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Original Article

Synergistic antitumor efficiency of docetaxel and curcumin against lung cancer

Haitao Yin[1]†, Rui Guo[2]†, Yong Xu[3]†, Yulong Zheng[3], Zhibo Hou[1], Xinzheng Dai[4], Zhengdong Zhang[5], Donghui Zheng[3]*, and Hua'e Xu[6]*

[1]Department of Oncology, the Affiliated Zhongda Hospital of Southeastern University, Nanjing 210009, China
[2]Department of Ophthalmology, the First Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing 210029, China
[3]Department of Nephrology, Huai'an Hospital Affiliated to Xuzhou Medical College and Huai'an Second Hospital, Huai'an 223002, China
[4]Department of General Surgery, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
[5]Department of Occupational Medicine and Environmental Health, Nanjing Medical University, Nanjing 210029, China
[6]Department of Pharmacy, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
†These authors contributed equally to this work.
*Correspondence address. Tel: +86-517-83668282; Fax: +86-517-83668282; E-mail: zygrsw@yahoo.com.cn (D.Z.)/Tel: +86-25-68136979; Fax: +86-25-68136979; E-mail: xuhuae@yahoo.com.cn (H.X.)

Curcumin (Cum), the principal polyphenolic curcuminoid, obtained from the turmeric rhizome *Curcuma longa*, is recently reported to have potential antitumor effects *in vitro* and *in vivo*. Docetaxel (Doc) is considered as first-line chemotherapy for the treatment of non-small cell lung cancer. Here we report for the first time that Cum could synergistically enhance the *in vitro* and *in vivo* antitumor efficacy of Doc against lung cancer. In the current study, combination index (CI) is calculated in both *in vitro* and *in vivo* studies to determine the interaction between Cum and Doc. In the *in vitro* cytotoxicity test, media-effect analysis clearly indicated a synergistic interaction between Cum and Doc in certain concentrations. Moreover, *in vivo* evaluation further demonstrated the superior anticancer efficacy of Cum + Doc compared with Doc alone by intravenous delivery in an established A549 transplanted xenograft model. Results showed that Cum synergistically increased the efficacy of Doc immediately after 4 days of the initial treatment. Additionally, simultaneous administration of Cum and Doc showed little toxicity to normal tissues including bone marrow and liver at the therapeutic doses. Therefore, *in vitro* and *in vivo* evaluations demonstrated the satisfying synergistic antitumor efficacy of Cum and Doc against lung cancer and the introduction of Cum in traditional chemotherapy is a most promising way to counter the spread of non-small cell lung cancer.

Keywords docetaxel; curcumin; synergistic interaction; lung cancer

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Introduction

Lung cancer is one of the most common serious respiratory cancers. It is reported that an estimated 215,020 new cases of lung cancer are expected in 2008, accounting for ~15% of cancer diagnoses [1]. Moreover, lung cancer accounts for the most cancer-related deaths in both men and women [2].

Docetaxel (Doc) has demonstrated extraordinary anticancer effects *in vitro* and *in vivo* against a variety of tumors, including lung, ovaries, breast cancers, etc. [3–5]. As recommended by the National Comprehensive Cancer Network, Doc is considered as first-line chemotherapy for the treatment of non-small cell lung cancer [6]. However, chemotherapeutics sometimes lead to severe toxicity at their therapeutic dose even though the response rate of single drug chemotherapy remains <20% [7]. In order to achieve higher antitumor efficacy and minimize the emergence of resistance, to search novel chemotherapy sensitizers become the focus in the field of cancer therapy.

Recent advances in the research of traditional Chinese medicine paved the way in the discovery of novel adjunct to chemotherapy. Curcumin (Cum), the principal polyphenolic curcuminoid, obtained from the turmeric rhizome *Curcuma longa*, has been reported for its potential chemopreventive and chemotherapeutic activity through influencing the various aspects, including cell cycle arrest, differentiation, and apoptosis in a series of cancers [8,9]. For example, *in vitro* and *in vivo* experiments showed Cum could inhibit skin squamous cell carcinoma growth and block tumor progression [10]. In addition, there are other evidences that Cum effectively delays uterine leiomyosarcoma cells' growth through the protein kinase B-mammalian target of

rapamycin pathway, targets cell cycle, and promotes cell apoptosis to suppress malignant pleural mesothelioma growth *in vitro* and *in vivo* [11,12].

