The differentiation-inducing effect of Nordy on HPV-16 subgenes-immortalized human endocervical cells H8

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The lipoxygenase inhibitor nordihydroguaiaretic acid, a natural product purified from Larrea divaricata and Guaiacum officinale, showed a marked capacity to induce differentiation of various human malignant tumor cell lines. Nordy, a derivative of nordihydroguaiaretic acid, has been shown to effectively inhibit the growth of malignant human tumors transplanted in mice. However, it is unknown whether Nordy plays an important role in inducing the differentiation effects of human papillomavirus (HPV)-16 subgenes-immortalized human endocervical cells H8. In this study, we showed that Nordy arrested H8 cells in the G_0/G_1 phase, promoted cell differentiation in morphology, downregulated the expression of HPV-16 E6 mRNA and nuclear antigen Ki67, and inhibited telomerase activity on HPV-16 subgenes-immortalized human endocervical cells H8. Taken together, Nordy could suppress proliferation by inhibiting proliferation-related events and promote cell differentiation in H8 cell. The results suggested that Nordy might be a potential tumor therapeutic agent. *Anti-Cancer Drugs* **19:713–719 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

In China, the incidence of cervical carcinoma, especially in women over 35 years, has been observed to increase rapidly in recent years. Cervical carcinoma has been one of the most frequent female malignant diseases [1]. In most cases, human papillomavirus (HPV) genes can be integrated into the chromosomes of host cells, and they inactivate the p53 or Rb protein in host cells by activating the relevant oncogenes, the virus-transforming proteins and products of antioncogene, which leads to the uncontrollable growth of host cells [2]. In this study, H8 cells are an immortalized cell line derived from Chinese women's cervical epithelium infected by HPV-16 E6 subgenes. They may become carcinoma cells under the impact of some promotion factors. So, H8 cells may be regarded as a kind of precancerous cells [3,4].

With the successful treatment of human acute promyelocytic leukemia with retinoic acids, cell differentiation therapy has been considered an important addition to the repertoire of cancer treatment methods [5,6]. Many new compounds, which may promote tumor cell differentiation, have been reported. Nordy is a synthesized chiral compound, which is based on the structure of a natural lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) [7], a natural product purified from Larrea divaricata and Guaiacum officinale. Previous studies showed that NDGA possessed a marked capacity to induce differentiation of various human malignant tumor cell lines, suggesting its potential as a tumor therapeutic agent [8]. Nordy has been shown to effectively inhibit the growth of malignant human tumors transplanted in mice. However, until now, there has been no report about the effects of Nordy on cervical immortalized cells. Therefore, in this study, we first investigated whether Nordy could inhibit the proliferation of HPV-16 subgenes E6-immortalized human endocervical epithelial cells (H8 cell) and promote its differentiation. Then, we investigated the possible mechanisms of the effect of Nordy.

Materials and methods

Materials and reagents

The department of immunology and microbiology at Sanxia University (Hubei, P.R. China) supplied the H8 cell line. 3-(4,5-dimethy-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide (MTT) and dimethylsulfoxide were obtained from Sigma (St. Louis, Missouri, USA). RPMI-1640 was from Sigma. Fetal bovine serum was from HyClone (Logan, Utah, USA). Tartrate-resistant acid phosphatase-enzyme linked immunosorbent assay kit was purchased from Huamei Corp. (Shanghai, P.R. China). The mouse monoclonal anti-Ki67, immunohistochemistry SP kit and 3,3′-diaminobenzidine were obtained from Santa Cruz Corp (Santa Cruz, California, USA). The RT-PCR kit was purchased from TaKaRa Corp. (Shiga, Japan).

Cell cultures

H8 cells were cultured in RPMI-1640 containing 10% fetal bovine serum and antibiotic-antimycotic. At 80%

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confluence, the cells were washed once with phosphate-buffered saline and incubated for 24 h in 0.05% fetal bovine serum-RPMI followed by a treatment with Nordy (99.9% HPLC purity, Institute of Pathology, Southwest Hospital, Chongqing, P.R. China).

