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Effect and mechanism of nociceptin/orphanin FQ reversing multi-drug resistance in K562/ADM cell

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Objective: To investigate the effect and mechanism of nociceptin/orphanin FQ (OFQ) reversing multi-drug resistance of K562/ADM cells *in vitro*. *Methods:* MTT assay, Wright staining, flow cytometry, transmission electron microscope and gel electrophoresis were used to evaluate the effect and mechanism of OFQ in reversing multi-drug resistance of K562/ADM cells. *Results:* OFQ could time-dependently reverse the ADM resistance of K562/ADM cell. After treatment with OFQ (1×10^{-7} mol · L^{-1}), K562/ADM cells were cultured for 24, 48 and 72 h. The reversal index (RI) was 1.33, 1.42 and 1.53, respectively. Furthermore, OFQ significantly increased the intracellular accumulation of ADM in K562/ADM cells and percentage apoptosis in K562/ADM cells. OFQ down-regulated the level of P-gp time-dependently, while the level of Fas and FasL were up-regulated. There were evidently significant differences compared with the control ($P < 0.01$). After treating K562/ADM cells with OFQ (1×10^{-7} mol · L^{-1}) and ADM (20 μg · ml^{-1}) for 48 hours, the cells showed apoptotic nuclear fragmentation, which was characterized by the appearance of a DNA ladder pattern in genomic DNA gel electrophoresis. *Conclusion:* OFQ can reverse the ADM resistance of K562/ADM cells. The mechanism involves OFQ up-regulating the expression of Fas/FasL, down-regulating the level of P-gp, and decreasing the intracellular level of calcium in K562/ADM cells.

1. Introduction

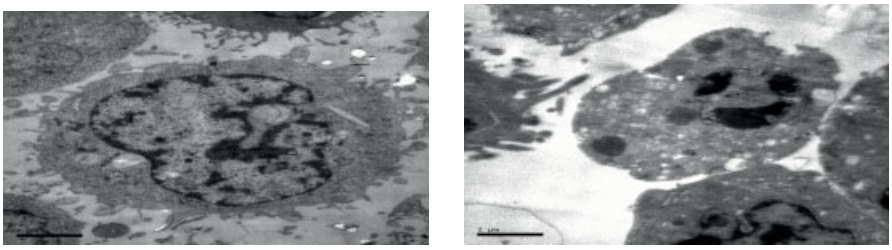
The treatment of cancer with chemotherapeutic drugs is frequently impaired or is in effective as a result of intrinsic or acquired resistance of the tumor cells. Primary or acquired resistance of cancers to cytotoxic drugs is in part due to the phenomenon described as multi-drug resistance (MDR). MDR results from the expression of a 170 kDa membrane glycoprotein (P-gp), which acts as a drug-efflux pump. P-gp or its encoding gene (*mdr 1*) has been detected in many human cancers, in both untreated and previously treated tumors. Reversal of drug resistance has been obtained by various agents, which usually act as competitive inhibitors. These resistance modifying agents include verapamil, quinine derived compounds and cyclosporin.

Endogenous opioid peptides have opioid activity in the mammalian brain. Previously, the study of opioids and their receptors has focused on the neuro-pharmacology and neurobiology of molecules such as morphine, which is used as a powerful analgesic agent after operations. But many studies have suggested that opioids play an important role in the nervous, endocrine and immune systems and can inhibit tumor cell proliferation and induce cell apoptosis. Morphine has recently been shown to induce apoptosis in human lung cancer cells, suggesting involvement of opioid ligands in signal-induced tumor cell death. Many normal and transformed cells are known to bind with opioid peptides. Met-enkephalin induces cytolytic processes of apoptotic type in K562 human erythroid leukemia cells. Moreover, some studies have shown that opioid can decrease levels of intracellular Ca^{2+}, and consequently diminish cellular efflux and increase the level of intracellular cytotoxic drugs. There has been no such report regarding OFQ, an endogenous opioid peptide. This study aimed to examine whether OFQ has a resistance-reversing effect on leukemia cells and to explore the possible use of OFQ for treating leukemia and the mechanism involved.

2. Investigations and results

2.1. Morphologic changes of K562/ADM cells treated with OFQ

OFQ and ADM reversed K562/ADM cell multi-drug resistance time-dependently as assessed by the MTT assay and the maximum effect was obtained using 10^{-7} M OFQ and



A B

Fig. 1:
Morphology of K562/ADM cells under transmission electron microscope (10000×). A, Control group showing cytoplasm shrunk. B, K562/ADM cells treated with OFQ group for 72 hours; plasma membrane remained well defined, chromatin condensed and located along nuclear envelope or forming irregularly shaped crescents at nuclear edges

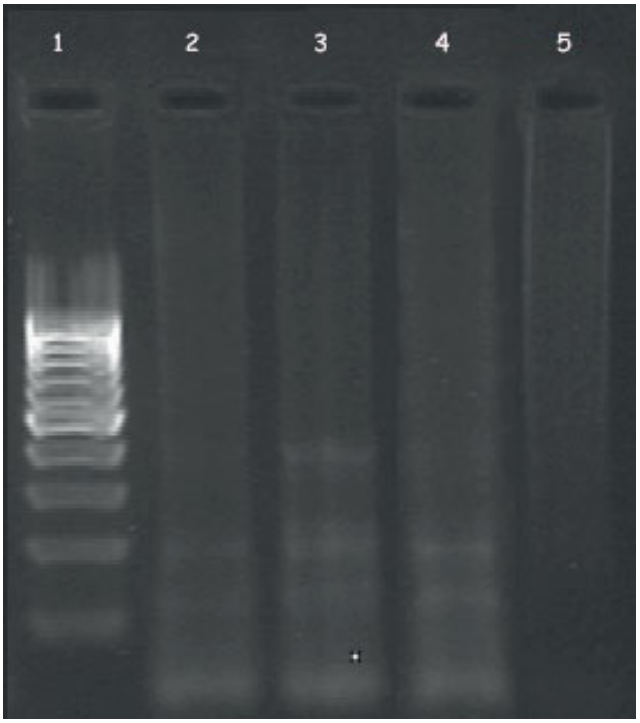


Fig. 2: DNA gel electrophoresis. Lanes 1 ~ 5: marker, ADM, ADM + OFQ, OFQ, control

