—Regular Article—

Effects of Novel All-Trans Retinoic Acid Retinamide Derivatives on the Proliferation and Apoptosis of Human Lung Adenocarcinoma Cell Line A549 Cells

Shu-Yu GUI,[a,b] Fei-Hu CHEN,[c] Qing ZHOU,[b] and Yuan WANG*,[b]

[a]*Department of Respiratory Medicine, First Affiliated Hospital,* [b]*Laboratory of Molecular Biology and Department of Biochemistry, Key Laboratory of Anti-inflammatory and Immunological Pharmacology, Ministry of Education and Key Laboratory of Gene Resource Utilization for Severe Disease of Anhui Province, and* [c]*College of Pharmacy, Anhui Medical University, Hefei, Anhui, P. R. China*

(Received August 17, 2010; Accepted June 16, 2011)

The aim of the present study was to synthesize a series of retinamide derivatives using all-trans retinoic acid (ATRA) as raw material and observe their effects on the differentiation and apoptosis of human lung adenocarcinoma A549 cells. Four new synthesized ATRA retinamide derivatives were structurally confirmed by spectral analysis, including ^{1}H-NMR, ^{13}C-NMR, and MS. The results showed that the new ATRA retinamide derivatives significantly decreased the carcinoembryonic antigen secretion of A549 cells, significantly decreased the proliferation of A549 cells in a dose- and time-dependent manner, and promoted the apoptosis of A549 cells compared with ATRA. The Western blot assay indicated that the expression of Bcl-2 was decreased more in A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide than that in A549 cells treated with ATRA. The results also showed that the effects of *N*-(3-trifluoromethylphenyl) retinamide on differentiation and apoptosis were the strongest among the newly synthesized ATRA retinamide derivatives. Our results suggested that the effects of novel ATRA retinamide derivatives on increasing the differentiation, decreasing the proliferation, and promoting the apoptosis of A549 cells were greater than those of ATRA. The apoptosis of A549 cells induced by *N*-(3-trifluoromethylphenyl) retinamide may be related to downregulating the expression of Bcl-2.

Key words——all-trans retinoic acid; retinamide-ester derivative; proliferation; apoptosis; human lung adenocarcinoma A549 cell

INTRODUCTION

All-trans retinoic acid (ATRA) is a natural vitamin A metabolite and plays a major role in regulating the proliferation, growth, and differentiation of both normal and malignant cells.[1] Initially, the strong antitumor activity of ATRA against acute promyelocytic leukemia was reported.[2] Recently, evidence has been obtained about the therapeutic potential of retinoids against various other cancers, such as lung, bladder, prostate, and breast cancer, glioma, neuroblastoma,[3] and skin cancer.[4] Gander and Gurney[5] reported a series of retinoic acid ester and amide derivatives, in which fenretinide [*N*-(4-hydroxyphenyl) retinamide, 4-HPR] had superior antitumor activity, but its poor solubility limited clinical application. Recently, Mershon and coworkers[6] have synthesized new 4-HPR derivatives. Among them, *N*-(2-hydroxy-4-nitrophenyl)-retinamide can induce cell apoptosis. The nitrophenyl in this compound has an electron-withdrawing effect, and the nitro group may be potentially harmful, including increasing oxidative stress, endothelial dysfunction, and cardiac autonomic dysfunction.[7] In the present study, we designed and synthesized a novel series of ATRA derivatives with another electron-withdrawing unit, trifluoromethylphenyl or trifluoromethoxyphenyl, and analyzing their effects on the proliferation and apoptosis of lung adenocarcinoma A549 cells, in the hope of increasing their antitumor activity.

MATERIALS AND METHODS

Reagents ATRA, trifluoromethylphenyl, dicyclohexylcarbodiime (DCC), dimethylaminopridine (DMAP), para-toluene sulfonic acid (PTSA), and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT) were purchased from Sigma (St. Louis, MO, USA). ATRA and retinamide derivatives were dissolved in dimethyl sulfoxide (DMSO) at concentrations of 10 mg/ml, to make stock solutions, which were stored at −20°C until use.

Synthesis of ATRA Retinamide Derivatives The

*e-mail: wangyuan@ahmu.edu.cn

target compounds were prepared using ATRA and trifluoromethylphenyl or trifluoromethoxyphenyl derivatives as the starting materials by condensation with DCC and DMAP, respectively.[8,9] Using the synthetic procedure for retinoic amide derivatives, we have improved the operation method and used DMAP-PTSA as a catalyst. The structures of target compounds were characterized by ^{1}H-NMR, ^{13}C-NMR, and HR-MS.

Cell Culture Human lung adenocarcinoma cell line A549 cells (ATCC, Manassas, VA, USA) were routinely grown in DMEM medium supplemented with 10% FBS, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM of L-glutamine (all from Invitrogen Co., Carlsbad, CA, USA) in 5% CO_2 and humidified air at 37°C.

