RESEARCH ARTICLE

Curcumin Analogue A501 induces G2/M Arrest and Apoptosis in Non-small Cell Lung Cancer Cells

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Abstract

Curcumin and its analogues have been reported to exert anti-cancer activity against a variety of tumors. Here, we reported A501, a new curcumin analogue. The effect of A501 on cell viability was detected by MTT assay, the result showed that A501 had a better inhibiting effect on the four non-small cell lung cancer (NSCLC) cells than that of curcumin. Moreover, Colony forming experiment showed A501 significant restrained cell proliferation. Flow cytometry displayed A501 can cause G2/M arrest and induce apoptosis. Western blotting showed that A501 decreased the expression of cyclinB1, cdc-2, bcl-2, while increased the expression of p53, cleaved caspase-3 and bax. In conclusion, curcumin analogues A501 played antitumor activity by inhibiting cell proliferation and inducing apoptosis of NSCLC cells. And it was likely to be a promising starting point for the development of curcumin-based anticancer drugs.

Keywords: NSCLC - curcumin - antitumor - cycle arrest - apoptosis

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Introduction

Non-small cell lung cancer (NSCLC) is the highest incidence and mortality rates of cancers in the world. Chemotherapy is the main treatment for advanced lung cancer patients, but many patients are resistant to chemotherapy (Dempke et al., 2010; Starakis et al., 2012) .Therefore, the development of new chemotherapy drug for the treatment of lung cancer is particularly imminent. The exploitation of chemotherapy drugs based on natural product is a common way to develop new drugs (Afaq et al., 2002; Zhang et al., 2012; Fang et al., 2013).

Curcumin is a phenolic compound extracted from curcuma longa rhizome belongs to ginger family. Numerous studies have shown that curcumin had broad biological functions such as anti-inflammatory, anti-oxidant and anti-tumor effects, and had non-toxic to human body (Strimpakos et al., 2008). Curcumin has been demonstrated to have good inhibition effects on wide variety of tumor cells (Kunnumakkara et al., 2008), such as lung cancer (Wu et al., 2010; Li et al., 2013), liver cancer (Dai et al., 2013, Li et al., 2014), breast cancer (Mo et al., 2012), bladder cancer (Kamat et al., 2009), biliary cancer (Prakobwong et al., 2011) and pancreatic cancer (Kunnumakkara et al., 2007). The postulated antitumor activities of curcumin include induction of tumor apoptosis and inhibition of tumor proliferation, invasion, angiogenesis and metastasis. In addition, curcumin has entered phase III human clinical trials. However, there is still no related success in clinical trials. The clinical application of curcumin has been significantly limited due to its poor bioavailability, instability and fast metabolism *in vivo* (Anand et al., 2007; Sharma et al., 2007). The presence of β-diketone moiety in its structure is the key reason (Liang et al., 2009; Zhao et al., 2013). To our delight, the chemical structure modification of curcumin is an effective approach for obtaining the better bioavailability and anti-tumor activity (Selvam et al., 2005; Ohori et al., 2006; Subramaniam et al., 2008; Xiao et al., 2010). Among these analogs, mono-carbonyl analogs of curcumin (MACs) have been designed, which not only effectively improved the stability, but also exhibited better pharmacokinetic activity *in vivo*.

In our previous work, our team has designed and synthesized three series of MACs, 1, 5-diaryl-1, 4-pentadiene-3-ones, together with cyclohexanone and cyclopentanone analogues, by displacing β-diketone moiety with a single carbonyl group (Zhao et al., 2013), and have screened multiple analogs with better anti-tumor activity, such as B19 (Wang et al., 2011), which induced non-small cell lung cancer cells and ovarian cancer cells apoptosis through the endoplasmic reticulum (ER) stress

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pathway. In subsequent work, we further designed and synthesized MACs, whose central linker was piperid-4-one structural unit, and found that these compounds generally have better anti-tumor activity. Among these compounds, A501 had better effect against NSCLC cell lines than curcumin, it exerted its anti-tumor activity via cycle arrest and induction of apoptosis.