Some small molecules from traditional Chinese medicine, such as tetrandrine and gambogic acid, are able to restore Doc sensitivity in gastric cancer cells by inhibiting the expression of drug associated genes that is involved in Doc resistance [13,14]. Moreover, emerging data demonstrate the potential of Cum as chemosensitizers. Previous reports indicated that Cum could enhance adriamycin-induced human liver-derived hepatoma G2 cell death through activation of mitochondria-mediated apoptosis and autophagy [15]. Sensitization of head and neck cancer to cisplatin is achieved by the application of Cum [16]. These findings showed the potential of Cum to be a novel adjunct to chemotherapy.

The current study aims to investigate whether Cum could enhance the antitumor efficiency of Doc in the treatment of lung cancer. To assess the potential anticancer efficacy of Cum and Doc, A549 cells were used to test the *in vitro* cytotoxicity. Meanwhile, the *in vivo* antitumor efficiency of Cum and Doc was evaluated by intravenous delivery in the A549-xenograft model. These mice were sacrificed to detect the influence of the two drugs on the peripheral blood parameters and liver and kidney functions.

Materials and Methods

Materials and animals

Doc was kindly provided by Jiangsu Hengrui Pharmaceutical Co. Ltd (Lianyungang, China). Cum was purchased from Sigma (St Louis, USA). All other chemicals were of analytical grade and used without further purification. Human lung cancer cell line A549 was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China).

Male and female nude mice (nu/nu; 6–8 weeks old and weighing 18–22 g) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The mice were housed and maintained in the animal facility of the Animal Center of Nanjing Medical University (Nanjing, China). The animal protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Cell lines and cell culture

Cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 100 U/ml penicillin–streptomycin at 37°C in a water-saturated atmosphere with 5% CO_2. Tumor cells growing in log-phase were trypsinized, seed at 2×10^3 cells/well into 96-well plates, and allowed to attach overnight. Cells were then treated with a series of drugs for 48 h. The medium-containing drug was decanted and the IC_{50} doses of each drug were determined by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described below. Each experiment was allocated 10 wells containing drug-free medium for the control and performed at least three separate occasions.

In vitro cytotoxicity studies

Cytotoxicity of Cum against A549 cells was assessed by MTT assay. Briefly, cells were seeded in 96-well plates with a density ~5000 cells/well and allowed to adhere for 24 h prior to the assay. Cells were exposed to a series of doses of Cum and Doc at 37°C. After 48 h of incubation, 50 μl of MTT indicator dye (5 mg/ml in phosphate-buffered saline, pH 7.4) was added to each well. Then the cells were incubated for 2 h at 37°C in the dark. The medium was withdrawn and 200 μl acidified isopropanol (0.33 ml HCl in 100 ml isopropanol) was added in each well, followed by agitating thoroughly to dissolve the formazan crystals. The solution was transferred to 96-well plates and immediately read on a microplate reader (Bio-Rad, Hercules, USA) at a wavelength of 490 nm. Absorption was measured at 550 nm in Microkinetics reader BT2000 (BioTek Instruments, Winooski, USA) and growth inhibition was calculated as a percentage of the controls, which were not exposed to drugs. All experiments were repeated three times.

Determination of synergism and antagonism *in vitro*

Subconfluent A549 cells were seeded at 2×10^3 cells/well in 96-well plates. Drugs (Cum and Doc) were added concomitantly with seven different concentrations of the single agents and six different concentrations of both agents at their fixed ratio based on their respective individual IC_{50} values for 48 h. The fractional inhibition of cell proliferation was calculated by comparison to control cultures. Dose–response curves were obtained for each drugs, and for multiple dilutions of a fixed-ratio combination of the two drugs.

