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# Synergistic antitumor effect of curcumin and dinitrosyl iron complexes for against melanoma cells

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ABSTRACT

The aim of this study was to examine whether combining curcumin, a chemoprevention agent, and dinitrosyl iron complexes (DNICs) would have a synergistic cytotoxic effect on mouse melanoma B16-F10 cells in vitro. Three synthesized DNICs-[PPN] $[(NO)_2Fe(SCH_2CONHCH_3)_2]$ (**NC01**), [PPN] $[(NO)_2Fe(SCH_2CON(CH_3)_2)_2]$ (**NC02**), and [Na]$[(NO)_2Fe(SCH_2CON(CH_3)_2)_2]$ (**NC03**) were tested in this study. In vitro DNA cleavage assay showed all three DNICs could cause plasmid DNA damage through releasing NO under UV irradiation. The cytotoxicity assay demonstrated these DNICs were toxic to B16-F10 cells in vitro, and the estimated values of $LD_{50}$ (24 h of incubation) of **NC01** and **NC02** were 1 μM, while the values of $LD_{50}$ of **NC03** was 200 μM. No synergistic cytotoxicity effect was noted in the treatments of the combinations of curcumin and DNICs. On the contrary, in the presence of **NC03**, the toxicity of curcumin was reduced. Using UV–Visible spectroscopy and fluorescence microscopy, we found **NC03** might interact with curcumin and reduce the accumulation of curcumin in cells. Further experiments using the pretreatment of curcumin for 4 h followed by the treatment of **NC03** showed the synergistic cytotoxic effect, while, the pretreatment of **NC03** followed by the treatment of curcumin did not have any effect. This study provides the basis for further investigation on the effects of combinations of curcumin and other NO donors.



## 1. Introduction

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione], is a polyphenolic compound extracted from the rhizomes of the Indian spice turmeric (*Curcuma longa*). For a long time, curcumin has been used as a traditional medicine to treat abdominal spasms, diarrhea, fever, headache and vomiting. It is also sold as a dietary herbal supplement. Recently, the biological and pharmaceutical properties of curcumin such as antitumor [1], antioxidant [2–5], and anti-inflammatory [6–8] have been demonstrated. The toxicity of curcumin to microbes [9,10], parasites [3,11–13], and zebrafish [14] has also been revealed. However, the use of curcumin as a therapeutic agent has not yet been approved [15]. The major problem of curcumin being used clinically is curcumin has relatively low bioavailability because of its low aqueous solubility and highly instability under light exposure. Moreover, curcumin is poorly absorbed through oral administration [16,17]. The studies using rats as animal models have shown most orally administered curcumin is excreted in the feces, with only trace amount of unchanged curcumin found in the blood [18–20].

Strategies such as preparation of new synthetic curcumin analogues, new formulations, and combinations of curcumin and other drugs have been used to improve the bioavailability of curcumin [15,21]. Dinitrosyl iron complexes (DNICs), the endogenous nitroso compounds are known as S-nitrosothiols (RSNO) as two possible naturally occurring forms for storage and delivery of NO in biological system [22,23]. They release NO spontaneously regulated by the environment such as ligation mode of iron or under light exposure [24,25]. Increasing evidence indicates NO has multiple physiologic and pathologic effects. In carcinogenesis, for example, NO plays two opposite roles of promoting or inhibiting the growth of cancer cells, according to its concentration. Exposure to lower NO concentrations (1–100 nM) results in increasing angiogenesis and proliferation of endothelial cells [26]. In addition, at this concentration range of NO, the activity of anti-apoptotic genes in tumors is also increased, which might protect the tumor cell from apoptosis [27–30]. At NO concentrations greater than 300 nM, phosphorylation of p53 is increased which leads to apoptosis [27–30]. Also,