Proliferation Study The MTT assay was performed using the method described previously.[10] The A549 cells were plated on a 96-well cluster dish at a density of 1×10^4 cells/well. Twenty-four hours later, the medium contained various concentrations of different ATRA retinamide derivatives. After incubation for 48 h, the medium was removed and 5 mg/ml MTT solution was added to each well. Cells were incubated for 4 h at 37°C in 5% CO_2 and then 10% sodium dodecyl sulfate (SDS) solution was added and the mixture was incubated for 2 h to dissolve formazan crystals. The absorbance was measured at a wavelength of 570 nm in a microplate reader (Bio-TEK Model ELx800, Bio-TEK instruments, Inc., Winooski, VT, USA). The survival rate of cells was calculated according to the following formula: survival rate of cells (%) = (test well A_{570} − control well A_{570}) / test well A_{570}) × 100%.

Assessment of DNA Fragmentation A549 cells treated with ATRA and retinamide derivatives (10 μg /ml) for 72 h were removed by Trypsin-EDTA treatment, washed twice with cold PBS, and the cell pellet was resuspended in 1 ml of hypotonic PI solution (50 mg/ml PI, 0.1% Triton X-100, 0.1% sodium citrate) for 30 min at 4°C. The percentage of hypodiploid cells present in each sample was then determined using flow cytometry by displaying cell cycle distribution according to DNA content.

Detection of Chromatin Condensation Chromatin condensation was detected by nucleus staining with Hoechst 33258 as described previously.[11] Hoechst 33258 (5 mg/l) was directly added to the culture medium by gently shaking at 4°C for 5 min. Stained nuclei were visualized with a Nikkon fluorescence microscope (Nikon E800, Japan) at 400× magnification with an excitation wavelength of 355 to 366 nm and an emission wavelength of 465 to 480 nm. In this way, apoptotic cells are stained bright blue because of their chromatin condensation, while normal cells are stained only slightly blue.

CEA Assay The supernatants of cells treated with ATRA and retinamide derivatives (10 μg/ml) for 72 h were collected and carcinoembryonic antigen (CEA) levels were measured with the electrochemiluminescence (ELC) method on a commercially available ELECSYS 2010 analyzer (Roche).

Western Blot Analysis The A549 cells treated with different concentrations of ATRA or *N*-(3-trifluoromethylphenyl) retinamide for 72 h were rinsed twice in ice-cold PBS and then lysed in RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, NaCl 150 mM, sodium phosphate buffer 10 mM, pH 7.2, EDTA 2 mM, PMSF 2 mM, leupeptin 10 mg/ml, aprotinin 10 mg/ml, sodium orthovanadate 2 mM, sodium pyrophosphate 10 mM, and sodium fluoride 20 mM). The lysates were centrifuged at 15000×*g* for 30 min at 4°C, and the supernatants were used for Western blotting. The total protein concentration of each supernatant was measured using the MicroBCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). The same amount of lysate from each line in SDS sample buffer was run with 10% SDS-polyacrylamide gel electrophoresis and electroblotted onto a PVDF membrane, which was then blocked with 5% fat-free milk in PBS and 0.1% Tween 20(PBST) for 2 h at room temperature. Anti-Bcl 2, Bax, Bak, NF-kb, ERk, pERK antibody (1:500–1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated overnight at 4 °C, followed by incubation with 1:2000 diluted HRP-conjugated goat antibody against rabbit or mouse IgG (Santa Cruz, Biotechnology), and staining with enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). Densitometric scanning of the exposed X-ray film was used for semiquantitative measurement of the protein bands (Image-Pro plus, MediaCybernetics, Silver Springs, MD, USA). Three independent experiments were performed, and the results were reproducible.

Statistical Analysis Values are expressed as mean ± standard deviation (S.D.). Data were analyzed using statistical software (SPSS 12.0; LEAD

Technologies, Inc., Chicago, IL, USA). Statistical significance of the differences among groups was assessed by one-way analysis of variance (ANOVA). Spearman's correlation was used as a test of correlation between two continuous variables. A p value of <0.05 was considered to be statistically significant.

