



Original Article

Synergistic cytotoxicity of 1,3-bis(2-chloroethyl)-1-nitrosourea and Rana catesbeiana ribonuclease-6 in hepatoma cells

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ABSTRACT

Objective: To demonstrate that a combination of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and Rana catesbeiana ribonuclease-6 (RC6) exerts synergistic cytotoxic effects on human hepatoma cells.**Materials and Methods:** Human hepatoma cells (J5 and HepG2) were treated with various concentrations of BCNU or RC6. The survival rate was determined by XTT assay. Apoptosis was determined by fluorescence-activated cell sorting analysis with propidium iodide/annexin-V double stain. Caspase activation was determined by Western blot assay.**Results:** BCNU and RC6 are able to inhibit the cell growth of hepatoma cells in a dose-dependent manner. BCNU combined with RC6 exerts a synergistic cytotoxic effect on hepatoma cells. Normal cells had less cytotoxicity than on hepatoma cells with BCNU/RC6 treatment. In addition, apoptosis was observed in hepatoma cells with BCNU treatment, RC6 treatment, and combination treatment. Our data also showed that combination treatment can activate the caspase-9/caspase-3 cascade obviously in hepatoma cells. **Conclusion:** Combination treatment with BCNU and RC6 exerts a synergistic cytotoxic effect on hepatoma cells.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common human tumors worldwide. HCC treatments involve surgery, radiation, and chemotherapy [1–3]. Surgery and radiation are only effective when tumors are diagnosed at an early stage [4,5]. Although chemotherapy is a conventional method, it has been associated with cytotoxicities under high-dose treatment [6,7]. Therefore, combination treatment with low-dose drugs has been considered [8–10].

1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), an alkylating agent, has been used for tumor treatment [11,12]. Many reports and our previous study have demonstrated that BCNU can interact with DNA, RNA, and proteins [13] and can induce DNA crosslinks; S-phase arrest; downregulation of Bcl-2; inhibition of glutathione

reductase activity; and dysfunction of DNA, RNA, and protein [14–17]. Based on these mechanisms, BCNU can affect tumorigenesis resulting in tumor cell death. However, high dosages of BCNU can cause many side effects, such as myelosuppression and lung fibrosis [18,19]. Moreover, previous studies indicated that BCNU can be detoxified by glutathione-dependent enzymes and O6-alkylguanine-DNA alkyltransferase, which are found in some hepatoma cells [20,21]. For these reasons, a combination of BCNU with other drugs has been investigated for hepatoma therapy [22,23].

RC6 is homologous to onconase and belongs to the ribonuclease family [24]. These ribonucleases have anticancer effects [25,26]. Onconase has been used as an anticancer drug in clinical trials [27,28]. Previous studies and our data indicated that the cytotoxic ribonucleases may affect tumorigenesis by inducing RNA cleavage, decreasing protein synthesis, inducing mitochondria dysfunction, and downregulating Bcl-2, Bcl-xL, p53, p21, cyclin, and p16 [9,24,29–31].

Our previous studies have indicated that hepatoma cells are inhibited by high-dose BCNU alone and high-dose RC6 alone. However, under these concentrations, BCNU and RC6 are cytotoxic to normal cells. Therefore, combination treatment with low-dose

