isothiocyanate. First-strand cDNA was reverse-transcribed from  $2 \mu\text{g}$  of RNA in a total volume of  $20 \mu\text{L}$  with AMV reverse transcriptase  $12 \text{ U}$ , at  $42 \text{ }^\circ\text{C}$  for 30 min. The *bcl-2* sense primer was 5'-GACTTCTCCC-GCCGCTAC-3', and *bcl-2* antisense primer was 5'-CA-CCAGGGCCAAACTGAGCAG-3'. The expansion length is 375 bp. To test the efficiency of reverse transcription, RT-PCR for  $\beta$ -actin mRNA was performed. The sense primer was 5'-CTCCTTAATGTCACGCACGATTTC-3', and the antisense primer was 5'-GTGGGGCGCCCC-AGGCACCA-3'. The expansion length is 548 bp. PCR procedures were carried out in a total volume of  $25 \mu\text{L}$  containing the first-strand cDNA as a template corresponding to total RNA  $0.5 \mu\text{g}$ , Tris-HCl  $10 \text{ mmol} \cdot \text{L}^{-1}$ , KCl  $50 \text{ mmol} \cdot \text{L}^{-1}$ ,  $\text{MgCl}_2 1.5 \text{ mmol} \cdot \text{L}^{-1}$ , and dNTP  $0.2 \text{ mmol} \cdot \text{L}^{-1}$ , each of antisense and sense primer  $25 \text{ pmol}$ , and *Taq* DNA polymerase  $1 \text{ U}$  with the following cycling conditions: 30 cycles of 30 s at  $94 \text{ }^\circ\text{C}$  (denatura-

tion), 40 s at 61 °C (annealing), and 1 min at 72 °C (extension).

**Statistics** Data were expressed as  $\bar{x} \pm s$  and statistically compared by ANOVA and equation was fitted by SAS software.

## RESULTS

**Formation of complexes of DOSPER and G3139** Mixtures of G3139 and DOSPER formed complexes. It showed that part of the mixtures stayed at the point of loading, indicating the mixtures contained complexes of different charge (data not shown).

**Cellular uptake of G3139 in HL-60 cells** After treatment with FITC-labeled G3139 complexed to different amounts of DOSPER at the concentration of  $0.3 \mu\text{mol} \cdot \text{L}^{-1}$  for 2 h, the cell-associated fluorescence intensity varied greatly when the DOSPER/G3139 ( $\mu\text{g}:\mu\text{g}$ ) ratio was between 2:1 and 3:1 (data not shown). Having defined the best DOSPER/G3139 ratio (2:1), DOSPER increased uptake of G3139 about 20 times compared with application of G3139 alone. The cell-associated fluorescence intensity was concentration-dependent without reaching a plateau at the range of G3139  $0.3 \mu\text{mol} \cdot \text{L}^{-1}$  to  $3.6 \mu\text{mol} \cdot \text{L}^{-1}$  in the presence or absence of DOSPER (Fig 1). The peak value of fluorescence intensity was observed after treatment for about 2 h in the presence of DOSPER, and it was about 4 h after treatment with G3139 alone (Fig 2).

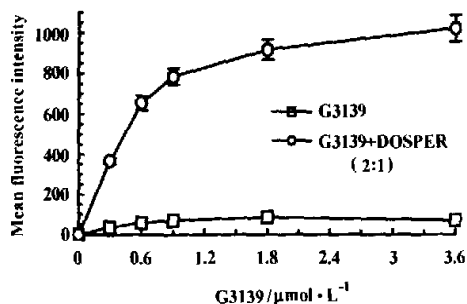


Fig 1. Cell-associated fluorescence intensity after treatment with various doses of G3139 in the absence or presence of DOSPER for 4 h in HL-60 cells.  $n = 3$ .  $\bar{x} \pm s$ .

**Exocytosis of FITC-labeled G3139 from HL-60 cells** After treatment with FITC-labeled G3139  $2.0 \mu\text{mol} \cdot \text{L}^{-1}$  for 4 h in the absence or presence of

DOSPER, the medium was removed and the cells were treated with fresh medium for the indicated time. The decrease of cell-associated fluorescence intensity was computer-fitted and the best fit was achieved by a double exponential function of form  $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ <sup>(9)</sup>. The coefficients A and B represented the size of the individual compartments, and the terms  $\alpha$  and  $\beta$  represented the kinetic constants for the loss of G3139 from the cells. For the treatment with G3139 alone, a dramatic decrease of G3139 bound to cells was observed at the first phase with a half-life about 18 min. The equation could be fitted by  $C(t) = 64.8e^{-2.27 t} + 35.2e^{-0.04 t}$  (% of initial value). However, for the treatment with G3139 mediated by DOSPER, the first phase was relatively slow with a half-life approximately 1.1 h. The equation could be fitted by  $C(t) = 68.2e^{-0.60 t} + 31.8e^{-0.02 t}$  (Fig 3).