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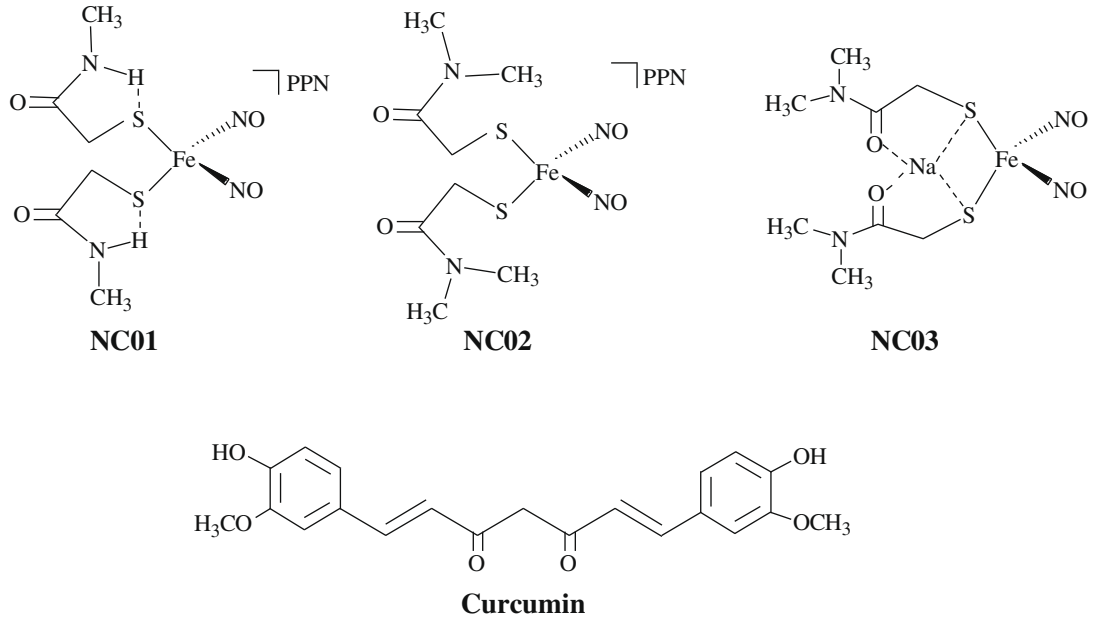


**Fig. 1.** The chemical structures of **NC01**, **NC02**, **NC03** and curcumin.

these higher levels of NO promote nitrosation of amines, thiols, and tyrosines of proteins, and result in inhibition of enzyme activities, subsequently, leading to cell death [31,32].

Here, we reported the cytotoxicity of three DNICs [PPN] $[(NO)_2Fe(SCH_2CONHCH_3)_2]$ (**NC01**), [PPN] $[(NO)_2Fe(SCH_2CON(CH_3)_2)_2]$ (**NC02**), and [Na]$[(NO)_2Fe(SCH_2CON(CH_3)_2)_2]$ (**NC03**) (Fig. 1) alone or combined with curcumin (Fig. 1) against mouse melanoma B16-F10 cells. We found no benefit from the co-treatments of DNICs and curcumin, however, the pretreatment of curcumin following the treatment of **NC03** showed a synergistic antitumor effect against B16-F10 cells in vitro.

## 2. Experimental

### 2.1. Chemicals

**NC01**, **NC02**, and **NC03** were synthesized as previously described [33]. Dimethyl sulfoxide (DMSO) and curcumin were purchased from Sigma (St. Louis, USA). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) was purchased from Merck (Darmstadt, Germany). pBR322 plasmid DNA was purchased from Promega (Madison, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin–EDTA, penicillin and streptomycin were purchased from Gibco BRL (Grand Island, USA).

### 2.2. DNA cleavage

The plasmid DNA cleavage reactions were performed by incubating 50 ng pBR322 plasmid DNA with **NC01** (20 μM), **NC02** (10 μM), or **NC03** (20 μM), and various concentrations of PTIO (0, 0.2, 2, 5, and 10 mM) in 10 μl reaction buffer (10 mM Tris–HCl, pH 7.4). The reaction mixtures were exposed to UV light of 312 nm for 5 min. After exposure, a stop solution containing 1 μl KCN (1 M) and 1 μl EDTA (0.04 M) was added into each reaction mixture. The reaction mixtures were incubated at 60 °C for 30 min. After incubation, nicked circular and supercoiled plasmid DNA in each reaction mixture was separated on a 1% agarose gel by electrophoresis, and visualized by ethidium bromide staining. The images of stained gels were pictured by a digital camera.

### 2.3. Cell culture and MTT assay

Mouse melanoma B16-F10 cells were purchased from the Culture Collection and Research Center (Hsinchu, Taiwan). The cells were seeded in tissue culture flasks (Nunc, 25 $cm^2$) containing DMEM, 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. The flasks were maintained at 37 °C in a humidified 5% $CO_2$ incubator. For evaluating the cytotoxicity of DNICs, the cells were harvested using phosphate-buffered saline (PBS) containing 0.15% trypsin and 0.08% EDTA, and placed in 96-well plates ($1.5 \times 10^4$ cells/well in 100 μl of medium). After incubation for 15 h, **NC01** (0.1, 1, 10, and 100 μM), **NC02** (0.1, 1, 10, and 100 μM), or **NC03** (0.2, 2, 20, and 200 μM) and curcumin (10 and 40 μM) dissolved in DMSO were added to each well. The final