Materials and Methods

Chemical synthesis

The piperidin-4-one (2mmol) and 2, 4-dichlorobenzaldehyde (4 mmol) was dissolved in the mixed solvent of distilled water (2mL) and ethanol (16mL), then 40% sodium hydroxide (catalytic, 0.5mL) was added. The mixture was stirred at 5-8°C for 3h and monitored by the silica gel TLC (thin layer chromatography). When the reaction ended, water was added to precipitate the product. The structure of A501 was shown in Figure 1A. The purification method was carried out by column chromatography using the mixture solvent (petroleum ether : ethyl acetate=2:1) as eluent, and the purity of A501 is greater than 97%. The structures of A501, (3E, 5E)-3, 5-bis (2, 4-dichlorobenzylidene) piperidin-4-one, were characterized by ESI-MS, ESI-HRMS and ^{1}H-NMR. The spectral data of A501 is as follow: brown-yellow power, 63.5% yield, mp 162.6-164.5°C. ^{1}H-NMR ($CDCl_3$), δ: 7.876 (s, 2H, Ar-CH=C×2), 7.479 (d, J=1.8Hz, 2H, Ar-H^3×2), 7.282 (dd, J=1.8, 8.4Hz, 2H, Ar-H^6×2), 7.145 (d, J=7.8Hz, 2H, Ar-H^5×2), 3.969 (s, 4H, CH_2-N-CH_2). ESI-MS m/z: 414.0, 412.1, 416.0 $(M+1)^+$, calcd for $C_{19}H_{13}C_{l4}NO$: 413.12.

In vitro assay, A501 was dissoloved in DMSO, and deployed into different concentrations.

Assay of the stability of compound

0.01 mM of Compound A501 was dissolved in 1mL DMSO and diluted by DMSO to a final concentration of 1mM, then to the separate eppendorf tube, 2μl of 0.01mM solution of A501 and 98μl of phosphate buffer (pH 7.4) was added and mixed well. The OD values from 250 to 600 nm were determined using a spectrum Max M5 (Molecular Devices, USA). Taking 5 minutes as intervals, the absorption curve was recorded for over 25 minutes. The measured setting was at 25°C at varying time interval in a 1 cm path-length quartz cuvette. In this experiments, the decline of absorption spectrum at different moments, meant the degradation of compound, it reflected the stability of compound in a certain degree.

Cell Lines and Reagents

The human NSCLC cell line H460 was purchased from ATCC (Manassas, VA). A549, H1975, HCC827 and normal liver cell line HL7702 were purchased from The Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Atlanta Biologicals Inc., Lawrenceville, GA) and 100 U/mL penicillin and streptomycin (Mediatech Inc., Manassas, VA) at 37°C with 10% CO_2. Apoptosis annexin V kit was purchased from Clotech Bio Company (Mountain View, CA). Antibodies including anti-cyclinB1, anti-cdc-2, anti-p53, anti-caspase-3, anti-bax, anti-bcl-2, anti-GAPDH, goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell proliferation assay

The effect of A501 or curcumin on the proliferation of A549, H460, H1975, HCC827 and HL7702 were determined by 3-[4, 5-dimethylthiazol-2-yl]- 2, 5-diphenyl tetrazolium bromide method (MTT). Briefly, 24h after being cultured in 96-well plate, NSCLC cells and HL7702 cell were treated with A501 or curcumin (0.096, 0.48, 2.4, 12, 60μM) for 72h. After incubation for 72h, cells were treated with the MTT solution for 4 hours. The dark blue formazan crystals formed in intact cells were solubilized with DMSO, and the absorbance was recorded at 490nm using microplate reader.

Clonogenic assay

The clonogenic assay was used to test every cell in a given population for its ability to undergo 'unlimited' division and form colonies. The A549 and H460 cells were treated with 4μM A501 or 4μM curcumin. After 24 hours, cells were transferred to the normal medium and allowed to forming colonies. After 6 days, colonies were stained with 0.5% crystal violet and counted manually.

