

Mechanisms of the antitumor action of the new antibiotic NC0604, a member of the bleomycin family

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Abstract. NC0604, the new antibiotic of the bleomycin family, was isolated from the fermentation broth of *Streptomyces verticillus* var. pingyangensis n.sp. In this study, we investigated its mechanisms of antitumor action *in vitro* and *in vivo*. The results showed that the colony formation inhibition of NC0604 was 2-10 times higher than that of bleomycin to several tumor cell types. In comparison to bleomycin, a better antitumor efficacy of NC0604 was observed in the BALB/c mice loading mouse hepatoma H22 tumors. Flow cytometry assay detected an increase of intracellular reactive oxygen species and arrest at G₂/M phase in human hepatoma HepG2 cells after exposure to NC0604. The comet of DNA damage was also observed in the NC0604-treated cells using single cell gel electrophoresis assay. The chromatin condensation appeared in the NC0604-induced apoptotic cells. The activation of apoptotic caspase pathway and increase of apoptosis-modulated protein expression, such as p53, Bax, were also detected by Western blot analysis. Taken together, the anti-tumor actions of NC0604 involve activation of the apoptotic pathway and it is valuable for further drug development.

Introduction

Bleomycin (BLM), a potent antitumor antibiotic, has long been used in first line of clinical cancer chemotherapy. It is widely used for several malignant tumor types including squamous cell carcinoma, malignant lymphoma, testicular cancer, ovarian cancer, and in combination with other antitumor agents (1). The mechanism of its action is mediated by the generation of reactive oxygen species

(ROS), and then degraded DNA and RNA in a selective manner (2,3). The advantage of BLM is that it does not cause suppression of bone marrow and immune system. However, its induction of pulmonary fibrosis retards further application in clinic. Therefore, exploring new antibiotics belonging to the BLM family with more effective and lower pulmonary toxicity becomes important.

The new antibiotic NC0604, a member of the BLM family, was first reported by us (4), it was found in the culture broth of *Streptomyces verticillus* var. pingyangensis n.sp. The clinically used form of BLM contains mixture of BLM A and B. By contrast, NC0604 has a single chemical structure with a different terminal amine moiety (Fig. 1). It exhibits a broad-spectrum antibacterial activities and stronger antitumor activities towards many human tumor cell lines. Moreover, lower pulmonary toxicity was also observed compared with BLM (4). However, it remains unknown how NC0604 inhibits tumor cells.

Accumulating evidence shows that induction of apoptosis plays a very important role in tumor chemotherapy. Both extrinsic and intrinsic apoptotic signaling pathways are triggered by many antitumor agents (5,6). Previous studies showed induction of apoptosis by BLM is accomplished by mitochondria-mediated apoptotic pathway (intrinsic), without involving Fas receptor-mediated apoptotic pathway (7,8). Increase of P53 expression after treatment with BLM was detected in malignant and normal cells (9,10).

In the present study, we investigated the mechanisms of antitumor action of NC0604 *in vitro* and *in vivo*. The results show that it has more potent efficacy than that of BLM. Activation of apoptotic caspase pathway contributes to its action towards tumor cells. NC0604 is valuable for further drug development.

Materials and methods

Reagents. NC0604 was extracted from the fermentation broth of *Streptomyces verticillus* var. pingyangensis n.sp and purified according to the method reported previously (4). The purity was >90% by the HPLC examination. It was prepared into a 2 μ M solution with PBS buffer and stored at -20°C before use. BLM was purchased from Nippon Kayaku (Tokyo, Japan) and dissolved in PBS at 10 mg/ml. DMSO, 3-(4, 5-dimethyl-2-thiazoyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT), Hoechst 33342, propidium iodide (PI) were purchased

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from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA). H2DFFDA, RPMI-1640 medium was obtained from Invitrogen (Carlsbad, CA, USA).

Cell lines and culture condition. Human hepatoma HepG2 cells, human breast cancer MCF-7 cells and human aryngocarcinoma KB cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Tianjin Haoyang Biotech Company, Tianjin, China), 2 mM glutamine, 100 U/ml penicillin and streptomycin, respectively. The cells were incubated at 37°C in humidified 5% CO₂.

