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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.

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 tumorgenesis 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 tumorgenesis 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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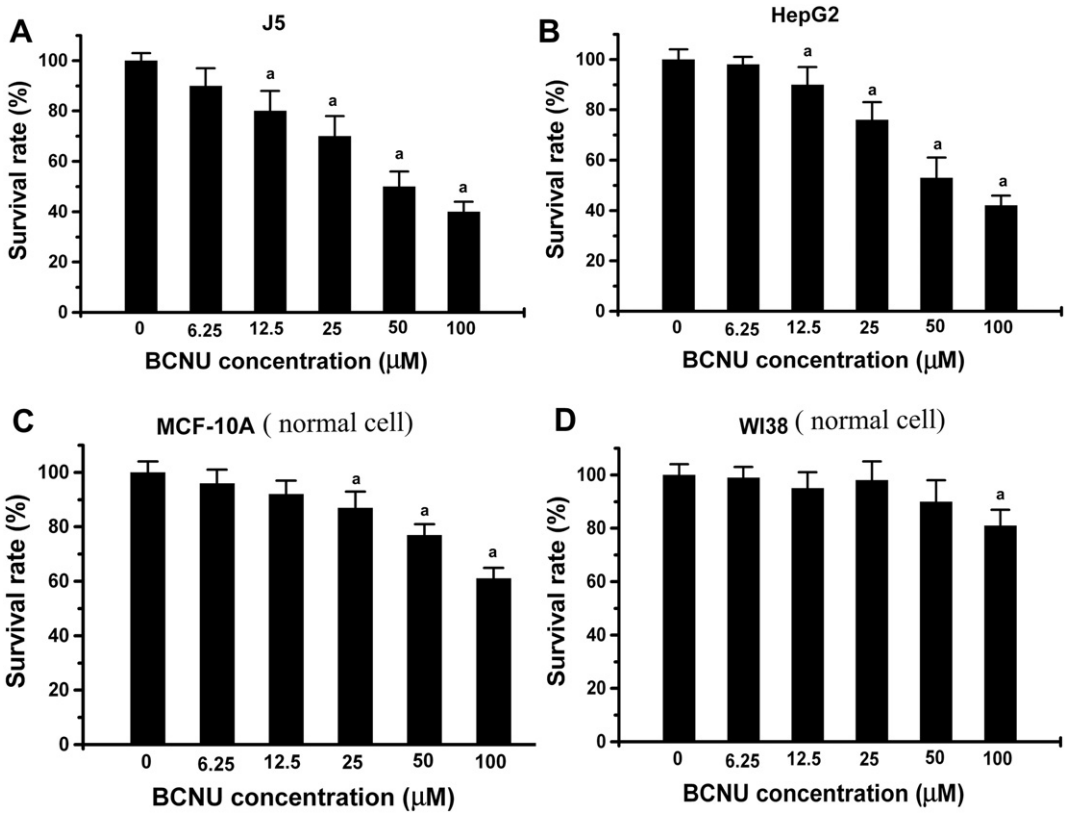

Fig. 1. BCNU inhibited the cell growth of human hepatoma cells. (A) J5; (B) HepG2; (C) MCF-10A; and (D) WI38 cells were treated with various concentrations of BCNU. After 48 hours of incubation, the survival rates of the treated cells were measured by XTT assay. The results were obtained from four independent triplicate experiments and are presented as mean ± 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 Bioresources 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 μg/mL bovine pancreatic insulin, 20 ng/mL epidermal growth factor, and 0.5 μg/mL hydrocortisone (GIBCO). These cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

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)