Flow cytometry

NSCLC cells were cultivated in a 6mm plate for 24h, and then treated with A501 or curcumin or DMSO for 24h and after the treatment period all the cells were collected eventually. For cell cycle arrest, the cells were fixed in 75% ethanol and placed in -20°C for 30mins and then centrifuged in order to get rid of alcohol. Then 500μl of PI dye was added and incubated for 10mins in dark at 4°C and analyzed with FACS flow cytometer. For quantification of apoptotic cells, the cells treated with the drug for 24h were prepared as a suspension in 500μl of cold PBS, centrifuged for 5mins at 1000×g then resuspended in 100μl cold binding buffer and added 3μl Annexin-V FITC and incubated for 10mins at room temperature in dark and then centrifuged at 1000×g for 5mins at room temperature, removed the supernatant and gently resuspended in 500μl of binding buffer and added 1μl PI dye. Flow cytometric analysis was performed using a fluorescence-activated cell sorter (Beckman Coulter, Inc., Fullerton, CA).

Western Blotting

A549 and H460 cells were plated at 6-well plate and cultured for 24h. Then they were treated with A501 or curcumin at different concentrations. After treatment, cells were washed with PBS and suspended in a protein lysis buffer at 0°C. After 10mins, the protein concentration was determined by Bio-Rad protein assay reagent using bovine serum albumin as a standard. Cellular extracts containing equal amounts of proteins were separated by 10-14% SDS-PAGE and transferred to a poly vinylidene difluoride (PVDF) membrane. The membranes were blocked with freshly prepared 5% non-fat milk in TBST for 90mins at room temperature then incubated with

specific antibodies (1:300 or 1:1000) in TBST with gentle shaking for overnight at 4°C. After washing three times with TBST, the membranes were incubated with the goat anti-mouse IgG-HRP or goat anti-rabbit IgG-HRP (1: 3000) for 1 hour at room temperature and washed again. The blots were visualized using chemiluminescence detection kit ECL-PLUS.

Statistical Analysis

All results in this study were performed at least three times. Data are expressed in a mean±standard deviation (SD) manner. Statistical comparisons were analyzed by Student's t-test. $p<0.05$ was considered to be statistically significant.

Results

Assessment of chemical stability of A501

We have analyzed the chemical stability of A501 and curcumin in phosphate buffer (pH 7.4) using an absorption spectrum assay. As shown in Figure 1B, the UV visible absorption spectrum of curcumin displayed an intense peak with an absorption maximum close to 425nm, and the absorption intensity of the curcumin spectrum decreases significantly in phosphate buffer (pH 7.4) with increase in time. Within 25mins of its incubation in phosphate buffer, curcumin lost more than 45% of its original intensity, while A501 degraded much less than curcumin. A501 showed almost complete stability in phosphate buffer in 25mins of incubation period. This result indicated that A501 was much more stable than curcumin *in vitro*.

Anti-proliferation effect of A501 on NSCLC cells

MTT assay was performed to test the anti-proliferative effect of compound A501 on the four strains of NSCLC cells. As shown in Figure 2A, IC_{50} of A501 on these four strains of NSCLC cells were 2.0±0.8, 1.2±0.4, 3.1±0.3 and 5.2±0.3μM respectively, and were found to be significantly less than that of curcumin (17.5±2.7, 17.7±2.1, 26.2±2.1 and 21.3±1.8μM). The effect of A501 and curcumin on normal liver cells HL7702 was also examined with IC_{50} of 10.4±2.8 and 28.7±7.4μM respectively, indicating that the inhibitory effect of A501 on normal liver cells was less than the effect on NSCLC cells.

In accordance with the results of IC_{50}, A549 and H460 cell lines were selected for colony forming experiment to further validate the effects of A501 against proliferation. A501 and curcumin (4μM) were added into the A549 and H460 cells respectively, and cell colonies were counted after a week. The results showed (Figure 2B) that A501 significantly inhibited the cell colony formations, while curcumin at the same concentrations had no significantly affect on the colonies.