ADM for 48 h. After incubation with OFQ for 48 h, Wright-stained cells showed clear apoptotic characteristics such as nuclear condensation, fragmentation, and many vacuoles. Electron microscopy revealed that, in comparison with untreated cells, the OFQ-treated cells showed cytoplasmic shrinkage, well-defined plasma membranes, and chromatin condensation and location along the nuclear envelope or in the form of irregular crescents at the nuclear edges (Fig. 1).

2.2. Detection of DNA ladder

After treatment with OFQ (1×10^{-7} mol · L^{-1}) and ADM (20 μg · ml^{-1}) for 48 h, the cells appeared to show the characteristic "DNA Ladder" of apoptosis (Fig. 2). A comparison with molecular weight markers indicated that the fragments were multiples of 180–200 bp.

2.3. Comet electrophoresis

Electropherograms of DNA from control group cells stained with ethidium bromide adopted a regular round shape and showed no fragmentation (Fig. 3A), whereas those from OFQ-treated cells were comet shaped with long tails which suggests severe fragmentation (Fig. 3B).

2.4. Detection of pre-apoptosis cells using FITC-annexin/PI double dye

It was shown that OFQ could significantly increase percentage apoptosis in K562/ADM cells compared with control cells (Table 1) and (Fig. 4).

Table 1: Percentage apoptosis in K562/ADM cells (Mean ± SD)

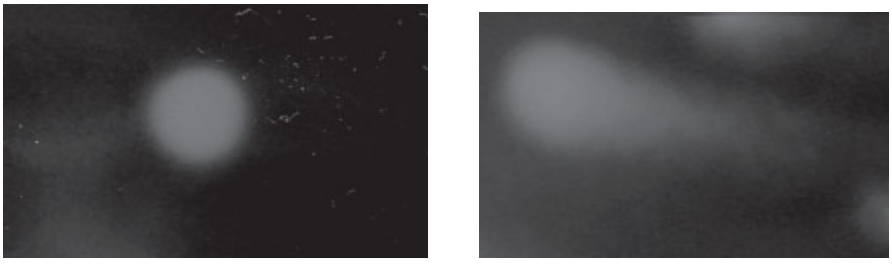
Group	Apoptosis rate
Control	3.14 ± 0.19
K562/ADM + OFQ[a]	3.48 ± 0.47
K562/ADM + ADM[b]	6.88 ± 1.60
K562/ADM + ADM + OFQ[c]	34.58 ± 4.95

Compared with control cells. a, $P > 0.05$; b, $P < 0.01$; c, $P < 0.01$; b, Compared with c, $P < 0.01$

Table 2: Effect of OFQ on expression positive rate of P-gp in K562/ADM cells (Mean ± SD)

Group	Result
Control	98.23 ± 2.39
10^{-8}[a]	63.47 ± 12.12
10^{-7}[b]	25.40 ± 3.86
10^{-6}[c]	14.30 ± 2.26

Compared with control cells. a, $P > 0.05$; b, $P < 0.01$; c, $P < 0.01$; b, Compared with c, $P > 0.05$



A B

Fig. 3:
Comet assay of K562/ADM cells treated with OFQ (200×). A, DNA of control group cells stained with ethidium bromide with round regular shaped and not fragmented. B, K562/ADM cell treated with OFQ for 48 hours. OFQ-treatment group cells comet shaped with a long tail, showing DNA of OFQ-treatmed cells severely fragmented

