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Reversal effect of nimodipine on cytarabine-resistant HL60 cells associated with triggering apoptosis

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Intrinsic or acquired drug resistance is the major reason for failure of cancer therapy. Multifactorial mechanisms are involved in multidrug resistance (MDR). Various arrays of drugs are indentified that sensitize multidrug–resistant cells to chemotherapy. The present study aimed to study whether nimodipine (NMDP) could reverse MDR and what mechanism is involved in the reversal effect. Cytarabine (Ara-c)-resistant HL60 leukemia cells (HL60/Ara-c) were treated with free Ara-c, free NMDP or combination of Ara-c with NMDP. The apoptotic effects were enhanced with increase of concentration, showing a dose-dependent manner. Combination of Ara-c with NMDP arrest more cells in G0/G1 phase. Bcl-2 mRNA and protein expression significantly decreased in co-administration group, while that of Bax increased. The activity of caspase-3 was remarkably activated by combination of Ara-c with NMDP, which was correlated with modulating Bcl-2/Bax. Intracellular Ca^{2+} concentration was significantly increased over culture time in co-administration group. In the xenograft model, antitumor activity of NMDP with Ara-c was signifanctly enhanced. In conclusion, the reversal effect of NMDP on HL60/Ara-c cells is associated with modulating proapoptotic and antiapoptotic protein expression changes and initiating a cascade of caspase activation. Combination of anticancer agents with a modulator may provide a novel strategy for improving the chemotherapeutic effects.

Key words: Nimodipine, cytarabine, multidrug resistance, leukemia.

INTRODUCTION

The prognosis of children diagnosed with acute leukemia has improved markedly during the past decades. However, approximately 25~40% of affected children relapse and cannot be cured with current chemotherapy(Pui et al., 2008). Intrinsic or acquired drug resistance is the major reason for failure of cancer therapy (Gottesman et al., 2002; Litman et al., 2001). Multidrug resistance (MDR) is referred to a situation in whereby a drug elicits a response in tumor cells resulting resistance to a large variety of structurally and functionally unrelated chemotherapeutic drugs. A growing number of research shows that MDR is multifactorial, including enhanced expression of cellular transporters, reduced drug uptake, enhanced repair processes, changes in cell cycle regulation and alterations in apoptotic pathways (Fojo and Menefee, 2007; Kruh,

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2003). Various arrays of drugs are indentified that sensitize multidrug–resistant cells to chemotherapy (Bates et al., 2002; Robey et al., 2008). Some drugs have been selected for the initial clinical studies and approved for clinical use, which are also called reversal agents, such as verapamil, immunosuppressors (in particular cyclosporin A), tamoxifen, quinidine and other agents.

Verapamil, as one kind of calcium channel blockers, has first been reported to sensitize the response of cancer cells to chemotherapy *in vitro*, while dose-limiting cardiovascular toxicity associated with its administration prevented its progress in the clinic (Barattin et al., 2010; Noviello et al., 1997; Song et al., 2009). Nimodipine (NMDP), analogue of verapamil, has its characteristic features. It showed that NMDP is a cerebroselective Ca^{2+} antagonist investigated for various cerebral pathologies, such as enhancing cerebral blood flow, improving memory retention and/or memory recall process, and possessing anticonvulsant properties (Horn et al., 2001; Li et al., 2009; Yanpallewar et al., 2004). All cerebral effects above mentioned may be due to its strong liposolubility. Our previous studies demonstrated that NMDP increased HL60 cell apoptosis induced by cytarabine (Ara-c) (Sun and Gao, 2005; Sun et al., 2007). On this basis, it was therefore reasonable to hypothesize that NMDP may reverse MDR, in particular for central nervous system leukemia. However, the underlying mechanisms involved in the reversal effect of NMDP on MDR HL60/Ara-c cells were undefined. Toward this end, our present study was aimed to investigate reversal-related mechanisms in order to provide the theoretical basis for rationally applying the chemotherapy drugs in clinic.

MATERIALS AND METHODS

Cell line

Ara-c-resistant HL60 cells (HL60/Ara-c, IC50 of Ara-c: 5.36μg/ml) was obtained from Wuhan PriCells Co. and maintained in RPMI 1640 medium (Gibco, China, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS),2 mM glutamine, 100 U/ml penicillin, and 5 μg/ml gentamicin at 37°C in a humidified atmosphere of 5% CO_2 in air. HL60/Ara-c cells were re-subcloned by limiting dilution techniques to ensure clonality of each clone and were maintained in complete media containing 5 μg/ml Ara-c (Cytosar, Pharmacia N.V./S.A). Cells were grown for 7 days in the absence of drugs prior to use in specific experiments.

Effect of NMDP on multidrug resistance in HL60/Ara-c

HL60/Ara-c cells were treated with free Ara-c (0.1~64μg/ml) or free nimodipine (NMDP, 0.5~160μg/ml), and trypan blue exclusion assay was performed to detect IC50 of different agents. The values of IC50 were used as an indicator for HL60/Ara-c cells. According to the results of trypan blue exclusion assay, four groups were used to study the changes of different factors. Three groups were treated with free Ara-c alone (4μg/ml), free nimodipine alone (NMDP, 10 μg/ml) or combination of Ara-c (4 μg/ml) and NMDP (10 μg/ml), respectively. HL60/Ara-c cells treated with culture medium without any drug was regarded as the negative control group.