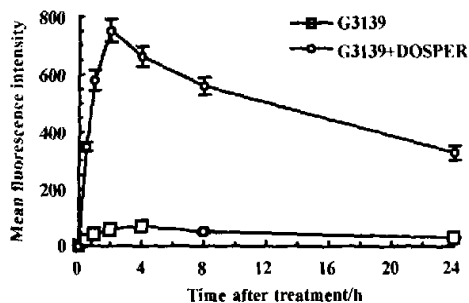


Fig 2. Cell-associated fluorescence intensity after treatment with G3139  $0.3 \mu\text{mol} \cdot \text{L}^{-1}$  in the absence or presence of DOSPER ( $\mu\text{g}:\mu\text{g} = 2:1$ ) for different time in HL-60 cells.  $n = 3$ .  $\bar{x} \pm s$ .

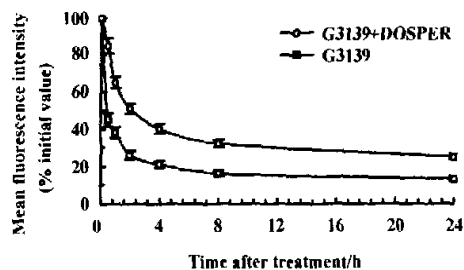


Fig 3. Efflux kinetics of FITC-labeled G3139 in HL-60 after removal of G3139 or complexes of G3139 and DOSPER ( $\mu\text{g}:\mu\text{g} = 2:1$ ).  $n = 3$ .  $\bar{x} \pm s$ .

**Subcellular distribution of FITC-labeled G3139** Incubation of HL-60 cells with FITC-labeled G3139  $1.0 \mu\text{mol} \cdot \text{L}^{-1}$  for 4 h resulted in a faint fusion

distribution of the G3139 in the cytoplasm in the absence of DOSPER (Fig 4A). On the contrast, cells incubated with G3139 and DOSPER exhibited a bright spotted nuclear fluorescence staining and a punctuated distribution in the cytoplasm (Fig 4B). And after treatment for 24 h, G3139 mainly accumulated in the nucleus (Fig 4C).

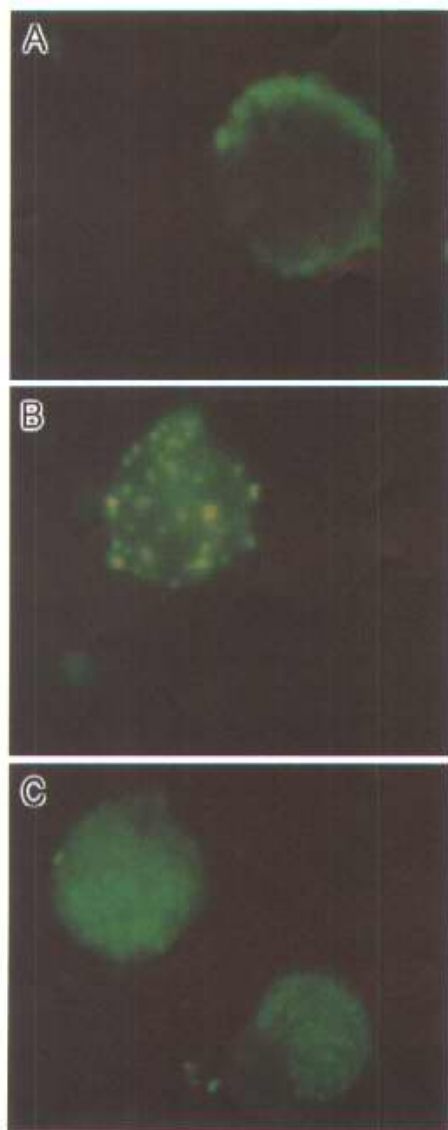


Fig 4. Subcellular distribution of FITC-labeled G3139 after treatment with G3139  $1.0 \mu\text{mol}\cdot\text{L}^{-1}$  in the absence or presence of DOSPER in HL-60 cells. A, incubation with G3139 alone for 4 h; B and C, incubation with complexes of G3139 with DOSPER ( $\mu\text{g}:\mu\text{g}=2:1$ ) for 4 and 24 h. The cells were observed with fluorescence microscopy.  $\times 1000$ .