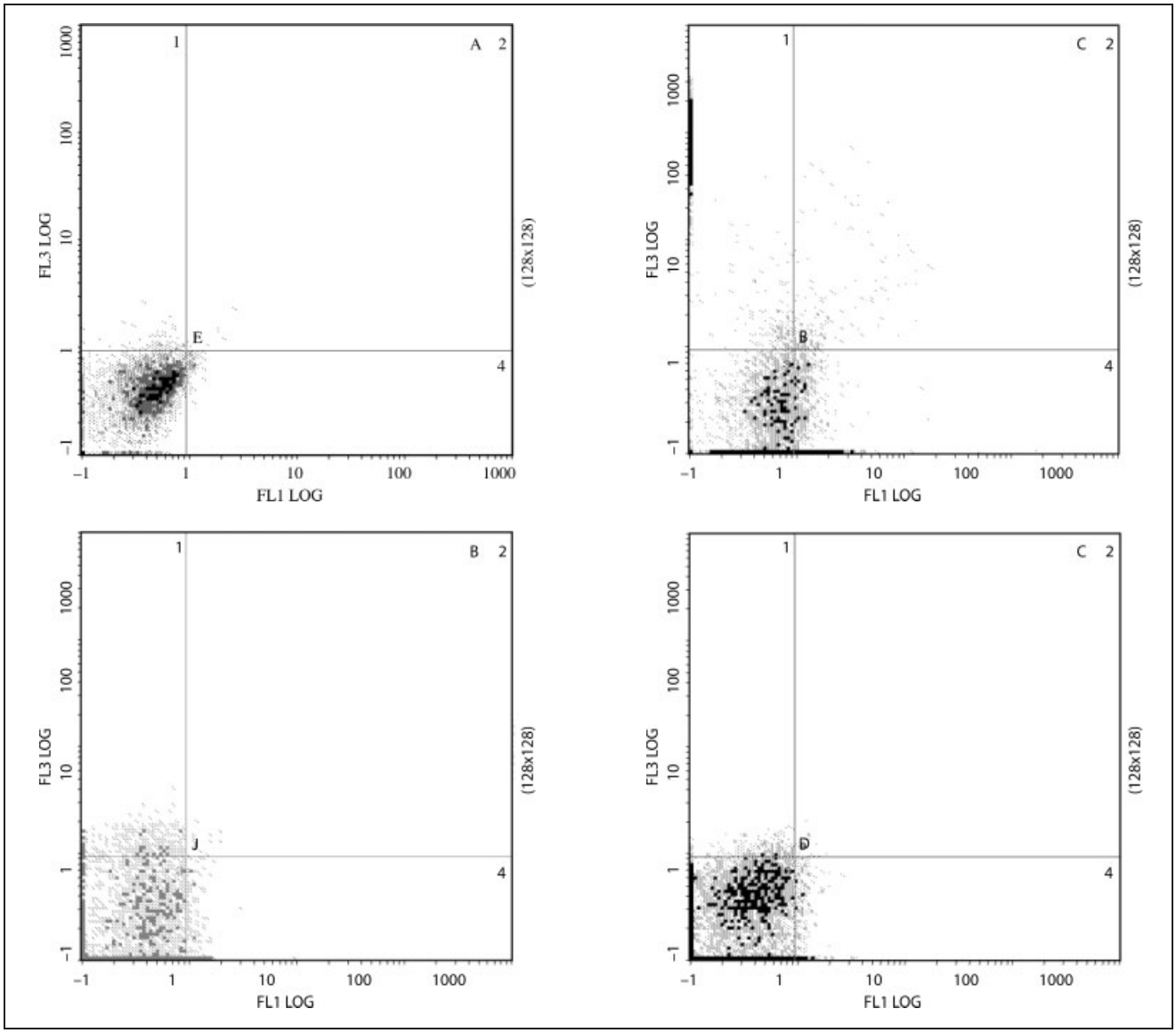


Fig. 4: Percentage apoptosis (AV/PI-FITC). A, K562/ADM cell (Control); B, K562/ADM cell treated with OFQ; C, K562/ADM cell treated with ADM; D, K562/ADM cell treated with (ADM + OFQ)

2.5. Detection of P-gp expression

As shown in Table 2, compared with control cells, P-gp protein expression was markedly decreased in OFQ-treated K562/ADM cells, which suggested that OFQ can dose-dependently down-regulate the level of P-gp (Fig. 5). There was a significant difference compared with the control group ($P < 0.01$).

Table 3: Mean fluorescence intensity of adriamycin in K562/ADM cells (Mean + SD)

OFQ (mol/L)	Fluorescence intensity of FasL			
	24 h	48 h	72 h	96 h
Control	14.00 ± 2.96	15.78 ± 3.16	15.50 ± 3.54	15.33 ± 3.70
1×10^{-7}	24.43 ± 4.45	34.18 ± 2.36	42.33 ± 2.11	52.88 ± 3.76

Compared with control cells, $P < 0.01$. K562/ADM cells treated with OFQ, 24 h compared with 48 h, $0.01 < P < 0.05$; others $P < 0.01$

Table 4: Mean fluorescence intensity of Fas in K562/ADM cells (Mean ± SD)

OFQ (mol/L)	Fluorescence intensity of Fas			
	24 h	48 h	72 h	96 h
Control	1.06 ± 0.04	1.21 ± 0.09	1.43 ± 0.05	1.42 ± 0.09
1×10^{-7}	1.11 ± 0.03[a]	2.93 ± 0.11[b]	3.20 ± 0.04[c]	3.43 ± 0.15[d]

Compared with control cells, $P < 0.01$. K562/ADM cells treated with OFQ. b compared with c, $0.01 < P < 0.05$; others $P < 0.01$

