

Brucine induces the apoptosis of U266 multiple myeloma cells by phosphorylation of c-Jun

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Abstract. The aim of this study was to investigate the mechanism of the apoptotic effect of brucine on human multiple myeloma (MM) cells. U266 cells (5×10^4) were plated in the presence or absence of brucine (0, 0.05, 0.1, 0.2 and 0.4 mg/ml) in 96-well culture plates for 24–72 h. The anti-proliferative response to brucine was assessed by MTT assay. Analysis of the cell cycle of U266 cells treated with or without brucine was performed using flow cytometry. The expression change of c-Jun following treatment with brucine or brucine plus the c-Jun N-terminal kinase (JNK)-specific inhibitor SP600125 was detected using RT-PCR. Brucine appeared to have an effect on apoptosis in a dose- and time-dependent manner. Cell cycle analysis using flow cytometry revealed the accumulation of cells at the sub-G₀/G₁ phase. The apoptotic rates were 4.137, 10.55, 12.31, 27.67 and 29.67% (0, 0.05, 0.1, 0.2, 0.4 mg/ml brucine, respectively; $P < 0.01$). The gray scale values were 0.7961 ± 0.007 and 0.4683 ± 0.003 (mRNA expression of c-Jun of U266 cells with or without SP600125, respectively). Concentrations of ≤ 0.4 mg/ml brucine induced apoptosis in U266 cells. Thus, brucine-induced apoptosis in U266 cells occurs via the JNK signaling pathway and phosphorylation of c-Jun.

Introduction

Multiple myeloma (MM) is a malignant clone of plasma cells. The synthesis and secretion of monoclonal immunoglobulin uniform structure and/or light chains alone, accompanied by a reduction in normal immunoglobulin levels, is a feature of MM. The activation of osteoclasts, upsetting the balance of osteoclasts and osteoblasts, appearance of extensive osteolytic lesions and/or malignant tumors and osteoporosis are also characteristic of MM (1). Although high-dose chemotherapy with autologous stem cell transplantation and other therapies of

enhancing response and survival rates have improved, due to the low proportion of tumor cells (typically less than 1.5%) (2) and multidrug resistance of tumor cells, MM treatment response is poor and the disease remains incurable, ultimately leading to drug resistance and relapse.

Plants of the *Strychnos* genus have been used to treat typhoid fever and sore throat, as described in the 'Compendium of Materia Medica'. The 'Chinese Medicine chi' reported that plants of the *Strychnos* genus can treat fever, swelling, ulcers and sores. Deng *et al* investigated the effect of *Strychnos* on HepG2 cells and found that the *Strychnos* alkaloid treatment of liver cancer is due to its direct cytotoxicity (3). *Strychnos* alkaloids mostly consist of biologically active substances, but also contain pharmacological and toxicological components, of which 80% are strychnine and brucine (4). A previous study (5) found that brucine induces apoptosis via the death receptor pathway in multiple myeloma. The present study used reverse transcription polymerase chain reaction (RT-PCR) and flow cytometry to analyze the apoptotic signaling pathway.

Materials and methods

Cell culture and cell proliferation assay. U266 cells were maintained in RPMI-1640 culture medium with 10% fetal calf serum, 100 mg/l penicillin and 100 mg/l streptomycin, in a 37°C incubator supplied with 5% CO₂ at room temperature. The anti-proliferative response of brucine was assessed by MTT assay. U266 cells were plated at a final concentration of 5×10^4 cells/ml in the presence or absence of brucine (0, 0.05, 0.1, 0.2 and 0.4 mg/ml) in a 96-well plate. Then 20 μ l MTT (5 mg/ml) were added at 24, 48 and 72 h following treatment. For the MTT assay, the supernatant was discarded and 200 μ l DMSO was added, and the 96-well plate was agitated on a micro-vibrator for 10 min. The optical density of each well was measured at $\lambda 492$ nm by an enzyme-immunoassay instrument.

