

The effect of co-treatment with DAPT and oxaliplatin on the biological behavior of human ovarian cancer stem cells

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Abstract

To assess the effects of the combination of DAPT and oxaliplatin on the biological behavior of human ovarian cancer stem cells. In vitro cultured human ovarian cancer stem cells were randomly divided into DAPT, L-OHP, DAPT + L-OHP, and control groups. MTT assays were measured to assess the ability of inhibit proliferation. Inverted microscopy, flow cytometry, and in vitro invasion assays were performed to assess cell morphology, apoptosis, and cell invasion, respectively. Then, western blotting was used to detect Notch-1 and LRP1 expression in the four groups of cells. MTT assay revealed that DAPT and L-OHP monotherapy could inhibit the proliferation of ovarian cancer stem cells in a time- and dose-dependent manner. Inverted microscopy showed that untreated ovarian cancer stem cells had an oval or polygonal morphology, with a plump shape and large nuclei. After treatment with DAPT or L-OHP, cells shrank and cracked, with an irregular shape and increased shedding. Flow cytometry revealed that apoptosis was significantly higher in the DAPT and L-OHP groups compared with the control group; the DAPT + L-OHP group had a significantly higher rate of apoptosis than either the DAPT or L-OHP groups. An in vitro invasion assay revealed that DAPT + L-OHP inhibited cell invasion to a greater extent than either DAPT or L-OHP alone. Western blotting revealed that, compared with control, L-OHP had no effect on Notch-1 protein expression, whereas DAPT and DAPT + L-OHP significantly reduced Notch1 protein levels. In addition, cells treated with DAPT + L-OHP expressed much less Notch than those treated with DAPT alone. In the L-OHP group, LRP protein levels were increased significantly, whereas levels were decreased significantly in the DAPT and DAPT + L-OHP groups. DAPT inhibits the proliferation of ovarian cancer stem cells, promotes their apoptosis, weakens their invasive ability, and functions synergistically with L-OHP.

Background

Ovarian cancer has the highest mortality rate among all gynecological malignancies because early diagnosis is rare and the treatment options available are poor. As such researchers have obtained an in-depth understanding of the biological behavior and mechanisms of ovarian cancer growth in an attempt to identify better treatments. In recent years, progress in cancer stem cytology-based techniques has helped characterize the biological behavior of ovarian cancer from a novel perspective [1]. To further explore the role of ovarian cancer stem cells in the occurrence and development of ovarian cancer, we developed a serum-free suspension culture method with paclitaxel in media containing EGF, bFGF, Noggin and LIF. This method was used both *in vitro* and *in vivo* to successfully screen the CD133+, CD117+ expression characteristics of ovarian cancer stem cells and identify their specific markers and biological characteristics [2].

The current comprehensive treatment program for ovarian cancer is generally surgery supplemented with chemotherapy. Ovarian cancer chemotherapy commonly utilizes platinum drugs, among which oxaliplatin has gained increased clinical emphasis due to its high efficiency and low toxicity [3]. The Notch signaling pathway has been studied extensively in recent years, and was confirmed to be closely associated with the occurrence, development, and drug resistance of several tumors [4]. Among the Notch

receptor family members, the Notch-1 receptor is studied most frequently, and it plays a very important role during Notch signaling. Use of the g-secretase inhibitor DAPT, which also inhibits the Notch-1 receptor, has revealed its important function in regulating tumor cell growth, proliferation, and apoptosis [5]. The aim of the current study was to assess the effect of the combination of DAPT and oxaliplatin on the biological behavior of ovarian cancer stem cells to attempt to identify novel strategies for the treatment of ovarian cancer.

Methods

Materials

Human ovarian cancer stem cells were screened and stored in the Gynecology Department at the Affiliated Hospital of Xuzhou Medical College[2]. MTT and apoptosis detection kits were purchased from Nanjing KeyGen Biotechnology Development Company. Serum-free medium was purchased from Hangzhou Evergreen Biological Engineering Materials Co. DAPT and L-OHP was purchased from Wuhan Biochempartner Co. Ltd. Notch-1, and lung resistance protein (LRP) primary and secondary antibodies were purchased from Chemicon (USA). Invasion chambers were purchased from Sigma (USA).

Cell culture and experimental grouping

Human ovarian cancer stem cells were cultured in serum-free medium containing EGF, bFGF, Noggin, and LIF at 37°C, with 5% CO₂ and saturated humidity in a closed thermostat incubator. Cells were randomly divided into five DAPT groups (20, 40, 80, 100, and 120 µmol/L), four L-OHP groups (2.5, 5, 7.5, and 10 µg/ml), nine DAPT + L-OHP groups (2.5, 5, 7.5 µg/mL DAPT combined individually with 20, 40, and 80 µmol/L L-OHP), and one control group.