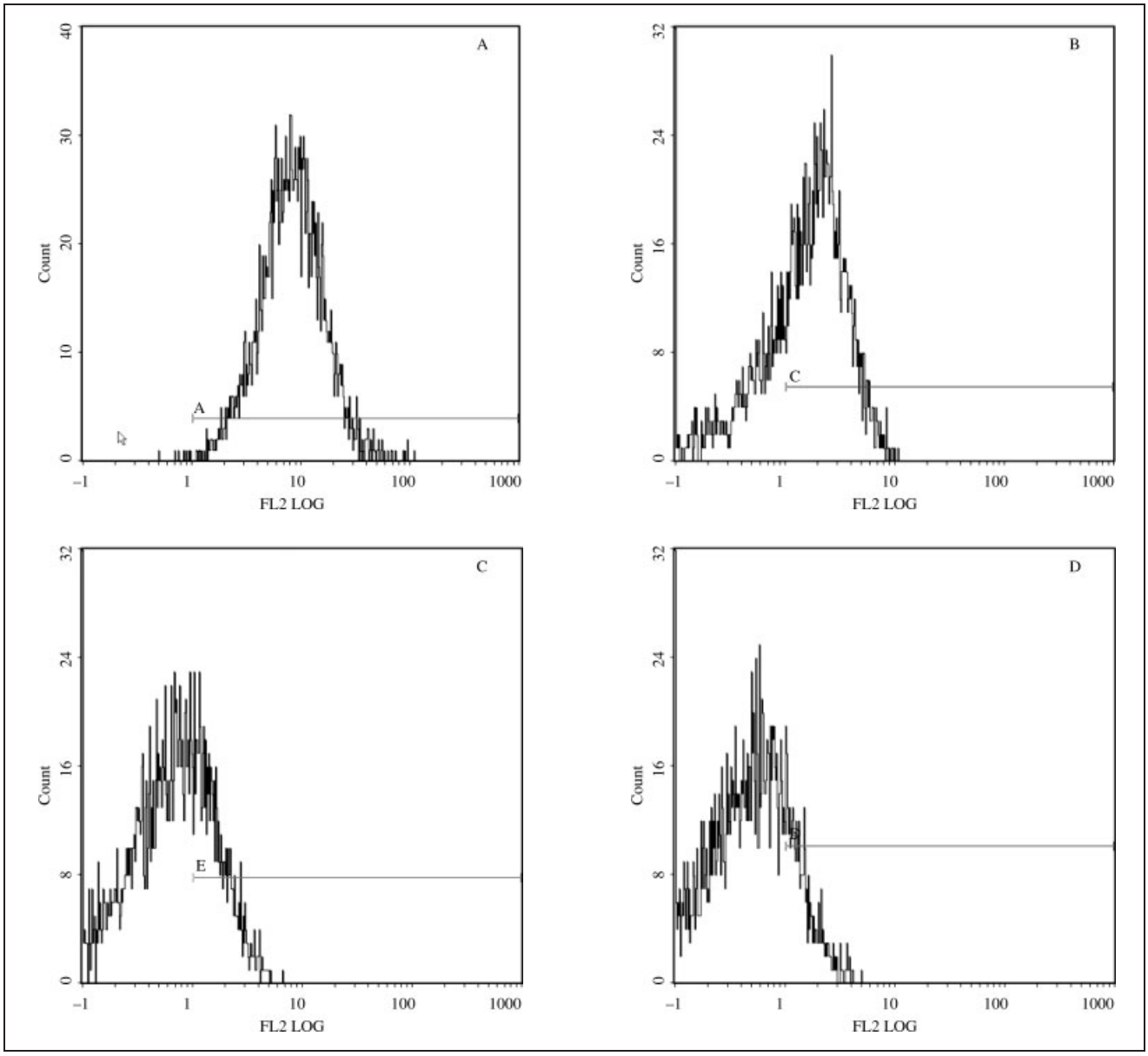


Fig. 5: P-gp expression (FCM) in K562/ADM cells. A, treated with K562/ADM cells (Control); B, treated with OFQ (10^{-8} mol/L); C, treated with OFQ (10^{-7} mol/L); D, treated with OFQ (10^{-6} mol/L)

Table 5: Mean fluorescence intensity of FasL in K562/ADM cells (Mean ± SD)

OFQ (mol/L)	Fluorescence intensity of FasL			
	24 h	48 h	72 h	96 h
Control	34.05 ± 0.95	34.60 ± 0.72	40.76 ± 1.35	43.07 ± 0.25
1×10^{-7}	55.27 ± 0.65[a]	58.40 ± 0.62[b]	60.27 ± 0.57[c]	65.93 ± 0.15[d]

Compared with control cells, $P < 0.01$. K562/ADM cells treated with OFQ; b compared with c, $0.01 < P < 0.05$; others $P < 0.01$ (h)

Table 6: Mean fluorescence intensity of Ca^{2+} in K562/ADM cells (Mean ± SD)

OFQ (mol/L)	Fluorescence intensity of Ca^{2+}			
	24 h	48 h	72 h	96 h
Control	2.59 ± 0.12	2.63 ± 0.13	2.60 ± 0.15	2.62 ± 0.14
1×10^{-7}	2.70 ± 0.15[a]	2.06 ± 0.08[b]	1.82 ± 0.02[c]	1.68 ± 0.03[d]

Compared with control cells. a $P > 0.05$; others $P < 0.01$. K562/ADM cells treated with OFQ, b Compared with c, $0.01 < P < 0.05$; others $P < 0.01$ (h)