Animals. Female BALB/c mice, 6 to 8-week old, weighing 20-22 g, were purchased from the Institute for Experimental Animals, Chinese Academy of Medical Sciences, Beijing. Animal care was in compliance with the regulation issued by the Beijing Committee of Laboratory Animals. Animals were maintained under specific pathogen-free conditions and provided with sterile food and water *ad libitum*. Before beginning the experiment, mice were allowed to acclimate for 3 days.

Colony formation assay. Cells were seeded in a 48-well plate at the density of 100 cells per well for 24 h and then treated with the drugs for 7 days. The cells were fixed in 1 ml methanol for 10 min and stained with 0.02% amido black for 10 min. The number of colonies with total cell number over 50 cells was scored. The colony formation efficiency was calculated as percentage of the control values. All experiments were performed in triplicate and were repeated twice.

In vivo therapeutic efficacy. Each mouse was injected with 2x10⁶ mouse hepatoma H22 cells into right lacteral chest wall of the axilla. After 5 days of inoculation, tumor-bearing animals were randomly allocated into 7 groups (n=9 per group). Mice were intraperitoneally administered 5 times every 3 days with BLM or NC0604 at 10, 5, 2 mg/kg, while control group were administered with saline. In addition, mice were weighed and tumor size was measured every 3 days. The tumor volume was calculated by the formula: $W^2L \times 1/2$, where *W* and *L* are the width and length of the tumor, respectively. On day 19 after tumor cell inoculation, mice were sacrificed, and tumors were removed and weighed. The ratio of tumor growth inhibition was calculated by the formula: $(A-B)/A \times 100\%$ (A: Average tumor weight of control group; B: Average tumor weight of experimental group).

Cell cycle analysis. The HepG2 cells were treated with the drugs for 24 h. The assay was carried out according to the previous methods reported by us (11).

Observation of chromatin condensation. Cells (2x10⁵ per well) were seeded in a 6-well plate for 24 h and treated with NC0604 for 48 h. The suspended and adherent cells were collected and incubated with 2 μg/ml Hoechst 33342 at 37°C for 30 min. Chromatin condensation was observed with a fluorescence microscopy (Nikon, Japan).

Measurement of ROS. Cells were seeded in a 6-well plate for 24 h. After the treatment with NC0604 for 12 h, the cells

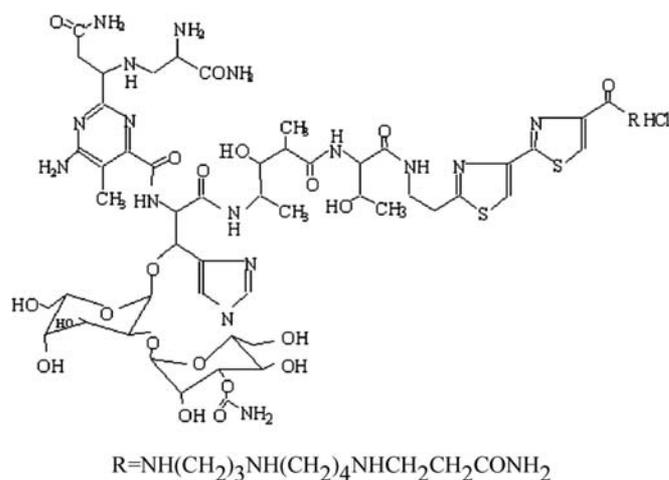


Figure 1. The chemical structure of NC0604.

were washed once with PBS and incubated with 10 μmol/l H2DFFDA for 30 min at 37°C. Thereafter, cells were washed with PBS and harvested by trypsinization. The fluorescent intensity was determined with the FACScalibur flow cytometer.