Median effect analysis using the combination index (CI) method of Chou and Talalay [17] was used to determine the interaction between Cum and Doc. The CI value is defined by the following equation: $CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2 + \alpha(D)_1(D)_2/(Dx)_1(Dx)_2$, in which $(Dx)_1$ and $(Dx)_2$ are the concentrations for D_1 (Cum) and D_2 (Doc) alone that gives x% inhibition, whereas $(D)_1$ and $(D)_2$ in the numerators are the concentrations of Cum and another drug that produce the identical level of effect in combination. $\alpha = 0$, when the drugs are mutually exclusive (i.e. with similar modes of action); while $\alpha = 1$, they are mutually non-exclusive (i.e. with independent modes of action). $CI > 1$ indicates antagonism, $CI < 1$ indicates synergy, and $CI = 1$ indicates additivity. Each CI ratio represented here is the mean value derived from at least three independent experiments. The *in vitro* drug-induced cytotoxic

effects were measured by the MTT reduction assay as mentioned above.

Analysis of synergism or antagonism of the two drugs *in vivo* and toxicity test

Nude mice implanted with A549 cells were used to qualify the antitumor efficacy of Cum and Doc, alone or in combination, through intravenous administration. The mice were raised under specific pathogen-free circumstances and all of the animal experiments were performed in full compliance with guidelines approved by the Animal Care Committee of Nanjing Medical University. The mice were subcutaneously injected at the left axillary space with 0.1 ml of cell suspension containing $4-6\times10^6$ A549 cells. Treatments were started after $7-8$ days of implantation. The mice whose tumor reached a tumor volume of 100 mm^3 were selected and this day was designated as 'Day 0'.

On Day 0, the mice were randomly divided into four groups, each group having six mice. The mice were treated intravenously with Cum and Doc, respectively, or in combination. Doc was administered at doses of 10 mg/kg. Cum was administered at doses of 15 mg/kg. All mice were tagged, and tumors were measured every other day with calipers during the period of study. The tumor volume was calculated by the formula (tumor volume $= W^2\times L/2$), where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point. Relative tumor volume (RTV) was calculated by the formula ($RTV = V_n/V_0$), where V_n is the tumor volume measured at the corresponding day, and V_0 is the tumor volume measure at Day 0. Another antitumor indicator is $T/C\%$ (tumor inhibition rate, TIR), which was calculated by the formula ($TIR = T_{RTV}/C_{RTV}$), where T_{RTV} is RTV of the experimental group, and C_{RTV} is RTV of the control group.

The combination index (Q) method reported previously was used to determine the interaction observed between Cum and Doc *in vivo* [18]. It is defined by the following equation: $Q = TIR_{A+B}/(TIR_A + TIR_B - TIR_A\times TIR_B)$, where TIR_{A+B} is the TIR of the combinational group, TIR_A is the TIR of the group receiving drug A and TIR_B is the TIR of the group receiving drug A. $Q < 0.85$ indicates antagonism, $Q > 1.15$ indicates synergy, and $0.85 < Q < 1.15$ indicates additivity.

Each animal was weighed at the time of treatment so that dosages could be adjusted to achieve the mg/kg amounts reported. Animals also were weighed every other day throughout the experiments. After 15 days of injections, the mice were sacrificed for the detection of peripheral blood parameters as well as liver and kidney functions.

Statistical analysis

Results were presented as the mean $\pm$ SD. Statistical comparisons were made by Student's t-test or analysis of variance analysis. The P value <0.05 was considered as significantly different.

Results

Cytotoxicity of Cum and Doc against A549 cells and the synergistic effects of the two drugs

According to the IC_{50} of Cum or Doc, sequential doses of Cum and Doc were applied singly and simultaneously to explore whether Cum could enhance the anticancer effects of Doc. The combination ratios were designed to approximate the IC_{50} ratios of the individual component compounds, so that the contribution of antiproliferative effect for each compound in the combinations is roughly the same [19].