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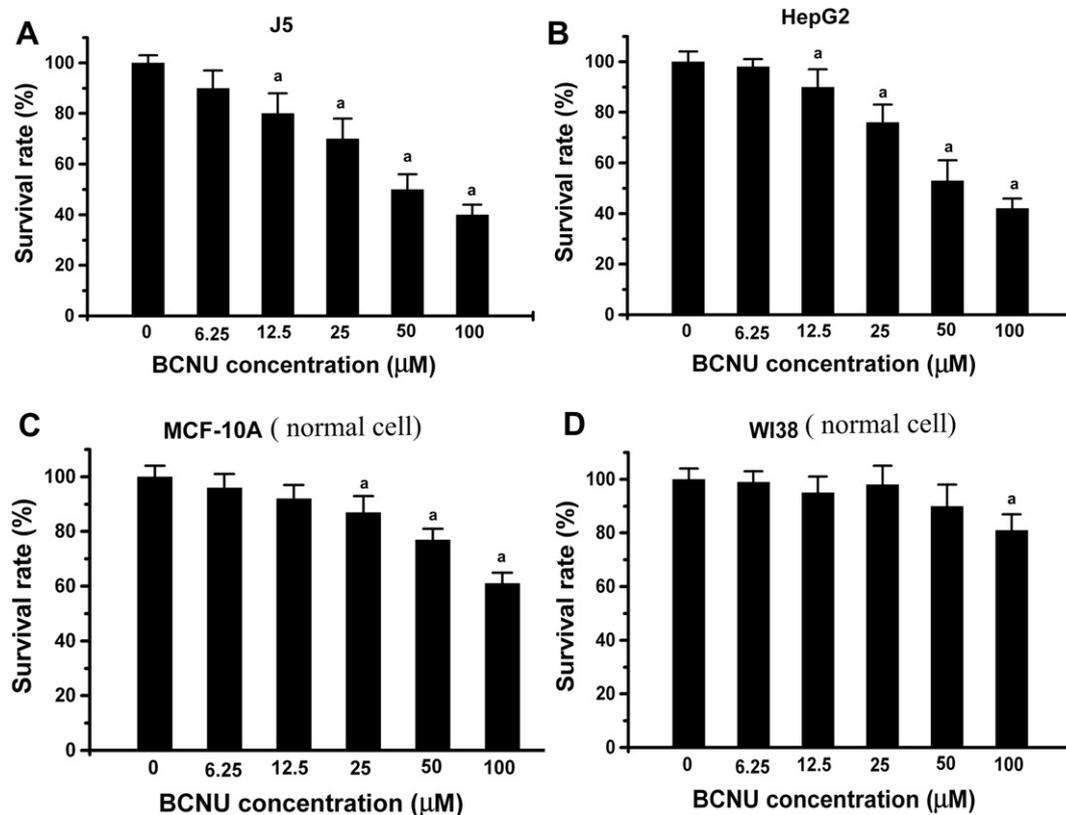


Fig. 1. BCNU inhibited the cell growth of human hepatoma cells. (A) J5; (B) HepG2; (C) MCF-10A; and (D) WI38 cells were treated with various concentrations of BCNU. After 48 hours of incubation, the survival rates of the treated cells were measured by XTT assay. The results were obtained from four independent triplicate experiments and are presented as mean \pm SD. ^a $p < 0.05$. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; SD = standard deviation.

BCNU and low-dose RC6 was investigated in hepatoma cells in this study. Our studies showed that combination treatment with low-dose BCNU and low-dose RC6 exerts a synergistic cytotoxic effect on hepatoma cells. Although combined low-dose BCNU and low-dose RC6 still exerted some cytotoxicity on normal cells, our data indicated that combination treatment with low-dose BCNU/RC6 was more toxic to hepatoma cells than normal cells. In addition, combination treatment with BCNU/RC6 can activate the caspase-9/caspase-3 pathway resulting in apoptosis in hepatoma cells. Overall, our study demonstrated that BCNU and RC6 exert a synergistic cytotoxic effect on hepatoma cells.

2. Materials and methods

2.1. Reagents and cell culture

RC6 was kindly given to us by Dr Jaang-Jiun Wang (Division of Pediatric Infectious Diseases, Emory University School of Medicine, Atlanta, USA). Caspase-3, caspase-8, and caspase-9 antibodies were purchased from Oncogene (Munich, Germany). Actin antibody was purchased from Millipore (Billerica, MA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA). The annexin-V FLOUS staining kit and XTT assay kit were obtained from Roche (Roche, Mannheim, Germany). Polyvinylidene fluoride membrane was purchased from Amersham Biosciences (Arlington Heights, IL, USA). HepG2 cells (well-differentiated human hepatoma cells) and WI38 cells (human fibroblasts) were obtained from Biosources Collection and Research Center (BCRC, Hsin Chu, Taiwan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA). J5 cells (intermediately differentiated

human hepatoma cells) were kindly given to us by Dr Jaang-Jiun Wang and maintained in Rosewell Park Memorial Institute-1640 medium (GIBCO). These cells were supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT, USA), 2mM L-glutamine, 100 IU/mL penicillin/streptomycin, and 0.1mM nonessential amino acids (GIBCO). MCF-10A (human epithelial cells) were given to us by Dr Yung-Luen Yu (Graduate Institute of Cancer Biology and Center for Molecular Medicine, China Medical University and Hospital, Taichung, Taiwan) and cultured in DMEM/F12 medium (GIBCO) supplemented with 5% horse serum, 1% penicillin/streptomycin, 10 μ g/mL bovine pancreatic insulin, 20 ng/mL epidermal growth factor, and 0.5 μ g/mL hydrocortisone (GIBCO). These cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

2.2. Cell survival assay

Cell survival was measured using an XTT assay kit that determines mitochondrial dehydrogenase activity. Briefly, 5×10^3 cells were grown in each well of a 96-well plate. On the second day, the cells were treated with various concentrations of BCNU (dissolved in ethanol) or RC6 (dissolved in PC buffer). XTT assays were carried out for 2 days according to the manufacturer's instructions. The absorbance at 492 nm and 620 nm was determined using a Microplate ELISA Reader (Ceres UV 900, Bio-Tek Instruments, Winoski, VT, USA).