Single cell gel electrophoresis assay. After the treatment with NC0604 for 6 h, the cells were harvested by trypsinization and collected at 1000 rpm for 5 min. Cells were suspended in 1 ml of 0.5% low melting agarose dissolved in PBS, and spread on the normal agarose-coated (1%) slides. The slides were put at 4°C to solidify the low melting agarose. After treatment with lysing solution (2.5 M NaCl, 100 mM Na₂-EDTA, 10 mM Tris base, 0.02 M NaOH, 1% Triton-X 100 and 10% DMSO) at 4°C for 1 h, the slides were transferred to an alkaline buffer (1 mmol/l Na₂-EDTA, 300 mmol/l NaOH, pH >13) for 20 min to unwind the DNA, and run electrophoresis in the same buffer for 20 min at 20 V at 4°C. The solution was neutralized with 0.4 mol/l Tris buffer (pH 7.5). Thereafter, all slides were stained with 2 mg/ml PI and examined under a fluorescence microscope.

Western blot analysis. After the treatment with NC0604 for 48 h, the HepG2 cells were harvested. The analysis was carried out following the previously reported method (11). Antibodies against bcl-2, Bcl-xL, Bax, Survivin, cleaved-caspase-9, procaspase-3 and PARP were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-P53 and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistics analysis. Results are indicated as the means ± SD. Comparison between two groups was analyzed by using the Student's t-test, with P<0.05 considered to be statistically significant.

Results

Inhibition of colony formation by NC0604 in tumor cells. In our previous study, potent growth-inhibitory effects of NC0604 was observed with MTT assay (4). To further compare the

Table I. The IC₅₀ value of colony formation inhibited by NC0604 and BLM on tumor cells.

Cell lines	NC0604 (IC ₅₀ , nM)	BLM (IC ₅₀ , nM)
HepG2	0.64±0.04	6.70±0.10
KB	0.76±0.08	3.70±0.05
MCF-7	0.50±0.02	2.16±0.07
MCF-7/DOX	0.27±0.07	6.28±0.09

The cells were seeded in a 48-well plate (100 cells per well) for 24 h and then treated with the drugs for 7 days. The number of colonies (>50 cells) was scored. The colony forming efficiency was calculated as percentage of the control values. All experiments were performed in triplicate and were repeated twice.

inhibitory action of NC0604 and BLM in tumor cells, colony formation assay was used. The IC₅₀ values of NC0604 for HepG2 and KB were 0.64 nM and 0.76 nM, respectively (Table I). It is noteworthy that the multidrug resistant MCF-7/DOX cells were more sensitive to NC0604 than the MCF-7

cells. Consistent with the results obtained by MTT assay, the growth-inhibitory effect of NC0604 was stronger than that of BLM towards these tumor cell lines (Table I). As hepatoma HepG2 cells are more sensitive to NC0604, it prompted us to further study the mechanism of NC0604 on hepatoma.

Potent inhibition of mouse hepatoma by NC0604 in vivo. We next assessed the inhibitory effect of NC0604 on tumor growth in the BALB/c loading mouse hepatoma H22 tumors. Results revealed that the tumor volumes were significantly suppressed after administration of NC0604 and BLM, compared with the control group (Fig. 2A and B). After mice were sacrificed, tumors were taken and weighed. In NC0604-treated groups, the inhibition ratios of tumor growth reached 91.5, 79.7 and 70.0% at the doses of 10, 5 and 2 mg/kg, higher than that of BLM, respectively (Fig. 2C). The data suggest that NC0604 treatment gave superior antitumor effects over that of BLM. In addition, there was no significant body weight loss in the NC0604 and bleomycin-treated groups compared with the control group (data not shown).

Arrest of HepG2 cells at G₂/M phase by NC0604. It has been reported that BLM retarded the cells at G₂/M phase. It can be