**Functional effects of G3139** After treatment with sense G3139  $4 \mu\text{mol}\cdot\text{L}^{-1}$  for 24 h, the percentage

of cells with positive staining for Bcl-2 protein was  $95 \pm 3 \%$  or  $94 \pm 4 \%$  in the absence or presence of DOSPER, respectively. The treatment with G3139 alone at a concentration of  $1 \mu\text{mol}\cdot\text{L}^{-1}$  for 24 h failed to inhibit *bcl-2* mRNA and protein expression (Tab 1 and Fig 5). However, treatment with G3139  $1 \mu\text{mol}\cdot\text{L}^{-1}$  in the presence of DOSPER for 24 h reduced *bcl-2* mRNA level markedly and Bcl-2 protein was decreased from  $97 \pm 4 \%$  to  $70.6 \pm 2.1 \%$ . G3139  $0.5 \mu\text{mol}\cdot\text{L}^{-1}$  reduced Bcl-2 protein to  $75.6 \pm 2.0 \%$ , different from application of G3139 alone at the concentration of  $2 \mu\text{mol}\cdot\text{L}^{-1}$  or  $4 \mu\text{mol}\cdot\text{L}^{-1}$  (Tab 1 and Fig 5).

Tab 1. Percentage of cells with positive staining for Bcl-2 protein in HL-6 cells after treatment with different concentration of G3139 in the absence or presence of DOSPER ( $\mu\text{g}:\mu\text{g}=2:1$ ) for 24 h.  $n=3$ .  $\bar{x} \pm s$ ;  $^{\text{b}}P < 0.05$ ,  $^{\text{c}}P < 0.01$  vs control.  $^{\text{d}}P < 0.01$  vs G3139 group.

G3139/ $\mu\text{mol}\cdot\text{L}^{-1}$	G3139/%	G3139 + DOSPER/%
0.0(control)	$97 \pm 4$	$96 \pm 3$
0.5	$95 \pm 3$	$75.6 \pm 2.0^{\text{d}}$
1.0	$92.5 \pm 2.0^{\text{b}}$	$70.6 \pm 2.1^{\text{d}}$
2.0	$88.5 \pm 2.8^{\text{c}}$	$62.5 \pm 2.0^{\text{d}}$
4.0	$85.3 \pm 2.2^{\text{c}}$	$56.2 \pm 1.6^{\text{d}}$

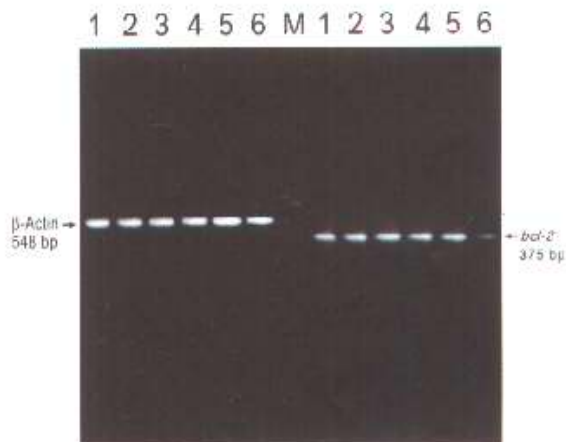


Fig 5. Expression of *bcl-2* mRNA after treatment with ODN (G3139 or sense ODN)  $1.0 \mu\text{mol}\cdot\text{L}^{-1}$  in the absence or presence of DOSPER ( $\mu\text{g}:\mu\text{g}=2:1$ ) for 24 h. *bcl-2* and  $\beta$ -actin mRNA expression were detected by RT-PCR. Lane M, DNA marker (1543, 994, 695, 515, 377, 237 bp); Lane 1, control; Lane 2, G3139; Lane 3, sense ODN; Lane 4, DOSPER  $20 \mu\text{mol}\cdot\text{L}^{-1}$ ; Lane 5, complexes of sense ODN and DOSPER; Lane 6, complexes of G3139 and DOSPER.

## DISCUSSION

Cationic lipids-mediated transfer of DNA into cells was a well-documented approach for gene therapy. However, the mechanisms by which these lipids enhanced the ODN uptake was not well known. It has been postulated that the cationic portion of lipid mixture interact with the negatively charged ODN, and the hydrophobic part associated with the lipid bilayer of the membrane, resulting in fusion with the bilayer and subsequent released into the cytosol<sup>[10]</sup>. Furthermore, some studies showed that cationic lipids formed a complex with ODN, which then bound to and penetrated the cell membrane via endocytosis<sup>[11]</sup>. In the present study, mixtures of DOSPER and G3139 were visualized on a 3 % agarose gel. Negatively charged G3139 were directed toward the positive current site. A portion of G3139 stayed in the loading well, implying only a part of G3139 could form complexes with DOSPER.