2.3. Propidium iodide/annexin-V double staining and fluorescence-activated cell sorting analysis

Apoptosis was determined by using propidium iodide (PI)/annexin-V staining and a fluorescence-activated cell sorting (FACS)

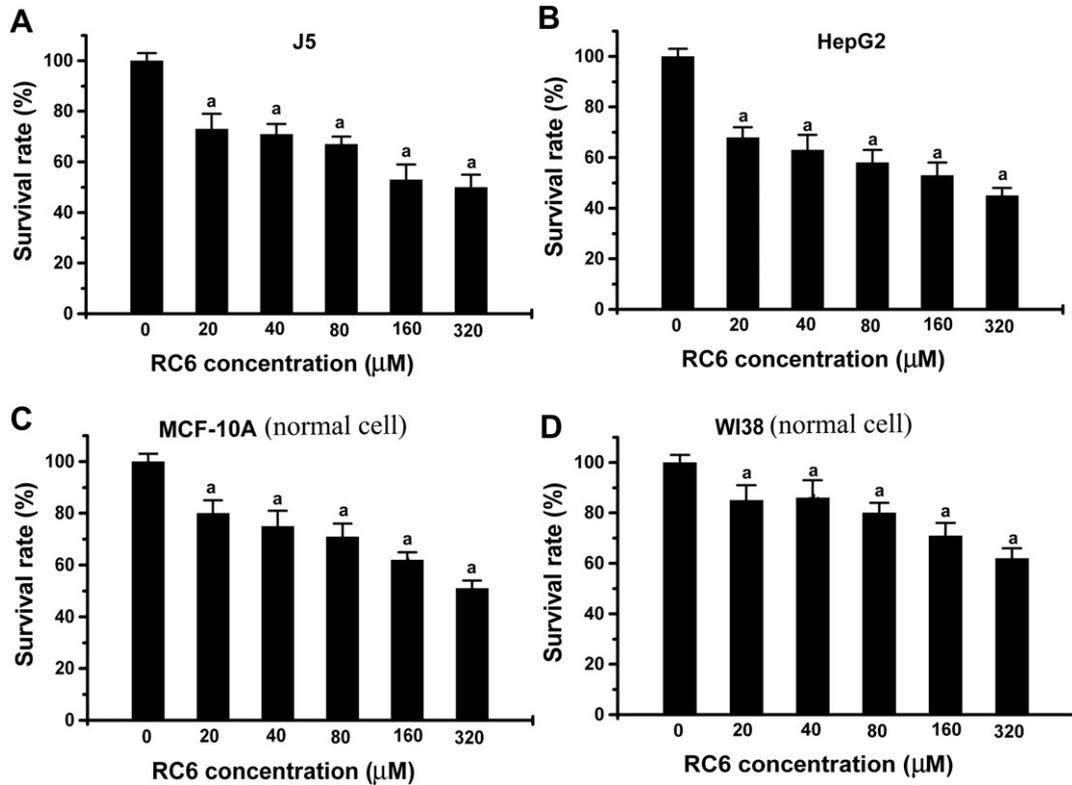


Fig. 2. RC6 inhibited the cell growth of human hepatoma cells. (A) J5; (B) HepG2; (C) MCF-10A; and (D) WI38 cells were treated with various concentrations of RC6. After 48 hours of incubation, the survival rates of the treated cells were measured by XTT assay. The results were obtained from four independent triplicate experiments and are presented as mean \pm SD. ^a $p < 0.05$. RC6 = Rana catesbeiana ribonuclease 6; SD = standard deviation.

analysis method as previously described [32] to detect phosphatylserine and membrane integrity. Briefly, the cells were stained with 10 μ g/mL annexin V-FLOUS kit and 20 μ g/mL PI. After staining, cells were resuspended in binding buffer at a concentration of 2×10^5 cells/mL before analysis by flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed on WinMDI version 2.8 software. The percentage of cells undergoing apoptosis was determined by three independent experiments.