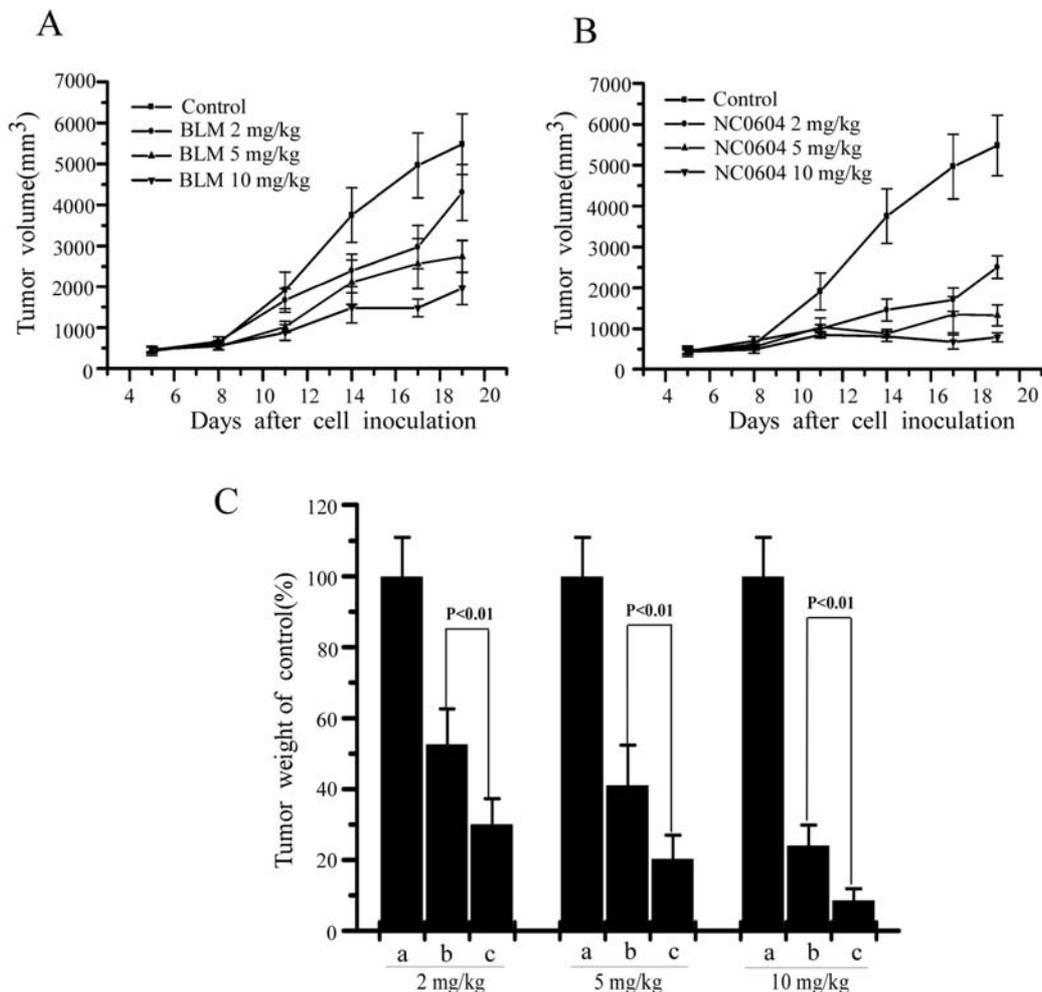


Figure 2. Inhibition of mouse hepatoma H22 tumor growth by NC0604 and BLM. After 5 days of inoculating hepatoma H22 cells, tumor-bearing BALB/c mice were intraperitoneally administrated 5 times every 3 days with BLM or NC0604 at 10, 5, 2 mg/kg, while control group was administered with saline. The mice were weighed and tumor size was measured every 3 days. On day 19 after tumor cell inoculation, mice were sacrificed, and tumors were removed and weighed. (A) Change of tumor volume after administration with BLM (n=9); (B) change of tumor volume after administration with NC0604 (n=9); (C) the percentages of tumor weight (control 100%) are shown as per the last day of the animal experiment. a: control group, b: BLM group, c: NC0604 group.