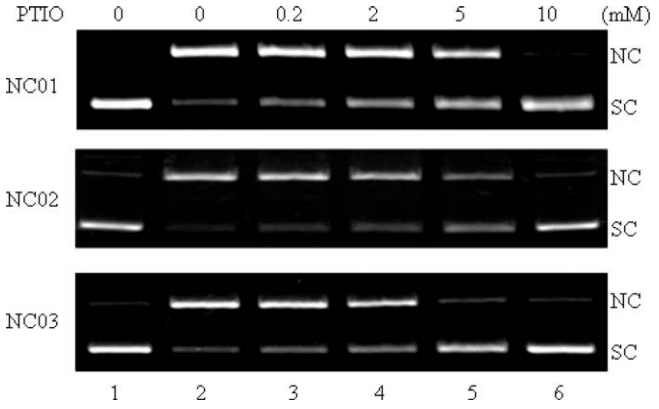


**Fig. 2.** DNA cleavage by **NC01**, **NC02**, and **NC03**. pBR322 plasmid DNA was incubated with **NC01** (20 μM), **NC02** (10 μM), or **NC03** (20 μM) with or without various concentrations of carboxy-PTIO. Lane 1: solvent control; lane 2: **NC01**, **NC02**, and **NC03** alone; lanes 3–6: **NC01**, **NC02**, and **NC03** with PTIO (0.2, 2, 5, and 10 mM). NC: nicked circular plasmid DNA; SC: supercoiled plasmid DNA.

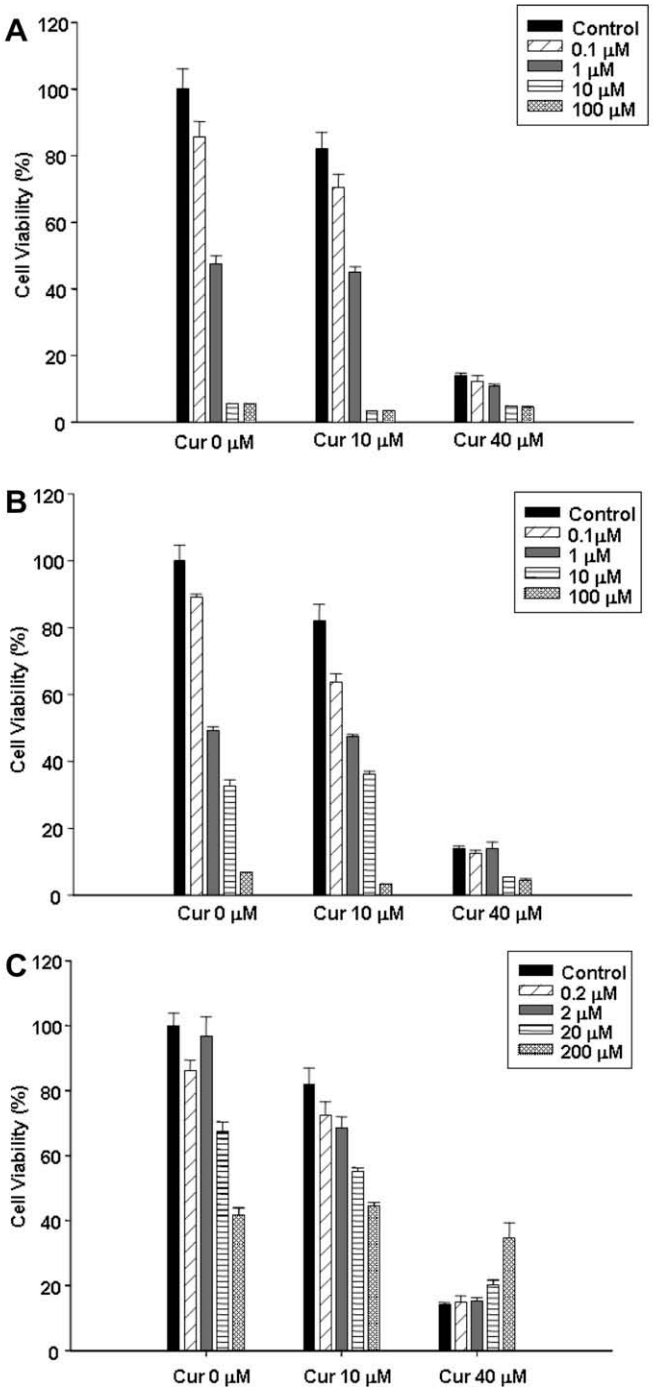


**Fig. 3.** The cytotoxicity of **NC01** (A), **NC02** (B) and **NC03** (C) alone or combined with curcumin. The B16-F10 cells were incubated with **NC01**, **NC02**, and **NC03** with different concentration in the absence or presence of 10 and 40 μM curcumin. After 24 h incubation, the relative survival rates were measured by MTT assay.

concentration of DMSO in the culture medium was 1% (v/v). The cells were incubated for 24 h. For the pretreatment experiments, curcumin (0, 10, and 15 μM) was added to each well, and the cells were incubated for 4 h. After incubation, the medium was removed and the cells were washed with PBS. The fresh medium containing **NC03** (0, 0.2, 1, 5, and 10 μM) was added to each well and the cells were incubated for 24 h. After incubation, the cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The medium was removed and the cells were washed by PBS twice. The fresh medium (100 μl) containing 0.5 mg/ml was added to each well and the cells were incubated for 4 h. After incubation, the reduced MTT product was dissolved in 200 μl DMSO. The absorbance was measured using a microplate reader (BioTek MQX200) at 570 nm. All experiments were performed with three replicates.