Analysis of cell cycle changes by flow cytometry

The percentage of cells in each phase of the cell cycle was analyzed by flow cytometry (FCM) by measuring the DNA content of nuclei labeled with propidium iodide (PI, including RNase), according to manufacturer's instructions (Sigma Aldrich Co.). Briefly, HL60/Ara-c cells were treated with different agents for 24 h. Cells were collected, centrifuged and then fixed in 70% cold ethanol overnight at 4°C. Finally, PT stain solution (50 μg/ml, including RNase 20μg/ml and 1% Triton X-100) was added and incubated in the dark.at room temperature for 30 min. Samples were immediately analyzed by the FCM and Flowjo 7.5 software. The experiment was performed three times. The ratio of cells in G0/G1, S, and G2/M phase were expressed as mean±SD.

Caspase-3 activity assay

The activity of caspase-3 was assessed by Caspase-3 Colorimetric Assay Kit (KenGen Co., Nanjing) according to the manufacturer's instructions. Following treatment for 8, 16 and 24 h, $3\sim5*10^6$ cells were collected, washed with ice-cold phosphate buffered saline (PBS) and lysed in a lysis buffer (including 5 mM DTT) at 4°C for 20 min. Lysed cells were centrifuged at 10000 rpm for 1 min and the protein concentrations were determined by Bradford method. 50 μl solution including 100 μg proteins from each sample were assessed by use of a caspase-specific peptide conjugated with the color reporter molecule. The optical density was quantified with a spectrophotometer at a wavelength of 405 nm.

Real-time quantitative polymerase chain reaction (RT-PCR)

RT-PCR was used to detect Bcl-2, Bax and caspase-3 gene expressions. After HL60/Ara-c cells were treated with different agents for 24 h, total RNA was isolated from cells by use of Trizol reagent (Invitrogen, USA). RNA concentration and purity were measured with a spectrophotometer at A260 and A280, respectively. The value of A260/A280 was at the range of 1.8 to 2.1. According to Primer Premier 5.0 software, the sequences of primers of Bcl-2, Bax and caspase-3 were as follow: sense primer 5' GGGCAACAGAGAACCATC 3' and antisense primer 5' GTCCCCAATTTGGAAAGTG 3' for Bcl-2, sense primer TTGCTTCAGGGTTTCATC 3' and antisense primer 5' ACACTCGCTCAGCTTCTTG 3' for Bax, sense primer 5' ACTCCTTCCATCAAATAG 3' and antisense primer 5' AATTCATAGCACAGCATC 3' for caspase-3. β-actin was used as an internal standard, whose primers were sense primer 5' CGTGACATTAAGGAGAAGCTG 3' and antisense primer GGAC 5' CTAGAAGCATTTGCGGTGGAC 3'. RNA was reverse-transcribed into cDNA by use of Promega MMLV reverse transcriptase, 10 mmol/l dNTPs, 1 unit/μl RNase inhibitor and 100 μmol/l OligodT for 30 min at 42°C, and then 10 min at 70°C. The reverse transcription product was amplified by use of the SYBR® Premix Ex Taq™ (TaKaRa). The conditions for PCR were: 94°C for 4 min, 94°C for 30 s, 56°C for 30 s, 72°C for 40 s, and 40 cycles. The amplification specificity was checked by melting curve analysis. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative abundance of target gene expression generated by Rotor-Gene Real-Time Analysis software 6.1.81. The relative level of target gene mRNA compared with β-actin was calculated for each sample.

Western blotting analysis

Total proteins were extracted from cell lysates of cultures. After the cells were treated with different agents for 24 h, the cells were collected and homogenized by RIPA cell lysis solution at 4°C for 30

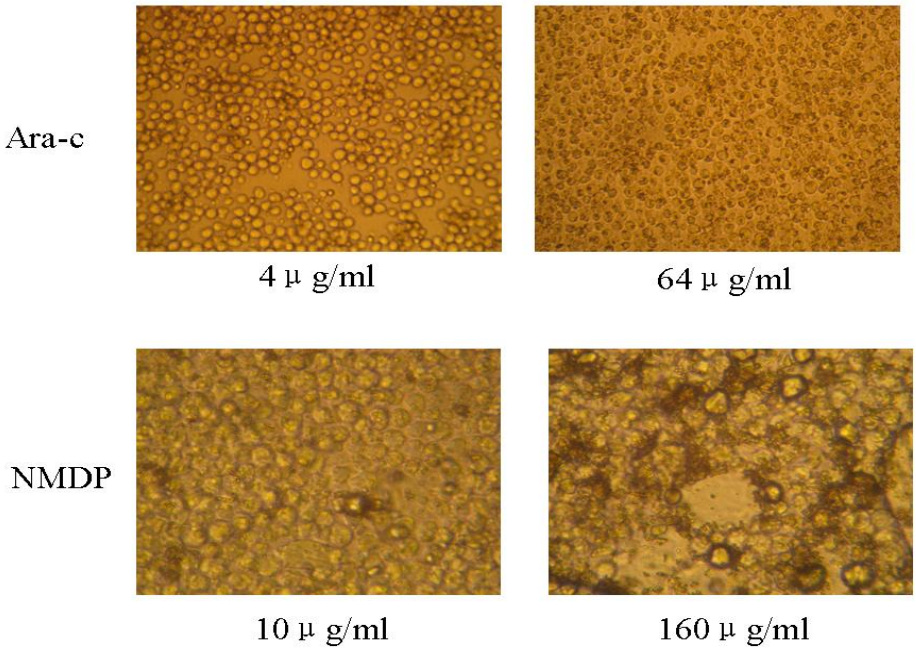


Figure 1. Cytotocxicity effect assayed by trypan blue exclusion. Cells were incubated with free Ara-c (0.1~64μg/ml) or free NMDP (0.5~160 μg/ml) for 24 h, and cell viability was measured by use of the trypan blue exclusion.