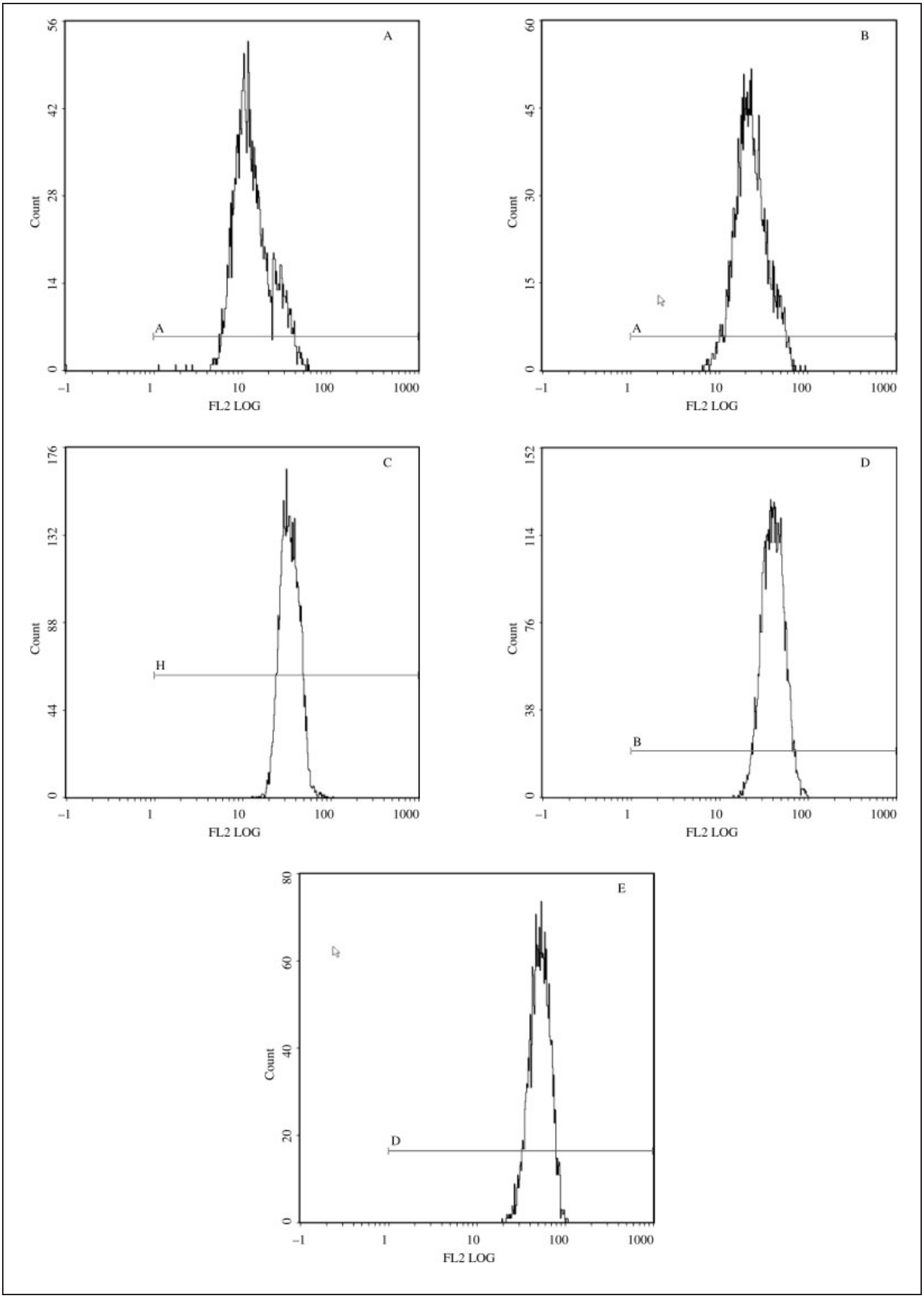


Fig. 6: Fluorescence intensity of ADM in K562/ADM cells. A, control; B, treated with OFQ (1×10^{-7} mol/L) for 24 h; C, treated with OFQ (1×10^{-7} mol/L) for 48 h; D, treated with OFQ (1×10^{-7} mol/L) for 72 h; E, treated with OFQ (1×10^{-7} mol/L) for 96 h

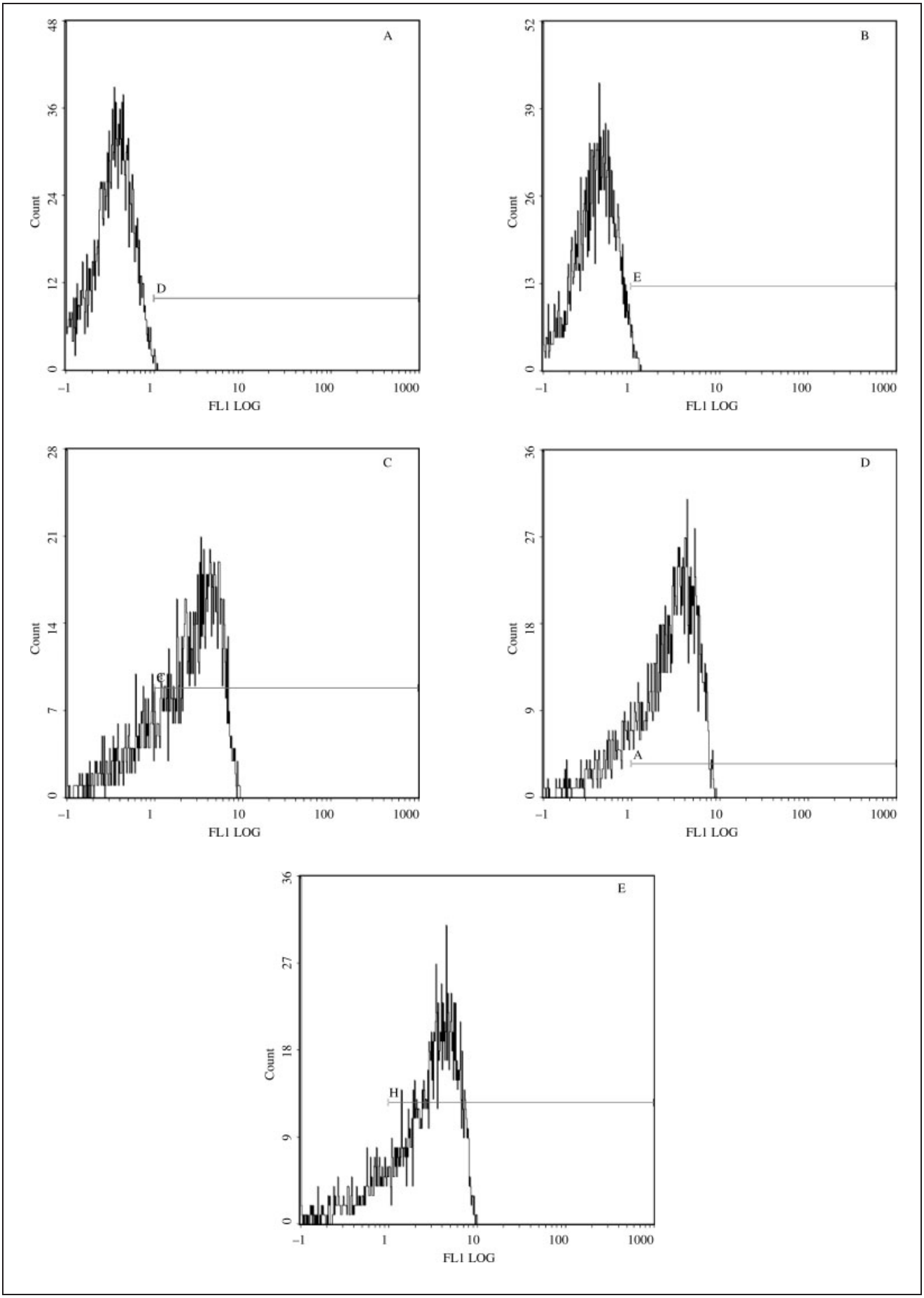


Fig. 7: Expression of Fas in K562/ADM cells A, Control; B, treated with OFQ (1×10^{-7} mol/L) for 24 h; C, treated with OFQ (1×10^{-7} mol/L) for 48 h; D, treated with OFQ (1×10^{-7} mol/L) for 72 h; E, treated with OFQ (1×10^{-7} mol/L) for 96 h