It is obvious to locate three dose–response curves corresponding to single and combinational application of Cum or Doc. Clearly, cells exposed to combinational administration of Cum and Doc underwent more death than single exposure to Cum or Doc. It is indicated in **Fig. 1(A)** that Cum and Doc inhibited cell growth with a dose-dependent manner against A549 cells. The IC_{50} of Cum was 10.25 $\pm$

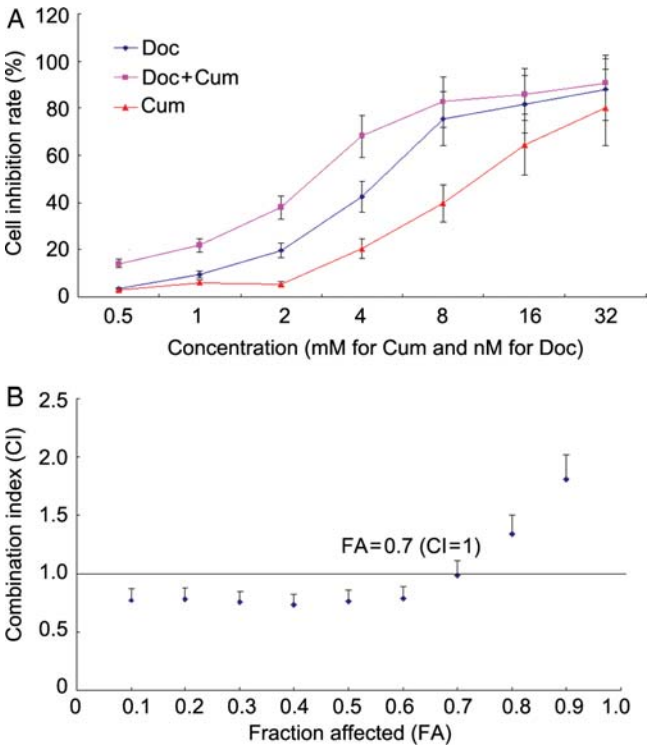

Figure 1 Analysis of synergy between Doc and Cum against A549 cells (A) Dose–response curve of Doc and Cum against A549 cells. (B) CI values at different level of growth inhibition effect (FA). Experiments were done at least three independent times.

1.03 μM while the IC_{50} of Doc was 4.26 ± 0.51 nM against A549 cells (Table 1). Moreover, the IC_{50} of Cum + Doc against A549 cells were 2.81 ± 0.27 μM (Cum) and 2.81 ± 0.34 nM (Doc), respectively, (Table 1). To fully evaluate the interaction between Cum and Doc, we analyzed the combination of drugs using media-effect analysis, which resolves the degree of synergism, additivity, or antagonism at various levels of cell death.

Figures 1 and 2 illustrated the multiple drug effect for A549 cells that were treated simultaneously with Cum and Doc and represented as fractional cell growth inhibition (fraction affected, FA). The combination of two drugs generated more cell death than the drugs used singly. The cell inhibition rate of ~40% was detected when exposed to the combination of Cum (2 μM) and Doc (2 nM) while an inhibition rate of <10% or 20% was observed when cells were treated by the same dose of Cum or Doc, respectively. More obviously, combinational treatment of 0.5 μM Cum and 0.5 nM Doc induced a nearly 20% cell death while <5% cell death was observed when Cum or Doc was administered at the same dose singly [**Fig. 1(A)**]. CI analysis indicated that CI values were below 1 when FA was <0.7 [**Fig. 1(B)**]. According to Chou and Talalay [17], media-effect analysis demonstrated a synergistic anticancer effect of Cum and Doc in certain concentrations.

Table 1 The IC_{50} values of Cum or Doc against A549 cells

Group	IC_{50} values
Cum	10.25 ± 1.03 μM
Doc	4.26 ± 0.51 nM
Cum + Doc	2.81 ± 0.27 μM (Cum)/2.81 ± 0.27 nM (Doc)

Data were represented as the mean ± SD.