A501 induced cycle arrest on NSCLC cell lines

Previous studies have shown that curcumin and its analogues affected the cell cycle changes (Selvendiran et al., 2007; Lin et al., 2008). In order to determine whether A501 can alter cell cycle distribution of NSCLC cells, flow cytometric analysis was performed. The statistics of the cell numbers in G0/G1 phase, S phase and G2/M phase respectively were analyzed after stimulation with compound. Under stimulation with 2μM A501 or curcumin on A549, H460, H1975 and HCC827 respectively, the rate of cells at G2/M phase cells was significantly greater in cells treated with A501 (18.9±9.1, 10.2±2.6, 19.0±0.6, 12.7±0.9μM) than these of curcumin (6.9±3.3, 8.8±4.7, 12.4±4.2, 10.0±3.1μM) (Figure 3A, B). These results revealed that the proliferation inhibition effect of the compound by A501 may be due to appreciable arrest of cells in G2/M phase.

To further examine the detailed mechanism of cell cycle inhibition at the G2/M phase, we carried out western blotting to check the correlation between the effect of A501 or curcumin on the expressions of cycle related proteins such as cyclin B1 and cdc-2. Treatment with A501 (0.5, 1, 2, 4μM) leaded to a dose dependent decrease in the expressions of cyclinB1, cdc-2 in the H460 cells (Figure 3C), and the similar results were observed in A549 (Figure 3D). Curcumin (4μM) had no significant effect on the expression of these proteins. It suggested that G2/M phase arrest of A501 maybe associated with reduced expression of cycle related proteins.

A501 induced apoptosis on NSCLC cell lines

Many studies have shown that curcumin or its

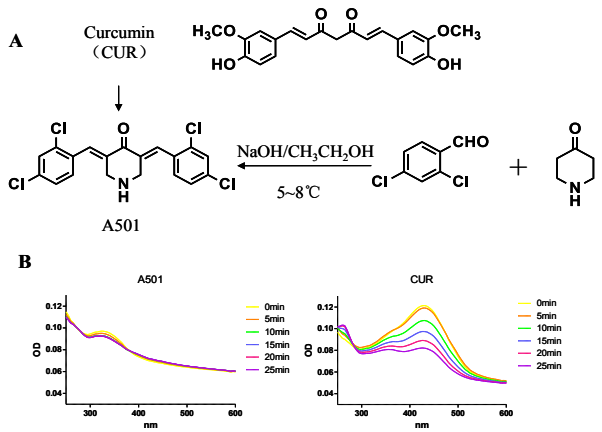

Figure 1. Design, Synthesis and Stability Assay of Compound A501. (A) The structure of A501 and curcumin (CUR) and the chemical synthesis reaction of A501. **(B)** UV-Visible absorption spectra of A501 and CUR

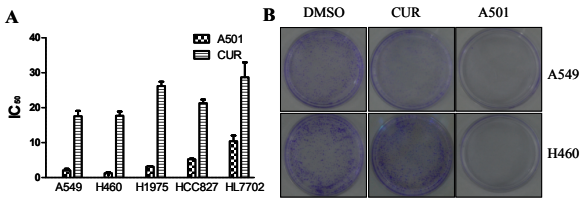

Figure 2. Effects of A501 and CUR on Cell Proliferation of Non-small Cell Lung Cancer Cells. (A) A549, H460, H1975, HCC827 and normal liver cells HL7702 were treated with various amounts of A501 (0.5, 1, 2, 4, 8μM) or curcumin (0.096, 0.48, 2.4, 12, 60μM) for 72h, detected the inhibition rate of tumor cells by MTT, then calculated the IC_{50}. The mean of three values was determined. **(B)** 4μM A501 or curcumin were incubated with A549 and H460 cells for 24 h, then replaced with fresh culture continue to fostered one week, stained by crystal violet and counted colony. DMSO was used as a negative control, the experiment was repeated three times