Cell proliferation assay

Cell growth was measured by a modified MTT assay. About 5×10^4 cells (in 200 µl) were subcultured in 96-well plates overnight. Cells were then treated with 10, 25, 50, and 75 µmol/l Nordy for 24, 48, 72, and 96 h. 20 µl MTT was added to each well and further incubated at 37°C for 4 h. Cell supernatant was removed and 200 µl dimethylsulfoxide in isopropanol was added to each well. The spectrometric absorbance at 570 nm (A_{570}) wavelength was measured on a microplate reader (Sigma). The negative control well contained the medium only. The ratios of the absorbance of the treated groups relative to those of the control group were calculated and expressed as a percentage of growth inhibition.

Morphological observation

Cells were planted in a 50-ml plastic culture flask. In the absence or presence of 12.2 µmol/l Nordy, incubation was carried out at 37°C, in 5% CO_2 for 96 h. Routine hematoxylin and eosin staining was conducted and observed under a light microscope. Mitotic figures were accounted for under per 10 high power fields (HPFs) and the median value was calculated by 50 HPFs. Mias-2000 image analysis system (Institute of Image & Graphics, Sichuan University, P.R. China) was used to quantitate the nuclear cytoplasmic ratio. Cells were collected into 1.5 ml EP tubes and fixed by 2.5% paraformaldehyde 1.5 ml for 5 min. The sections were prepared and photographed under an electron microscope (Hitach 600, Tokyo, Japan).

Flow cytometry

Cells were planted in 50-ml plastic culture flask at the density of 5×10^4cells/ml, in the absence or presence of 12.2 µmol/l Nordy. After treatment for 48, 72, and 96 h, 1×10^6 cells were harvested, pelleted, and washed in phosphate-buffered saline, fixed with 70% cold alcohol at 4°C, and stained by propidium iodide (ClonTECH, Mountain View, California, USA). Specimens were analyzed by FASCan (Becton Dickinson, New Jersey, USA) to estimate the percentage of each phase in cell cycle.

Reverse-transcriptase polymerase chain reaction

Expression of HPV-16 E6 mRNA was analyzed by reverse-transcriptase polymerase chain reaction (RT-PCR) in H8 cell line. For RT-PCR, total RNA was extracted from H8 cell line treated with 0, 10, 25, 50, and 75 µmol/l Nordy by the Trizol method. The total mRNA was reverse-transcribed into cDNA with 200 U of RT (TaKaRa Corp.). cDNA was amplified with AmpliTaq Gold (TaKaRa Corp.) and 10 pmol/µl from the specific primers (see below) with a thermal cycler (Hybaid, LabX Midland, Ontario, Canada). An initial denaturation step at 95°C for 5 min was used, followed by denaturation at 94°C for 45 s, an annealing phase at 52°C for 45 s, and a synthesis phase at 72°C for 1 min. Thirty-five cycles were performed, ending with an extension phase at 72°C for 5 min.

The following primers were used for amplifying a fragment of 338 bp. 5′-CGACCCAGAAAGTTACCA CAGT-3′(sense); 5′-GGTCCACCGACCCCTTATATT-3′ (antisense). PCR products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide. The photos were taken under ultraviolet radiation. Optical density values of expression of HPV-16 E6 mRNA were analyzed with Quantity one 4.0 software (Bio-Rad, Hercules, California, USA).

Immunocytochemistry

H8 cells treated with 12.2 µmol/l Nordy for 96 h were fixed with stock acetone. Immunostaining was carried out as described in the kit protocol. The Ki67 positive cells were defined when there was an aggregation of brown particles in the nucleus of the cells. The rate of positive expression = (the positive cells/500 cells) × 100%.

Tartrate-resistant acid phosphatase enzyme linked immunosorbent assay assay

Cells were planted in 50-ml plastic culture flask at a density of 5×10^4 cells/ml, in the absence or presence of 12.2 µmol/l Nordy. After treatment for 48, 72, and 96 h, 1×10^6 cells were harvested. The telomerase was extracted and its activity with tartrate-resistant acid phosphatase enzyme linked immunosorbent assay was tested according to the description in the kit.