Cell cycle analysis. U266 cells (2×10^6) were treated with or without brucine (0, 0.05, 0.1, 0.2 and 0.4 mg/ml) for 48 h. The cells were harvested, washed with PBS at 1,000 rpm for 5 min, fixed with 70% alcohol, washed again with PBS and stained with propidium iodide in the presence of 100 μ l RNase A for 30 min prior to analysis by flow cytometry.

RT-PCR. Total RNA was extracted using a TRIzol reagent. cDNA was amplified from 6 μ l of total RNA using

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Table I. mRNA expression of caspase-3 and c-Jun of U266 cells with or without brucine for 12, 24 and 48 h (mean \pm SD).

Group	Caspase-3 ^a	c-Jun ^a
Control	0.2597 \pm 0.020	0.1354 \pm 0.0016
12 h	0.5488 \pm 0.016	0.2603 \pm 0.0032
24 h	0.6205 \pm 0.006	0.4874 \pm 0.0068
48 h	0.6533 \pm 0.009	0.5965 \pm 0.0089

^aP<0.01.

Table II. mRNA expression of caspase-3 and c-Jun of U266 cells with or without SP600125 (mean \pm SD).

Group	Caspase-3 ^a	c-Jun ^a
Brucine	0.7683 \pm 0.0050	0.7961 \pm 0.007
Brucine+SP600125	0.5723 \pm 0.0055	0.4683 \pm 0.003

^aP<0.05.

ThermoScript RT-PCR System with 1 μ l oligo(dT)18 (0.5 μ g/ μ l), 1 μ l TransScriptTM RT/RI enzyme, 10 μ l 2X TS Reaction mix, 2 μ l RNase-free water, analyzed on 2% agarose gel and confirmed by nucleotide sequencing. The primer pairs used for RT-PCR were: caspase-3 (151 bp): 5'-TTTTTCAGAGGGGATCGTTG-3' and 5'-CGGCCTCCACTGGTATTTTA-3'; c-Jun: 5'-CCCCAAGATCCTGAAACAGA-3' and 5'-CCGTTGCTGGACTGGATTAT-3'; GADPH: 5'-TGAACGGGAAGCTCACTGG-3' and 5'-TCCACCACCCTGTTGCTGGA-3'.

Statistical analysis. Data were expressed as the mean \pm SD and analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). The statistical methods involved two independent sample t-test and analysis of variance (ANOVA). P<0.05 was considered to indicate a statistically significant result.

Results

Growth inhibitory effects of brucine on U266 cells. The effect of brucine on the proliferative response of U266 cells was analyzed by treating cells with different concentrations of brucine (0, 0.05, 0.1, 0.2 and 0.4 mg/ml) for 24, 48 and 72 h. The growth inhibitory effects of brucine treatment were assessed by MTT assay. The treated cells showed a significant decrease in proliferation in a dose- and time-dependent manner (P<0.05; Fig. 1). The IC₅₀ value of brucine at 48 h was 0.16 mg/ml.

Cell cycle analysis. The appearance of the sub-G₀/G₁ cell is characteristic of apoptosis. The results of brucine treatment showed that the sub-G₀/G₁ phase population significantly increased following brucine treatment in a dose-dependent manner. The sub-G₀/G₁ phase population increased to 4.137,

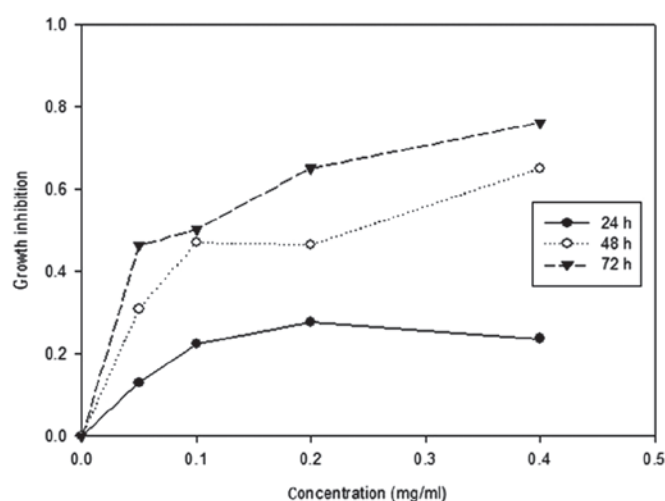


Figure 1. Effect of brucine on U266 cells with different concentrations (0, 0.05, 0.1, 0.2, 0.4 mg / ml) for 24, 48 and 72 h. The treated cells showed a significant decrease in proliferation in a dose- and time-dependent manner.