MTT assays

Cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well. After 1, 2, 3, 4, 5, and 6 days in culture, 20 µL MTT-working solution was added, and the cells were cultured for an additional 4 h in a CO₂ incubator at 37°C. Dimethyl sulfoxide was then added to stop the reaction, and the optical absorbance (A) at 490 nm of each well was measured using an enzyme-linked immunosorbent detector. The rate of inhibition of proliferation was calculated according to the following formula: inhibition (%) = $(1 - \text{OD of the experimental group} / \text{OD of the control group}) \times 100\%$. The Q method described by Zhang SQ [6] was used to calculate the interaction index of the two drugs: $q = E(A + B) / (EA + EB - EA \times EB)$, where EA and EB are the inhibition rate of each drug alone. $Q > 1.15$ defines synergism, $q = 0.85 - 1.15$ represents a simple additive effect, and $q < 0.85$ represents antagonistic action. Based on these results, the optimal conditions for DAPT, L-OHP, DAPT + L-OHP, and the control group were selected and used for subsequent analyses.

Inverted microscopy

Proliferating stem cells were harvested, and seeded into 6-well culture plates at a density of 4×10^5 cells/ml. Changes in morphology were then observed under an inverted microscope after 24 h in culture.

Flow cytometry

Cells were harvested using 0.25% trypsin, washed with PBS, centrifuged, and diluted to 1.5×10^5 cells/ml. They were then stained with 5 μ l Annexin V-FITC and 5 μ l PI for 10 min in the dark at room temperature, and analyzed by flow cytometry.

In vitro invasion assay

Transwell chambers were used for *in vitro* invasion assays, with a Matrigel invasion film placed between the upper and lower chambers. A single-celled suspension was plated on the invasion film in 200 μ l per well (approximately 10^5 cells), and cells were cultured at 37°C, with 5% CO₂ for 12 h. The cells and Matrigel were then removed from the membrane by wiping, and the invasion film was fixed ready for staining with hematoxylin and eosin (H&E). Cell number was counted using a microscope, and the rate of invasion inhibition was calculated using the following formula: invasion inhibition (%) = $(1 - \text{cell number in experimental group} / \text{cell number in the control group}) \times 100\%$.

Western blotting

Cells in the exponential growth phase were harvested and lysed in 200 μ l of cell lysis buffer on ice. The protein content was quantified using a bicinchoninic acid (BCA) assay. An equal amount of protein was then electrophoresed on 10% sodium dodecyl sulfonate sodium-polyacrylamide gels (SDS-PAGE), and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk for 60 min, and then incubated with 1:1,000 anti-Notch-1 or anti-LRP antibodies (rabbit anti-human) at 4°C overnight, followed by horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:10,000) at room temperature for 2 h. Enhanced chemiluminescence (ECL) reagents were then added, and the signal exposed and developed using highly sensitive X-ray film in a dark room.

Statistical analysis

SPSS16.0 software (Chicago, IL, USA) was used for all statistical analyses, and experimental data are presented as $\pm$ s. Comparisons of mean values between two groups were performed using one-way ANOVA. The difference between two sets of data was compared using *t*-tests. Correlations were analyzed using Pearson correlation coefficient. $P < 0.05$ was considered to indicate statistical significance.

Results

Effects of L-OHP and DAPT, alone or in combination, on the proliferation of human ovarian cancer stem cells

Different concentrations of L-OHP inhibited the proliferation of ovarian cancer stem cells to varying degrees. This effect was significant when compared with the control group ($P < 0.05$). Pearson correlation analysis revealed clear dose- ($r = 0.773$) and time-dependent effects ($r = 0.591$; Table 1). Similarly, different concentrations of DAPT also inhibited the proliferation of ovarian cancer stem cells to varying degrees. Compared with control, these effects were statistically significant ($P < 0.05$). Pearson correlation analysis revealed obvious dose- ($r = 0.819$) and time-dependent effects ($r = 0.520$; Table 2).

Next, the effect of co-treatment with L-OHP (2.5, 5, and 7.5 $\mu\text{g/ml}$) and DAPT (20, 40, 80 and $\mu\text{mol/L}$) was assessed in ovarian cancer stem cells. Increasing drug concentrations inhibited cell proliferation significantly ($P < 0.05$) compared with the control group and the corresponding monotherapy groups. According to the Q method reported by Zhang SQ [6], co-treatment with 2.5 $\mu\text{g/ml}$ L-OHP and 7.5 $\mu\text{g/ml}$ DAPT had a clear additive effect ($q = 0.85 - 1.15$). When 5 $\mu\text{g/ml}$ L-OHP was used, synergistic effects occurred after co-treatment with 20, 40, or 80 $\mu\text{mol/L}$ DAPT ($q > 1.15$). Treatment with 5 $\mu\text{g/ml}$ L-OHP alone inhibited proliferation by 38.24%; co-treatment with 20, 40, and 80 $\mu\text{mol/L}$ DAPT inhibited the 24 h proliferation rate of ovarian cancer stem cells by 60.13%, 68.17%, and 71.33%, respectively. Therefore, the inhibition rate increased with increasing concentrations of DAPT. This result demonstrates that DAPT synergizes with L-OHP to inhibit the proliferation of ovarian cancer stem cells (Table 3).