2.4. Western blot

Cells were resuspended in lysis buffer (50mM Tris-HCl; 120mM NaCl; 1mM EDTA; 1% NP-40, pH 7.5) supplemented with protease inhibitors. Cell lysates were obtained after centrifugation ($15,000 \times g$) for 20 minutes at 4°C. Cell lysates were run in 13.3% sodium dodecyl sulfate polyacrylamide gel electrophoresis and

then transferred to a polyvinylidene fluoride membrane. After the membrane was blocked with 5% nonfat milk in Tris buffered saline with Tween (0.8% NaCl; 0.02% KCl; 25mM Tris-HCl; 0.05% Tween 20, pH 7.4), the membrane was reacted with primary antibodies (20–50 ng/mL) at 4°C overnight. The next day, the membrane was incubated with biotinylated antimouse immunoglobulin G or antirabbit immunoglobulin G antibodies (0.5–2 ng/mL), and was subsequently incubated with streptavidin-horseradish peroxidase conjugates (0.25 ng/mL). Labeled proteins were visualized by the Super Signal Chemiluminescent-HRP substrate system (Pierce, Rockford, IL, USA).

2.5. Caspase substrate cleavage activity assay

To analyze the activity of various caspases, cells were lysed with lysis buffer (50mM Tris-HCl, 120mM NaCl, and 1mM EDTA, 1%

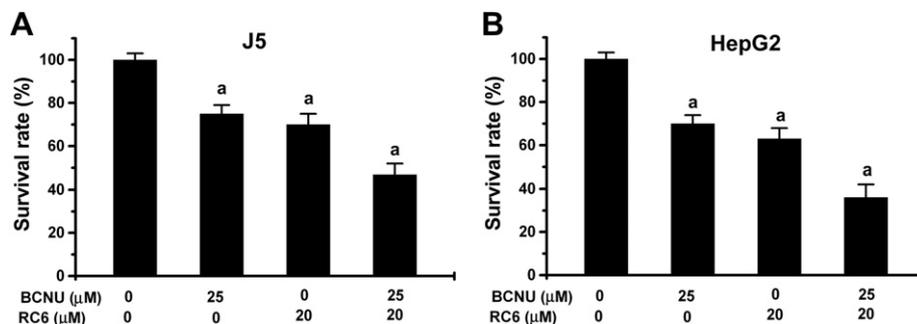


Fig. 3. BCNU with RC6 synergistically inhibited the cell growth of human hepatoma cells. J5 (A) and HepG2 cells (B) were treated with indicated concentrations of BCNU and RC6. After 48 hr of incubation, the survival rates of the treated cells were measured by XTT assay. The results were obtained from four independent triplicate experiments and are presented as mean \pm SD. ^a $p < 0.05$. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; RC6 = Rana catesbeiana ribonuclease 6; SD = standard deviation.

Table 1
The 2-day survival rate of cells treated with BCNU

BCNU concentration (μM)	HepG2 survival rate (%)	J5 survival rate (%)	MCF-10A survival rate (%)	WI38 survival rate (%)
6.25	98 \pm 3	90 \pm 7	96 \pm 5	99 \pm 6
12.5	90 \pm 7	79 \pm 8	92 \pm 5	95 \pm 7
25	76 \pm 7	69 \pm 8	87 \pm 6	98 \pm 8
50	53 \pm 8	51 \pm 6	79 \pm 4	90 \pm 6
100	42 \pm 4	40 \pm 4	63 \pm 4	81 \pm 7

BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea.

Table 2
The 2-day survival rate of cells treated with RC6

RC6 concentration (μM)	HepG2 survival rate (%)	J5 survival rate (%)	MCF-10A survival rate (%)	WI38 survival rate (%)
20	68 \pm 4	73 \pm 6	83 \pm 5	85 \pm 6
40	63 \pm 6	71 \pm 4	75 \pm 6	86 \pm 7
80	58 \pm 5	67 \pm 3	71 \pm 5	80 \pm 4
160	53 \pm 5	53 \pm 6	66 \pm 3	71 \pm 5
320	45 \pm 3	49 \pm 5	52 \pm 3	62 \pm 4

RC6 = Rana catesbeiana ribonuclease 6.