min. Proteins from cell lysates of cultures were quantified spectrophotometrically by use of the Bradford method, separated by 10% sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) electrophoresis and tranfered to a polyvinylidenefluoride (PVDF) nitrocellulose membrane (Millipore, USA). Membranes were incubated into the Botto blocking solution at room temperature for 1.5 h, and then incubated with the primary mouse monoclonal antibodies for Bcl-2 and Bax (mouse anti-human Bcl-2 from Invitrogen, mouse anti-human BAX from Biolegend) at a dilution of 1:1000 at 4°C overnight. The membrane was washed with TBST three times, and incubated with the horseradish perodixase-conjugated anti-mouse secondary antibody (Biolegend) diluted 1:5000 at room temperature for 2 h. The immunoreactive protein was visualized with an enhanced chemiluminescence substrate Western Lightning®-ECL (Perkin Elmer, NEL100001EA).

Analysis of intracellular calcium ion ([Ca^{2+}]) concentration

The intracellular Ca^{2+} concentration, in particular cytosol free Ca^{2+} concentration, was determined by flurescent Ca^{2+} indicator Fura-2/AM ester (Kamouchi et al., 2007). The cells treated with different agents for 8, 16 and 24 h were collected and incubated with 2 μM Fura-2/AM ester at room temperature for 30 min. Flurescence intensity of Fura-2 was measured by fluorescence spectrophotometer (Shimadzu, Japan) at an emission wavelength of 510 nm and alternative excitation wavelength of 340 and 380 nm. The ratio of fluorescence intensity at 340 and 380 nm (F=F340/F380) was calculated. Meanwhile, the maximal and minimal fluorescence was determined by use of Triton X-100 and ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid (EGTA). Flurescence measurements were converted to the calcium concentration according to the following formula:

$$Ca^{2+}]_i = Kd[(F-Fmin)/(Fmax-F)$$

Where, Kd represents constant quantity of Fura-Ca^{2+} complex and is 224nM.

Tumor growth inhibition assay *in vivo*

To examine the MDR reversal effect of NMDP *in vivo*, male BALB/C nude mice, 6 weeks old, initially weighing 18-20g, were obtained from Shanghai SLAC Lab Animal CO. LTD and divided into four groups（n=6 in each group）for tumor-bearing recipients. Approximately $1*10^7$ HL60/Ara-c tumor cells suspended in 0.1 ml of serum-free cell culture medium were subcutaneously injected into the right armpits of mice. On the 2nd, 4th and 6th days after tumor challenge, physiological saline, free Ara-c alone (4 mg/kg), free NMDP alone (10 mg/kg), and combination of Ara-c (4mg/kg) with NMDP (10 mg/kg) were administered via tail vein. Growth of solid tumor (in mm^3) was monitored daily by caliper measurement according to the formula: ($width^2$*length)/2. The weight of mice and the tumor volumes were recorded every three to four days. On the 20th day, tumor-bearing mice were sacrified and the tumor volume was calculated.

Statistical analysis

Data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to determine significance among groups. A value of $P<0.05$ was considered to be significant.

RESULTS

Cytotoxicity assay by trypan blue exclusion

As shown in Figure 1, the antiproliferative effect was

enhanced with the increase of Ara-c or NMDP concentration, showing a dose-dependent manner. As a representive value, the apoptotic rate induced by free Ara-c (4μg/ml) or free NMDP (10μg/ml) was close to 45%.

Cell cycle arrest

Cell cycle arrest of HL60/Ara-c cells induced by Ara-c alone, NMDP alone or combination of Ara-c with NMDP was determined by detecting PI-staining (Figure 2). Combination of Ara-c with NMDP significantly increased the number of cells in G0/G1 phase and decreased the number of cells in the S phase, indicating that NMDP could enhance HL60/Ara-c cell apoptosis induced by Ara-c by arresting G0/G1 cell cycle in HL60/Ara-c cells.

NMDP increased Ara-c-induced HL60/Ara-c cell apoptosis by modulating Bcl-2/Bax and activating caspase

In order to understand the activation of the cascade during reversal effect of NMDP on HL60/Ara-c cells, we here investigated caspase-3 activity after the cells were treated with free Ara-c alone, free NMDP alone, and combination of Ara-c with NMDP for 8, 16 and 24 h. As shown in Figure 3A, the results of colorimetric assay revealed that caspase-3 activity increased significantly in HL60/Ara-c cells treated with with Ara-c. Addition of NDMP attenuated the effect of Ara-c on caspase-3 activity, indicating that activation of caspase-3 activity was involved into the antitumor effect of NMDP and Ara-c on HL60/Ara-c cells. Furthermore, to better understand the reversal effect of NMDP, antiapoptotic (Bcl-2), proapoptotic (Bax) mRNA and protein expressions were analyzed by RT-PCR and western blotting method. RT-PCR analysis revealed that addition of NMDP signifcicantly upregulated Bax (Figure 4A) and caspase-3 mRNA expression (Figure 3B), and downregulated Bcl-2 mRNA expression (Figure 4A). On the other hand, Bcl-2/Bax protein expression assayed by western blotting showed that significant differences in Bcl-2/Bax protein expression were observed in combination of Ara-c with NMDP, compared with the control group, free Ara-c group and free NMDP group (Figure 4B). Together, this data suggested that NMDP and Ara-c played synergetic effect on HL60/Ara-c cells apoptosis.