Effects of L-OHP and DAPT, alone or in combination, on the morphology of human ovarian cancer stem cells

Under an inverted microscope, control cells exhibited an oval or polygonal morphology, with a round shape and a large nucleus. However, after 24 h treatment with 5 $\mu\text{g/ml}$ L-OHP, blurred cell membranes were observed, with an enlarged cell gap, and increased apoptosis. Similarly, 24 h treatment with 40 $\mu\text{g/ml}$ DAPT resulted in fusiform, shrunk, and cracked cells with a reduced cell density, enlarged cell gaps, an increased number of nuclear particles, and enhanced apoptosis. After 24 h of treatment with the combination of 40 $\mu\text{g/ml}$ DAPT and 5 $\mu\text{g/ml}$ L-OHP, the cell number was reduced further; in addition, more cells had disintegrated and were undergoing apoptosis (Fig.1).

Increased apoptosis in human ovarian cancer stem cells treated with L-OHT and DAPT alone and in combination

Flow cytometry revealed that the apoptosis rate of cells in the L-OHP ($17.7 \pm 0.6\%$) and DAPT groups ($15.3 \pm 0.2\%$) was increased significantly compared with the control group ($8.1 \pm 0.6\%$; $P < 0.05$). As expected, the apoptosis rate in the DAPT + L-OHP group ($36.5 \pm 0.3\%$) was significantly higher than in the L-OHP or DAPT groups ($P < 0.05$; Fig.2).

Synergistic effects of DAPT and L-OHP on inhibiting the invasion of human ovarian cancer stem cells

The rates of invasion inhibition in the DAPT and L-OHP alone groups were $40.3 \pm 4.2\%$ and $50.2 \pm 7.8\%$, respectively. However, co-treatment inhibited invasion by $75.1 \pm 2.9\%$. This suggests that L-OHP could enhance the DAPT-mediated inhibition of cell invasion in ovarian cancer stem cells (Fig.3).

Effects of L-OHP and DAPT, alone or in combination, or Notch-1 and LRP expression in human ovarian cancer stem cells

There were no significant differences in Notch-1 protein expression between the L-OHP (0.849 ± 0.02) and control groups (0.861 ± 0.01 ; $P > 0.05$). However, Notch-1 expression was reduced significantly in the DAPT (0.644 ± 0.02) and DAPT + L-OHP groups (0.517 ± 0.05 ; both $P < 0.05$). In addition, the DAPT + L-OHP group had significantly lower Notch-1 expression than the DAPT group ($P < 0.05$).

LRP protein levels in the L-OHP group (0.811 ± 0.03) were significantly higher than in the control group (0.686 ± 0.03 ; $P < 0.05$). In contrast, levels in the DAPT (0.487 ± 0.01) and DAPT + L-OHP groups (0.318 ± 0.04) were significantly lower than in the control and L-OHP groups (both $P < 0.05$). Finally, LRP expression was significantly lower in the DAPT + L-OHP group than in the DAPT group ($P < 0.05$; Fig.4).

Discussion

Currently, the standard treatment for ovarian cancer is effective cytoreductive surgery in combination with chemotherapy. Although most patients obtain varying degrees of disease relief after this treatment regimen, most will suffer relapse or become resistant to chemotherapy and consequently die because of the ineffectiveness of the existing antineoplastic therapies.

Ovarian cancer stem cells can cause ovarian cancer; their ability for self-renewal, unlimited proliferation, and differentiation is considered to be a root cause of the occurrence, abnormal proliferation, metastasis, and recurrence of ovarian cancer [7]. Therefore, the search for effective chemotherapy drugs and chemotherapy programs for the ablation of ovarian cancer stem cells has become urgent. Platinum-based drugs, particularly cisplatin and carboplatin, play a very important role in chemotherapy for ovarian cancer; however, the effectiveness of chemotherapy is often unsatisfactory because of drug resistance. Oxaliplatin is a third generation, broad-spectrum platinum-based anticancer drug that produces alkylating conjugates to damage DNA and inhibit DNA synthesis. It has completely different pharmacokinetic characteristics from, and consequently no cross-resistance with, carboplatin and cisplatin; therefore, ovarian cancer patients who are resistant to cisplatin or carboplatin remain sensitive to oxaliplatin [8,9].

