

Selective killing effect of oxytetracycline, propafenone and metamizole on A549 or Hela cells

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Objective: To determine the selective killing effect of oxytetracycline, propafenone and metamizole on A549 or Hela cells.

Methods: Proliferation assay, lactate dehydrogenase (LDH) assay, apoptosis detecting, flow cytometry and western blot were performed.

Results: It was found that treatment with propafenone at the concentration of 0.014 g/L or higher for 48 h could induce apoptosis in Hela cells greatly, while it was not observed in oxytetracycline and metamizole at the concentration of 0.20 g/L for 48 h. Oxytetracycline, propafenone and metamizole all displayed evident inhibitory effects on the proliferation of A549 cells. The results of LDH assay demonstrated that the drugs at the test range of concentration did not cause necrosis in the cells. Propafenone could elevate the protein level of P53 effectively ($P < 0.01$).

Conclusions: Oxytetracycline, propafenone and metamizol (dipyrone) all displayed evident inhibitory effects on the proliferation of A549 cells. Propafenone also displayed evident inhibitory effects on the proliferation of Hela cells.

Keywords: Cancer; drug; oxytetracycline; propafenone; metamizol



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Introduction

In the course of drug discovery, it is a very time-consuming process (it may take more than 15 years before a drug receives approval), and some promising compounds were failed because of the failure of clinical trials, although more than millions of dollars had been spent (1,2). While, when a new function of a clinical drug is found, it could have much more chance to be used clinically. It is commonly called “old drugs for new applications”.

Aspirin is a typical nonsteroidal anti-inflammatory drug (NSAID) used to relieve pain and reduce inflammation (3). Then it was found by clinical practice that it has the function to prevent generation of cerebral thrombosis. It was also used to prevent next attack of myocardial infarction (4). Later, its anticancer effect was also found and studied (5,6).

Berberine (BR), the main alkaloid component in Huang Lian and other medicinal herbs, was used mainly as an

antibacterial drug. Then, by clinical practice, it was found that it has the function of treating type II diabetes (7). The most promising actions of BR are its inhibition on cell growth and induction of apoptosis in several human cancer cells (8,9).

Vitamin C takes part in amino acid metabolism and synthesis of collagen. It can also decrease permeability of capillary and accelerate blood coagulation. Later on, it was found that it has the function of antitumor. According to experiments *in vitro*, vitamin C can only display antitumor effect at high concentrations (10-12). It was reported that vitamin C can enhance the generation speed and effect of some induced pluripotent stem cells. The results will make vitamin C be used in a new pathway (13,14).

Many drugs have multiple functions. So, we had screened more than 60 kinds of clinical drugs, which had no obvious anticancer function reported, to test whether they

could inhibit proliferation of tumor cells, such as A549 and HeLa cells. Finally, we found three kinds of clinical drugs could inhibit proliferation of A549 or HeLa cells greatly. They were oxytetracycline, propafenone, and metamizol (dipyrone).

Oxytetracycline is a broad-spectrum antibiotic produced by *Streptomyces rimosus* (15). About 30 years ago, it was reported that the proliferation of Zajdela tumor cells, grown *in vivo* in Wistar rats, was arrested by low amounts of oxytetracycline because of the inhibition of mitochondrial protein synthesis (16). Propafenone is a Vaughan-Williams class Ic antiarrhythmic drug frequently used in the management of atrial fibrillation and other supraventricular arrhythmias (17). Metamizol (dipyrone), a potent drug also centrally acting as a non-opioid analgesic and antipyretic agent with an additional spasmolytic effect, is widely used in humans to relieve postoperative pain alone and in combination with opioids (18). Although it is not available in all countries (e.g., USA and Sweden), there is a wide experience in the use of metamizol, particularly in the management of postoperative pain in animals (19).

Materials and methods

Materials

All reagents were of analytical grade or chemically pure. DMEM and RPMI 1640 were obtained from Gibco invitrogen Co. Bovine calf serum was provided by Ding Guo Biotechnology (Beijing, China). Sulforhodamine B (SRB) (Cat. No. S-9012) was provided by Sigma-Aldrich (USA). Tablets of oxytetracycline, propafenone and metamizole were purchased from Shandong Jinyang Drug Company, Shandong Renhetang Drug Company, and Peking Shuguang Drug Company, respectively.