### 2.4. Analysis of the interaction of curcumin and *NC03*

Curcumin (10 μM) was mixed with various concentrations of **NC01**, **NC02**, and **NC03** (30, 40, and 50 μM) at room temperature. The mixtures were scanned using a UV–Visible spectroscopy. The values of absorbance were the averages of three replicates.

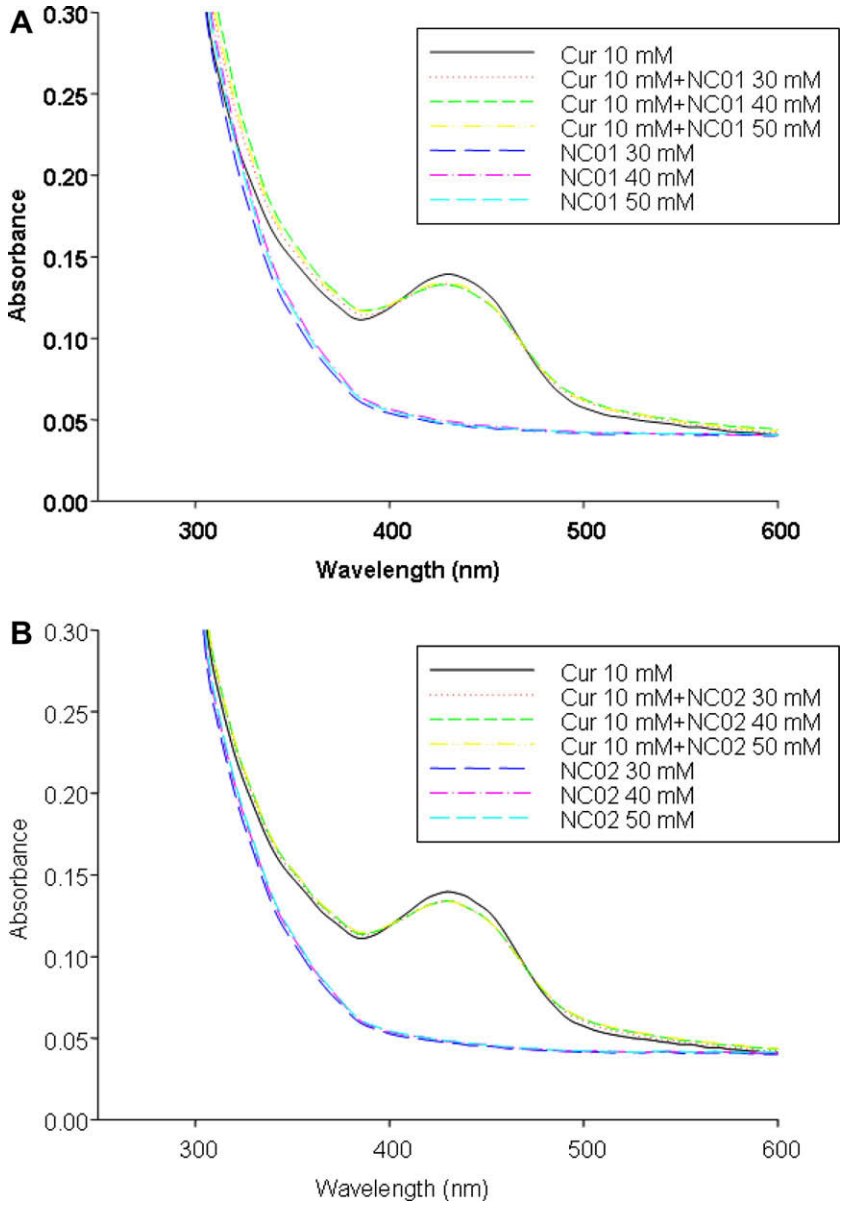


**Fig. 4.** The UV–Visible spectra of curcumin alone, or combined with **NC01** (A), **NC02** (B), and **NC03** (C). (D) The IR spectra of **NC03** and curcumin in the ratio of 1:1 measured in 0, 5, 30, 60 and 90 min, respectively.