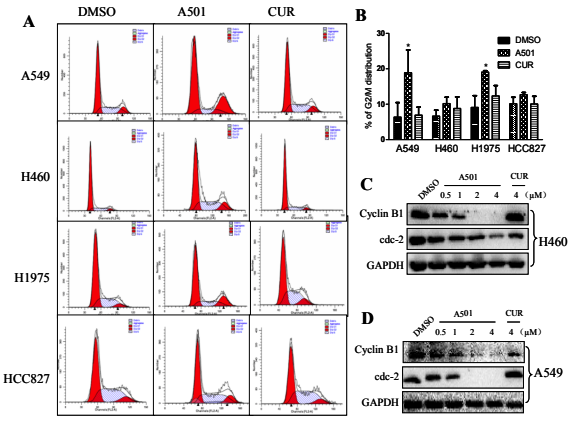

Figure 3. Effects of A501 and CUR on Cell Cycle Arrest of Non-small Cell Lung Cancer Cells. **(A)** A549, H460, H1975 and HCC827 were treated with 2μM A501 or CUR for 24h, detected the cell number of G0/G1 phase, S phase, G2/M phase by flow cytometry. **(B)** Histogram illustrating the rate of G2/M phase from FACS analysis. **(C, D)** Western blotting analysis for cell cycle arrest. cyclin B1 and cdc-2 were shown in H460 and A549 cells treated with various amounts of A501 (0.5, 1, 2, 4μM) or curcumin (4μM) for 24h

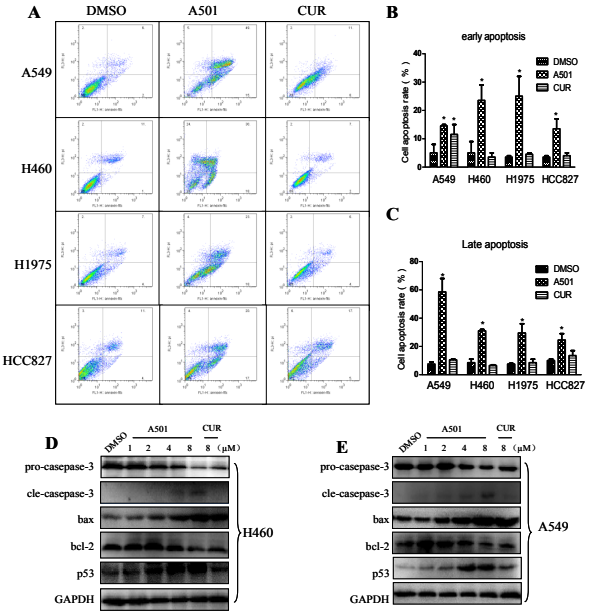

Figure 4. Effects of A501 and CUR on Cell Apoptosis of Non-small Cell Lung Cancer Cells. **(A)** A549, H460, H1975 and HCC827 were treated with 8μM A501 or CUR for 24, then stained with Annexin V-FITC/propidium iodide and detected by flow cytometry. **(B, C)** Histogram illustrating the rate of cell apoptosis from FACS analysis. **(D, E)** Western blotting analysis for cell apoptosis. The expressions of pro-caspase 3, cleaved-caspase 3, p53, bcl-2 and bax were shown in H460 and A549 cells treated with various amounts of A501 (1, 2, 4, 8μM) or curcumin (8μM) for 12h

analogues can induce tumour cells apoptosis (Tong et al., 2006; Selvendiran et al., 2007; Xiao et al., 2010; Prakobwong et al., 2011). Flow cytometry was used to determine whether the compound A501 can induce apoptosis of NSCLC cells. Annexin-V/PI staining was performed to determine early and late apoptotic cells followed by treatment with 8μM of A501 and curcumin respectively on NSCLC cells (A549, H460, H1975 and HCC827). As shown in Figure 4A, B and C, early and late apoptotic rates of tumor cells induced by the A501 group were significantly higher than those of vehicle and curcumin-treated cells, which indicated the induction of apoptosis by compound A501.

In the process of cell apoptosis, some family proteins play very important roles, such as bcl-2 family and caspases family. Some drugs can down-regulate bcl-2 and up-regulate bax, thereby activate caspase cascade reaction, ultimately leaded to cell morphological changes and apoptosis. Research has found that curcumin inhibited cancer cell growth through the regulation of bcl-2/bax and affect the mitochondrial apoptosis pathway (Li et al., 2013). Curcumin also exerted anti-cancer effects by inducing apoptosis via the caspase signaling pathway (Li et al., 2014). To find out whether induction of apoptosis by A501 via modulating apoptosis related proteins in NSCLC cells, we examined the expression of pro-caspase-3, cleaved caspase-3, p53, bcl-2 and bax.