Statistics analysis

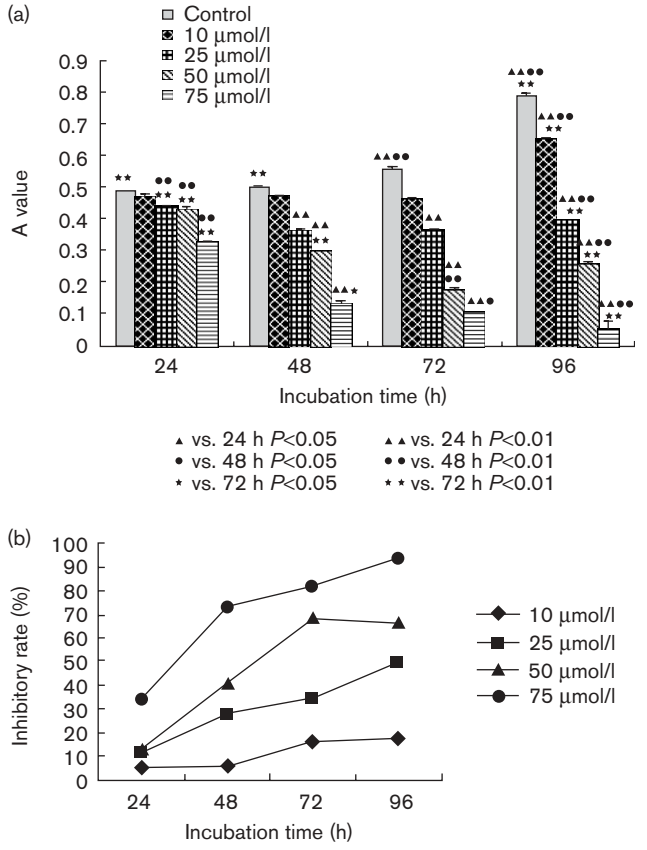
All experiments were performed at least three times and representative results are presented. Data were analyzed with computer aided SPSS 12.0 statistical software (Chicago, Illinois, USA). The unpaired dependent-samples *t*-test was used to compare the difference between two groups. One-way analysis of variance was used to determine the statistical significance of the differences among multiple groups. For comparison of protein expression levels, independent-samples *t*-test was used. Statistical significance was set at $P < 0.05$.

Results

Effects of Nordy on proliferation of H8 cells

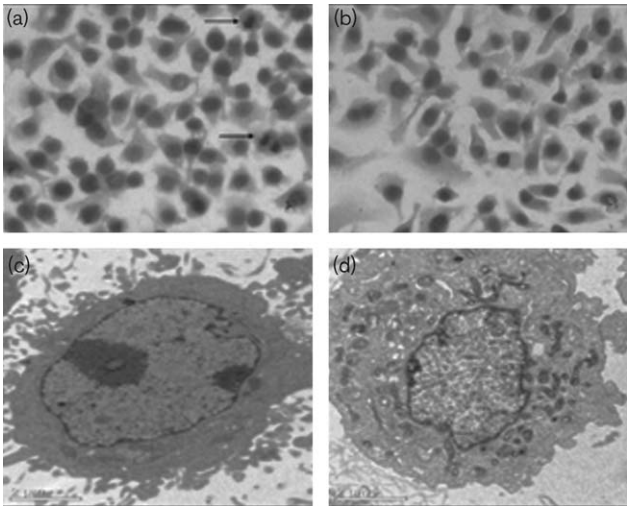
To examine the effects of Nordy on cell proliferation in the cultured H8 cells, Nordy was applied at 10, 25, 50, and 75 µmol/l *in vitro* for 24, 48, 72, and 96 h and analyzed by the MTT assay. Treatment of H8 cells with Nordy resulted in dose-dependent and time-dependent anti-proliferative effects (Fig. 1). Compared with the control group, the growth of H8 cells in the treated group was inhibited significantly in a dose-dependent and time-

Fig. 1



Antiproliferative effect of Nordy. Dose-dependent and time-dependent response: (a) H8 cells were treated with or without a series concentrations (10, 25, 50, and 75 μmol/l) of Nordy for 24, 48, 72, and 96 h. Cell viability was determined with spectrometric absorbance at 570 nm (A_{570}) wavelength by microplate reader. (b) H8 cells were treated with 10, 25, 50, and 75 μmol/l Nordy for 24, 48, 72, and 96 h. The inhibitory effect of the growth of H8 cells was described by the inhibitory rate. vs., versus.