### *2.5. Observation of the accumulation of curcumin in cells*

Taking advantage of the fluorescent property of curcumin, we were able to monitor its accumulation in cells using fluorescence microscopy. B16-F10 cells ($1 \times 10^5$ cells/ml) were seeded in 6-well plates and incubated for 16 h. Curcumin (40 μM), **NC03** (100 μM), or a mixture of 40 μM curcumin and 100 μM **NC03** were added into the wells. After incubation for 4 h, the cells were washed by PBS, and examined under a Zeiss microscope and pictured by a Pixera CCD camera in light and fluorescent fields.

## 3. Results and discussion

### *3.1. DNA cleavage and cytotoxicity of DNICs*

Our previous study has demonstrated **NC01**, **NC02**, and **NC03** could release NO in dark or under UV irradiation [33]. To investigate the biological activities of NO released from these DNICs, we performed the DNA cleavage assay using plasmid DNA as a template. Fig. 2 shows all three DNICs could cause plasmid DNA damage under UV irradiation. To verify the plasmid DNA was cleaved by NO, we tested whether plasmid DNA cleavage could be inhibited by carboxy-PTIO, which acts as a NO scavenger. The result showed increasing the concentrations of carboxy-PTIO decreased the nicked circular plasmid DNA. At 10 mM carboxy-PTIO, the plasmid cleavage was completely inhibited.

Since several synthetic NO donors such as the nitrobenzene derivatives [34] and Roussin's black salt [35,36] have been shown to induce cell death in cancer cells, we examined whether these DNICs also have toxic effects against mouse melanoma B16-F10 cells in vitro. Using MTT assay, we found all three DNICs displayed cytotoxicity against B16-F10 cells in a dose-dependent manner. The estimated $LD_{50}$ (24 h of incubation) of **NC01**, **NC02**, and **NC03** were 1, 1, and 200 μM, respectively