Intracellular Ca^{2+} concentration

Effect of free Ara-c alone, free NMDP alone or combination of NMDP with Ara-c on the intracellular Ca^{2+} concentration of HL60/Ara-c cells are shown in Figure 5. Results showed that significant differences were observed in free Ara-c alone and combination of Ara-c with NMDP for 8 h, as compared to control group and free NMDP alone. The intracellular Ca^{2+} concentration increased markedly over culture time. Intracellular Ca^{2+} concentration in HL60/Ara-c cells treated with combination of Ara-c with NMDP for 24 h was highest.

Addition of NMDP inhibited murine xenograft tumor growth in leukemia models

Effect of NMDP with Ara-c on the HL-60/Ara-c xenografts in nude mice is shown in Figure 6. Two days following tumor challenge, mice were administered with different agents. Monitoring of tumor growth in all groups clearly demonstrated that the time of tumor appeared in combination of NMDP with Ara-c group was significantly later than that in other groups. The tumor masses appeared on the 15^{th} day for the group administered with NMDP and Ara-c, on the 13^{th} day for the group administered with free Ara-c, and on the 10^{th} day for the group administered with physiological saline or free NMDP, respectively. Ara-c offered restriction of tumor growth, but significantly lesser extent than group treated with combination of Ara-c with NMDP. Together, these data were consistent with *in vitro* findings, indicating that addition of NMDP could inhibit HL60/Ara-c cell tumor growth *in vivo*.

DISCUSSION

In this study, reversal effect of NMDP on Ara-induced apoptosis was evaluated in drug resistant HL60/Ara-c cells. Both *in vivo* and *in vitro* studies demonstrated that combination of NMDP with Ara-c inhibited significantly the proliferation of Ara-c-resistant HL60 leukemia cells (HL60/Ara-c), indicating that NMDP and Ara-c had a synergetic effect on HL60/Ara-c cells. This is consistent with the previous reports on reversal effect of other calcium channel blockers. Verapamil, the well-known calcium channel blocker, is a reference for P-gp inhibition. The recent research shows that verapamil and its derivatives trigger apootosis through glutathione extrusion by multidrug resistance protein MRP1 (Trompier et al., 2004). Amlodipine derivatives have been reported to be applied for reversal of P-gp-mediated MDR in doxorubicin-resistant human myelogenous leukemia (K562/Dox) cells (Ji et al., 2005; Li et al., 2006).

The potency and effectiveness of Ara-c was shown in acute myeloid leukemia (AML). However, it's reported that 40~50% AML patients relapsed, and even it is difficult for those treated with high dose Ara-c to achieve complete remission (Funato et al., 2004; Shipley and Butera, 2009). Multifactorial mechanisms were involved in MRD. New strategies for reversing MDR, including antibody and other drug agents (FLT3, tyrosine kinase inhibitors and iRNA targeting MDR-associated genes),