10.55, 12.31, 27.67 and 29.67% following the exposure of the cells to 0, 0.05, 0.1, 0.2 and 0.4 mg/ml brucine concentrations, respectively. This accumulation of cell population at the sub-G₀/G₁ phase in a dose-dependent manner indicated an induction of apoptosis.

Caspase-3 is activated in brucine-induced U266 cell apoptosis. Since caspase-3 is an executor of apoptosis, caspase-3 expression was measured in brucine-treated U266 cells by RT-PCR after 12, 24 and 48 h. A brucine-induced time-dependent increase of caspase-3 was detected (Fig. 2). The gray value of caspase-3 was 0.2597 \pm 0.020 in the control group and increased in the brucine-treated (0.16 mg/ml) group to 0.5488 \pm 0.016, 0.6205 \pm 0.006 and 0.6533 \pm 0.009 on exposure of the cells at 12, 24 and 48 h, respectively (Table I).

Effects of brucine on c-Jun expression. c-Jun is a downstream product of the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) signaling pathway. To investigate the effect of c-Jun on the brucine-induced U266 cell apoptosis, the expression of c-Jun was measured in brucine-treated U266 cells by RT-PCR at 12, 24 and 48 h (Fig. 3). The gray value of c-Jun was 0.1354 \pm 0.0016 in the control group and increased in the brucine-treated (0.16 mg/ml) group up to 0.2603 \pm 0.0032, 0.4874 \pm 0.0068 and 0.5965 \pm 0.0089 upon exposure of the cells at 12, 24 and 48 h, respectively (Table I).

Caspase-3 and c-Jun expression. To verify that the activation of caspase-3 and c-Jun is associated to the JNK signaling pathway, the expression of caspase-3 and c-Jun was measured after the specific inhibitor of the JNK signaling pathway, SP600125, was added by RT-PCR (Fig. 4). The gray values of caspase-3 and c-Jun were 0.7683 \pm 0.0050 and 0.7961 \pm 0.007, respectively, in the brucine-treated (0.16 mg/ml) group. By contrast, a decrease in the brucine (0.16 mg/ml) and SP600125 group down to 0.5723 \pm 0.0055 and 0.4683 \pm 0.003 was detected following treatment for 24 h (Table II).

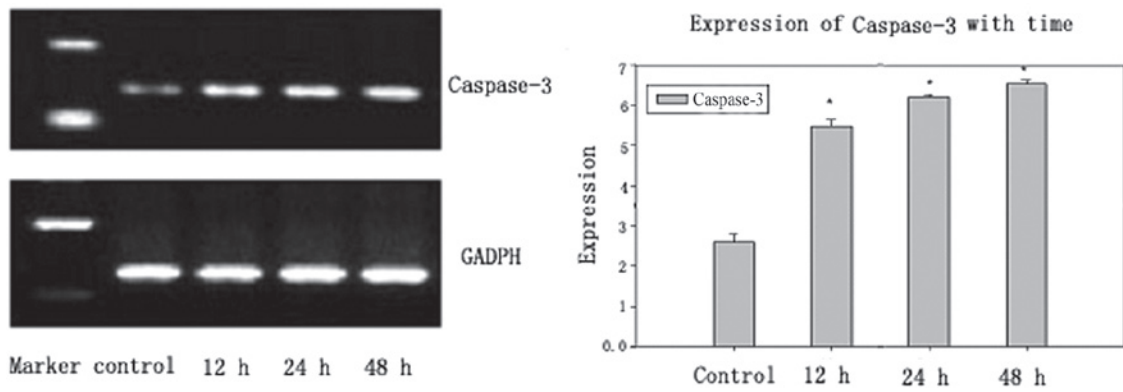


Figure 2. Effects of brucine on caspase-3 expression at various time points. GADPH was used as an internal reference. Each experiment was performed in triplicate.