Fig. 2



Morphological changes caused by Nordy: (a) large and dark-colored nucleus, mitoses were frequent (control group) (HE × 400). (b) The ratio of nuclear to cytoplasm was decreased (12.2 μmol/l Nordy for 96 h) (HE × 400). (c) Rich euchromatin and few organelles could be found (control group) (TEM × 10 000). (d) Some mature organelles such as mitochondria and endoplasmic reticulum could be found in cytoplasm (12.2 μmol/l Nordy for 96 h) (TEM × 10 000). HE, hematoxylin and eosin; TEM, transmission electron microscopy.

dependent manner. After treatment for 96 h, the inhibition rate was in the range of 17.5–93.7%. $IC_{50(96\,h)}$ = 24.4 μmol/l. Statistical analysis indicated that there were significant differences among the different time and dose groups ($P < 0.05$).

Effects of Nordy on morphological changes of H8 cells

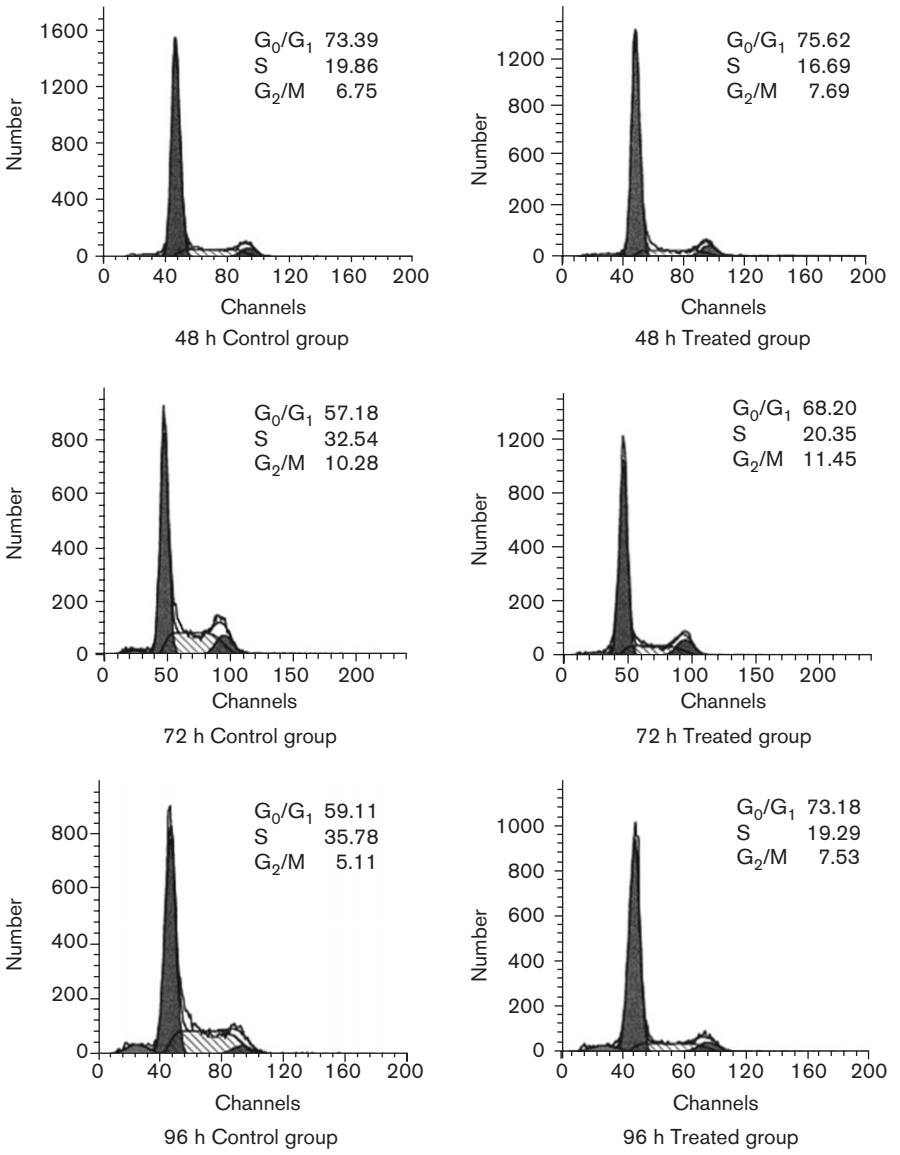
To determine whether Nordy could induce differentiation of H8 cells, morphological changes were also observed. Under light microscopy, the H8 cells were polygonal and large-sized, with large and dark-colored nucleus. Nuclear mitoses could be easily observed (Fig. 2a). After treatment with Nordy for 96 h, the cells became smaller and rounder. Both the nuclear cytoplasmic ratio and the number of nuclear mitoses decreased (Fig. 2b). The result showed that the mean nuclear–cytoplasm ratios in the test group and the control group were 0.74 ($256.97 \pm 28.56\,\mu m^2$: $349.28 \pm 31.63\,\mu m^2$) and 0.82 ($329.14 \pm 54.71\,\mu m^2$: $401.21 \pm 38.41\,\mu m^2$), respectively, and the ratios were not statistically different ($P > 0.05$). The median value of mitotic figures in the test group was 3.2 per 10 HPFs (ranged from 0–5) instead of the control group 4.6 per 10 HPFs (ranged from 1–7). The values of the mitotic figures were statistically different ($P < 0.05$).