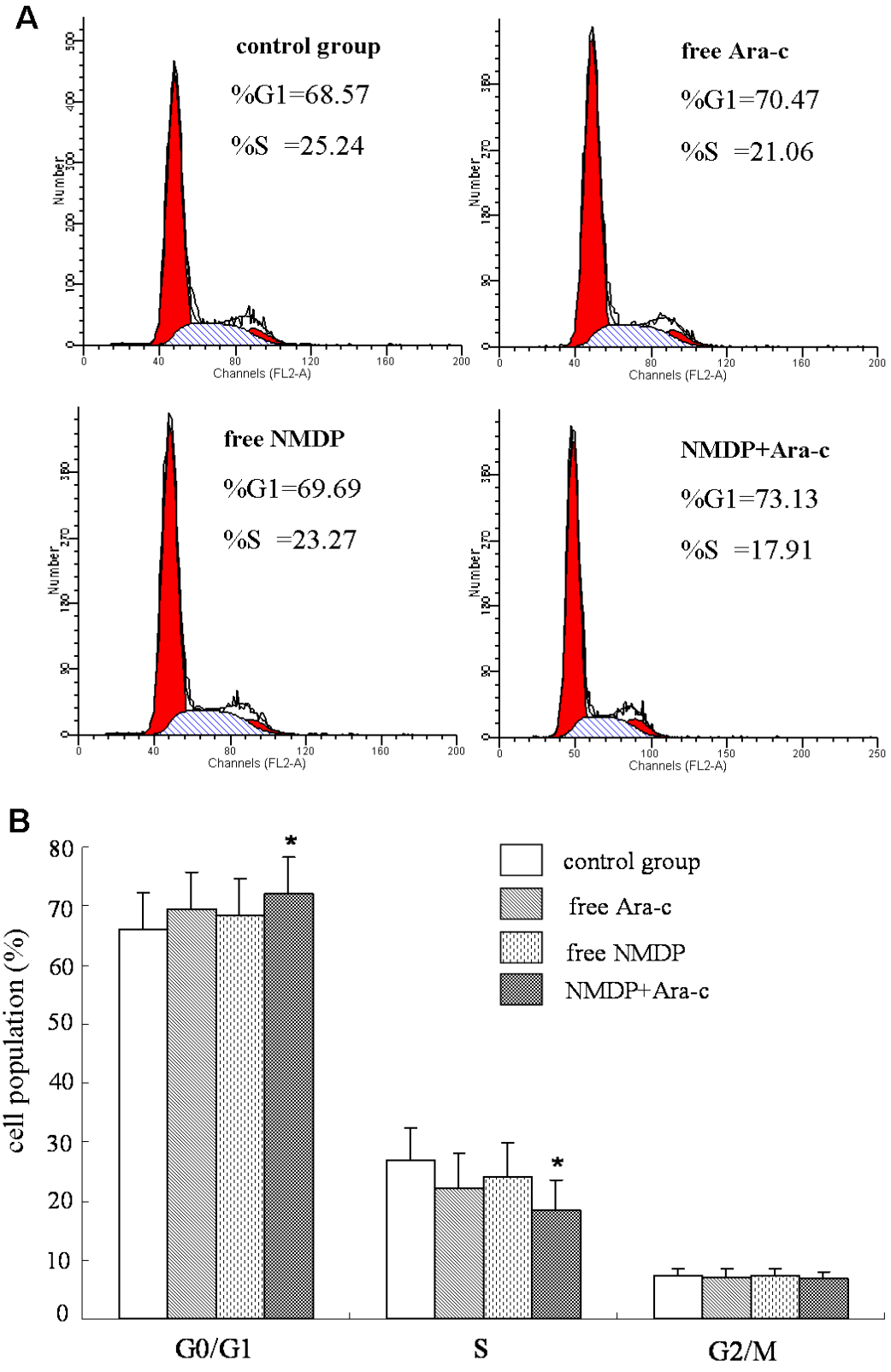
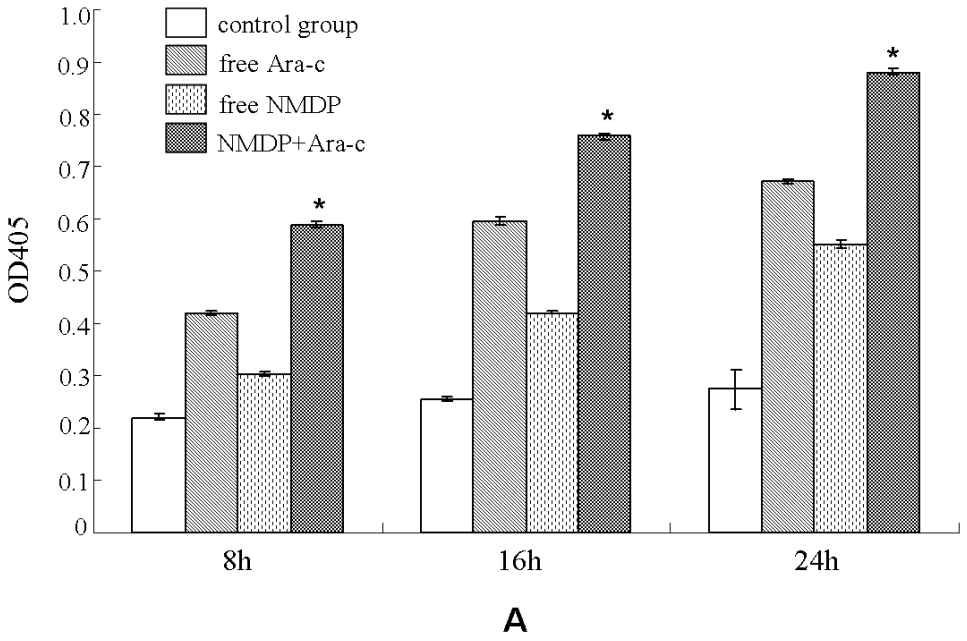


Figure 2. Effect of NMDP and Ara-c on cell cycle arrest in HL60/Ara-c cells by FCM. A, Histograms of HL60/Ara-c cells treated with RPMI-1640, free Ara-c, free NMDP, and combination of NMDP with Ara-c, respectively; B, combination of NMDP with Ara-c significantly increased the proportion of HL60/Ara-c cells in G0/G1 phase and decreased the proportion of cells in S phase. Results were shown as mean ± standard (n=3). *P<0.05, compared with control group.