RESULTS

Synthesis of ATRA Retinamide Derivatives Compound **3a-01**: mp186.1–187.6℃. ^{1}H-NMR ($CDCl_3$), δ (ppm): 1.03 (s, 6H), 1.72 (s, 3H), 2.01 (s, 3H), 2.43 (s, 3H), 1.46+1.63 (t, 4H), 2.02 (m, 2H), 5.79 (s, 1H), 6.12–6.30 (m, 4H), 7.03 (m, 1H), 7.55 (d, 1H), 7.68 (d, 1H); ^{13}C-NMR ($CDCl_3$), δ (ppm): 13.01, 13.90, 19.31, 21.82, 29.04, 33.21, 34.36, 39.71, 120.48, 128.98, 129.45, 130.18, 131.23, 134.98, 137.27, 137.80, 139.95, 152.13, 165.34, 119.26, 126.33, 141.50. High-resolution MS (m/z): 443.2444 [calcd. for $C_{27}H_{32}F_3NO$, 443.2436, to substituue for *N*-(4-trifluoromethylphenyl) retinamide]. Compound **3a-02**: mp 89.7–92.5℃. ^{1}H-NMR ($CDCl_3$), δ (ppm): 1.03 (s, 6H), 1.72 (s, 3H), 2.01 (s, 3H), 2.44 (s, 3H), 1.47+1.62 (t, 4H), 2.02 (m, 2H), 5.78 (s, 1H), 6.15–6.30 (m, 4H), 7.03 (m, 1H), 7.72 (d, 1H), 7.22 (s, 1H), 7.33, 7.45 (m, 1H), N-H 7.88 (s, 1H), ^{13}C-NMR ($CDCl_3$), δ (ppm): 13.00, 13.88, 19.31, 21.82, 29.04, 33.21, 34.36, 39.71, 116.47, 129.49, 129.53, 130.15, 131.11, 135.06, 137.29, 137.81, 139.84, 151.92, 165.39, 138.95, 128.90, 125.33, 122.63 (d), 120.50, 131.29 (q). High-resolution MS: m/z: 443.2437 Compound [calcd. for $C_{27}H_{32}F_3NO$, 443.2436 to substituue for *N*-(3-trifluoromethylphenyl) retinamide]. Compound **6a-01**: mp 172.5–174.5℃. ^{1}H-NMR ($CDCl_3$), δ (ppm): 0.96 (s, 6H), 1.64 (s, 3H), 1.94 (s, 3H), 2.35 (s, 3H), 1.40+1.55 (t, 4H), 1.95 (m, 2H), 5.70 (s, 1H), 6.05–6.23 (m, 4H), 6.95 (m, 1H), 7.51 (d, 1H), 7.11 (d, 1H), N-H 7.09 (s, 1H); ^{13}C-NMR ($CDCl_3$), δ (ppm): 13.01, 13.86, 19.32, 21.83, 29.05, 33.21, 34.37, 39.72, 121.83, 128.86, 129.50, 130.13, 130.97, 135.11, 137.32, 137.82, 139.74, 151.59, 165.14, 120.64, 120.86, 137.04. High-resolution MS: m/z: 459.2379 [calcd. for $C_{27}H_{32}F_3NO_2$, 459.2385, to substituue for *N*-(4-trifluoromethoxyphenyl) retinamide]. Compound **6a-02**: mp 139.2–140℃. ^{1}H-NMR ($CDCl_3$), δ (ppm): 0.96 (s, 6H), 1.65 (s, 3H), 1.94 (s, 3H), 2.36 (s, 3H), 1.40+1.56 (t, 4H), 1.95 (m, 2H), 5.70 (s, 1H), 6.05–6.23 (m, 4H), 6.95 (m, 1H), 7.22–7.29 (m, 1H), 6.86–6.88 (m, 1H), N-H 7.10 (s, 1H); ^{13}C-NMR ($CDCl_3$), δ (ppm): 13.02, 13.88, 19.32, 21.83, 29.05, 33.22, 34.37, 39.72, 120.53, 128.89, 129.50, 130.14, 130.09, 135.07, 137.31, 137.82, 139.83, 151.89, 165.15, 112.51, 116.13, 117.64, 119.08, 121.06, 130.02, 149.72. High-resolution MS: m/z: 459.2377 [calcd. for $C_{27}H_{32}F_3NO_2$, 459.2385, to substituue for *N*-(3-trifluoromethoxyphenyl) retinamide] (Fig. 1).

Differentiation of A549 Cells Induced by ATRA Retinamide Derivatives The results showed that ATRA retinamide derivatives significantly decreased the secretion of CEA in A549 cells compared with ATRA ($p<0.01$, Table 1).

Proliferation of A549 Cells Decreased by ATRA Retinamide Derivatives A proliferation assay was carried out to compare the effects of the new ATRA retinamide derivatives with those of ATRA on A549 cells. *N*-(4-trifluoromethylphenyl) retinamide, *N*-(3-

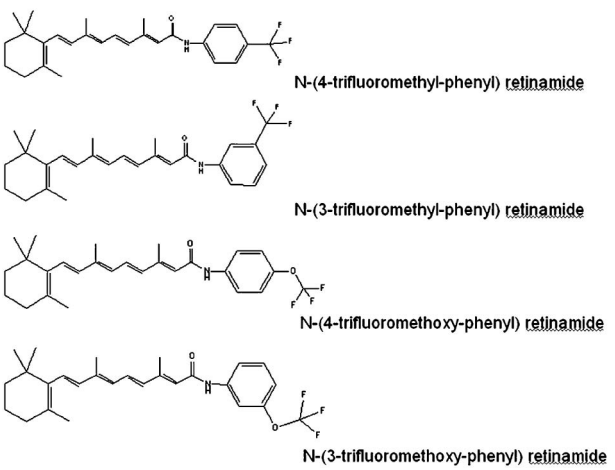

Fig. 1. Structures of ATRA Retinamide Derivatives

Table 1. The Effect of ATRA Retinamide Derivatives on CEA Secretion of A549 Cells