Hopfer et al. [10] revealed that the expression of activated Notch-1 was significantly higher in ovarian cancer than in benign ovarian tumors and normal ovarian tissues. In addition, Notch-1 is expressed in the ovarian cancer cell line, suggesting that Notch signaling, and particularly Notch-1, is closely associated with the occurrence, development, and drug resistance of ovarian cancer. Likewise, other investigators demonstrated the importance of the abnormal regulation of Notch signaling in several characteristics of other tumor cells including invasion, metastasis, and drug resistance [11,12].

g-secretase is a multi-molecule complex and a key enzyme during activation of the Notch signaling pathway. As such, g-secretase inhibitors inhibit the activation of Notch signaling [13,14]. As well as directly inhibiting the Notch signaling pathway in tumor cells, g-secretase inhibitors also interfere with Notch signal transduction between tumor cells and tumor vascular epithelial cells, thereby inhibiting

angiogenesis [15]. DAPT is a synthetic g-secretase inhibitor that blocks the cleavage of Notch receptors, which is mediated by g-secretase. This inhibits the conversion of Notch receptors into active fragments, therefore suppressing the activation of the Notch signaling pathway [16,17].

Based on the above findings, the current study assessed the biological effects of co-treating ovarian cancer stem cells with DAPT and L-OHP, and explored its possible mechanism of action. Cells were treated with different concentrations of DAPT and L-OHP for different lengths of time. MTT assays revealed that monotherapy with DAPT or L-OHP alone inhibited the proliferation of ovarian cancer stem cells *in vitro* in a clear dose- and time-dependent manner. Flow cytometry demonstrated that DAPT or L-OHP monotherapy also induced apoptosis, which was confirmed by morphology observations using inverted microscopy. An *in vitro* invasion assay revealed that either monotherapy also inhibited the invasiveness of ovarian cancer stem cells. When the combination of DAPT and L-OHP was used to treat human ovarian cancer stem cells, the effects on the inhibition of proliferation and the induction of apoptosis were enhanced significantly compared with either monotherapy. Similarly, the invasion ability of ovarian cancer stem cells was declined significantly compared with either monotherapy. These results above support the hypothesis that DAPT could enhance the sensitivity of ovarian cancer stem cells to L-OHP, and synergize with L-OHP to inhibit proliferation and invasion, and promote apoptosis in ovarian cancer stem cells.

Western blotting showed that Notch-1 protein levels were comparable in the L-OHP and control groups. However, levels were reduced significantly in the DAPT and DAPT + L-OHP groups compared with the control and L-OHP groups. Notably, the DAPT + L-OHP group had more reduced expression. The above results suggest that DAPT might inhibit proliferation and induce apoptosis in ovarian cancer stem cells by downregulating Notch-1 expression; these effects are more pronounced after co-treatment with DAPT and L-OHP.

The human LRP gene is located on the short arm of human chromosome 16; the molecular weight of the encoded LRP is 110 kD. LRP plays roles in tumor cell multidrug resistance (MDR) via two mechanisms: (1) pumping out drugs that target nuclei or inhibiting their transition through the nuclear pore into the nucleus; and (2) engulfing drugs into vesicles in the cytoplasm and discharging them to the extracellular compartments via exocytosis. Therefore, LRP mediates the resistance to DNA-targeting drugs such as platinum-based and DNA-alkylating agents [18-20]. In the current study, western blotting revealed that LRP protein levels were increased significantly in the L-OHP group compared with control. In contrast, levels were reduced significantly in the DAPT and DAPT + L-OHP groups compared with the control and L-OHP groups; the reduction was greater in the DAPT + L-OHP group.

Taken together, these data suggest that the sensitizing effects of DAPT on L-OHP might be related to the downregulation of Notch-1 and LRP protein expression. However, additional experiments are required to verify any potential correlation between Notch signaling and LRP.

Based on the results of the current study, we propose that activation of the Notch signaling pathway might play an important role in the acquisition of chemotherapy resistance in ovarian cancer, as well as

the migration and invasion of cancer cells. This suggests that therapeutic interventions targeting the Notch signaling pathway might help improve the chemosensitivity of ovarian cancer stem cells and provide a novel perspective for the treatment of ovarian cancer.

Declarations

Compliance with Ethical Standards

Conflict of Interest

Author Nan Yu declares that she has no conflict of interest. Author Yuchen Yan declares that he has no conflict of interest. Author Lisha Fang declares that she has no conflict of interest. Author Yang Xu declares that she has no conflict of interest. Author Qiang Li declares that he has no conflict of interest. Author Hongchao Yan declares that he has no conflict of interest.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required

Availability of data and material

All data generated or analysed during this study are included in this published article

Authors' contributions

Nan Yu and Yuchen Yan are responsible for ovarian cancer stem cell culture, drug concentration screening, experimental grouping, cell proliferation test and Western blotting. Lisha Fang is responsible for inverted microscope observation experiments. Yang Xu is responsible for flow cytometry experiments. Qiang Li is responsible for in vitro invasion experiments. Hongchao Yan is responsible for the design and statistical processing of the whole experiment

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Tables

Table 1 Effect of L-OHP on the proliferation of human ovarian cancer stem cells ($\pm$ s)