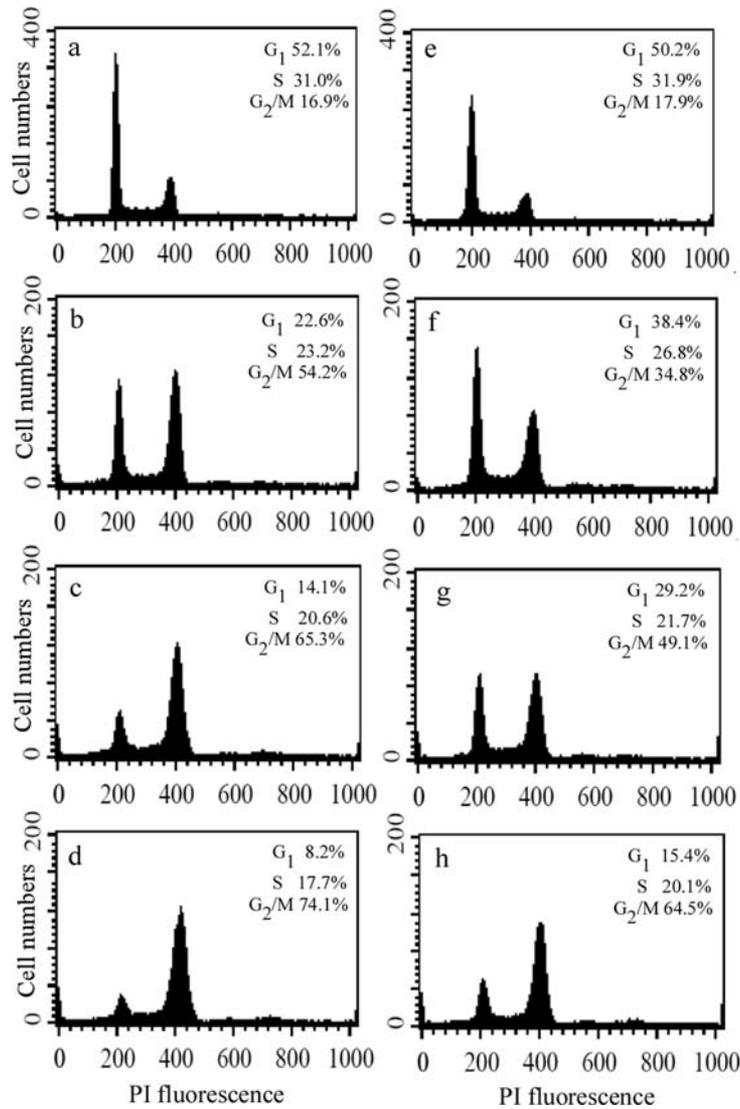


Figure 3. Arrest of HepG2 cells at G₂/M phase by NC0604 and BLM. The cells were treated with the drugs for 24 h: (a) and (e) control; (b) 0.2 μM NC0604; (c) 0.5 μM NC0604; (d) 1 μM NC0604; (f) 0.2 μM BLM; (g) 0.5 μM BLM; (h) 1 μM BLM. The fixed cells were stained with PI and detected by flow cytometry. The results are representative of two separate experiments.