	CEA (μg/l)
Control	5.8±0.9
ATRA (10 μg/ml)	4.5±0.8
N-(4-trifluoromethylphenyl) retinamide (10 μg/ml)	2.9±0.4*
N-(3-trifluoromethylphenyl) retinamide (10 μg/ml)	2.8±0.6*
N-(4-trifluoromethoxyphenyl) retinamide (10 μg/ml)	2.9±0.5*
N-(3-trifluoromethoxyphenyl) retinamide (10 μg/ml)	3.5±0.6*

* compare with ATRA group, $p<0.01$.

trifluoromethylphenyl) retinamide, *N*-(4-trifluoromethoxyphenyl) retinamide, and *N*-(3-trifluoromethoxyphenyl) retinamide significantly suppressed A549 cell proliferation in a concentration-dependent manner with IC_{50} values of 0.202, 0.047, 0.313, and 0.459 mmol/l, respectively (Table 2). At the ATRA retinamide derivative concentration of 10 μg/ml, the proliferation of A549 cells was also decreased significantly with time (Table 3).

Apoptosis of A549 Cells Promoted by ATRA Retinamide Derivatives After treatment with ATRA or ATRA retinamide derivatives (10 μg/ml) for 72 h, apoptosis was triggered in A549 cells and the effect was statistically significant ($p<0.05$) for all treatment groups compared with the control group. There was no DNA fragmentation associated with necrosis, since supernatants did not show significant DNA fragments, implying that plasma membranes were intact at the times and concentrations examined (Fig. 2). The apoptosis rate of the control, ATRA, *N*-(4-trifluoromethylphenyl) retinamide, *N*-(3-trifluoromethylphenyl) retinamide, *N*-(4-trifluoromethoxyphenyl) retinamide, and *N*-(3-trifluoromethoxyphenyl) retinamide groups was 2.95%, 29.3%, 17.6%, 40.9%, 30.5%, and 9.79%, respectively. Apoptosis analysis with Hochest 33258 staining revealed that some apoptotic bodies appeared in A549 cells treated with ATRA and ATRA retinamide derivatives (Fig. 3).

***N*-(3-trifluoromethylphenyl) Retinamide Decrease the Expression of Bcl-2 and Phosphorylation of ERK** ATRA 10 μg/ml produced a decrease in Bcl-2 to less than 20% of control levels. The results in Fig. 4(a) and (b) show that in A549 cells, *N*-(3-trifluoromethylphenyl) retinamide 1 μg/ml and 10 μg/ml most potently decreased the levels of Bcl-2 to less than 41% and 53% of control levels, respectively, with the effect being maximally observed 72 h post-treatment. *N*-(3-trifluoromethylphenyl) retinamide also decreased the phosphorylation of ERK. Quantitative analysis of there data by densitometric scanning normalized to the internal β-actin control is shown in Fig. 4(a) and (b).

DISCUSSION

Lung cancer is one of the most common malignantcies worldwide. With advances in science and technology, the treatment of lung cancer ranges from surgery

Table 2. The Dose-effect of ATRA Retinamide Derivatives on A549 Cells Proliferation

		OD ($\bar{x}\pm s$)	Inhibition ratio (%)	CI50 (mmol/l)
Control		1.92±0.16		
ATRA (10 μg/ml)		1.72±0.11	10.42±5.73	
N-(4-trifluoromethylphenyl) retinamide	1 μg/ml	1.60±0.07	16.84±3.55	0.202
	2.5 μg/ml	1.50±0.10	21.70±5.24*	
	5 μg/ml	1.42±0.06	25.87±3.14*	
	7.5 μg/ml	1.37±0.09	28.47±4.81*	
	10 μg/ml	1.31±0.05	31.94±2.35*	
N-(3-trifluoromethylphenyl) retinamide	1 μg/ml	1.60±0.08	16.49±3.91	0.047
	2.5 μg/ml	1.54±0.10	19.79±5.29	
	5 μg/ml	1.50±0.17	21.70±8.87*	
	7.5 μg/ml	1.25±0.07	34.72±3.66*	
	10 μg/ml	0.97±0.13	49.65±6.78*	
N-(4-trifluoromethoxyphenyl) retinamide	1 μg/ml	1.69±0.06	11.81±2.87	0.313
	2.5 μg/ml	1.68±0.16	12.67±8.34	
	5 μg/ml	1.64±0.04	14.76±1.97	
	7.5 μg/ml	1.51±0.18	21.53±9.47*	
	10 μg/ml	1.36±0.05	28.99±2.35*	
N-(3-trifluoromethoxyphenyl) retinamide	1 μg/ml	1.70±0.17	11.63±8.87	0.459
	2.5 μg/ml	1.65±0.07	14.24±3.66	
	5 μg/ml	1.58±0.10	17.88±5.32	
	7.5 μg/ml	1.52±0.17	21.01±8.97*	
	10 μg/ml	1.45±0.13	24.65±6.88*	

* compare with ATRA, $p<0.01$.