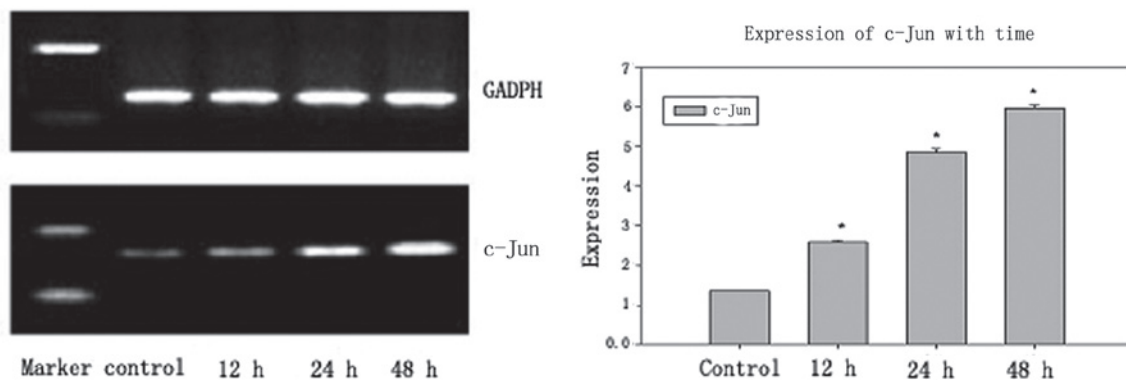


Figure 3. Effects of brucine on c-Jun expression at various time points. GADPH was used as an internal reference. Each experiment was performed in triplicate.

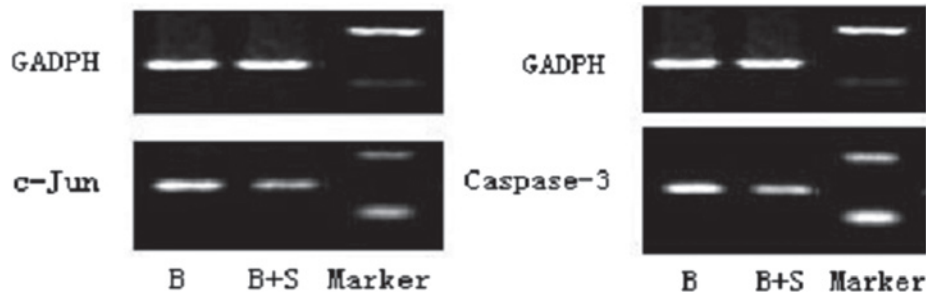


Figure 4. Expression of caspase-3 and c-Jun after adding the inhibitor SP600125. GADPH was used as an internal reference. Each experiment was performed in triplicate. B, brucine; B+S, brucine+SP600125.

Discussion

The process of programmed cell death (PCD), or apoptosis, is required to maintain homeostasis (6). Apoptosis is closely correlated with the occurrence of a variety of diseases (including autoimmune disease and cancer) (7). Apoptosis is distinct from necrosis and is a process in which cells are actively involved in initiating a series of gene activations to adapt to the environment. MM is an incurable plasma cell neoplasm characterized by the accumulation of malignant plasma cells in the bone marrow. The occurrence and development of MM is associated to abnormal proliferation and the inhibition of apoptosis of tumor cells.

This study was performed to evaluate the response to brucine using the human MM line cell, U266. These results confirmed the anti-proliferative effect of brucine on the U266 cell line, with an IC_{50} value of 0.16 mg/ml at 48 h (Fig. 1). Furthermore, the flow cytometric analysis was performed on brucine-treated U266 cells to analyze the type of cell death process (apoptosis or necrosis). The results demonstrated that the sub- G_0/G_1 cell population increased in a dose-dependent manner.