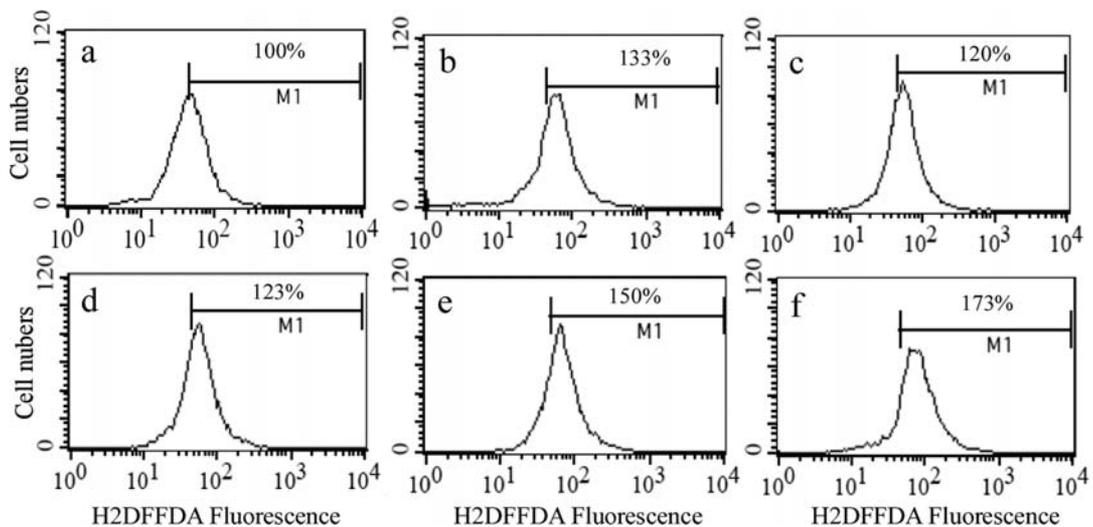


Figure 4. Accumulation of intracellular ROS triggered by NC0604 and BLM. The HepG2 were treated with the drugs for 12 h: (a) control; (b) 2 μM BLM; (c) 0.2 μM NC0604; (d) 0.5 μM NC0604; (e) 1 μM NC0604; (f) 2 μM NC0604. The cells were incubated with H2DFFDA for 1 h and detected by flow cytometry. The results are representative of two separate experiments.

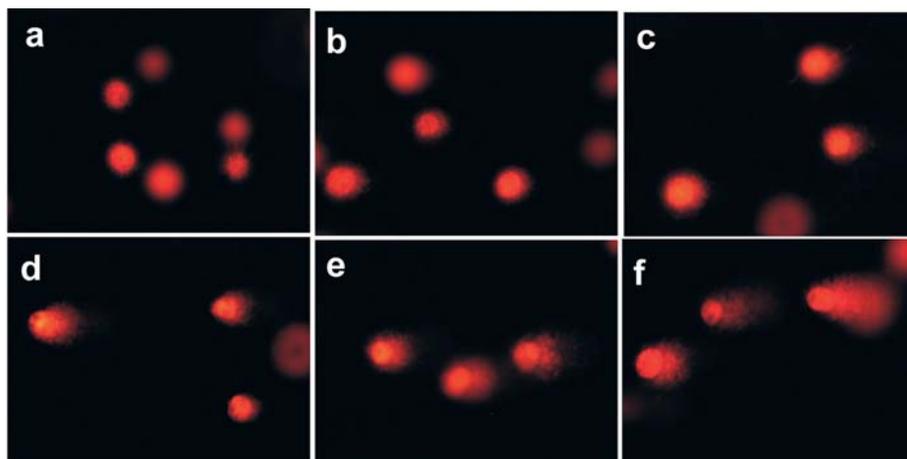


Figure 5. Comets of DNA lesion damaged by NC0604 in the HepG2 cells. The cells were treated with NC0604 for 12 h: (a) control; (b) 0.2 μM ; (c) 0.5 μM ; (d) 1 μM ; (e) 2 μM ; (f) 5 μM BLM. The data were obtained with single cell electrophoresis assay. All slides were stained with 2 mg/ml PI and examined under a fluorescence microscope. The results are representative of three separate experiments.