Table 3. The Time-effect of ATRA Retinamide Derivatives on A549 Cells Proliferation

	Time	OD ($\bar{x}\pm s$)	Inhibition ratio (%)
Control	1d	0.917±0.048	0.0±0.0
	2d	1.463±0.086	0.0±0.0
	3d	0.927±0.045	0.0±0.0
	7d	2.074±0.053	0.0±0.0
ATRA (10 μg/ml)	1d	0.883±0.062	3.7±0.58*
	2d	1.360±0.052	7.0±1.42*
	3d	0.845±0.034	8.9±1.9*
	7d	1.835±0.030	11.5±2.6*
N-(4-trifluoromethylphenyl) retinamide (10 μg/ml)	1d	0.680±0.055	25.8±4.6**
	2d	1.042±0.046	28.8±4.1**
	3d	0.623±0.006	32.8±2.8**
	7d	1.325±0.028	36.1±2.4**
N-(3-trifluoromethylphenyl) retinamide (10 μg/ml)	1d	0.617±0.072	32.7±8.9**
	2d	0.703±0.025	51.9±2.1**
	3d	0.229±0.010	75.3±1.2**
	7d	0.106±0.035	94.9±3.7**
N-(4-trifluoromethoxyphenyl) retinamide (10 μg/ml)	1d	0.646±0.019	29.6±2.0**
	2d	0.909±0.065	37.8±5.6**
	3d	0.567±0.057	38.9±4.9**
	7d	1.243±0.024	40.1±3.1**
N-(3-trifluoromethoxyphenyl) retinamide (10 μg/ml)	1d	0.774±0.030	15.6±3.4**
	2d	1.065±0.045	27.2±4.2**
	3d	0.408±0.028	56.1±2.7**
	7d	0.666±0.049	67.9±5.3**

* compare with control, $p<0.01$. ** compare with ATRA, $p<0.01$.

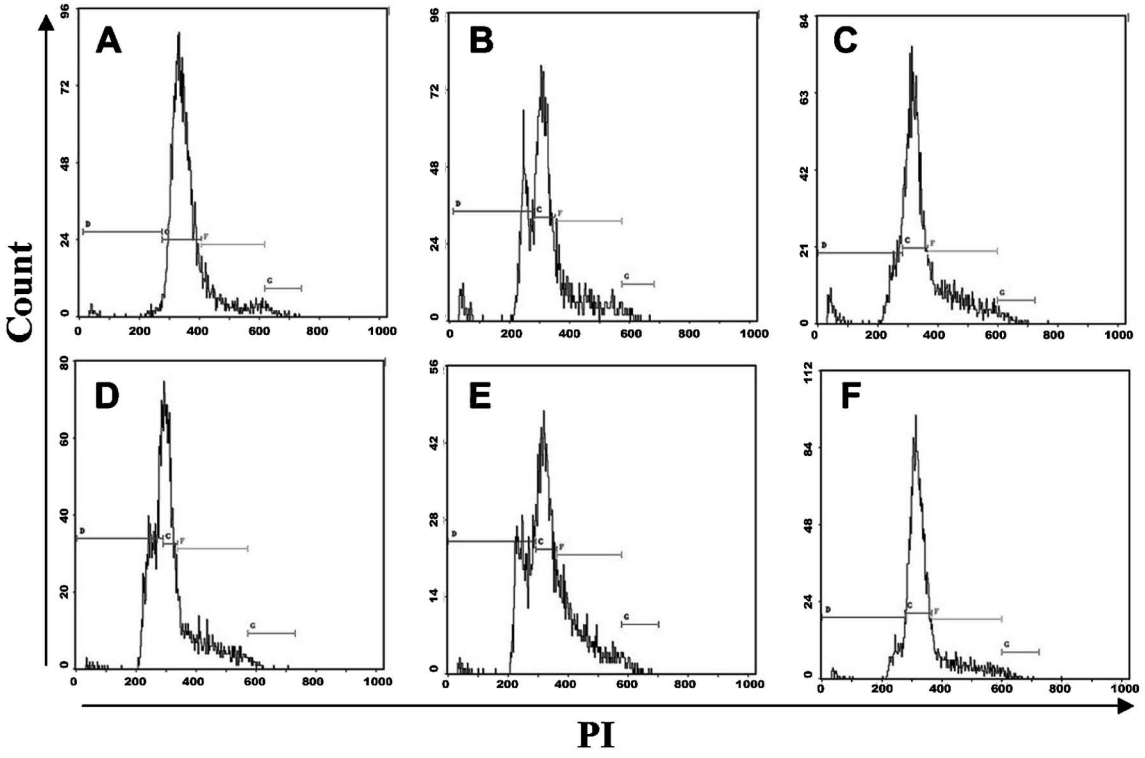

Fig. 2. Flow Cytometric Analysis of Apoptosis in A549 Cells Treated with 10 μg/ml of ATRA Retinamide Derivatives

A, A549 cells; B, A549 cells treated with ATRA 10 μg/ml; C, A549 cells treated with *N*-(4-trifluoromethylphenyl) retinamide 10 μg/ml; D, A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide 10 μg/ml; E, A549 cells treated with *N*-(4-trifluoromethoxyphenyl) retinamide 10 μg/ml; F, A549 cells treated with *N*-(3-trifluoromethoxyphenyl) retinamide 10 μg/ml.