In vivo antitumor evaluation of Cum and Doc, singly or in combination, against A549 xenograft and influence of Cum and Doc, singly or in combination, on peripheral blood parameters

Antitumor efficacy of Cum and Doc, when delivered singly or in combination, was investigated in A549 human lung cancer xenografts in nude mice. The focus of this work was to evaluate whether the combination delivery of

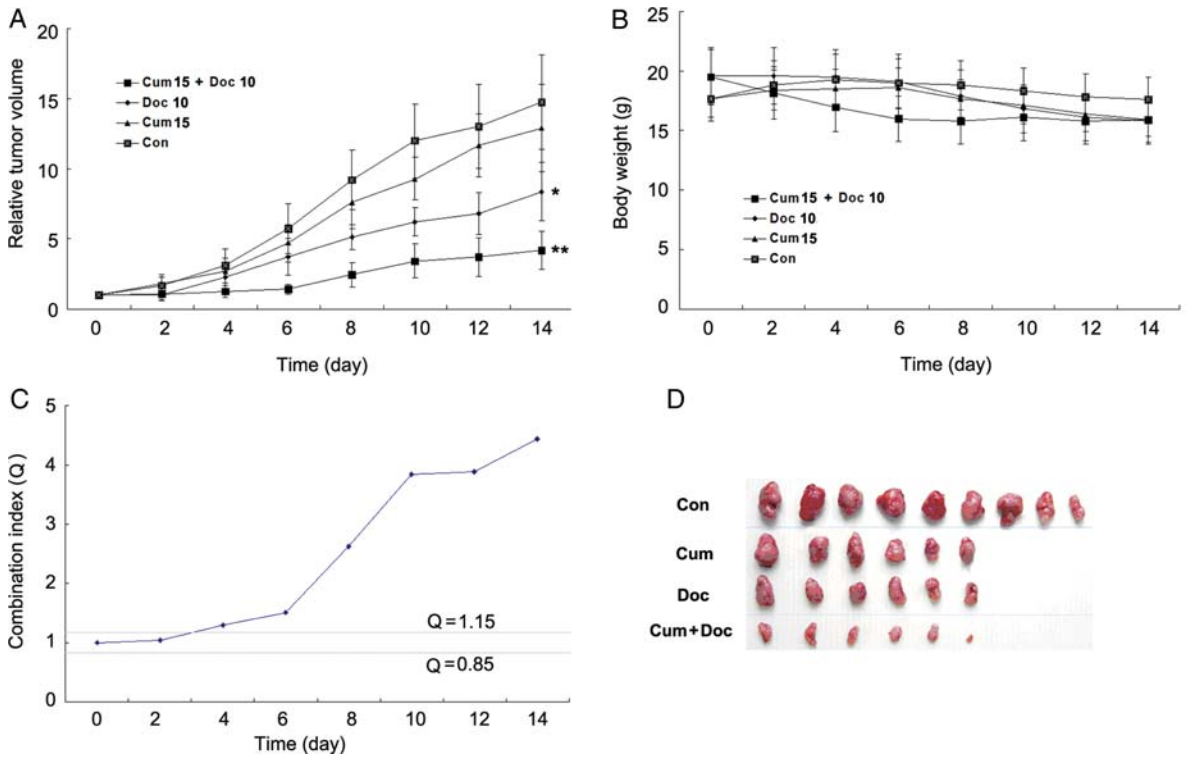

Figure 2 Variation of tumor volume, body weight, CI, and tumor images of mice established A549 xenografts during therapy with different doses of Doc or Cum (A) Tumor volume of established A549 xenografts in nude mice during therapy under different treatments. Mice were treated with different protocols on Day 0 as showed in the figure. The mice were treated intravenously with Cum and Doc, respectively, or in combination. Doc was administered at doses of 10 mg/kg. Cum was administered at doses of 15 mg/kg. Different agents were delivered through intravenous pathway when tumor volume measured 100 mm^3. Data are presented as the mean ± SD ($n = 6$). *$P < 0.05$ vs. the control group. **$P < 0.05$ vs. the group treated with 10 mg/kg Doc. (B) Body weight change of nude mice with different treatments during therapy. Data are presented as the mean ± SD ($n = 6$). (C) The interaction of combined therapy determined by the Q values. (D) The images of excised tumors at the time of sacrifice from the subcutaneous A549 lung cancer xenograft-bearing male nude mice after 15 days of single dose therapy.