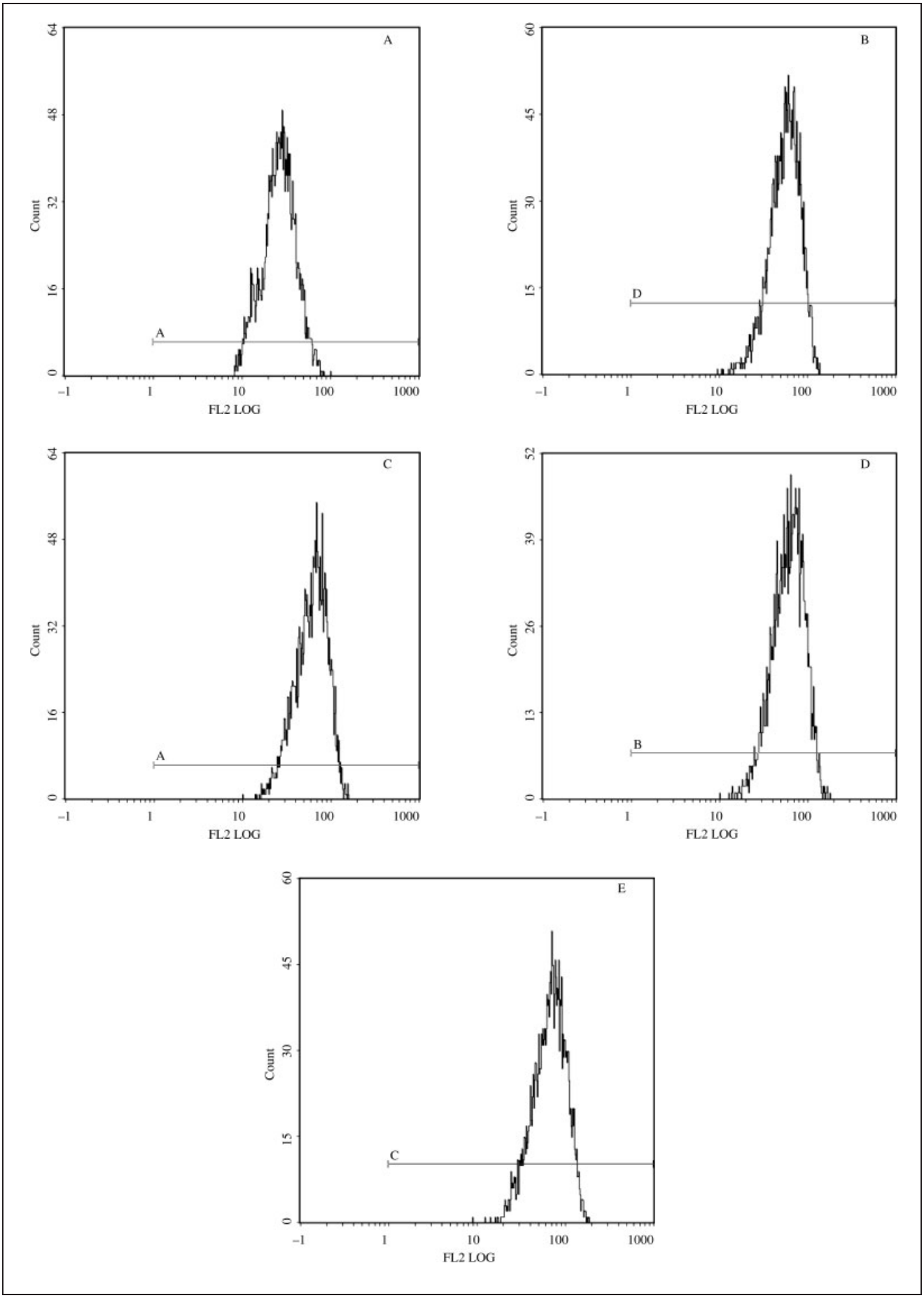


Fig. 8: Expression of FasL in K562/ADM. A, control; B, treated with OFQ (1×10^{-7} mol/L) for 24 h; C, treated with OFQ (10^{-7} mol/L) for 48 h; D, treated with OFQ (1×10^{-7} mol/L) for 72 h; E, treated with OFQ (1×10^{-7} mol/L) for 96 h