Similarly, in the present study, we found significant changes have emerged in apoptotic morphology of H460 cells treated with compound for 12h. As shown in Figure 4D, pro-caspase-3 expression was reduced along with the increase in drug concentration of A501, while cleaved caspase-3 was appeared at high concentrations of A501. With the treatment by A501, the expression of p53 and bax were also increased in a dose-dependent manner, while the expression of anti-apoptosis protein bcl-2 was decreased. Curcumin (8μM) had no significant effect on the expression of these proteins. A501 exhibited the similar results on the A549 cells (Figure 4E).

Discussion

NSCLC is one of the highest incidence of malignant tumors around the world, and the worldwide incidence and mortality of NSCLC have been on the rise, therefore, the clinical development of new therapies for NSCLC was urgent needed, such as some new therapy drugs based on natural products (Aggarwal et al., 2006; Pal et al., 2010). The development of such a therapy is a long-term objective and hotspot of the present study.

Curcumin is a natural product extracted from curcuma longa, some researchers have shown that curcumin play a protective role on a variety of diseases. In some clinical trials, curcumin has also been shown to be nontoxic, but its poor bioavailability limit its clinical application. Previous studies have found that the stabilities of MACs were greatly enhanced *in vitro* and their pharmacokinetic profiles were also significantly improved *in vivo* (Liang et al., 2009; Liu et al., 2014). We found that analogue A501 also had a higher stability than curcumin. In addition , A501 had a good inhibitory effect against NSCLC cell lines, the IC_{50} of A501 on NSCLC cells achieved micromol/L level, which reached the ideal level of active MACs (Zhao et al., 2013), whereas the antitumor activity of curcumin was weaker than A501.

The growth of cells involves a series of procedures, wherein the cell cycle arrest and apoptosis play a very

important role in the survival of the cell growth process. Several studies have indicated that cell cycle arrest and induction of apoptosis of cancer cells by curcumin and its analogues might be an important mechanism responsible for the impact on tumor cell viability. Our study further evidenced, A501 could cause cell cycle arrest and apoptosis. This indicated that A501 has a good pharmacological effect. Curcumin can cause tumor cells cycle arrest through inhibiting nuclear factor-κB (NF-κB) function and regulating some cycle related genes and proteins expression (Nagai et al., 2005), such as mdm-2 (Selvendiran et al., 2007), cdc-2, cyclin D1 and some cell survival proteins (Prakobwong et al., 2011). Our results indicated that A501 can reduce the expression of cyclinB1, cdc-2 in a dose-dependent manner, and the effect of A501 is better than that of curcumin at the same concentration.

Mitochondrial pathway is an important pathway to leading cell apoptosis, and it would be activated under the drug treatment. In this cascade, bcl-2 was suppresed, and bax was released, resulting in the discharge of cytochrome C, and caspase cascade activation, and then cell apoptosis. It had been reported that curcumin analogue could induced apoptotis through mitochondrial membrane depolarization in prostate cancer cells, with up-regulation of bax and down-regulation of bcl-2 proteins and the activation of caspase 3 (Valentini et al., 2009; Dai et al., 2013). Our research showed that A501 can inhibit the expression of bcl-2 and promote bax protein expression, thus promote cell apoptosis. At the same time, Caspase-3 was activated in a dose-dependent manner in lung cells after treated with A501. Curcumin had no significant effect at the same concentration. A501 was more efficacious than curcumin in the induction of caspase-3. This suggested that A501 may induce apoptosis via a pathway mediated by a caspase-dependent signaling pathway.

In conclusion, our study reported a new curcumin analogue A501 which had a better stability than curcumin. A501 resulted in G2/M arrest by down-regulating the expression of cycle related protein, and induced the apoptosis by reducing the expression of anti-apoptosis protein and increasing the expression of apoptosis protein. A501 exhibited a stronger anti-cancer effect *in vitro* than that of curcumin. Our study indicated that A501 might be a potent and promising agent to combat NSCLC, and deserves further evaluation and development. Further studies are needed to clarify its bioavailability and antitumor *in vivo*.

Acknowledgements

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