Table 2 Tumor growth inhibition effect of Doc and Cum against A549 xenografts

Group	Dose (mg/kg)	Tumor volume (mm^3)		RTV ($x \pm$ SD)	*T*/*C* (%)	*P* value
		Day 0	Day 14			
Control	–	215 ± 67	2935 ± 874	14.8 ± 6.8	–	–
Cum	15	142 ± 22	1840 ± 991	12.9 ± 6.5	87.2	0.09
Doc	10	159 ± 44	1235 ± 384	8.3 ± 3.3	56.4	0.03
Doc + Cum	10 + 15	143 ± 25	574 ± 132	4.2 ± 1.4*	28.3*	0.001

*$P < 0.05$ vs. the group of Doc.

Table 3 Influence of Doc and Cum on peripheral blood parameters

Group	WBC (10^9/l)	RBC (10^9/l)	Hb (g/l)	Plt (10^9/l)
Control	24.3 ± 4.3	7.9 ± 1.3	111.3 ± 13.2	153.3 ± 22.4
Cum	24.2 ± 3.1	7.3 ± 1.4	109.2 ± 15.9	142.5 ± 20.2
Doc	26.5 ± 2.9	7.6 ± 1.7	89.7 ± 10.5*	105.5 ± 15.3*
Doc + Cum	23.9 ± 3.9	7.5 ± 0.9	119.6 ± 18.5	151.3 ± 17.8

*$P < 0.05$ vs. saline.

Table 4 Influence of Doc and Cum on liver (ALT) and kidney (BUN, CRE) parameters

Group	Alanine aminotransferase (ALT) (U/l)	Blood urea nitrogen (BUN) (mM)	Creatinine (CRE) (mM)
Control	49.3 ± 11.1	7.9 ± 1.5	35.6 ± 6.7
Cum	50.4 ± 12.5	7.7 ± 2.1	41.7 ± 5.9
Doc	97.7 ± 16.8*	8.3 ± 3.8	39.7 ± 7.1
Doc + Cum	51.3 ± 14.6	7.4 ± 0.9	47.8 ± 8.6

*$P < 0.05$ vs. saline.

Cum and Doc could generate superior antitumor efficiency compared with single administration of either drug.

Tumor growth curves showed that 15 mg/kg of Cum could hardly inhibit the growth of lung cancer ($P = 0.09$ vs. control) while 10 mg/kg of Doc delayed tumor growth moderately ($P = 0.03$ vs. control). The combination of Cum and Doc inhibited the growth of tumor more efficiently than single delivery of Cum or Doc ($P = 0.01$ vs. Doc) [**Fig. 2(A)**]. As shown in **Table 2**, RTV and *T*/*C* analysis confirmed that groups Doc or Doc + Cum demonstrated significantly higher antitumor efficiency. The group treated with the combination of Doc and Cum was observed to maintain the greatest amount of antitumor activity [**Fig. 2(A)** and **Table 2**]. At the end of treatment, the RTV and *T*/*C*% was 4.19 ± 1.39 and 28.3%, which was the lowest among all the groups indicating the strongest tumor inhibition. Statistical analysis revealed the significant differences between the group treated with Doc + Cum and the group treated with Doc or Cum, respectively. As shown in **Fig. 2(C)**, CI analysis indicated that Cum could synergistically enhance the antitumor efficacy of Doc since 4 days after initial treatment ($Q > 1.15$). **Figure 2(D)** showed the shrinkage of tumors among the different treatment groups. It could be observed clearly that the tumors from the mice treated with Doc + Cum were obviously smaller than those of other groups.

An analysis of body weight variations generally defined the adverse effects of the different therapy regiments [**Fig. 2(B)**]. No significance was observed between either two groups.