L-OHP	24h	48h	72h
(ug/ml)	Inhibition rate%	Inhibition rate%	Inhibition rate%
Control group			
2.5	32.12 $\pm$ 0.9 ^{ab}	38.94 $\pm$ 1.6 ^{ab}	38.88 $\pm$ 0.7 ^{ab}
5	38.24 $\pm$ 1.5 ^{ab}	62.26 $\pm$ 3.4 ^{ab}	79.18 $\pm$ 0.6 ^{ab}
7.5	49.64 $\pm$ 1.8 ^{ab}	72.06 $\pm$ 2.1 ^{ab}	92.97 $\pm$ 1.5 ^{ab}
10	71.89 $\pm$ 0.5 ^{ab}	88.08 $\pm$ 0.7 ^{ab}	96.02 $\pm$ 1.8 ^{ab}

a, the inhibitory effects of different drug concentrations were significantly different at the same time point ($P < 0.05$); b, the inhibitory effects of the same drug concentration were significantly different at different time points ($P < 0.05$).

Table 2 Effect of DAPT on the proliferation of ovarian cancer stem cells ($\pm$ s)

DAPT concentration	24h	48h	72h
μmol/ml	Inhibition rate%	Inhibition rate%	Inhibition rate%
Control group			
20	21.79±1.6 ^{ab}	27.48±0.8 ^{ab}	34.35±0.1 ^{ab}
40	32.06±1.3 ^{ab}	42.06±2.6 ^{ab}	53.32±1.5 ^{ab}
80	38.11±0.7 ^{ab}	49.96±1.6 ^{ab}	72.29±2.3 ^{ab}
100	51.02±1.1 ^{ab}	60.31±1.8 ^{ab}	79.96±0.8 ^{ab}
120	58.63±2.2 ^{ab}	68.34±2.8 ^{ab}	82.65±1.8 ^{ab}

a, the inhibitory effects of the same drug concentration were significantly different at different time points ($P < 0.05$); b, the inhibitory effects of different drug concentrations were significantly different at the same time point ($P < 0.05$).

Table 3 Effect of the combination of DAPT and L-OHP on the proliferation of human ovarian cancer stem cells ($\pm$ s)

L-OHP (ug/ml)	DAPT (umol/ml)			
	0	20	40	80
0	0.00±0.00 ^{ab}	21.79±1.6 ^{ab}	32.06±1.3 ^{ab}	38.11±0.7 ^{ab}
2.5	32.12±1.0 ^{ab}	53.55±1.2 ^{ab}	61.89±0.8 ^{ab}	65.27±1.7 ^{ab}
5	38.24±1.5 ^{ab}	60.13±1.5 ^{ab}	68.17±1.9 ^{ab}	71.33±1.7 ^{ab}
7.5	49.64 ±1.8 ^{ab} ±1.8 ^{ab}	63.26±2.1 ^{ab}	72.03±0.8 ^{ab}	74.73±1.2 ^{ab}

a, the inhibitory effects of one concentration of L-OHP in combination with different concentrations of DAPT were significantly different among groups ($P < 0.05$); b, the inhibitory effects of one concentration of DAPT in combination with different concentrations of L-OHP were significantly different among groups ($P < 0.05$).