Cell culture

A549 lung cancer cells were cultured in RPMI 1640 medium at 37 °C with 5% CO₂ and 95% air, supplemented with 10% (V/V) bovine calf serum and 80 U/mL gentamicin. HeLa cells were cultured in DMEM (high glucose) medium under the same condition as above. The cells were seeded onto 96-well plates or other appropriate dishes containing the medium at the density of about 6,000/cm² (20).

Antiproliferative activity

Screening of synthesized compounds was carried out using

HeLa cell line. Proliferation percentage was determined by the SRB assay. Cells were incubated with the drugs at the concentrations of 0.005-0.2 g/L for 48 h, and the cell proliferation/viability was determined using the survival percentage with the cells treated only with dimethyl sulfoxide (DMSO) at 0.1% as a reference (20-22). The results were expressed as the average of triplicate assays.

Lactate dehydrogenase (LDH) assay

Cell culture medium was collected after 48 h treatment with 0.1% DMSO (as control) or the drugs. LDH assay was performed using LDH kit (Nanjing Jiancheng Co., China) according to the manufacturer's instructions (20).

Hoechst 33258 staining to detect apoptosis

HeLa and A549 cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 10 min before staining with 2 µg/mL of Hoechst 33258 at 37 °C for 30 min. Subsequently, the cells were gently washed once with PBS, and then observed under a fluorescence microscope (Nikon). The condensed DNA of apoptotic cells was identified by intense local staining in the nucleus, in contrast to diffused staining of DNA in normal cells. A minimum of 500 cells was counted, and each experiment was performed in triplicate (23-25).

Flow cytometry analysis of cell cycle distribution

Cells were harvested, then fixed with 70% ethanol, and stained with 50 µg/mL propidium iodide (PI) containing 10 µg/mL RNase A at 4 °C for 1 h. The stained cells were analyzed using FACSCalibur flow cytometer (BD Bioscience, USA). Cell cycle distribution was analyzed with Modifit software (BD Bioscience, USA) (25-27).

Western blot analysis

Cells were washed twice with ice cold PBS, and then lysed in protein lysis buffer [1% sodium dodecyl sulfate (SDS) in 25 mmol/L Tris-HCl, pH 7.5, 4 mmol/L ethylene diamine tetraacetic acid (EDTA), 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin and 10 µg/mL soybean trypsin inhibitor]. The protein concentration of the cells was determined by Bradford method (28). Equal amount of protein was loaded onto 12% SDS-polyacrylamide gel. After