Cum had no adverse effect on the levels of peripheral blood parameters. On the contrary, 10 mg/kg Doc induced significant reduction of hemoglobin (Hb) (89.7 ± 10.5 g/l) ($P = 0.03$) and platelet (Plt) (105.5 ± 15.3 × 10^9/l) ($P = 0.02$) while Cum + Doc showed little influences on white blood cell and Hb (**Table 3**). Determination of liver parameters featured the abnormality of liver function with increased alanine aminotransferase (ALT) (97.7 ± 16.8 U/l) in mice treated with Doc alone, while in Cum + Doc treated mice showed no liver function damage (**Table 4**). In addition, Cum and Doc, no matter delivered singly or in combination, showed no side-effect on the kidney function because the parameters remained within normal ranges (**Table 4**).

Discussion

Here we report for the first time that Cum could substantially enhance the antitumor efficiency of Doc *in vitro* and *in vivo*, against lung cancer. Recently, traditional Chinese medicine attracted intensive interests for their potential antiproliferative properties and low toxicities. Moreover, several studies reported that these compounds, such as tetrandrine, gambogic acid, genistein, and emodin, are capable to sensitize cancer cells to chemotherapeutic agents [15,16,20,21]. Previous studies have demonstrated the potential anticancer effect of Cum *in vitro* and *in vivo* [10–14].

In the current report, combinational index is calculated in both *in vitro* and *in vivo* studies to determine the interaction between Cum and Doc [17,18]. In the *in vitro* cytotoxicity test, media-effect analysis clearly indicates a synergistic interaction between Cum and Doc in certain concentrations. Moreover, *in vivo* test shows that Cum significantly increases the efficacy of Doc immediately after 4 days of the initial treatment. The calculated Q value remains >1.15 from Day 4 to Day 14. Therefore, *in vitro* and *in vivo* evaluation demonstrates the satisfying synergistic antitumor efficacy of Cum and Doc against lung cancer, which provides the probability of Cum as potential chemotherapy sensitizer for clinical application.

Possible mechanisms underlying the synergistic antitumor effect of Cum and Doc may be related to the reactive oxygen species (ROS) and its downstream pathways. As reported in recent study, resistance to Taxols is proportional to cellular total antioxidant capacity [22]. It means that induction of ROS can enhance the cytotoxicity of Taxols. Moreover, there are evidences that Cum could lead to A549 cell apoptosis through an ROS-dependent mitochondrial signaling pathway [23]. Therefore, the probability emerges that induction of ROS by Cum may increase the cytotoxicity of Doc. The following studies are ongoing in our lab.

Moreover, simultaneous administration of Cum and Doc shows little toxicity to normal tissues including bone marrow and liver at its therapeutic dose. **Table 2** clearly indicates that Doc alone significantly lowers the level of Hb and Plt while increases ALT. On the contrary, Cum alone shows no obvious toxicity in our experiments. Most importantly, no severe toxicity is defined when the two drugs are simultaneously delivered through intravenous pathway. It implies that Cum could reverse effectively the toxicity of Doc.

Further study is warranted to confirm the possible mechanisms of Cum in reducing the side effect of Doc. Planned modifications with the current study are under active consideration as a part of this ongoing research. In addition, further development in searching for other small molecules with the potential of enhancing chemotherapy will be more fully reviewed in order to further expand the parameters of this current research. Further emphasis has been placed on specific elucidation in the possible mechanisms underlying the synergistic antitumor efficiency of Cum and Doc, which is still under active current review and requires further study. Therefore, introduction of Cum in traditional chemotherapy is a most promising way in countering the spread of non-small cell lung cancer, and continuing research will definitely advance the current study.

In conclusion, results from this work not only confirm the potential role of Cum in treating non-small cell lung cancer but also offer an effective way to improve the anticancer efficiency of Doc. Additionally, since other kinds of traditional Chinese medicine also possess antitumor effects, they could be potential drug sensitizers for the application of first-line chemotherapeutics. It is undoubtedly, however, that the development of traditional Chinese medicine as drug sensitizers warrants more intensive research in order to evaluate the feasibility and advantages of clinical applications.

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