The mechanisms by which antisense ODN might inhibit gene expression were proposed. These included prevention of new protein synthesis by translational arrest, promotion of mRNA degradation by ribonuclease H-dependent mechanism, inhibition of mRNA mutation, inhibition of mRNA transport out of the nucleus and inhibition of gene transcription by forming a triple helix structure<sup>[12]</sup>. The fact that ODN appeared to concentrate within the nucleus after introduction into the cytoplasm might have important ramification for mechanisms of ODN. In the present study, the FITC-labeled G3139 showed to be associated with cytoplasmic structures and the concentration of G3139 at  $4 \mu\text{mol} \cdot \text{L}^{-1}$  just decreased Bcl-2 protein to  $85.3 \% \pm 2.2 \%$  in the absence of DOSPER. On the contrary, in the presence of DOSPER, the labeled G3139 was localized mainly in the nucleus and G3139 reduced Bcl-2 protein to  $76.5 \% \pm 2.0 \%$  at the concentration of  $0.5 \mu\text{mol} \cdot \text{L}^{-1}$ . These results suggested that DOSPER reduce dose of G3139 by specially inhibiting Bcl-2 protein, and nuclear distribution of antisense ODN might be an important factor for enhancement of its activity.

Previous studies have shown that ODN bound to cells could be released from cells<sup>[9]</sup>, so it was important to determine the intracellular half-life of ODN in cells. In this study, the kinetic data on the efflux of cell-associated fluorescence intensity could be described in a mathematical model. The ODN bound to cells was released from cells rapidly at the first phase with a short half-life of 18 min after removal of G3139 in the absence of

DOSPER. On the contrast, after treatment with DOSPER, the half-life in the first phase could reach about 1.1 h. These kinetic data indicated that for inhibiting a gene expression which coded for a RNA and a protein with a single transfection of antisense ODN might not be sufficient and further transfection might be useful.

In summary, the present study proved that cationic lipids enhanced activity of *bcl-2* antisense oligodeoxynucleotide G3139 and it might be attributed to its enhancing the uptake and changing the subcellular distribution of G3139. Thus, cationic lipids have proved to be useful for delivery of antisense ODN into cells and could potentially be used for the delivery of antisense ODN into animals or human beings.

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### 阳离子脂质体增加 *bcl-2* 反义寡脱氧核苷酸 G3139 在 HL-60 细胞中的摄取及活性

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**关键词** 反义寡核苷酸类; 脂类; HL-60 细胞; *bcl-2* 基因

**目的:** 探讨阳离子脂质体 DOSPER 对 *bcl-2* 反义寡脱氧核苷酸 G3139 在 HL-60 细胞中的摄取和活性的影响. **方法:** 流式细胞仪测定细胞结合的平均荧光强

度和 Bcl-2 蛋白阳性细胞的百分数; 荧光显微镜观察细胞内 FITC 荧光素标记 G3139 的分布; RT-PCR 测定 *bcl-2* mRNA 的表达. **结果:** ① DOSPER 增加细胞对 G3139 的摄取, 当 DOSPER/G3139 ( $\mu\text{g}:\mu\text{g}$ ) 为 2:1 时, 细胞对 G3139 的摄取在 2 h 左右达到高峰, 为 G3139 直接作用时的 20 倍, 细胞核和细胞浆中的 G3139 为明亮的点状聚集; 而 G3139 直接作用时, G3139 则弥漫分布于细胞浆中; ② 细胞内的 G3139 可向胞外转运. DOSPER 介导转染 G3139 4 h 后, 细胞内的 G3139 符合方程  $C(t) = 68.2e^{-0.60t} + 31.8e^{-0.02t}$  (初值为 100%),  $t_{1/2}$  约为 1.1 h, 而 G3139 直接作用后, 细胞内的 G3139 符合方程  $C(t) = 64.8e^{-2.27t} + 35.2e^{-0.04t}$ ,  $t_{1/2}$  约为 18 min; ③ 在 DOSPER 介导转染下, G3139 在浓度  $1 \mu\text{mol}\cdot\text{L}^{-1}$  时, 特异性减少 *bcl-2* mRNA 的表达, 同时使 Bcl-2 蛋白阳性率从  $97\% \pm 4\%$  下降到  $70.6\% \pm 2.1\%$ . **结论:** DOSPER 增加细胞对 G3139 的摄取和改变 G3139 在细胞内的分布可能是其增强 G3139 活性的重要机制.

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