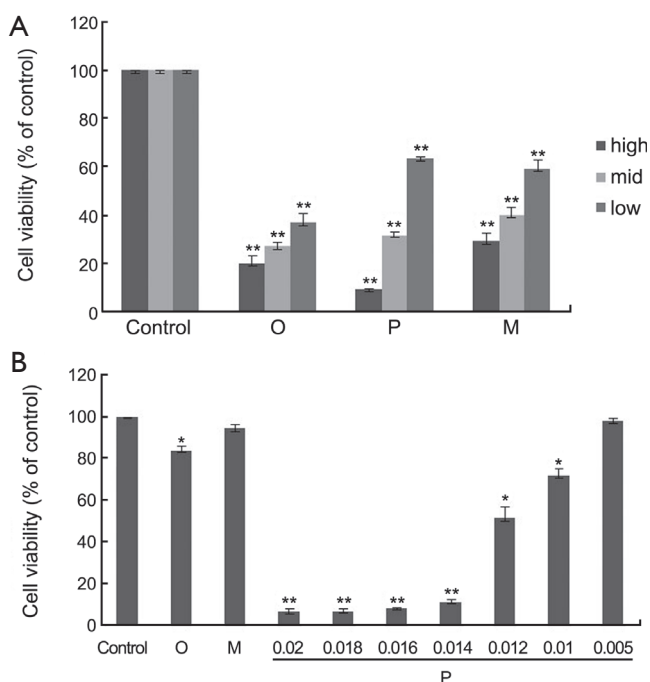


Figure 1 Effects of the drugs on the viability of A549 and HeLa cells. (A) A549 cells were treated with oxytetracycline, propafenone, metamizole or 0.1% DMSO (control) for 48 h. The concentrations of oxytetracycline was 0.15 (high), 0.1 (mid), and 0.05 g/L (low). The concentrations of propafenone is 0.03 (high), 0.02 (mid), and 0.01 g/L (low). The concentrations of metamizole is 0.20 (high), 0.1 (mid), and 0.05 g/L (low); (B) HeLa cells were treated with oxytetracycline, propafenone, metamizole or 0.1% DMSO (control) for 48 h. The concentrations of oxytetracycline and metamizole were 0.2 g/L. The concentrations of propafenone were 0.005–0.02 g/L respectively. O, oxytetracycline; P, propafenone; M, metamizole; *, $P < 0.05$ vs. control; **, $P < 0.01$ vs. control; $n = 3$.

electrophoresis, the resolved protein was electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membrane was incubated in TBST (10 mmol/L Tris-HCl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween-20) containing 2% (W/V) bovine serum albumin (BSA) and 2% (W/V) skim milk powder for 12 h at 4 °C. Subsequently, the membrane was probed with the primary antibody of P53 (sc-126, Santa Cruz, 1:200 diluted in TBST), LC3-II (sc-271625, Santa Cruz, 1:500 diluted in TBST), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (2118S, CST, 1:500 diluted in TBST) overnight at 4 °C, and was then washed twice with PBS-Tween 20 (PBST), each time for 5 min. The membranes were respectively incubated with peroxidase-conjugated anti-

Table 1 IC_{50} (g/L) of oxytetracycline, propafenone and metamizole at 48 h in A549 and HeLa cells

Cells	Drug		
	Oxytetracycline	Propafenone	Metamizole
A549	0.0255	0.0129	0.0709
HeLa		0.0106	

mouse IgG for P53 and LC3-II, and anti-rabbit IgG for GAPDH for 1 h at room temperature (secondary antibodies 1:5,000 diluted in TBST, Santa Cruz). After washed with PBST, the membrane was incubated with horseradish peroxidase (HRP) substrate until the protein strip could be visualized, and the luminous intensity was detected with X-ray films. Intensity of the protein bands was quantified using ImageJ software (National Institutes of Health) (29).

Statistical analysis

Data were presented as $\bar{x} \pm s$ from at least three independent experiments and analyzed by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Inhibitory effects of oxytetracycline, propafenone and metamizole on proliferation of A549 and HeLa cells

All the drugs selected were evaluated for their cytotoxicity against A549 and HeLa cells. Firstly, the assay incubated with drugs at the concentrations from 0.005 to 0.2 g/L for 48 h was carried out, and the cells were treated with SRB to measure their growth/viability (% of the untreated control) using a spectrophotometer as described previously. Oxytetracycline, propafenone and metamizole all displayed a most potent inhibitory effect on the growth of A549 cells in a dose-dependent manner (Figure 1A). Propafenone displayed an evident inhibitory effect on the growth of HeLa cells in a dose-dependent manner (Figure 1B).

The half maximal inhibitory concentrations (IC_{50}) (g/L) of the drugs at 48 h in A549 and HeLa cells are shown in Table 1.

Effects of drugs on morphology of A549 and HeLa cells

The morphological image of A549 cells treated with oxytetracycline, propafenone and metamizole at the concentration of 0.1, 0.02, or 0.1 g/L for 48 h was observed