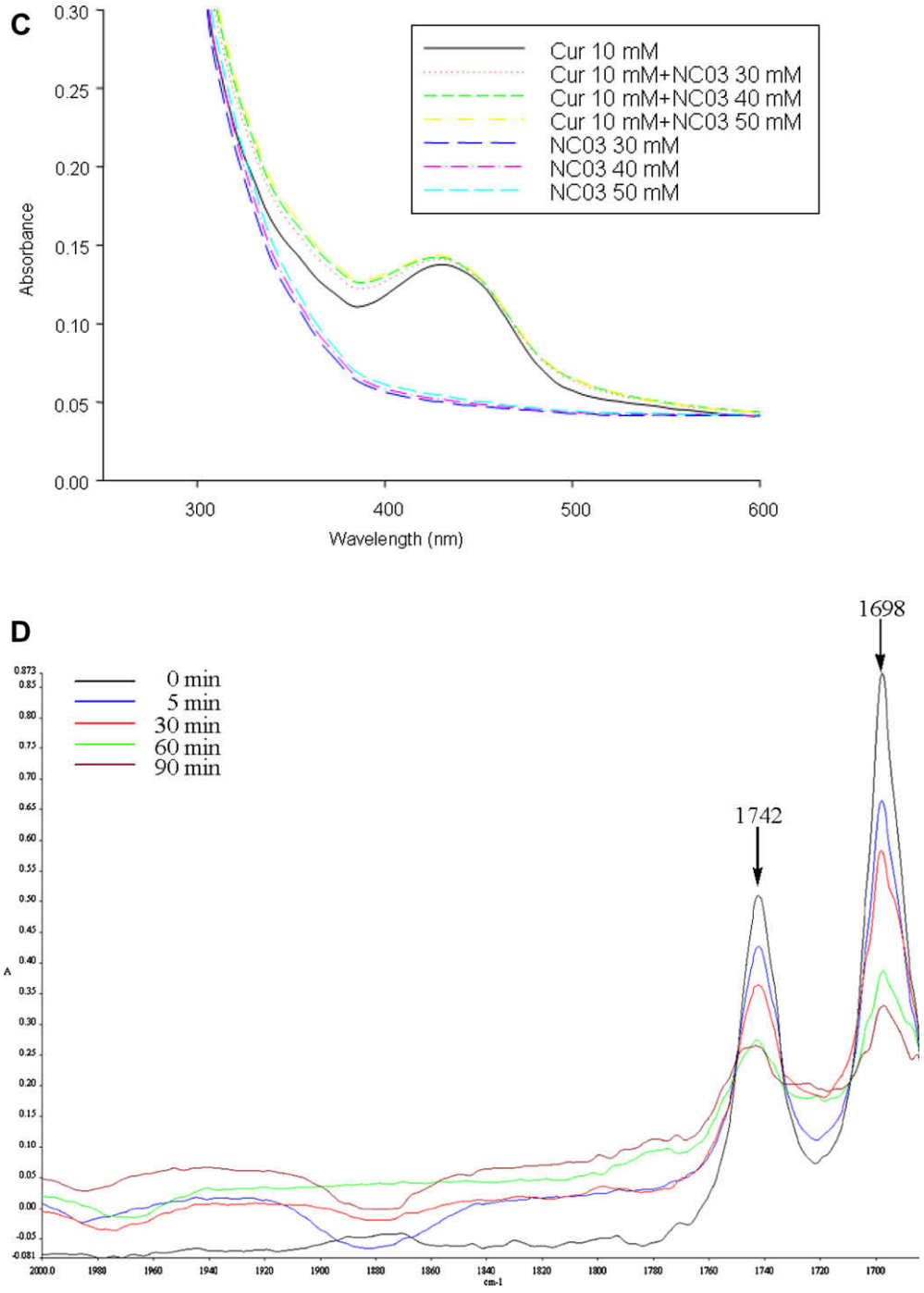


**Fig. 4** (*continued*)

(Fig. 3). **T**he cation, PPN of **NC01** and **NC02** displayed highly extent cytotoxicity (Supplementary 1) and caused the lower survival rates of cells than that of **NC03**. The solvent control (1% DMSO) had no effect on the survival of B16-F10 cells. Although the values of $LD_{50}$ of **NC01** and **NC02** were the same, the survival rate of cells treated with 10 μM **NC01** was 5.4 ± 0.2%, while the survival rate of cells treated with 10 μM **NC02** was 32.7 ± 1.9%. This result suggests that the concentration of NO released from **NC01** were higher than those from **NC02**. It also confirmed our previous finding that **NC01** has a higher trend to release NO [33].

### 3.2. Cytotoxicity of DNICs combined with curcumin

Studies have shown co-treatments of curcumin and gemcitabine [37], beta-phenylethyl isothiocyanate [38], epigallocatechin gallate [39], and taxol [40] were synergistically cytotoxic to cancer cells. We attempted to examine whether the combinations of curcumin and DNICs also had a synergistic cytotoxic effect against melanoma cells. As shown in Fig. 3, in the presence of 10 μM curcumin, it has lightly effect on the survivals of B16-F10 cells. The co-treatments of 10 μM curcumin with **NC01**, **NC02**, or **NC03** also have no synergetic toxic effect. We next examined whether higher

concentrations of curcumin combined with DNICs would have a synergetic toxic effect. In the presence of 40 μM curcumin alone, the survival rate of 24 h incubation was about 14.0 ± 0.8%. Again, no additive cytotoxic effect was noted in the co-treatments of 40 μM curcumin with **NC01**, **NC02**, or **NC03** (Fig. 3). Interestingly, we observed an unexpected result which was in the co-treatments of 40 μM curcumin with 20 or 200 μM **NC03**, the survival rates were 20.3 ± 1.4% or 34.5 ± 4.8%, respectively. These survival rates were higher than the survival rate of the treatment of 40 μM curcumin alone. Similar results were not found in the co-treatments of 40 μM curcumin with **NC01** or **NC02**. Therefore, we hypothesized **NC03**, but not **NC01** or **NC02**, could reduce the cytotoxicity of curcumin.

### 3.3. Interaction between curcumin and *NC03*

To examine whether the reduction in the cytotoxicity of curcumin by **NC03** resulted from the interaction of these two molecules, we performed UV–Visible spectroscopy analysis to detect the absorption of curcumin in the presence of various concentrations of **NC01**, **NC02**, or **NC03**. Fig. 4 shows curcumin had an absorption signal at a wavelength of 430 nm. No signal was observed in the samples of **NC01**, **NC02** and **NC03** at the concentrations we tested. We also found no change in the curcumin spectra when curcumin co-incubated with **NC01** or **NC02** (Fig. 4A and B). However, we noted increasing the concentrations of **NC03** enhanced the absorption signal of curcumin, suggesting **NC03** might interact with curcumin (Fig. 4C). The IR spectra of **NC03** and curcumin in the ratio of 1:1 measured in 0, 5, 30, 60 and 90 min, respectively showed the stretching frequency band of NO decreased (Fig. 4D). It has been known β-diketone and phenolic moieties of curcumin act as free radical scavengers [41,42]; especially, NO reacts with a phenol moiety to form the phenoxyl radical [43]. The results here imply that curcumin may play as a NO quencher in the beginning for **NC03** possessing more labile NO. After NO releasing, the β-diketone moiety of curcumin further chelates with Fe to form the complex as curcumin manganese complex (CpCpx) [43]. It reduces the accumulation of curcumin in the cells, thus, reducing the cytotoxicity of curcumin.