Under electron microscopy, the cells were large and round with large and irregular nucleus. Additionally, we found that the cells had rich euchromatin and more than one nucleolus with very little organelles (Fig. 2c). After being treated with Nordy, the cell size became smaller with regular nucleus and more heterochromatin. The number of nucleoli was markedly decreased and some mature organelles such as mitochondria and Golgi bodies could be found (Fig. 2d).

Effects of Nordy on cell cycle of H8 cells

The cell cycle was measured by flow-cytometric analysis. As shown in Fig. 3, compared with the control group, after being treated with 12.2 μmol/l Nordy (1/2 IC_{50}) for 48, 72, and 96 h, the percentage of G_0/G_1 phase cells was significantly increased, whereas the percentage of S phase cells was significantly decreased. These results suggest that the growth of H8 cells was arrested in G_0/G_1 phase. The difference of expression rate between the control group and the treated group was statistically significant ($P < 0.05$) (Table 1).

Fig. 3



The growth of H8 cells was arrested in G_1 phase by Nordy treatment. Cells were treated with or without 12.2 μmol/l Nordy for 48, 72, and 96 h. After treatment, the cells were fixed and stained with propidium iodide and the cell cycle distribution was determined by flow cytometry.

Effects of Nordy on expression of Ki67 in H8 cells

To test whether Nordy could induce cell proliferation on the cultured H8 cells, immunocytochemistry analysis was performed using anti-Ki67 antibody. After being treated with 12.2 μmol/l Nordy for 96 h, the expression rate of Ki67 decreased from 68.15 to 10.03%. The difference of expression rate between the control group and the treated group was statistically significant ($P < 0.05$) (Fig. 4).

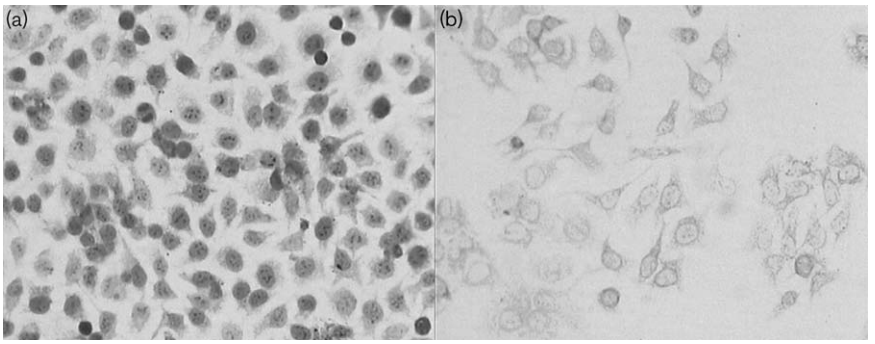
Table 1 Effects of Nordy on the cell cycle distribution of H8 cells ($\bar{x} \pm s$)

		Cell cycle distribution		
Groups	Time (h)	G_0/G_1	S	G_2/M
Control	48	73.39 ± 0.01	19.86 ± 0.01	6.75 ± 0.01
	72	57.18 ± 0.01	32.54 ± 0.01	10.28 ± 0.01
	96	59.11 ± 0.02	35.78 ± 0.01	5.11 ± 0.01
12.2 μmol/l Nordy	48	75.62 ± 0.02	16.69 ± 0.03	7.69 ± 0.02
	72	68.20 ± 0.01	20.35 ± 0.02	11.45 ± 0.02
	96	73.18 ± 0.01	19.29 ± 0.02	7.53 ± 0.02