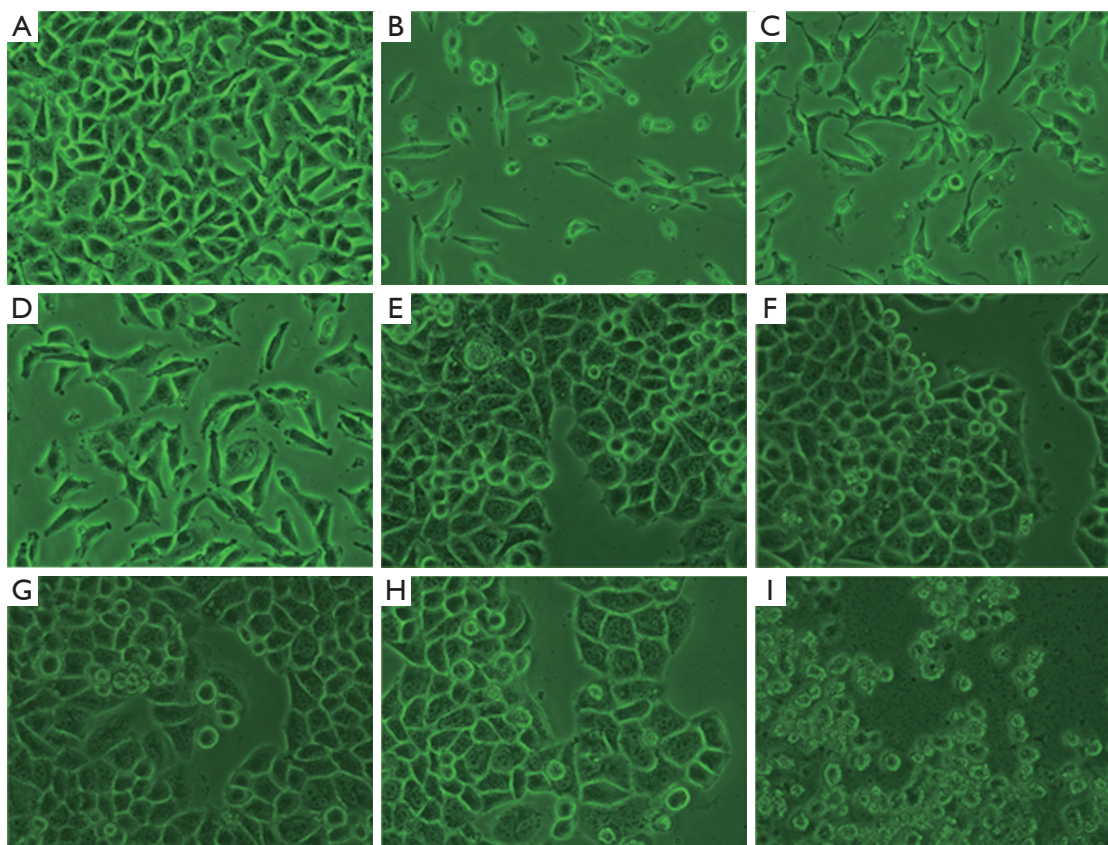


Figure 2 Morphology image of A549 and HeLa cells treated with oxytetracycline, propafenone, metamizole or 0.1% (V/V) DMSO (control) for 48 h (200 \times). (A-D) A549 cells; (E-I) HeLa cells. A and E were control, cells treated with DMSO as a vehicle control; (B-D) Cells treated with oxytetracycline, propafenone and metamizole at the concentration of 0.1, 0.02 and 0.1 g/L, respectively; (F) Oxytetracycline 0.2 g/L; (G) Metamizole 0.20 g/L; (H,I) Propafenone 0.012 and 0.014 g/L, respectively.

under an inverted phase contrast microscope (Nikon). It was found that the quantities of the cells treated with these drugs were decreased greatly. In addition, the shape of some A549 cells changed to globular. In the case of HeLa cells, there were some morphological changes such as volume shrinkage, fragment and changing to globular, when the concentration of propafenone is more than 0.014 g/L. Such morphological changes were not apparent in the control cells (*Figure 2*).

Oxytetracycline, propafenone and metamizole do not cause necrosis in A549 and HeLa cells

In order to determine whether the growth inhibitory effects were due to necrosis that is believed to be an unwanted side effect of cancer-fighting agents, LDH assay was carried out on cells treated with oxytetracycline, propafenone, or

metamizole at the concentration of 0.1, 0.02 or 0.1 g/L or 0.1% DMSO (as control) for 48 h. As shown in *Figure 3*, for A549 and HeLa cells, there were no significant differences in LDH release between the cells in the control group and the drug treatment group. The results demonstrated that the drugs at the test range of concentration did not cause necrosis in the cells.