Curcumin is a fluorescent compound with various spectrums in different kinds of organic solvent [44,45]. This fluorescent property has been applied to determine the levels of curcumin uptake and to trace its locations in cancer cells. Because there is a positive association between fluorescent intensity and the toxicity of curcumin [46], we next examined whether the interaction between **NC03** and curcumin could result in decreased intensity fluorescence of curcumin in cells. Using a fluorescent microscopy, we found after incubation of 4 h, the cells treated with DMSO or **NC03** did not display fluorescent signals. The fluorescence was only observed in curcumin treated cells. In the presence of **NC03**, however, the intensity of fluorescence in the cells was reduced (Fig. 5).

### 3.4. Effect of pretreatment of curcumin on cytotoxicity of *NC03*

To avoid the interaction of **NC03** and curcumin, we examined whether the cells separately treated with curcumin and **NC03** would have synergistic cytotoxicity effects. Two methods were performed. First, the cells were treated with curcumin for 4 h, and then curcumin was removed before adding **NC03** to the culture medium. After a further 24 h of incubation with **NC03**, the survival rates of the cells were determined by MTT assay. Fig. 6A shows the survival rates of the cells receiving the pretreatment of curcumin following the treatments of **NC03** were lower than the survival rates of the cells receiving the same concentrations of curcumin or **NC03** alone. To demonstrate the synergistic cytotoxic effect clearly, we converted the survival rates to the mortality and calculated the synergistic cytotoxic effect by subtracting the mortality of the cells received pretreatment curcumin followed by the treatments of **NC03** with the mortality of the cells received the treatment of curcumin and **NC03**, respectively. After calculation, we found a synergistic cytotoxic effect in this first strategy. For example, the mortality of the cells treated with 10 μM curcumin for 4 h or 0.2 μM **NC03** for 24 h were 10.7 ± 2.5% or 8.42 ± 3.5%; while, the mortality of the cells received the pretreatment of 10 μM curcumin for 4 h followed by the treatment of 0.2 μM **NC03** was 33.8 ± 3.1%. The synergistic effect, therefore, was 14.7%. It should be noticed that no synergistic effect was observed in the pretreatment of 10 or 15 μM curcumin followed by the treatment of 10 μM **NC03** (Supplementary 2).

The second method was **NC03** was added to the culture medium first, followed by curcumin. After a 4 h of incubation with **NC03**, the cells were washed with PBS, then incubated with curcumin for 24 h. Fig. 6B shows the survival rates of the cells receiving the pretreatment of **NC03** following the treatments of curcumin were similar to the mortality of the cells treated with curcumin alone (Supplementary 3). This result indicated no synergistic cytotoxic effect for this method.

We have reported NO released from DNICs and Roussin's red ester can kill cancer cell [47] and here pretreatment of **NC03** and co-treatment of **NC03** and curcumin did not show any synergistic cytotoxic effect may caused by the quenching of NO by curcumin, namely, pretreatment of curcumin exerts the anticancer effect followed by the NO released from **NC03** to enhance the effect against the tumor cells. These results also suggest that **NC03** may release NO outside the cells without directly interaction with curcumin. However, curcumin is an unstable compound in vivo. After incubation for 4 h, curcumin might undergo structure changes and no longer interact with **NC03**. In such situation, **NC03** still can pene-

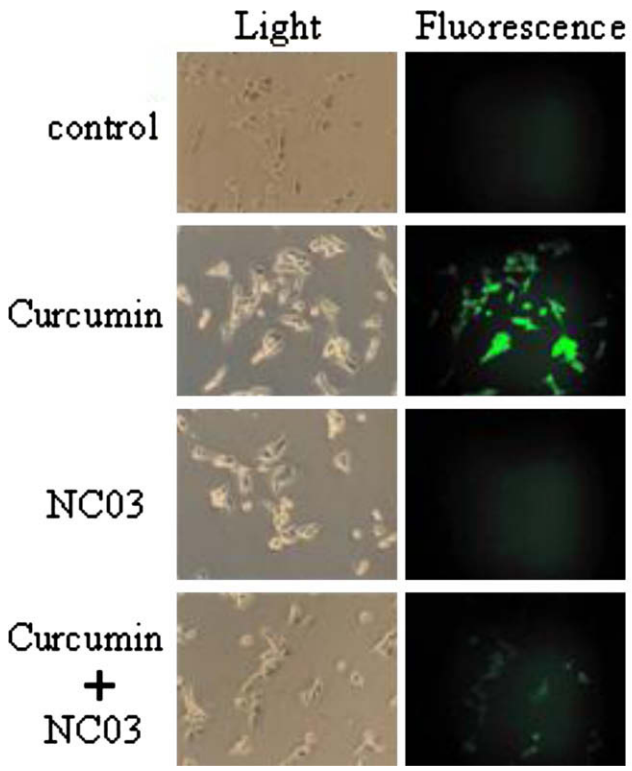


**Fig. 5.** The accumulation of curcumin in the cells. The B16-F10 cells were incubated with 40 μM curcumin, 100 μM **NC03**, or a combination of 40 μM curcumin and 100 μM **NC03** for 4 h. After incubation, the cells were pictured using a fluorescent microscope in the light and fluorescent fields.