NP-40, pH 7.5) in the presence of protease inhibitors. The cell pellets were discarded after centrifugation at 15,000 \times g for 30 minutes at 4°C. The caspase activity was assessed in a working reaction containing 40 μL cell lysate (80 μg total protein), 158 μL reaction buffer (20% glycerol, 0.5mM EDTA, 5mM dithiothreitol, 100mM HEPES, pH 7.5), and 2 μL fluorogenic substrate, either Ac-LEHD-pNA or Ac-DEVD-pNA (100 μM final concentration); the reaction was incubated at 37°C for 6 hours. Cleavage of the fluorogenic substrate released p-nitroanilide, which was measured at 405 nm in an ultramicroplate reader (Ceres UV 900; Bio-Tek). The fold increase in caspase activity was calculated using the following formula: (A405sample – A405control)/A405control.

2.6. Statistical analysis

Data were obtained from four independent triplicate experiments and are presented as the mean values of the chosen triplicate groups. These data are shown as means with standard deviations. The statistical differences were analyzed using student's *t* test for normally distributed values. A value of *p* less than 0.05 was considered statistically significant.

Table 3
The 2-day survival rate of cells treated with 25 μM BCNU and 20 μM RC6

Survival rate	HepG2	J5	MCF-10A	WI38
Survival rate (%)	36 \pm 6	47 \pm 5	82 \pm 4	86 \pm 7

BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; RC6 = Rana catesbeiana ribonuclease 6.

3. Results

3.1. BCNU-induced cell cytotoxicity in human hepatoma cells

To determine whether BCNU inhibited cell growth of human hepatoma cells, both J5 and HepG2 cells were treated with various concentrations of BCNU for 2 days. As shown in Fig. 1, BCNU was able to induce cytotoxicity in both types of cells and this effect was dose-dependent manner. The 2-day survival rate was below 50% only when cells were treated with 50–100 μM BCNU. However the 2-day survival rate was above 70% when cells were treated with 0–25 μM BCNU. That is, BCNU in the range 50–100 μM BCNU was able to effectively inhibit the cell growth of both types of hepatoma cells, but no obvious cytotoxicity was observed in hepatoma cells with 6.25–25 μM BCNU treatment.

3.2. RC6-induced cell cytotoxicity in human hepatoma cells

To further clarify whether RC6 inhibited the cell growth of human hepatoma cells, both J5 and HepG2 cells were treated with various concentrations of RC6 for 2 days. Our studies showed that RC6 was able to induce cytotoxicity in both types of cells and this effect was dose-dependent (Fig. 2), similar to the BCNU-induced cytotoxicity shown in Fig. 1. Our experiments also showed that only 160–320 μM RC6 was able to effectively inhibit the cell growth of both types of hepatoma cells resulting in a 2-day survival rate below 50%. Observations from Figs. 1 and 2 suggest that the cell growth of both J5 and HepG2 could be inhibited effectively when cells were treated with higher dosages of BCNU or RC6 alone.

3.3. Combination treatment with low-dose BCNU and RC6 exerted a synergistic cytotoxic effect in human hepatoma cells

RC6 treatments with dosages less than 80 μM inhibited the cell growth of normal cells (data not shown). To inhibit cell growth of hepatoma cells effectively using lower dosages of BCNU and RC6, combination treatment with BCNU and RC6 was applied in hepatoma cells. As shown in Figs. 1 and 2, the 2-day survival rates were above 70% in both J5 and HepG2 cells with 25 μM BCNU and 20 μM

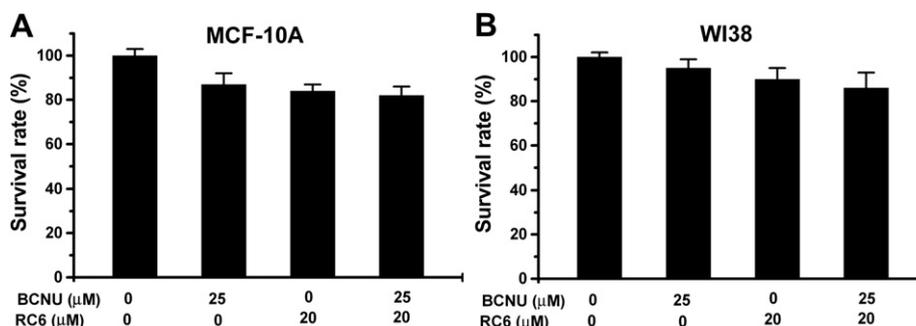


Fig. 4. Combination treatment with BCNU and RC6 did not induce cytotoxic effects on normal cells. (A) MCF-10A and (B) WI38 cells were treated with the indicated concentrations of BCNU and RC6. After 48 hours of incubation, the survival rates of the treated cells were measured by XTT assay. The results were obtained from four independent triplicate experiments and are presented as mean \pm SD. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; RC6 = Rana catesbeiana ribonuclease 6; SD = standard deviation.