Compounds induced apoptosis in A549 and HeLa cells

To detect whether oxytetracycline, propafenone and metamizole resulted in apoptosis of the cells, Hoechst 33258 staining assay was performed. It is well known that DNA fragmentation, chromatin condensation, cell shrinkage, and membrane blebbing are the characteristics of apoptotic cells. The chromatin condensation and DNA fragmentation in the cells were determined by Hoechst

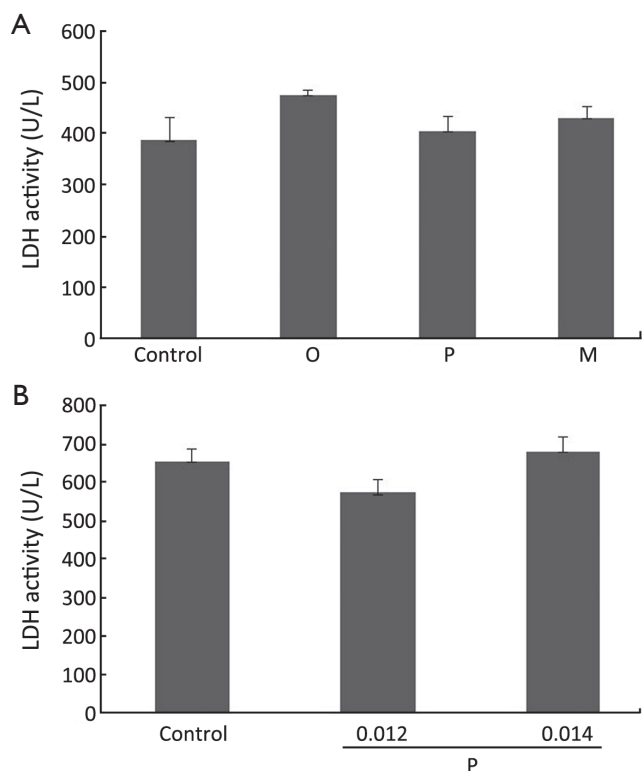


Figure 3 Effects of the drugs on LDH release in A549 and HeLa cells. (A) A549 cells were treated with oxytetracycline (0.1 g/L), propafenone (0.02 g/L), and metamizole (0.1 g/L) or 0.1% DMSO (control) for 48 h; (B) HeLa cells were treated with propafenone (0.012 and 0.014 g/L) or 0.1% DMSO (control) for 48 h. LDH assay results are presented as $\bar{x} \pm s$; $n=3$. O, oxytetracycline; P, propafenone; M, metamizole.

33258 staining under a fluorescence microscope. Treatment with oxytetracycline, propafenone and metamizole at the concentration of 0.1, 0.02 or 0.1 g/L for 48 h could not induce apoptosis in A549 cells greatly (Figure 4A). It was interestingly found that propafenone at the concentration of 0.014 g/L or higher for 48 h could induce apoptosis in HeLa cells greatly ($P < 0.05$ vs. control). While, treatment with oxytetracycline and metamizole at the concentration of 0.20 g/L for 48 h could not induce apoptosis in HeLa cells significantly (Figure 4A,B).

Cell cycle distribution

In order to get a more precise insight of growth inhibition, flow cytometry analysis was performed to detect whether oxytetracycline, propafenone and metamizole could induce

A549 cell cycle arrest. The results showed that treatment with 0.1 g/L, 0.02 g/L or 0.1 g/L of oxytetracycline, propafenone and metamizole for 48 h induced G1-phase arrest effectively (Figure 5). After 48 h treatment with 0.1 g/L, 0.02 g/L or 0.1 g/L, the G1-phase cell population was noticeably enhanced by about 29%, 21% or 19%, respectively, compared with control. The increase in the G1-phase cell population was accompanied by a decrease in S and G2-phase cell populations. The fact that oxytetracycline, propafenone and metamizole induced strong G1-phase arrest of A549 cells is concomitant with the growth inhibitory effect.

Western blot

To find out the inhibitory mechanism of propafenone on HeLa cells, the expressions of P53 and LC3-II were detected, after HeLa cells were treated with propafenone for 48 h. By way of western blot analysis, it could be seen that propafenone can elevate the protein level of P53 effectively ($P < 0.01$). Protein level, treated with propafenone, of LC3-II was not greatly altered (Figure 6).

Discussion

According to the application instruction, oxytetracycline tablets may be taken 0.25-0.5 g once and every 6 h for an adult. So, one adult can take at most 2.0 g every day. It was used at the concentration of 0.1 g/L in the experiment. We also found that when the tablet of oxytetracycline was dissolved with DMSO for more than 10 d at 4 °C, the solution became dark gradually and its antitumor effect also became stronger and stronger. In the experiment, we used the solution made within one week.