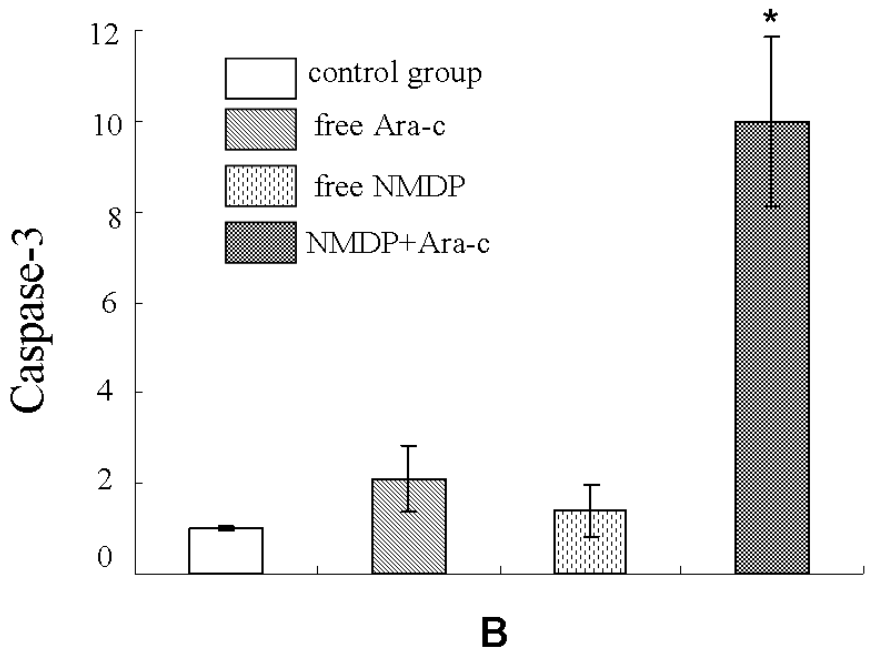


Figure 3. Effect of NMDP and Ara-c on caspase-3 activity in HL60/Ara-c by colormetric assay and RT-PCR. A, Colorimetric assay of caspase-3 activity. Caspase-3 activity was increased in groups treated with free Ara-c, free NMDP or combination of NMDP with Ara-c for 8h, compared with the control group. The activity of Caspase-3 enhanced over culture time. The activity was significantly increased in group co-treated with NMDP and Ara-c for 24h (*p<0.05); B, caspase-3 mRNA expression level in groups treated with different drugs for 24 h was assayed by RT-PCR. Results showed that combination of NMDP with Ara-c upregulated significantly the mRNA expression level of caspase-3 in MDR HL60/Ara-c cells (*p<0.01).

have been reported (Tallman, 2005; Tallman et al., 2005). In the present study, flow cytometry showed that combination of NMDP with Ara-c markedly arrested HL60/Ara-c cells in G0/G1 phase of cell cycle, suggesting that retardation of cell cycle progression may be one of the mechanisms underlying the reversal effect of co-

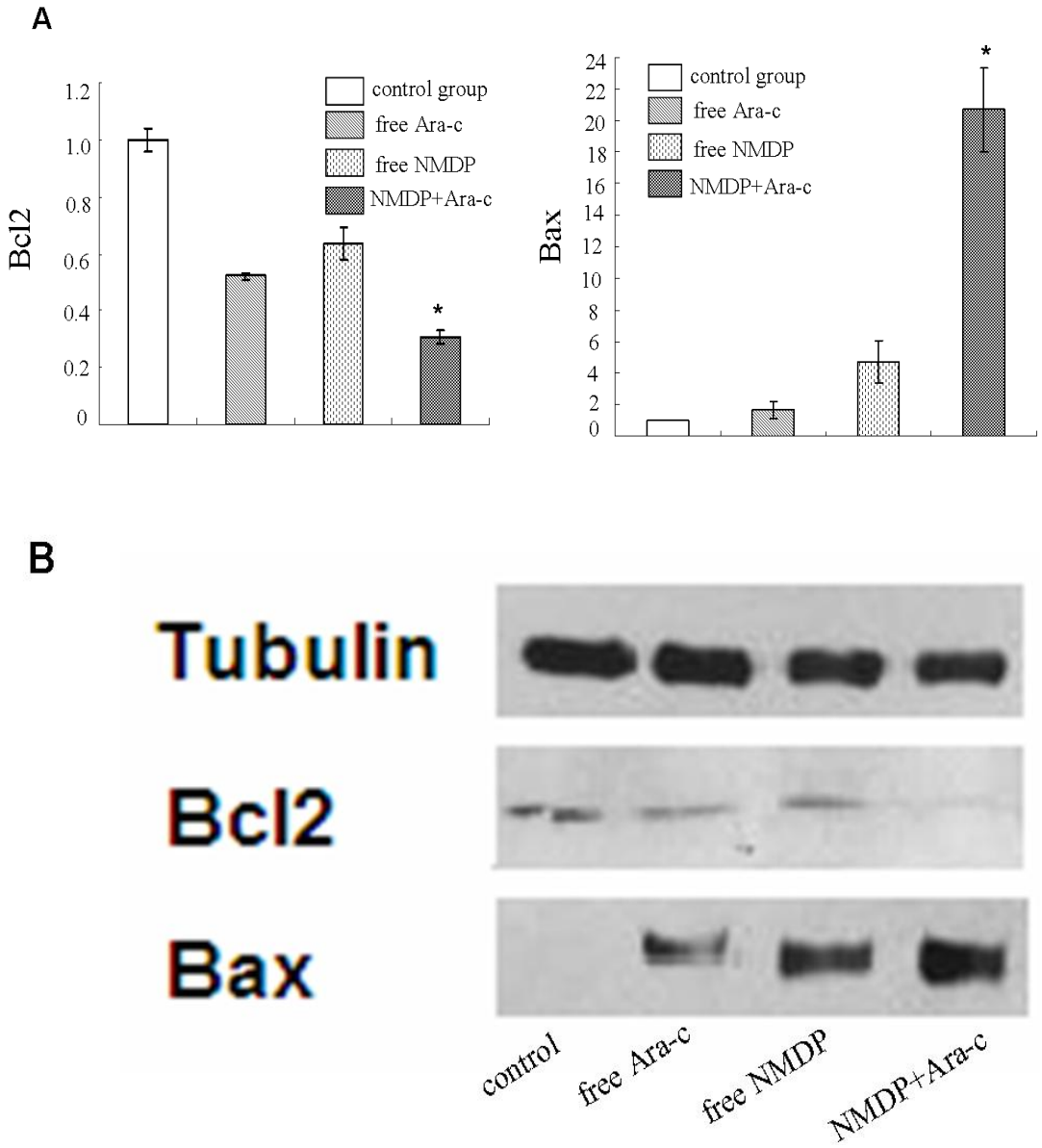


Figure 4. Bcl-2 and Bax mRNA and protein expression level assayed by RT-PCR and western blotting in HL60/Ara-c cells treated with different agents for 24 h. A, Co-administration of NMDP with Ara-c for 24 h upregulated significantly mRNA expression level of Bax (*p<0.01) and downregulated that of Bcl-2 (*p<0.05); B, protein expression level in MDR HL60/Ara-c cells detected by western blotting. Expression of Bax was increased remarkably in group treated with combination of NMDP with Ara-c, while that of Bcl-2 was decreased. B-tubulin was used as control.

administration. Consistent with the reversal effect via apoptotic processes, RT-PCR and western blotting results showed that combination of NMDP with Ara-c upregulated the expression of proapoptosis gene(Bax) and down-regulated the expression of antiapoptosis gene(Bcl-2), indicating that the reversal effect was associated with modulating Bcl-2 and Bax expression. Furthermore, caspase-3 activity assayed by colorimetric method and RT-PCR was remarkably activated by combination of NMDP with Ara-c. These results indicated the imbalance of apoptosis-associated proteins (Bcl-2/Bax) and caspase activation were involved in the reversal effect.