RC6 alone. To further determine whether combination treatment with lower dosages of BCNU and RC6 has a synergistic cytotoxic effect on hepatoma cells, both J5 and HepG2 cells were exposed to BCNU alone, RC6 alone, and BCNU/RC6. Our results showed that the survival rate was about 70% in both types of cells treated with 25 μ M BCNU and 20 μ M RC6 alone (Fig. 3). However, combination treatment with 25 μ M BCNU and 20 μ M RC6 resulted in about a 40% survival rate (Fig. 3). Our data suggest that combination treatment with lower dosages of BCNU or RC6 exerts a synergistic cytotoxic effect on human hepatoma cells. In addition, as shown in

Tables 1–3, the survival rates were below 50% when HCC cells (HepG2 and J5) were treated with 100 μ M BCNU alone or 320 μ M RC6 alone. However, when MCF-10A and WI38 cells were treated with 100 μ M BCNU, the survival rates were about 63% and 80% (Table 1). When MCF-10A and WI38 cells treated with 320 μ M RC6, the survival rates were around 52% and 62%, respectively (Table 2). HCC cells had survival rates below 50% with a combination of 25 μ M BCNU and 20 μ M RC6. Nevertheless, the survival rates of MCF-10A and WI38 cells exceeded 80% with this combination treatment. (Fig. 4 and Table 3).