According to the application instruction, propafenone tablets may be taken 100-200 mg once and every 6 h for an adult. So, one adult can take at most 0.8 g every day. It was used at the concentration of 0.02 g/L in the experiment. According to the application instruction, metamizole tablets may be taken 0.75-1.25 g for an adult every day. It was used at the concentration of 0.1 g/L in the experiment.

Preliminary biological evaluation showed that oxytetracycline, propafenone and metamizole could inhibit the growth of A549 cells in a dose-dependent manner. Propafenone could also inhibit the growth of HeLa cells in a dose-dependent manner.

Globally, cervical cancer is second only to breast cancer as the leading cause of cancer death in women. Despite

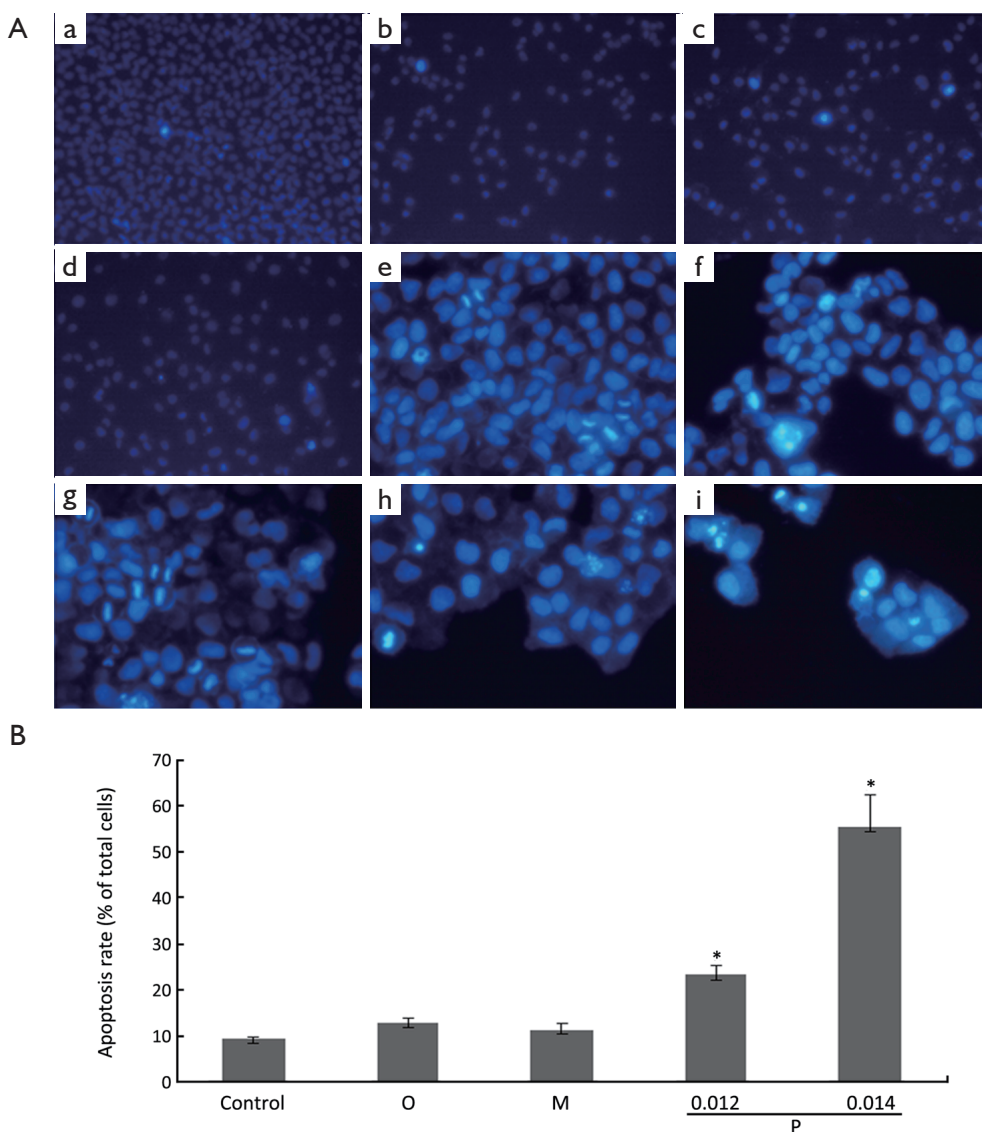


Figure 4 Apoptosis in A549 and HeLa cells induced by oxytetracycline, propafenone and metamizole. (A) Morphology image of apoptosis. Cells were treated with the drugs or 0.1% DMSO (control) for 48 h, and then were stained with Hoechst 33258. (a-d) A549; (e-i) HeLa; (a,e) Control; (b) Oxytetracycline 0.1 g/L; (c) Propafenone 0.02 g/L; (d) Metamizole 0.1 g/L; (f) Oxytetracycline 0.20 g/L; (g) Metamizole 0.20 g/L; (h,i) Propafenone 0.012 and 0.014 g/L, respectively; (B) Apoptosis rate of HeLa cells corresponding to e-i in A. Data are $\bar{x} \pm s$ from three independent experiments. *, $P < 0.05$ vs. control.

advances in the study of cervical cancer over the past 20 years, its pathogenesis is still not fully understood. HeLa cell line, which was established by Gey *et al.*, was derived from cervical adenocarcinoma of a black woman (30). The cell line is one of the most representative cervical squamous carcinoma cell lines, and is used widely (31). Lung cancer is one of the leading causes of death worldwide (32). A549 cells, as a kind of lung cancer cell line, were often used to

examine antitumor effects of some compounds (33,34).

P53 protein suppresses the accumulation of mutations in somatic cells and substantially decreases the probability of malignant diseases (35). Propafenone can elevate the protein level of P53 effectively ($P < 0.01$), indicating that the antitumor effect of propafenone is induced by way of protein P53.