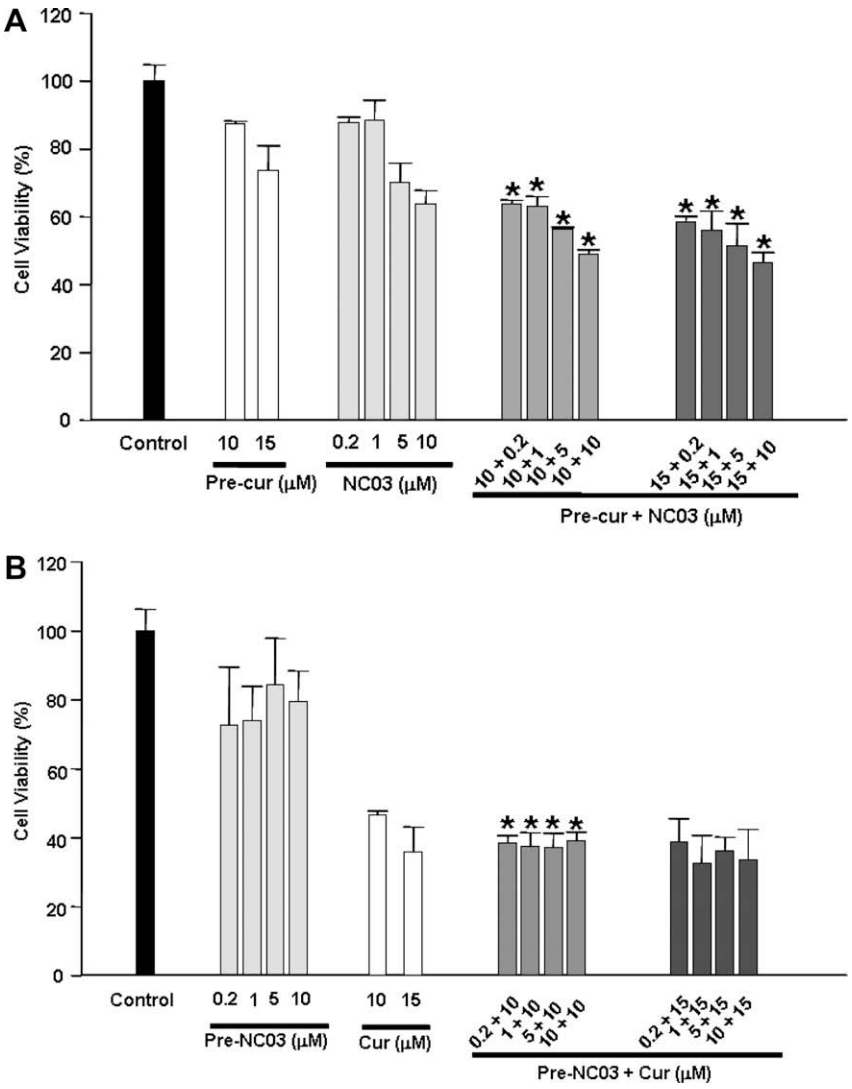


**Fig. 6.** **NC03** and curcumin synergistically induce cell death. (A) The viability of the cells receiving the pretreatment of curcumin for 4 h, the treatment of **NC03** for 24 h, or the pretreatment of curcumin for 4 h following the treatment of **NC03** for 24 h. (B) The viability of the cells receiving the pretreatment of **NC03** for 4 h, the treatment of curcumin for 24 h, or the pretreatment of **NC03** for 4 h following the treatment of curcumin for 24 h. These results were expressed as mean ± S.D. of triplicate assays. Differences in viability after exposure to **NC03** and curcumin separately and to their combination were analyzed by ANOVA. $^{*}P < 0.05$. $^{**}P < 0.01$, $^{***}P < 0.001$.

trate into the cells and release NO inside the cells without further decreasing the activity of curcumin. Hung et al. have found their DNIC can either directly permeate into cells or be transported into the cells by protein-bound or low-molecular-weight DNIC species [48]. More experiments have to be done to clarify whether or not **NC03** could penetrate into the cells.

## 4. Conclusion

Combination therapy is an attractive strategy which can enhance the activities of different drugs, and thus reduce the amount of the required dose and the side-effects. However, interactions between drugs in co-treatments may also decrease the activities of the drugs. In this study, we showed **NC03** interacts with curcumin and reduces its cytotoxicity against B16-F10 cells in vitro. By using the pretreatment of curcumin following the treatment of **NC03**, we noted a synergistic effect on inhibition of growth of B16-F10 cells in vitro. This finding provides the basis for further development of combination therapy using curcumin and other NO donors.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jorganchem.2009.10.041.

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