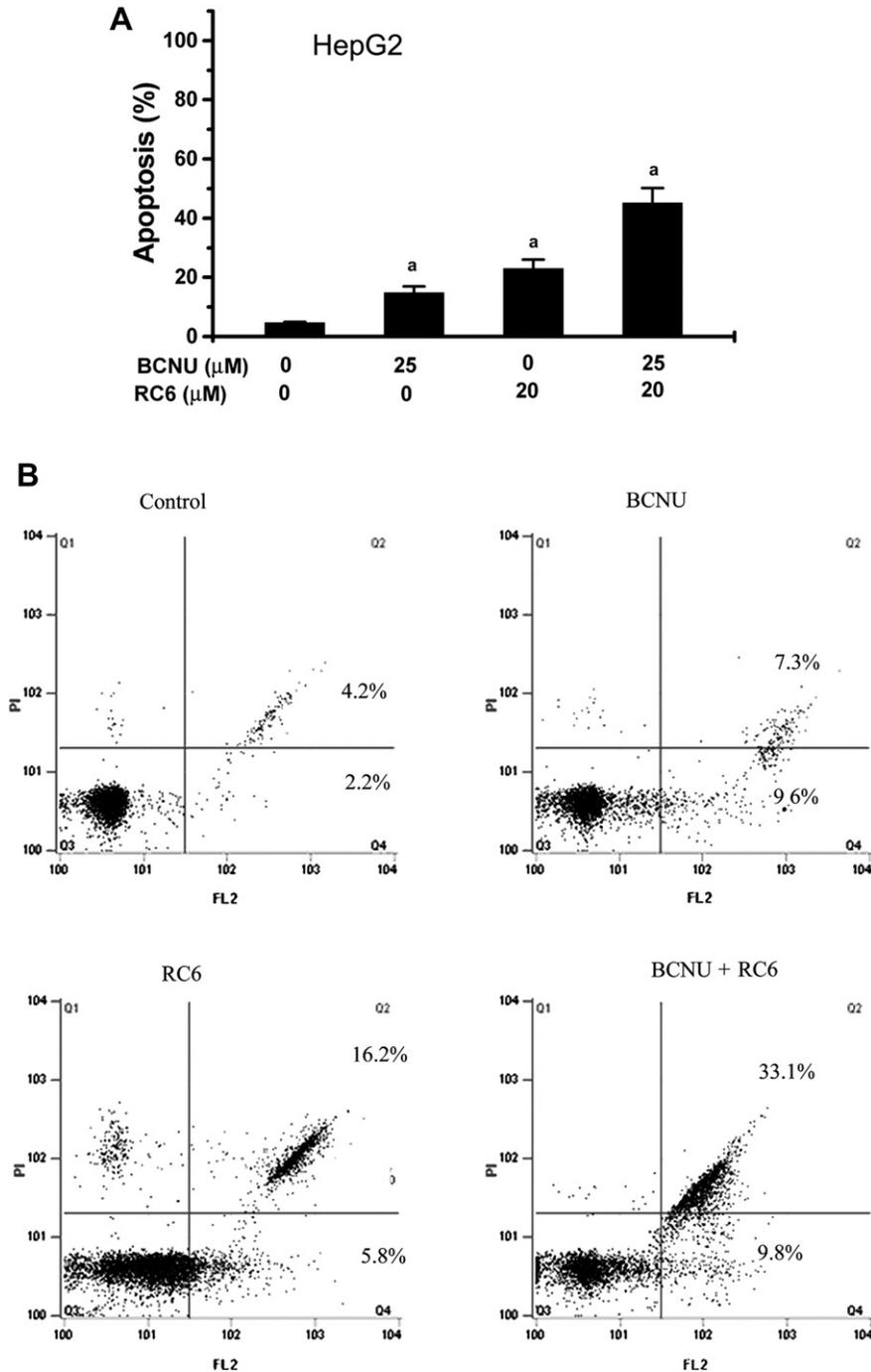


Fig. 5. (A) Apoptosis was induced in HepG2 cells with BCNU or RC6 treatment. Cells were double stained with PI/annexin-V, and the percentage of apoptosis was determined with FACS analysis; (B) The original FACS analysis diaphragms. ^a*p* < 0.05. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; FACS = fluorescence-activated cell sorting; PI = propidium iodide; RC6 = Rana catesbeiana ribonuclease 6.

3.4. BCNU and RC6-induced apoptosis and activated the caspase-9/caspase-3 cascade in hepatoma cells

After 2 days treatment with a single agent or combined treatment, HepG2 cells were collected and double stained with annexin-V/PI for FACS analysis. The percentage of apoptosis induced by BCNU and RC6 was about 17% and 20%, respectively (Fig. 5). However, combined exposure with BCNU and RC6 resulted in up to 50% apoptosis (Fig. 5). To further demonstrate whether the caspase-9/caspase-3 or caspase-8/caspase-3 cascade was induced in HepG2 cells with combination treatment, caspase-3, caspase-8, and caspase-9 activities were measured by Western blot (Fig. 6). After HepG2 cells were treated with a combination of BCNU and RC6, procaspase-9 was decreased and cleaved, but caspase-3 increased (Fig. 6, Lanes 4). However, there was no obvious decrease in procaspase-8 (Fig. 6). Moreover, caspase-9 and caspase-3 activities were found in combination treatment with BCNU and RC6 by substrate cleavage assay (Fig. 7). The apoptosis percentage and caspase activation were determined in J5 cells. Our study showed that J5 cells exposed to a combination of BCNU and RC6 had results similar to those for the HepG2 cells exposed to a combination of BCNU and RC6 (data not showed).

4. Discussion

Previously, Liao et al [24] showed that 20 μM RC6 has a strong cytotoxic effect on cervical cancers. However, our data showed that the 2-day survival rate was about 70% in J5 and HepG2 hepatoma cells with 20 μM RC6 treatment (Fig. 2). That is, RC6 is more toxic to cervical cancers than hepatoma cells. We suggest that RC6 has a different cytotoxic effect on different cell lines. In addition, our data showed that a high dosage of RC6 (160–320 μM) could effectively inhibit the growth of hepatoma cells (Fig. 2) and also the growth of normal cells. Therefore, we focused on the use of low-dose RC6 for hepatoma cell therapy.

BCNU has been used as a clinical anticancer drug [11,12]. Hepatoma cells could be inhibited under high dosage BCNU