Microtubule-associated protein 1 light chain 3- β

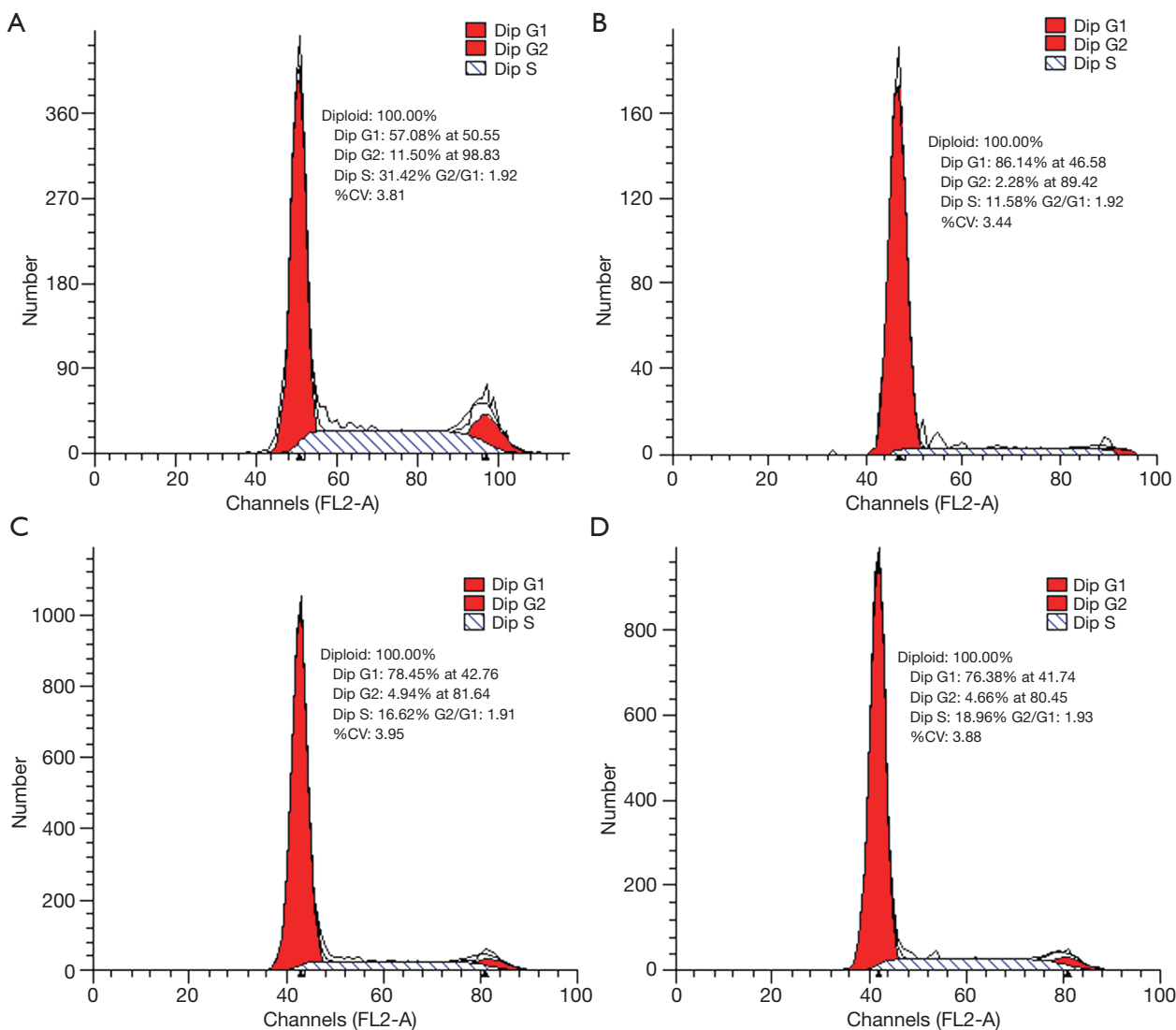


Figure 5 Effect of oxytetracycline, propafenone and metamizole on cell cycle distribution of lung cancer A549 cells. Cells were exposed to the drugs and incubated for 48 h, and then were stained with PI. Values are expressed as percentage of the cell population in the G1, S and G2 phases of cell cycle. (A) Control; (B) Oxytetracycline 0.1 g/L; (C) Propafenone 0.02 g/L; (D) Metamizole 0.1 g/L.

(MAP1LC3-II) is one kind of markers of autophagy. When the expression level of LC3-II is elevated, it usually indicates that autophagy is induced (36). After treatment with propafenone, the protein level of LC3-II was not greatly altered. So, the antitumor effect of propafenone is not induced by way of autophagy.

This study showed that oxytetracycline, propafenone and metamizol (dipyron) all displayed evident inhibitory effects on the proliferation of A549 cells. Propafenone also displayed evident inhibitory effects on the proliferation of Hela cells. At the same time, we found the new function of