Effects of Nordy on expression of human papillomavirus-16 E6 mRNA in H8 cells

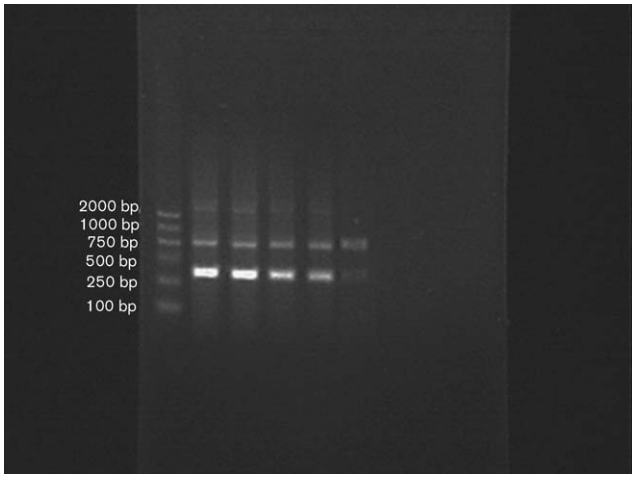
As HPV type 16 plays an important role in the occurrence of cervical carcinoma, the transcripts of HPV-16 E6 were analyzed by semiquantitative RT-PCR. RT-PCR analysis demonstrated the presence of mRNA for HPV-16 E6 in the H8 cell line. Consistently, after treatment with 10,

Fig. 4



Effects of Nordy on expression of Ki67: (a) positive expression of Ki67 of control group (DAB × 200). (b) Weakened expression of Ki67 after treatment with Nordy for 96 h (DAB × 200). DAB, 3,3′-diaminobenzidine .

Fig. 5



Reverse-transcriptase polymerase chain reaction analysis showed the presence of mRNA for human papillomavirus (HPV)-16 E6 in H8 cell line. After treatment with 10, 25, 50, and 75 μmol/l Nordy, the expression levels of HPV-16 E6 mRNA were reduced at all doses tested.

25, 50, and 75 μmol/l Nordy, the expression levels of HPV-16 E6 mRNA were reduced at all doses tested (Fig. 5). The difference was statistically significant between the control group and all doses tested except the 10 μmol/l group (Table 2).

Effects of Nordy on the activity of telomerase in H8 cells

It is well recognized that telomerase activity may be considered as a marker of early diagnosis and progression of cervical neoplasia. Therefore, we examined the effects of Nordy on the activity of telomerase in H8 cells. The activity of telomerase in the cells of the control group was remarkably high. After being treated with 12.2 μmol/l Nordy for 48, 72, and 96 h, the activity of telomerase was decreased in a time-dependent manner. At a different treatment time, the difference between the control group and the treated group was statistically significant ($P < 0.05$). When the treatment time was 96 h, the difference was statistically significant ($P < 0.01$) (Table 3).

Table 2 Effects of Nordy on the expression of HPV-16 E6 mRNA ($\bar{x} \pm s$)

	Control group	10 μmol/l	25 μmol/l	50 μmol/l	75 μmol/l
OD value	4.03 ± 0.47	3.54 ± 0.30	2.76 ± 0.31	2.67 ± 0.71	0.44 ± 0.18
P	1.00[a]	0.04[b]	0.03[c]	< 0.01[d]	

HPV, human papillomavirus; OD, optical density.
[a]Control versus 10 μmol/l Nordy.
[b]Control versus 25 μmol/l Nordy.
[c]Control versus 50 μmol/l Nordy.
[d]Control versus 75 μmol/l Nordy.

Table 3 Effects of Nordy on the activity of telomerase ($\bar{x} \pm s$)

Activity of telomerase (OD value)	48 h	72 h	96 h
Control group	0.62 ± 0.01	0.61 ± 0.04	0.62 ± 0.03
12.2 μmol/l Nordy	0.47 ± 0.01	0.34 ± 0.01	0.16 ± 0.01

OD, optical density.