Figures

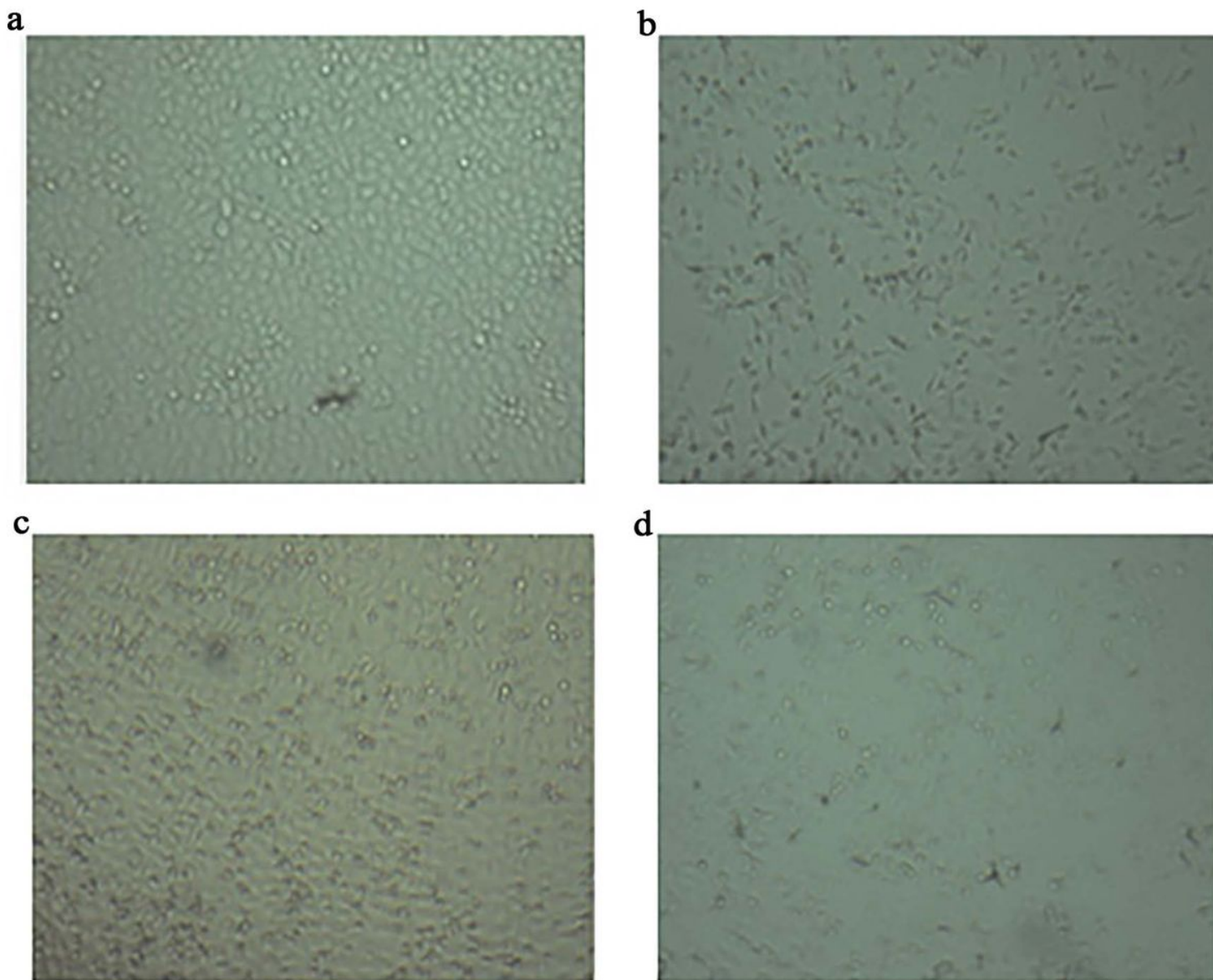


Figure 1

Morphological changes in cells in each group (200 ×) a:control group;b:DAPT group; c:L-OHP group; d:DAPT + L-OHP group