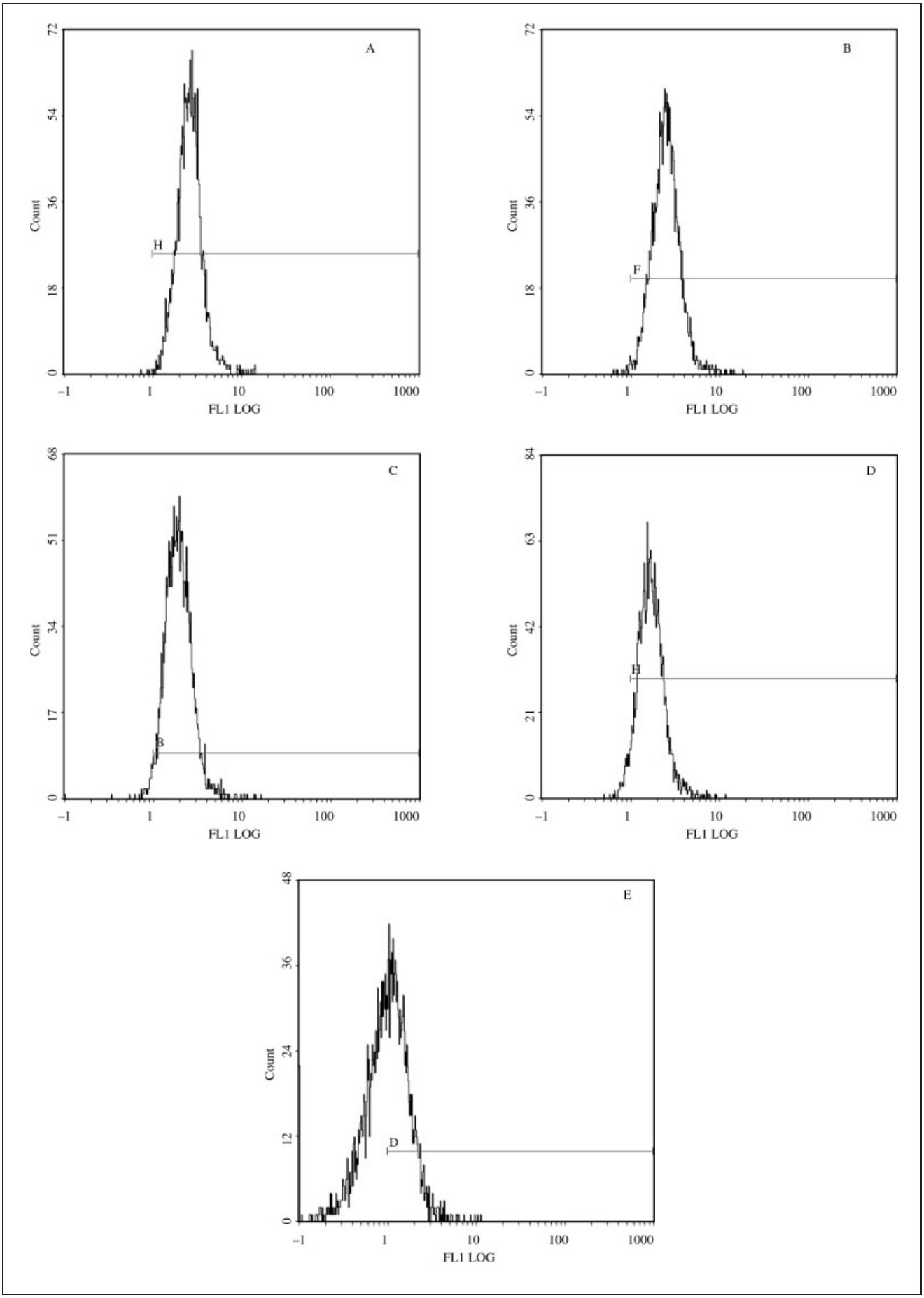


Fig. 9: Level of intracellular calcium in K562/ADM. A, control; B, treated with OFQ (1×10^{-7} mol/L) for 24 h; C, treated with OFQ (1×10^{-7} mol/L) for 48 h; D, treated with QFQ (1×10^{-7} mol/L) for 72 h; E, treated with OFQ (1×10^{-7} mol/L) for 96 h

2.6. Level of intracellular adriamycin by FCM

The results showed that OFQ significantly increased the intracellular accumulation of ADM in K562/ADM cells (Table 3, Fig. 6). There was a significant difference compared with the control group ($P < 0.01$).

2.7. Expression of Fas and Fas ligand

The results showed that OFQ increased the level of Fas and Fas ligand in OFQ-treated K562/ADM cells (Figs. 7–8, Tables 4–5). There was a significant difference compared with the control group ($P < 0.01$). OFQ up-regulated the level of Fas and Fas ligand dose-dependently.

2.8. Level of intracellular calcium by flow cytometry

The results showed that OFQ significantly decreased the intracellular level of calcium in K562/ADM cells (Table 6, Fig. 9). There was a significant difference compared with the control group ($P < 0.01$).

3. Discussion

Primary or acquired resistance of cancer cells to cytotoxic drugs is part of a larger phenomenon described as multi-drug resistance (Covelli 1995).

Studies have shown that OFQ (Thiebaut et al. 1987), a potential growth factor to normal cells, participates in tumor cell proliferation and differentiation by autocrine, paracrine, neurocrine, and endocrine mechanisms. In this study, data from MTT assay indicated that constant stimulation by OFQ could inhibit K562/ADM cell proliferation. The maximum effect was at a concentration of 10^{-7} M for 48 h. Furthermore, OFQ has distinct synergy with ADM. When K562/ADM cells were cultured in the presence of ADM, the median inhibition concentration of ADM (IC_{50}) was $46.99 \pm 0.25\ \mu g \cdot ml^{-1}$, whereas it was $23.11 \pm 0.29\ \mu g \cdot ml^{-1}$, when K562/ADM cells were treated with ADM and OFQ ($10^{-7}\ mol \cdot L^{-1}$). The reversal index (RI) was 2.03. Thus, OFQ can reverse multi-drug resistance in K562/ADM cells to some extent.

There are many reasons for MDR (Motoji et al. 2000), but numerous studies have shown that MDR is associated with increasing drug excretion from cells. This study showed that OFQ could significantly increase the intracellular accumulation of ADM in K562/ADM cells. The mechanisms of this phenomenon are associated with decreasing expression of P-gp (Ferrao et al. 2001) and the intracellular level of calcium in K562/ADM cells. This study indicated that OFQ could down-regulate the level of P-gp in a dose-dependent manner and decrease the intracellular level of calcium in K562/ADM cells. The well-known P-gp, which exhibits sequence similarities to some bacterial transport proteins and displays ATPase activity, appears to act as a relatively non-specific energy-dependent drug-efflux pump (Ling et al. 1978), and would therefore, be responsible for decreasing drug levels in MDR cells. Some studies have shown that increasing the intracellular level of calcium causes an increase in drug excretion from cells (Mernenko OA et al. 1996). Many researchers have demonstrated that the increase of intracellular calcium concentration plays an important role in cell apoptosis, and the apoptosis of cells is related to the growth of tumor cells. Some studies have shown that calcium antagonists can reverse multi-drug resistance.