Mitogen-activated protein kinase (MAPK) is a significant method of signal transduction in eukaryotes and is key in the regulation of gene expression (8). The classic MAPK signaling pathway is the MAPKKKs \rightarrow MAPKKs \rightarrow MAPKs continuous

enzymatic reaction (8,9). The MAPK family includes extracellular signal regulated kinase (ERK), JNK/SAPK and p38 categories (10). The dynamic balance of JNK, p38 and ERK determines cell survival and apoptosis (11). JNK is a serine/threonine protein kinase located in the cytoplasm with a molecular mass of 54 kDa (12). Due to its interaction with the N-terminal activation domain of c-Jun and the phosphorylation of serine 63 and 73, JNK has been designated as c-Jun N-terminal kinase (10). c-Jun belongs to the Jun subfamily (c-Jun, Jun B and Jun D) and forms the center of transcription factor-activated protein-1 (AP-1) as well as dimers of basic leucine zippers. c-Jun and Fos (v-Fos, c-Fos, FosB and FosL1, FosL2) form homo- or heterodimers, which activate transforming growth factor (ATF-2, LRF-1/ATF-3, B2ATF, JDP1 and JDP2) or fibrosarcoma tendon membrane protein (c-Maf, MafB, MafA, MafG/F/K and NRL). Phosphorylation of c-Jun and ATF-2, and activation of the transcription factor (AP-1), combined with Fas through the activation of caspase-8 (13). JNK induces apoptosis via the death receptor pathway. JNK may also act via the phosphorylation of Bcl-2 and Bcl-xL (14). The release of cytochrome C, and activation of caspase-9 induces apoptosis through the mitochondrial pathway (15). In the anti-Fas monoclonal antibody induction apoptosis of MM cells, JNK/SAPK in the cytoplasm and transcription factor c-Jun were induced (16,17). The roselle extract is known to activate JNK/p38MAPK (18). The phosphorylation of the target proteins of c-Jun activates the signal transduction of apoptosis-related proteins, including the Fas-mediated signal, and induces apoptosis (19).

Findings of our previous study (5) have verified that the apoptosis induced by brucine occurs via the death receptor pathway. To investigate the signaling pathways of apoptosis, the U266 cells treated with brucine for 12, 24 and 48 h were collected. c-Jun, the downstream product of JNK and caspase-3, which is the executor of apoptosis, was detected (Figs. 2 and 3). The results showed that the gray value in brucine-treated cells for 12, 24 and 48 h increased up to 0.5488 ± 0.016 , 0.6205 ± 0.006 , 0.6533 ± 0.009 for caspase-3 and 0.2603 ± 0.0032 , 0.4874 ± 0.0068 , 0.5965 ± 0.0089 for c-Jun, respectively (Table I; $P < 0.01$), and demonstrated that caspase-3 and c-Jun were activated in brucine-treated cells. In addition, caspase-3 and c-Jun were detected again following the addition of the specific JNK inhibitor, SP600125 (20). The results demonstrate that the gray value of caspase-3 and c-Jun were decreased to 0.5723 ± 0.0055 and 0.4683 ± 0.003 , respectively (Fig. 4, Table II), and that the activation of caspase-3 and c-Jun in brucine-treated cells is capable of inhibition by the specific JNK inhibitor SP600125. We suggest that brucine, via phosphorylation of c-Jun by the JNK signaling pathway, induces apoptosis in the human MM cell line U266.

In the present study, we suggest that the anti-proliferative activity of brucine on U266 cells is due to apoptosis. Preliminary analysis of the apoptosis mechanism demonstrated that the JNK signaling pathway was activated in the apoptotic U266 cells treated with brucine. The present study suggests that brucine has possible anti-cancer effects and provides a theoretical basis for the clinical treatment of MM. However,

the inhibitory growth effect of brucine on other myeloma cell lines and resistant cell lines as well as other signaling pathways remains to be determined. Additionally, the overall level of apoptosis and toxicity caused by brucine requires additional investigation.

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