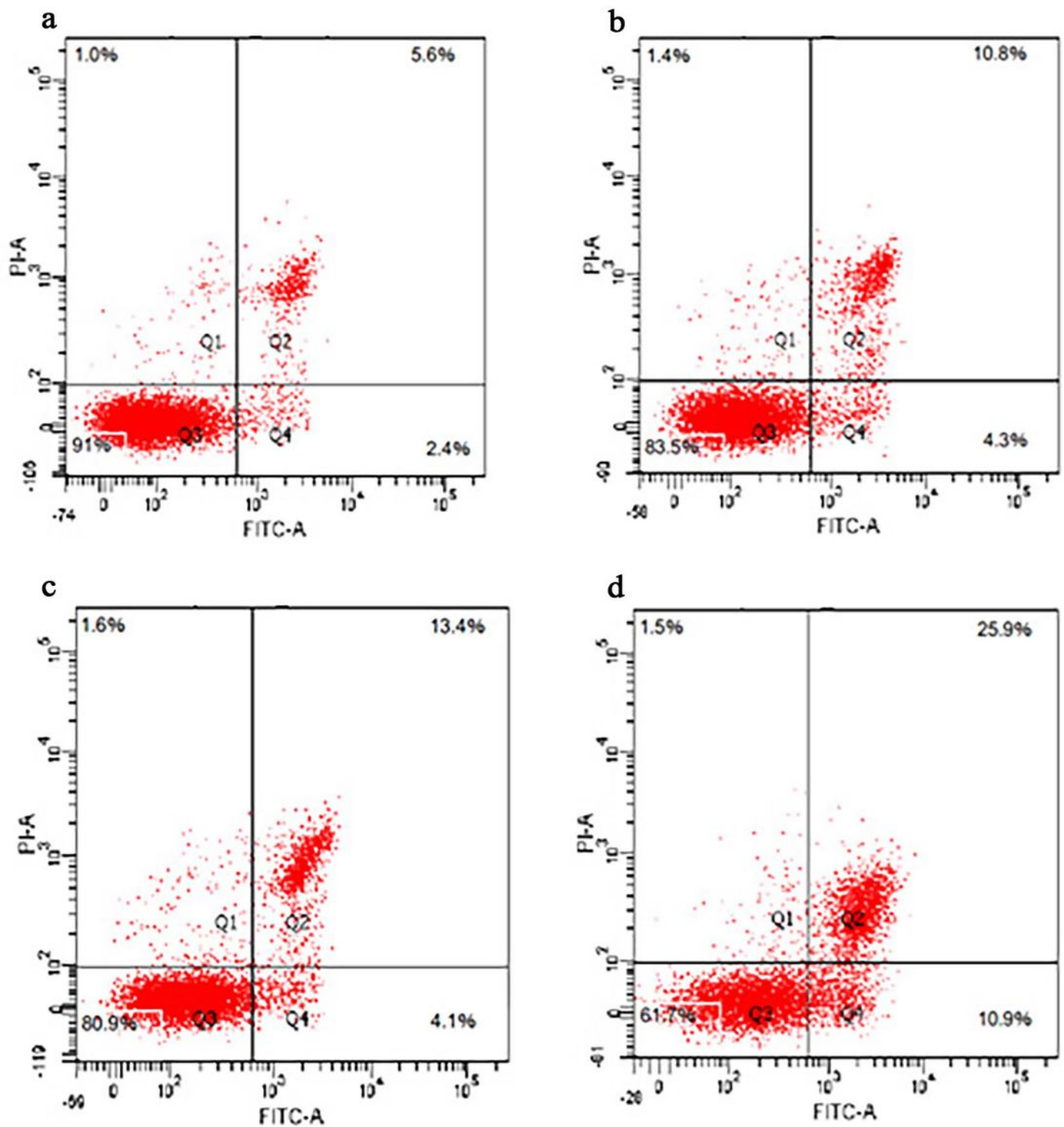


Figure 2

Rates of cellular apoptosis in each group a:control group; b:DAPT group; c:L-OHP group; d:DAPT + L-OHP group. The figures show representative dot plots from flow cytometry. The horizontal axes show Annexin V-FITC staining, and the vertical axes shows PI (propidium iodide) staining. Live cells are both Annexin V-FITC- and PI-negative (lower left quadrant); early apoptotic cells are Annexin V-FITC-positive, but PI-negative (lower right quadrant); necrotic cells and late apoptotic cells are both Annexin V-FITC- and PI-

positive staining (upper right quadrant). Events in upper left quadrant are normal detection errors within the permitted range.