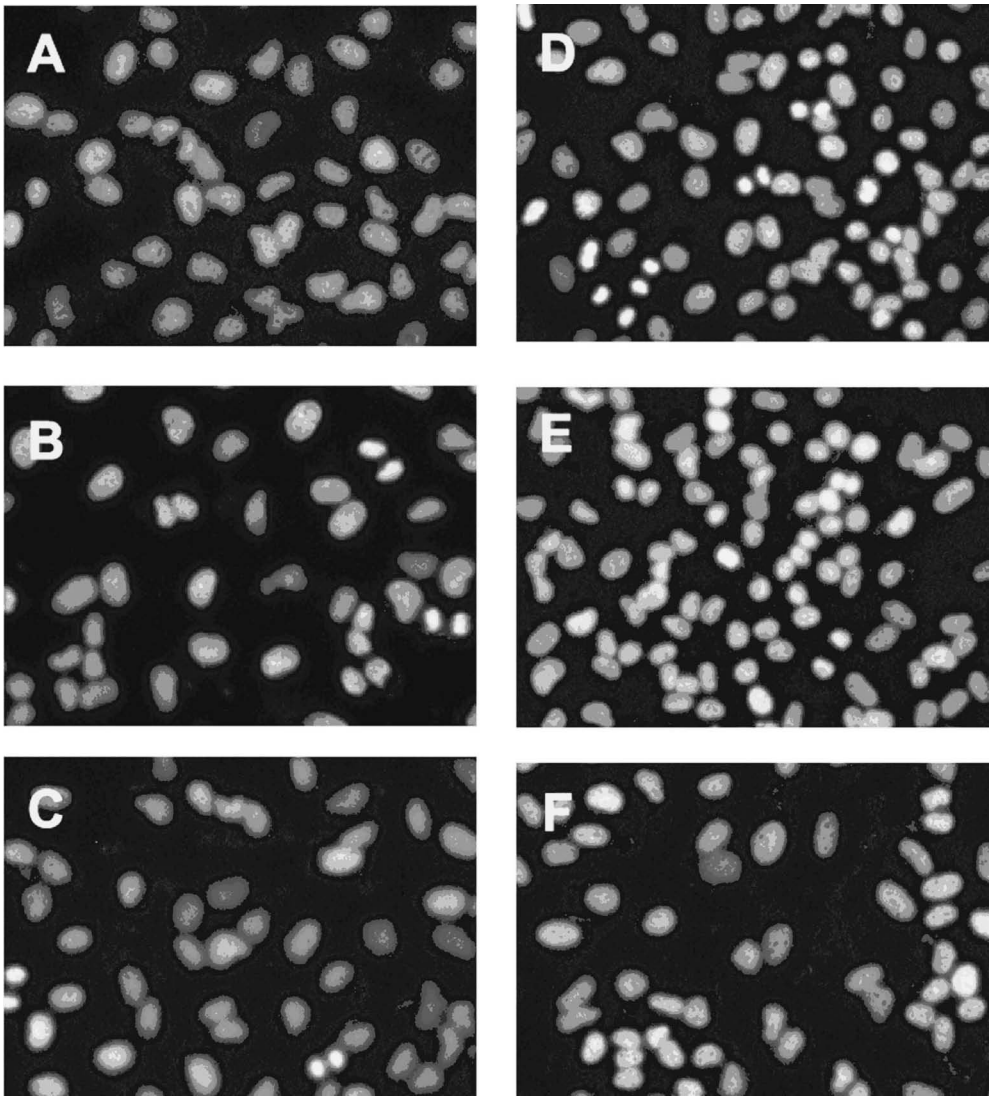

Fig. 3. Hoechst 33258 Staining Analysis of Apoptosis in A549 Cells Treated with 10 μg/ml of ATRA Retinamide Derivatives

A, A549 cells; B, A549 cells treated with ATRA10 μg/ml; C, A549 cells treated with *N*-(4-trifluoromethylphenyl) retinamide 10 μg/ml; D, A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide 10 μg/ml; E, A549 cells treated with *N*-(4-trifluoromethoxyphenyl) retinamide 10 μg/ml; F, A549 cells treated with *N*-(3-trifluoromethoxyphenyl) retinamide 10 μg/ml.

with postoperative radiotherapy and chemotherapy to molecular-targeted therapy, gene therapy, *etc.* Induction differentiation therapy is a new treatment strategy emerging in recent years. Its basic theory is the application of differentiation inducers in conjunction with traditional treatment to change cancer cells into normal or nearly normal cells.[12] The differentiation inducers commonly used are retinoic acid, tanshinone, butyric acid and its derivatives, *etc.*[12–14] For nearly 30 years, to discover agents with lower toxicity and higher efficacy, researchers have continued to alter the structure of ATRA, and nearly 2000 types of retinoic acid derivatives have been developed. The ATRA retinamide derivatives used in this study are the first-generation products developed *via* carboxyl transformation of the structure of ATRA.