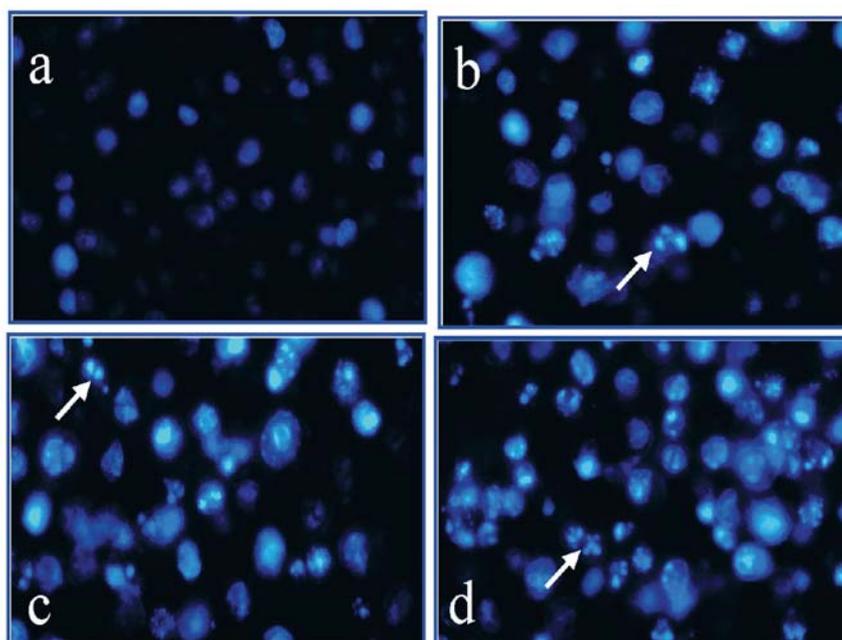


Figure 6. Chromatin condensation of HepG2 cells after exposure to NC0604 for 48 h. (a) Untreated cells, (b) the cells treated with 1 μM , (c) the cells treated with 2 μM , (d) the cells treated with 5 μM . The suspended and adherent cells were collected and stained with 2 $\mu\text{g/ml}$ Hoechst 33342. The chromatin condensation of the cells was visualized with a fluorescence microscope. Representative results from two independent experiments are shown.

predicted that NC0604 has the same effect on cell cycle distribution. Fig. 3 shows that NC0604 arrested the HepG2 cells at G₂/M phase in a concentration-dependent manner. Comparing to the same concentration treatments, the percentage of the cells at G₂/M phase arrested by NC0604 was more than that by BLM, further confirming that the efficacy of NC0604 is better than that of BLM.

The accumulation of ROS in NC0604-treated HepG2 cells. The intracellular accumulation of ROS is an early critical event in the mitochondrial apoptotic pathway. We next determined the level of intracellular ROS by flow cytometry. The significant ROS accumulation was detected in a concentration-dependent manner after the cells were exposed to increasing

concentrations (0.2, 0.5, 1, 2 μM) of NC0604 for 12 h (Fig. 4). When the cells were treated at the same concentration of 2 μM for 12 h, the level of intracellular ROS triggered by NC0604 increased 1.7-fold (Fig. 4f), whereas it only increased 1.3-fold after exposure to BLM (Fig. 4b).

Induction of DNA damage by NC0604 in the HepG2 cells. To further study the action of NC0604, we performed single cell gel electrophoresis assay to probe whether NC0604 induced DNA lesions in HepG2 cells. The intact cells appeared sphere shaped and the cells with damaged DNA as comets. As shown in Fig. 5, comet DNA tail increased in a concentration-dependent manner after exposure to NC0604 for 6 h, reaching maximum in the 5 μM NC0604-treated cells.

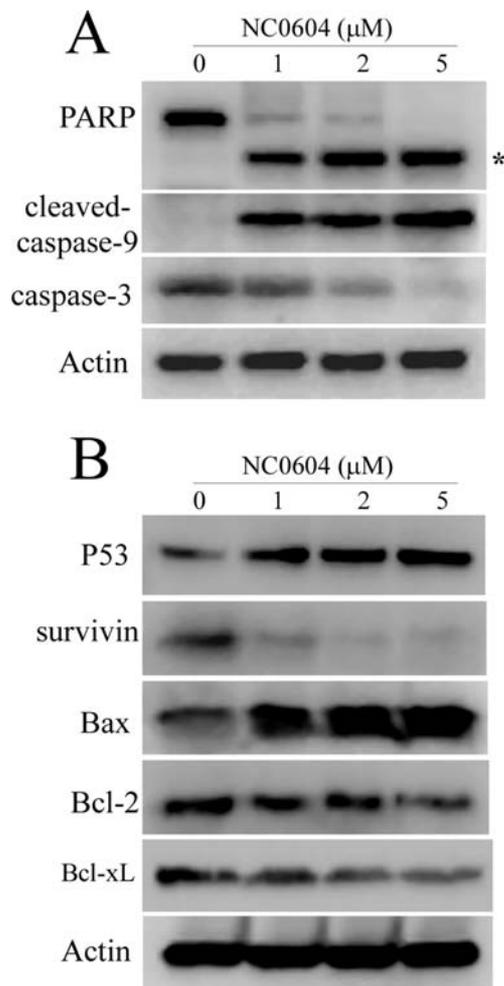


Figure 7. Activation of apoptotic caspase pathway and expressions of apoptosis-regulated proteins after exposure to NC0604. The cells were treated with NC0604 for 48 h. The protein levels were analyzed by Western blotting. (A) Activation of caspase pathway aroused by NC0604. Asterisk (*) indicates cleaved PARP fragments. (B) The expressions of apoptosis-regulated proteins. The experiment was repeated twice and similar results were obtained.