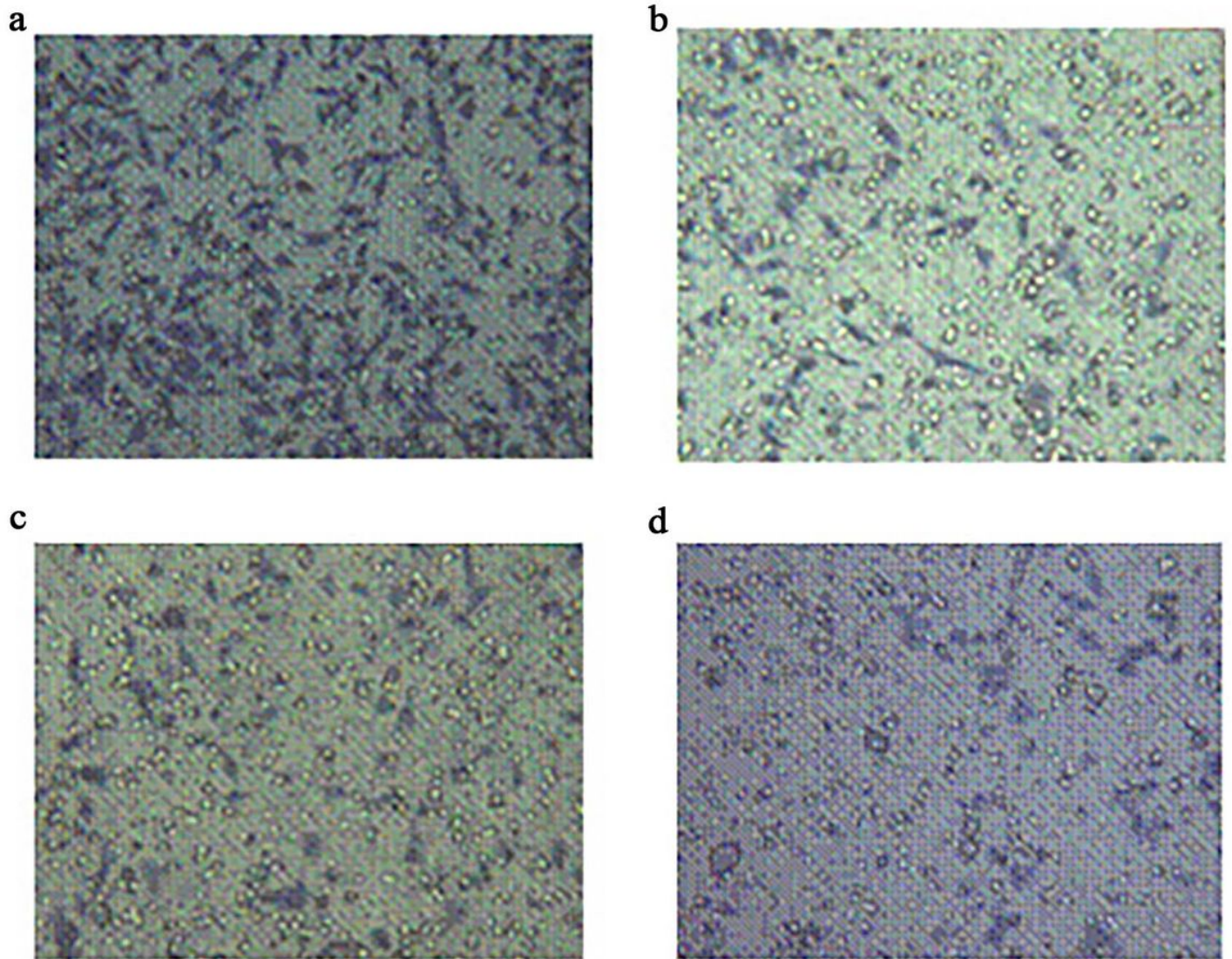


Figure 3

The invasive ability of cells each group (200 ×). a:control group;b:DAPT group; c:L-OHP group; d:DAPT + L-OHP group.

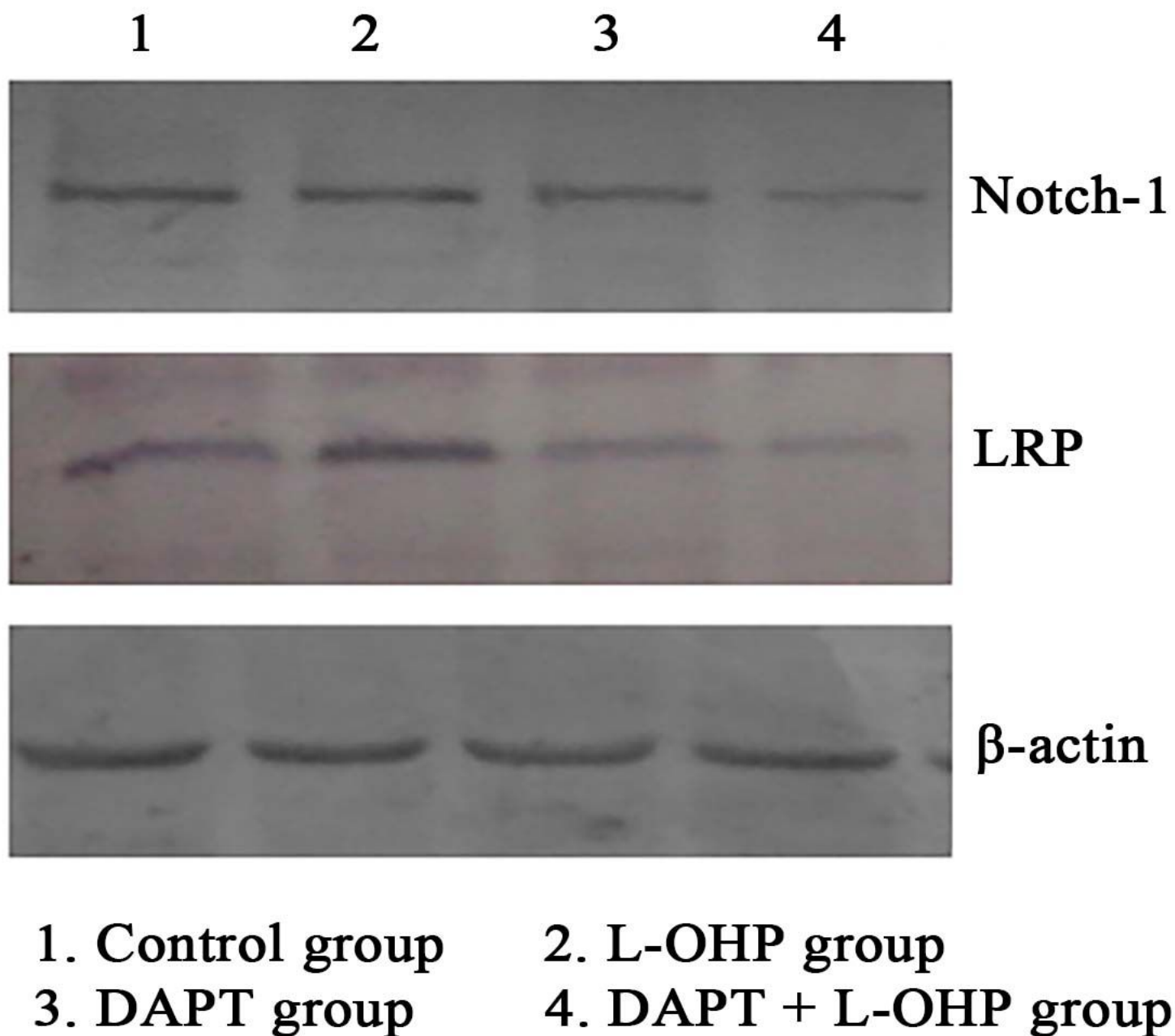


Figure 4

Notch-1 and LRP protein expression in cells from each treatment group. 1:control group; 2:L-OHP group ; 3:DAPT group; 4:DAPT + L-OHP group.