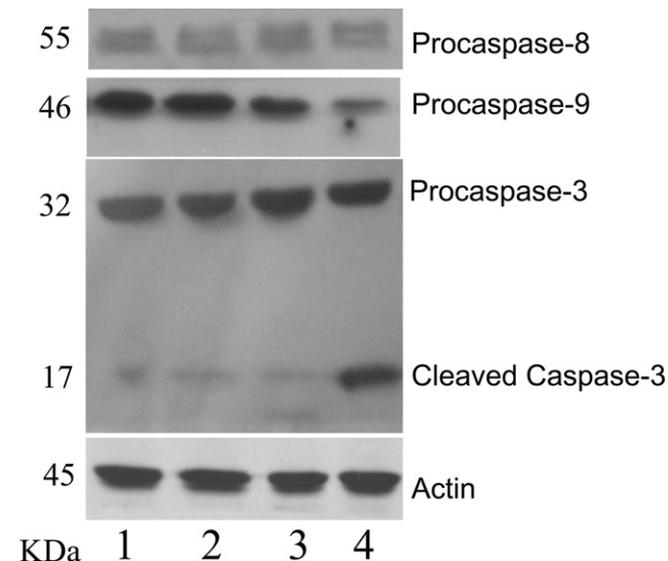


Fig. 6. Caspase activation was determined by Western blotting analysis. HepG2 cells were treated either with diluent (Lane 1), 25 μM BCNU (Lane 2), 20 μM RC6 (Lane 3) or 25 μM BCNU, and 20 μM RC6 (Lane 4). Two days after treatment, cells were lysed and subjected to SDS-PAGE followed by Western blotting analysis using antibodies against procaspase-8, procaspase-9, or procaspase-3. Actin served as a loading control. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; RC6 = Rana catesbeiana ribonuclease 6; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

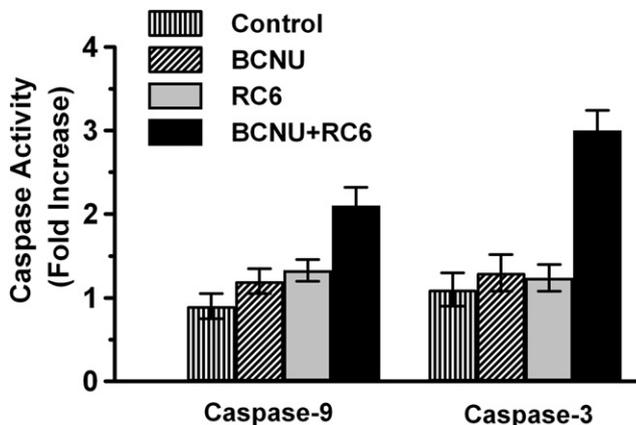


Fig. 7. Caspase-9 and caspase-3 activity were determined in HepG2 cells treated with 25 μM BCNU, 20 μM RC6, and a combination of 25 μM BCNU and 20 μM RC6. Note that caspase-9/caspase-3 activity was significantly increased in the combination treatment. The results are presented as mean ± SD from four independent triplicate experiments. BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; RC6 = Rana catesbeiana ribonuclease 6; SD = standard deviation.

treatment (Fig. 1). Similar results were also demonstrated in a previous study [10].

To avoid the side effects caused by high-dosage treatment, a combination treatment with low-dose BCNU and RC6 was considered in this study. As shown in Fig. 3, combination treatment with 25 μM BCNU and 20 μM RC6 had a strong synergistic cytotoxic effect on hepatoma cells. Previous studies and our primary data indicated that RC6 can cleave RNA, inhibit protein synthesis, and downregulate Bcl-2 and Bcl-xL [9,24,31]. BCNU can interact with DNA, RNA, and protein; induce DNA crosslinks and S-phase arrest; and inhibit cell division [13–17]. These mechanisms are factors in RC6/BCNU-induced cell death and can explain why RC6 and BCNU exert synergistic cytotoxic effects.

Cell death has been divided into the apoptosis and necrosis pathways [33]. DNA condensation, fragmentation, and the presence of phosphatidyl serine in the outer membrane are the major characteristics of apoptosis [9,10]. In this study, phosphatidyl serine was detected in the outer membrane in BCNU/RC6-treated hepatoma cells by annexin-V/PI analysis (Fig. 5). Therefore, combination treatment with BCNU and RC6 can induce apoptosis in hepatoma cells. In addition, many studies have shown that the caspase-3/caspase-8 and caspase-3/caspase-9 cascades are the major caspase pathways [34,35]. In the Western blot, caspase-9 and caspase-3 activities were induced and caspase-8 activity was not activated in BCNU/RC6 treated-hepatoma cells (Figs. 6 and 7). We suggest that combination treatment with BCNU and RC6 induces apoptosis through the caspase-9/caspase-3 cascade.

This study is the first to demonstrate that combination treatment with low-dose BCNU and RC6 has a synergistic cytotoxic effect on hepatoma cells and induces apoptosis through the caspase-9/caspase-3 cascade in hepatoma cells.

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