Induction of apoptosis by NC0604 in the HepG2 cells. The obvious changes in morphology such as shrinkage and floating cells were observed after the cells were exposed to a higher concentration of NC0604. To confirm the morphological features of the apoptotic cells we performed Hoechst 33342 staining for DNA. The results showed most of the NC0604-treated cells developed typical morphological changes such as chromatin condensation consistent with apoptosis, and the number of these cells increased in a concentration-dependent manner (Fig. 6).

To determine whether NC0604-induced apoptosis is mediated via the activation of caspase pathway, Western blot analysis was performed. The result showed that the activation of caspase-9, -3 and cleavage of PARP were detected in a concentration-dependent manner after exposure to NC0604 for 48 h (Fig. 7A). To further explore the molecular mechanism of NC0604-induced apoptosis in HepG2 cells, we investigated several key molecules known to regulate apoptosis. The significant increase of P53 and Bax expressions and rapid

down-regulation of survivin level were detected, whereas there was a slight decrease in Bcl-2 and Bcl-xL protein expression (Fig. 7B).

Discussion

In this study, we investigated the mechanism of antitumor action of the antibiotic NC0604, using several methods. The data demonstrated potent inhibition *in vitro* and *in vivo*, and the efficacy is stronger than that of BLM, used in clinic as antitumor agent. Our previous results showed that NC0604 exerts lower pulmonary toxicity than BLM (4). It can be concluded that NC0604 is a very promising antibiotic as a candidate drug for chemotherapy of human malignancies and deserves further investigation.

It is well known that the action of BLM on tumor cells includes: 1) binding with iron after entrance of the cells, 2) damaging DNA and RNA, 3) determining cell fate response to the damage (1,12). According to the data presented herein, the action of NC0604 is similar to that of BLM. The damage signals triggered by BLM led to generation of ROS and arrest of the cell cycle, finally initiation of the cell death programs, such as apoptosis or senescence (13,14). Our data revealed that intracellular ROS was accumulated, and caspase-9 and -3 were activated during the progression of NC0604-induced apoptosis, suggesting that it is mediated by the mitochondria apoptotic pathway. Besides activation of caspase apoptotic pathway, NC0604 also led to senescence in the tumor cells (data not shown).

Accumulating evidence shows that many molecules modulate process of apoptosis. The data presented herein showed that the NC0604 treatment resulted in an increase of P53 and Bax protein expressions, rapid down-regulation of survivin protein (Fig. 7B), consistent with previous data reported (15). The tumor suppressor and transcription factor P53 plays a central role in mitochondrial mediated apoptosis (16). It not only drive the expression of downstream target genes, such as Puma, Bax, but also translocates to the cytoplasm forming a complex with Bcl-2 and Bcl-xL, resulting in cytochrome c release and caspase activation (17). Moreover, p53 induces the inhibition of survivin at the transcriptional level. Survivin is a member of the inhibitor of apoptosis protein family and is believed to inhibit apoptosis through direct binding to caspase-3 and -7, preventing their activation, or inhibiting the AIF pathway (18,19).

Interestingly, NC0604 shows potent inhibition in hepatoma cells. Hepatocellular carcinoma is a critical global health issue (20,21). In China, it ranks second in cancer incidence. It requires further study as to why NC0604 exerts potent cytotoxicity in hepatoma cells at molecular level.

In summary, NC0604 induces apoptosis via damage of DNA and increases ROS generation. It is more efficient than that of BLM *in vitro* and *in vivo*.

Acknowledgements

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