This experiment investigated whether ATRA retinamide derivatives affected the proliferation, differentiation, and apoptosis of human lung adenocarcinoma A549 cells. We found that new synthetic ATRA retinamide derivatives significantly inhibited the proliferation, promoted the apoptosis, and promoted the differentiation of A549 cells, among which *N*-(3-trifluoromethylphenyl) retinamide was the most potent. The proliferation inhibitory rate of *N*-(3-trifluoromethylphenyl) retinamide at concentrations of 1, 5, and 10 μg/ml was 16.9$\pm$0.058%, 19.1$\pm$0.306%, and 49.7$\pm$0.028% at 72 h

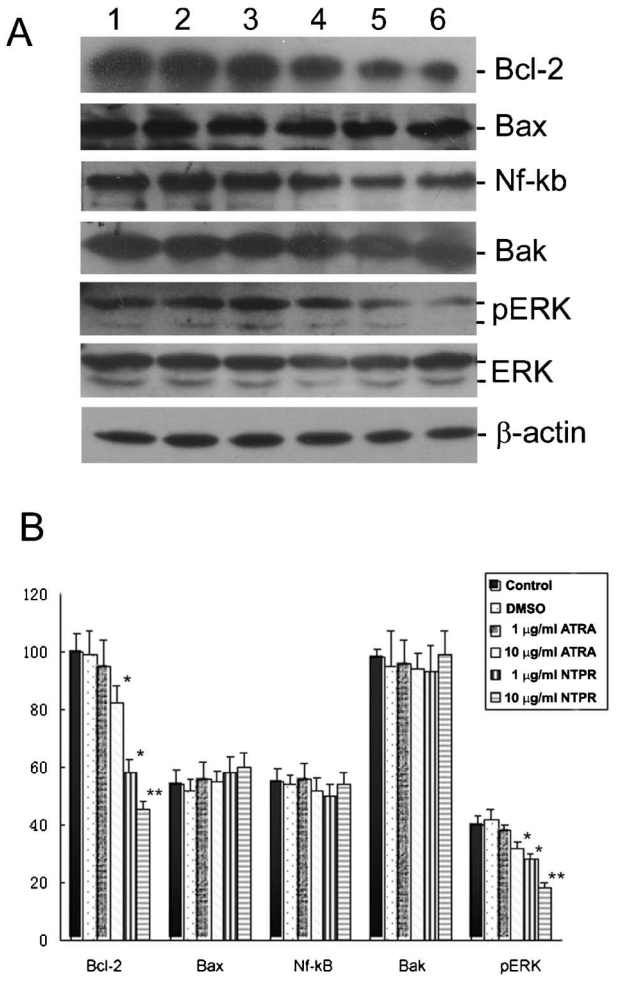

Fig. 4. Western Blot Analysis

A: 1, A549 cells; 2, A549 cells/DMSO; 3, A549 cells treated with ATRA 1 μg/ml; 4, A549 cells treated with ATRA 10 μg/ml; 5, A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide (NTPR) 1 μg/ml; 6, A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide 10 μg/ml. B: Bar graph of protein quantification plotted from at least three independent experiments. $^{*}p<0.05$ relative to controls; $^{**}p<0.05$ relative to ATRA.

posttreatment, respectively, which was higher than that of the same concentrations of ATRA. The highest inhibitory rate was 94.9±0.37% with 10 μg/ml of *N*-(3-trifluoromethylphenyl) retinamide after 7 days. The results showed that *N*-(3-trifluoromethylphenyl) retinamide significantly inhibited the proliferation of A549 cells in a time- and dose-dependent manner. When we used CEA as an indicator of the reversal the malignant phenotype of human lung cancer cells, it was found that *N*-(3-trifluoromethylphenyl) retinamide 10 μg/ml more effectively stimulated cell differentiation and decreased CEA secretion and malignant transformation of A549 cells than ATRA. The apoptosis rate of A549 cells treated with *N*-(3-trifluoromethylphenyl) retinamide 10 μg/ml was 40.9% over 3 days as shown by flow cytometry, in which the apoptosis peaks were clearly moved forward. The flow cytometry results showed that the ability to arrest the A549 cell cycle in the G_0/G_1 phase and G_2/M phase by ATRA (see Table) and ATRA derivatives was different, and thus the A549 cell growth inhibition rate and apoptosis rate appeared to be inconsistent. The exact mechanism requires further research. We chose *N*-(3-trifluoromethylphenyl) retinamide to study the apoptosis mechanism. The results showed that ERK phosphorylation and antiapoptotic protein Bcl-2 expression both decreased, which suggested that *N*-(3-trifluoromethylphenyl) retinamide induced apoptosis of A549 cells, which may occur through the ERK pathway and the Bcl-2 family proteins.