Results for measurements of intracellular Ca^{2+} concentrations in the present study showed that intracellular Ca^{2+} concentrations was significantly increased in HL60/Ara-c cells treated with combination of NMDP and Ara-c for 8, 16 and 24 h. The intracellular Ca^{2+} concentrations was enhanced over culture time, which indicated that inhibition of Ca^{2+} influx may be not involved

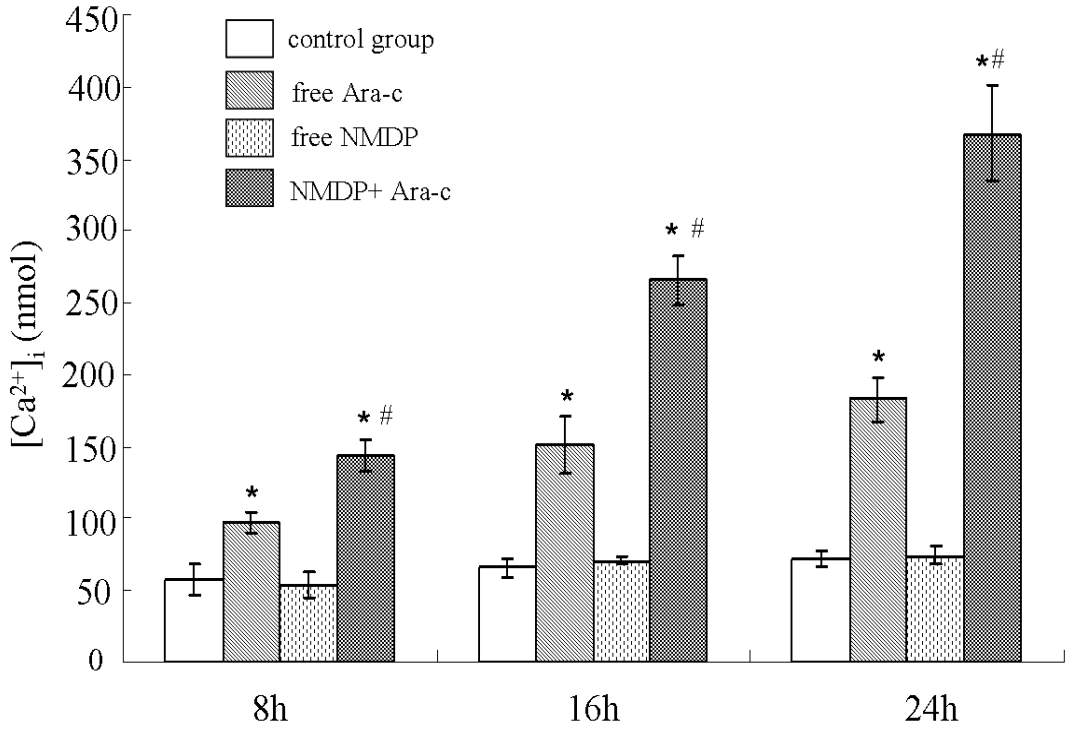


Figure 5. Effect of NMDP and Ara-c on $[Ca^{2+}]_i$ concentration changes in HL60/Ara-c cells by use of Fura/AM agent. Significant differences were observed in groups treated with free Ara-c and combination of NMDP with Ara-c for 8 h, compared with control group and free NMDP(*p<0.01). Combination NMDP with Ara-c significantly increased $[Ca^{2+}]_i$ concentration in comparison to free Ara-c(# p<0.05)., and the $[Ca^{2+}]_i$ concentration was the highest at 24 h.

in the reversal effect of NMDP and Ara-c. Meanwhile, It is not consistent with other reports (Florea and Busselberg, 2009). Previous studies reported that amlodipine and other Ca^{2+} channel blockers inhibited Ca^{2+} influx evoked by the passive depletion of internal Ca^{2+} stores, such as human epidermoid carcinoma A431 cells (Earnshaw et al., 1999; Li et al., 2006). This may be due to the diversity of different cancer cells and different drugs. On the other hand, under the trigger of external signals, calcium ions in endoplasmic reticulum was released into the cytoplasm, which activated the nitric oxide synthase of mitochondrial membrane to produce nitric oxide (Bischof et al., 1995; Sasaki et al., 2000; Xu et al., 1999), and then inhibited the respiration of cells and released cytochrome C and proapoptotic factors. These factors further induced apoptosis by activating caspase family. However, the underlying mechanisms, which were involved in the increase of intracellular calcium concentration in the apoptotic cells induced by combination of NMDP with Ara-c and whether apoptotic effect was associated with the increase of intracellular calcium concentration, are still undefined and need further study.