these old drugs by screen experiments, while, new functions of other old drugs were found usually by clinical practice. In addition, as the drug has already undergone clinical trials and tested to be bioactive, this strategy will greatly reduce the cost and increase the chance of drug discovery.

Acute arrest of bone marrow will be produced after treatment of some anticancer drugs, while oxytetracycline, propafenone and metamizol do not. This is an advantage of them. The three kinds of drugs may be used in treatment of lung cancer or cervical cancer along with normal anticancer drugs.

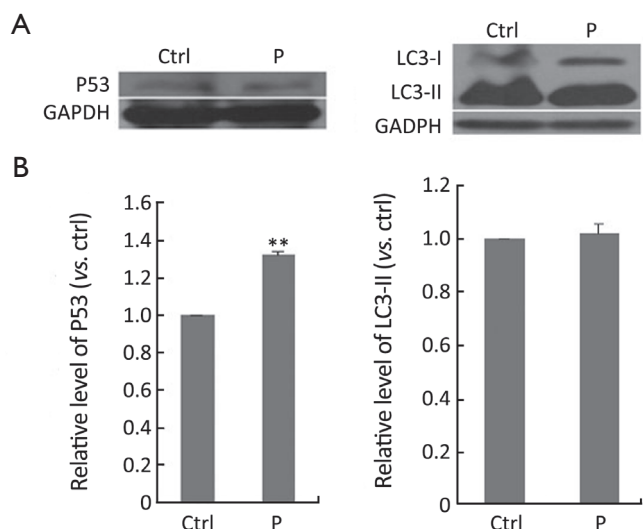


Figure 6 Effect of propafenone on expression of P53 and LC3-II in HeLa cells. (A) Western blot analysis of protein levels of P53, LC3-II, and GAPDH as a normalization control. HeLa cells were treated with propafenone at the concentration of 0.02 g/L for 48 h; (B) Relative quantification of P53 and LC3-II protein levels. **, $P < 0.01$ vs. control, $n = 3$.

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