Discussion

Nordy is a synthesized chiral compound, which is based on the structure of a natural lipoxygenase inhibitor NDGA. NDGA has been reported to have multiple functions including inhibition of growth of tumor cells and their grafts grown in mice [9–14]. Moreover, NDGA induced differentiation of rat and human glioma cells [15] and blocked differentiation of myoblast cells [16]. Our previous studies showed that NDGA inhibited the growth of human malignant glioma cell lines by promoting their differentiation [17]. The design of Nordy

is based on the structure of natural NDGA to reduce the potential cytotoxicity of NDGA and inhibit tumor growth more effectively. In this study, it is expected to clarify the differentiation-inducing effects of Nordy on endocervical immortalized cells to find and develop the potential and effective differentiation inducer.

A major feature of cell differentiation is the inhibition of cell proliferation. The MTT assay indicated that Nordy could inhibit the proliferation of H8 cells significantly in a time-dependent and dose-dependent manner. Cytodifferentiation may be demonstrated in morphology and function. By light microscopy and electron microscopy, after the treatment with Nordy, H8 cells became morphologically mature, which was indicated in smaller size of cell body and nucleus, less ratio of nuclear to cytoplasm, more heterochromatin, and less nucleoli. Meanwhile, some mature organelles such as mitochondria and Golgi bodies could be found. We further found that low-dose Nordy could disturb the cell cycle of H8 cells, increase the cell percentage in G_0/G_1 phase, and decrease the cell percentage in S phase. It suggested that Nordy could arrest H8 cells in G_0/G_1 phase, inhibit the synthesis of DNA, and slow down the velocity of cell division. Antigen Ki67 is a kind of nuclear antigen expressed in the proliferation phase and it plays a vital role in cell proliferation. It could be synthesized in G_1, G_2, S, and M phases but not in G_0 phase. Owing to its short half-life, Ki67 is able to reflect the proliferation activity exactly. Therefore, it is better than other indexes such as proliferating cell nuclear antigen index and DNA content [18,19]. Treatment with Nordy could reduce the expression of Ki67, which indicated the reduction of cell proliferation activity. Meanwhile, this was consistent with the results of flow cytometry. Reduction of cell proliferation is one of the results of differentiation in most cases. So, we thought that Nordy showed significant effects on promoting H8 cells differentiation.

It has been reported that HPV type16 plays an important role in the occurrence of cervical carcinoma. Their early nosogenetic genes E6 and E7 may not only transform endocervical cells directly but also interact with the regulative proteins, and then interfere with the normal cell cycle, which may lead to the excessive proliferation of infected cells and carcinogenesis [20]. By RT-PCR, we found that Nordy could decrease the expression of HPV-16 E6 mRNA, which is one of the possible mechanisms of Nordy-induced differentiation in H8 cells.

Telomerase activity has been detected in a wide variety of human tumors and tumor-derived cell lines, whereas it is present in a limited range of normal somatic tissue *in vivo* and normal cells *in vitro* [21,22]. Several recent reports have described that telomerase activity is present not only in cervical cancer lesions but also in precancerous lesions [23]. Wang *et al.* [24] have reported that telomerase activity was found to be significantly higher in cells from patients with cervical intraepithelial neoplasia than that from patients with cervicitis. Moreover, there is significant correlation between the severity of cervical lesions and the signal intensity of telomerase activity. Telomerase activity may be considered as a marker of early diagnosis and progression of cervical neoplasia. In this study, after the treatment with Nordy for 48, 72, and 96 h, the activity of telomerase was decreased in H8 cells. The inhibition was more notable after 72 and 96 h ($P < 0.05$, $P < 0.01$) in a time-dependent manner. The previous study showed there was significant correlation between the expression of telomerase activity and the cell cycle. Moreover, the telomerase activity was at the highest level when tumor cells were blocked in the S phase, whereas in G_0/G_1 and G_2/M phases it was significantly decreased. We found that Nordy could arrest H8 cells in G_0/G_1 phase, which indicated that Nordy might inhibit the DNA synthesis of H8 cells in S phase and downregulate the telomerase activity.

In conclusion, our results suggested that Nordy might be a promising differentiation-inducing agent of cervical intraepithelial neoplasia. Further studies will be needed to elucidate the mechanisms by which Nordy induces differentiation of tumor cells to develop more efficacious antitumor agents.

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