In conclusion, reversal effect of NMDP on HL60/Ara-c cells is associated with modulating proapoptotic and antiapoptotic protein expression changes and initiating a cascade of caspase activation. The enhanced antitumor activities by combination of NMDP with Ara-c in HL60/Ara-c cells *in vitro* and *in vivo* are mainly due to the potentiating apoptotic effect and overcoming the resistance by NMDP. So, combination of anticancer agents with a modulator may provide a novel strategy for improving the chemotherapeutic effects.

ACKNOWLEDGEMENT

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Abbreviations: NMDP, Nimodipine; **Ara-c**, Cytarabine; **MDR**, multidrug resistance; HL60/Ara-c, Ara-c-resistant HL60 leukemia cells; **FCS,** fetal calf serum; **FCM,** flow cytometry; **RT-PCR,** real-time quantitative polymerase chain reaction; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **PVDF,** polyvinylidene fluoride; **EGTA,** ethylene glycol-bis (2-aminoethylether)-N, N, N', N'-tetraacetic acid; **AML,** acute myeloid leukemia.

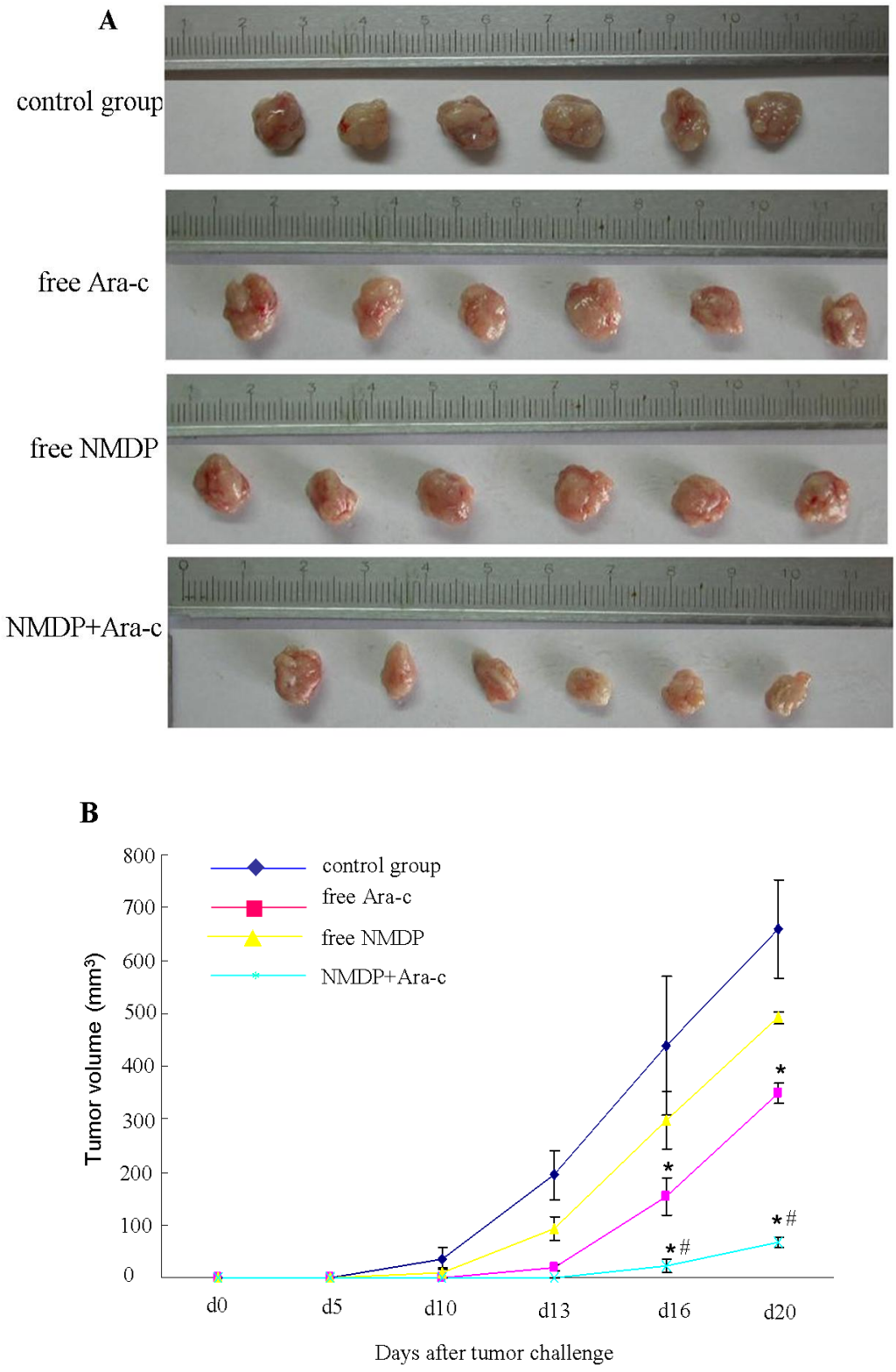

Figure 6. Tumor growth inhibited by different drugs. $1*10^7$ HL60/Ara-c cells were subcutaneously injected into the armpits of nude mice. On the 2nd, 4th and 6th day after tumor challenge, free Ara-c(4 mg/kg), free NMDP(10 mg/kg) or combination of NMDP (10 mg/kg)with Ara-c(4 mg/kg) were administered via tail vein, respectively. A, Tumor volume in four group of nude mice (n=6 in each group)on the 20th day; B, tumor growth curve, as monitored by caliper according to the formula: $(width^2*length)/2$. *p<0.05,versus control group and free NMDP. #p<0.05, versus free Ara-c.

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