Bcl-2 is a major member of Bcl-2 family that plays a role in antiapoptosis through a variety of protein–protein interactions.[15] In malignant tumors, disorder of the apoptotic mechanism is an important cause of drug resistance, and increasing antiapoptotic proteins is one of the main mechanisms to reduce the susceptibility of malignant tumors to chemotherapy agents. Our results indicated that *N*-(3-trifluoromethylphenyl) retinamide significantly decreased the expression of Bcl-2 in A549 cells. This suggests that part of the antitumor activity of *N*-(3-trifluoromethylphenyl) retinamide may be mainly mediated by promoting the apoptosis of A549 cells. The mechanism of the MAPK pathway may be affected by Bcl-2 family protein expression,[15,16] which promotes the release of cytochrome c and affects the expression of the caspase family,[17] ultimately leading to apoptosis.

In summary, newly synthesized retinoic acid derivatives may play a more important role in inducing the differentiation and inhibition of proliferation and apoptosis of A549 cells than ATRA at the same dose and treatment time. ATRA derivatives, especially *N*-(3-trifluoromethylphenyl) retinamide, may be a new differentiation inducer in the treatment of lung cancer.

Acknowledgments This work was supported by the National Natural Science Foundation of PR China (No. 30971226) and the Natural Science Foundation of Anhui province of PR China (No. 090413116).

REFERENCES

1) Tallman M. S., Wiernik P. H., *J. Clin. Pharmacol.*, **32**, 868–888 (1992).
2) Huang M. E., Ye Y. C., Chen S. R., Chai J. R., Lu J. X., Zhoa L., Gu L. J., Wang Z. Y., *Blood*, **72**, 567–572 (1988).
3) Smith M. A., Parkinson D. R., Cheson B. D., Friedman M. A., *J. Clin. Oncol.*, **10**, 839–864 (1992).
4) Lippman S. M., Meyskens F. L. Jr., *Pharmacol. Ther.*, **40**, 107–122 (1989).
5) Gander R. A., Gurney J. A., US Patent 4190594 (1980). http://www.docstoc.com/docs/43757182/Retinoic-Acid-Derivatives---Patent-4190594.
6) Mershon S. M., Anding A. L., Chapman J. S., Clagett-Dame M., Stonerock L. A., Curley R. W. Jr., *Bioorg. Med. Chem. Lett.*, **17**, 836–840 (2007).
7) Gori T., Parker J. D., *J. Am. Coll. Cardio.*, **52**, 251–254 (2008).
8) Sangmam C., Winum J. Y., Lucas M., Montero J. L., Chavis C., *Syn. Commun.*, **28**, 2945–2958 (1998).
9) Shen J., Shi J. B., Chen F. H., Wang Y., Ruan J. J, Huang Y., *Chin. Chem. Lett.*, **20**, 809–811 (2009).
10) Ren B., Wang Y., Ndebele K., Zhi Q., Chen F. H., Wang Y. Z., *Front. Biosci.*, **10**, 1089–1097 (2005).
11) Grusch M., Polgar D., Gfatter S., Leuhuber K., Huettenbrenner S., Leisser C., Fuhrmann G., Kassie F., Steinkellner H., Smid K., Peters G. J., Jayaram H. N., Klepal W., Szekeres T., Knasmüller S., Krupitza G., *Cell Death Differ.*, **9**, 169–178 (2002).
12) Tsiftsoglou A. S., Bonovolias I. D., Tsiftsoglou S. A., *Pharmacol. Ther.*, **122**, 264–280 (2009).
13) Wang J., Wang X., Jiang S., Yuan S., Lin P., Zhang J., Lu Y., Wang Q., Xiong Z., Wu Y., Ren J., Yang H., *J. Neurooncol.*, **82**, 11–21 (2007).
14) Domokos M., Jakus J., Szeker K., Csizinszky R., Csiko G., Neogrady Z., Csordas A., Galfi P., *Dig. Dis. Sci.*, **55**, 920–930 (2010).
15) Bolenz C., Becker A., Trojan L., Schaaf A., Cao Y., Weiss C., Alken P., Michel M. S., *Urol. Oncol.*, **25**, 476–482 (2007).
16) Wang B., Feng Y., Song X., Liu Q., Ning Y., Ou X., Yang J., Zhang X., Wen F., *Toxicology*, **258**, 176–183 (2009).
17) Li Y., He K., Huang Y., Zheng D., Gao C., Cui L., Jin Y. H., *Mol. Carcinog.*, **49**, 630–640 (2010).