Many studies have shown that multi-drug resistance is associated with inhibition of apoptosis (Eloisi et al. 2002). This study indicates that OFQ can up-regulate the level of Fas/FasL time-dependently in K562/ADM cells. Consequently, OFQ can induce apoptosis of K562/ADM cells. The mechanisms of this phenomenon are correlated with up-regulating the expression of Fas/FasL in K562/ADM cells. Moreover, P-gp can inhibit the activation of caspase8 and caspase3, while OFQ can down-regulate the level of P-gp, therefore OFQ can activate caspase8 and caspase3 and induce apoptosis.

4. Experimental

4.1. Reagents

Nociceptin/orphanin FQ was synthesized and purified by ourselves (Chang et al. 2005; Peng et al. 2006). RPMI 1640 was purchased from Gibco-BRL (USA). Fetal calf serum was provided by Hangzhou Sijiqing (Hangzhou, China). Adriamycin (ADM), MTT and DMSO were from Sigma (St Louis, MO.USA). Annexin V/PI was from Maxin Biotech. Fas and FasL were from Caltag. P-170 was from Coulter. Fluo 3/AM was from Calbiochem.

4.2. Cell lines and cell culture

Leukemia cell lines K562 and K562/ADM were from the central laboratory of the First Affiliated Hospital of Lanzhou University. The cells were maintained in RPMI1640 containing 20% fetal bovine serum, 1 mM L-Glu, $50\ U \cdot ml^{-1}$ penicillin and $50\ U \cdot ml^{-1}$ streptomycin, and cultured at 37 °C in a humidified 5% CO_2 incubator. To maintain drug resistance, cells were supplemented with adriamycin ($3\ \mu g \cdot ml^{-1}$) at regular intervals. Cells were harvested and used for experiments 1 week after removal of adriamycin.

4.3. Morphology

K562/ADM cells (5×10^6/ml) were treated with OFQ ($1 \times 10^{-7}\ mol \cdot L^{-1}$) for 48 h. K562/ADM cells and control cells were collected, washed twice with $0.1\ mol \cdot L^{-1}$ PBS, fixed with 0.25% glutaral for 24 h and fixed again with 1% quadr-oxidize-dio-osmium for 2 h. The cells were examined under a transmission electron microscope (TEM) (Li et al. 2005).

4.4. DNA fragment assay

K562/ADM cells (5×10^6/ml) were treated with OFQ ($1 \times 10^{-7}\ mol \cdot L^{-1}$) and ADM ($20\ \mu g \cdot ml^{-1}$) for 48 h. The cells were treated with cell lysate. The DNA was isolated by the supra-stele method by SK1261 (Shanghai Sangon), and analysed in 2% agarose gel by electrophoresis at 100 V (Shen et al. 2005).

4.5. Comet assay

Comet assay was performed according to a previously described method (Klaude et al. 1996).

4.6. Observation of early apoptosis by FITC-annexin V/PI

After treatment, 5 µl FITC-annexin-V and 5 µl PI ($250\ \mu g \cdot ml^{-1}$) was added to 490 µl K562/ADM cells suspension in an ice bath, cultured in the dark for 10 min, centrifuged twice and washed twice with $0.1\ mol \cdot L^{-1}$ PBS and suspended again (Singh et al. 1988).

4.7. Determination of P-gp by flow cytometry

K562/ADM cells (5×10^6/ml) were treated with OFQ ($1 \times 10^{-6}\ mol \cdot L^{-1}$, $1 \times 10^{-7}\ mol \cdot L^{-1}$ and $1 \times 10^{-8}\ mol \cdot L^{-1}$) for 48 h. The collected cells were incubated with PE anti-P-gp antibody (UIC2) or nonspecific IgG2a in the dark at 4 °C for 30 min. The percentage of P-gp positive and the mean fluorescence intensity (MFI) of the sample were analyzed by flow cytometry (Coulter Epics XL, Beckman-Coulter Inc, Fullerton, CA, USA).

4.8. Analysis of Fas and Fas ligand expression

K562/ADM cells (5×10^6/ml) incubated for 24, 48, 72 and 96 h with OFQ ($1 \times 10^{-7}\ mol \cdot L^{-1}$), were spun down and the pellet was incubated with 1 µg (2 µl) of anti-human Fas-PE (clone number DX2) or anti-human FasL (clone number NOK-1) for 30 min at 4 °C, washed twice with chilled PBS and resuspended in PBS containing 1% formaldehyde. For FasL analysis, after primary incubation, cells were spun down and incubated with a secondary antibody goat anti-mouse IgG-FITC. Human adsorbed analysis for surface immunofluorescence was performed on a flow cytometer (Motomura et al. 1998).

4.9. Determination of level of intracellular adriamycin by flow cytometry

This analysis was performed according to a published method (Lin et al. 2004).

4.10. Determination of intracellular calcium level by flow cytometry

The intracellular calcium level was determined using Fluo-3/AM (Molecular Probes, Eugene, OR) staining followed by flow cytometry. Briefly, cells were irradiated with UVA and then loaded with Fluo-3/AM (3 μM) at different times after exposure for 30 min at 37 °C. After extensive washing, cells were trypsinized and washed with cold PBS (calcium/magnesium-free) containing 0.1% bovine serum albumin. The cells were resuspended, and the changes in green fluorescence were detected by flow cytometry as an indicator of calcium mobilization.

4.11. Statistical analysis

Data were expressed as the mean ± standard deviation (S.D.). Comparison between groups was done using a one-way ANOVA followed by a Student-Newman-Keul's test, and the criterion of statistical significance was taken as $P < 0.05$ or $P < 0.01$. The median inhibitory concentration of ADM (IC_{50}) was computed by linear regression.

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