The Effects of *Crinum asiaticum* on the Apoptosis Induction and the Reversal of Multidrug Resistance in HL-60/MX2

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The present study investigated the anti-proliferative and chemosensitizing effects of *Crinum asiaticum* var. japonicum against multi-drug resistant (MDR) cancer cells. The 80% methanol extract, chloroform (CHCl₃) fraction and butanol (BuOH) fraction of *C. asiaticum* inhibited the growth of mitoxantrone (MX) resistant HL-60 (HL-60/MX2) cells. When HL-60/MX2 cells were treated with the CHCl₃ and BuOH fractions, DNA ladder and sub-G1 hypodiploid cells were observed. Furthermore, the fractions reduced Bcl-2 mRNA levels, whereas Bax mRNA levels were increased. These results suggest that the inhibitory effect of *C. asiaticum* on the growth of the HL-60/MX2 cells might arise from the induction of apoptosis. Treatment of HL-60/MX2 cells with the fractions markedly decreased the mRNA levels of the multidrug resistance protein-1 and breast cancer resistance protein. The CHCl₃ fraction and hexane fraction increased MX accumulation in HL-60/MX2 cells. These results imply that the CHCl₃ fraction of *C. asiaticum* plays a pivotal role as a chemosensitizer. We suggest that components of *C. asiaticum* might have a therapeutic potential for the treatment of MDR leukemia.

**Key words:** HL-60/MX2, *Crinum asiaticum*, Apoptosis, Chemosensitizer, MRP-1, BCRP.

**INTRODUCTION**

The development of multi-drug resistance (MDR) by tumor cells is a major obstacle to successful cancer chemotherapy (Riordan et al., 1979). MDR is the phenomenon by which exposure of tumor cells to a single cytotoxic agent results in cross-resistance to other, structurally unrelated, classes of cytotoxic compounds. MDR is multifactorial, and the strategies proposed to reverse MDR include targets from the apoptosis pathway, efflux transporters and so on. Increased expression of efflux proteins, which belong to the ATP-binding cassette (ABC) family of proteins, is a common feature of MDR (Allikmets et al., 1996; Gotteeman et al., 1993), and overexpression of efflux proteins is associated with resistance to numerous anticancer agents (Larsen et al., 2000). The best known and well studied are P-glycoprotein (P-gp; MDR1) encoded by the *mdr1* gene (Endicott et al., 1989), multidrug resistance-associated protein-1 (MRP-1) encoded by the *mrp1* gene (Cole et al., 1992), and breast cancer resistance protein (BCRP) encoded by the *abcg2* gene (Doyle et al., 2003).

Another mechanism of MDR is related to resistance to apoptosis of multi-drug resistant cells induced by cytotoxic agents. The cellular molecules involved in this mechanism include anti-apoptotic proteins such as Bcl-2, which are overexpressed, and pro-apoptotic proteins such as Bax, which are down-regulated (Seiler et al., 1999).

Many studies have shown a link between tumor hypoxia and MDR. Under hypoxic conditions, the cytotoxicity of chemotherapeutic agents such as cisplatin, etoposide, bleomycin and mitomycin C is reduced (Koch et al., 2003). Hypoxia-elicited chemotherapeutic resistance has been reported in a number of cell types, including fibroblasts, breast cancer cells, glioma cells and testicular germ cells (Koch et al., 2003; Kalra et al., 1993; Liang, 1996). Hypoxia-associated chemotherapeutic resistance is a broad phenomenon. Comerdord et
al. have reported that MDR1 gene expression and subsequent functional P-gp expression are dramatically up-regulated in a hypoxia inducible factor-1 (HIF-1)-dependent manner in response to hypoxia (Comerford et al., 2002).

Studies of *Cfium asiaticum* var. *japonicum*, a member of the tribe Amaryllidaceae, have described that it contains several phenanthridine alkaloids, triterpene alcohols and flavonoids (Sakurai et al., 1976; Takagi et al., 1997; Min et al., 2001). Alkaloids isolated from the bulbs of Amaryllidaceae have shown various pharmacological effects, such as antiviral (Gabrielsen et al., 1992), antimalarial (Likhitwitayawud et al., 1993), cytotoxic (Likhitwitayawud et al., 1993; Abdel-Halim et al., 2004; Petti et al., 1990; Xie et al., 2004), anti-inflammatory (Samud et al., 1999) and antineoplastic activities (Furusawa et al., 1988), as well as effects on diseases of the nervous system (Cakici et al., 1997). In particular, cinnarizine from *C. asiaticum* has been reported to induce apoptosis in hepatoma cancer cells (McNulty et al., 2007) and inhibit HIF-1 activity (Kim et al., 2006). In spite of chemotherapeutic resistance related to the activity of HIF-1, there are few reports on the anti-proliferative effect of *C. asiaticum* in chemotherapeutic agent resistant cells.

In this paper, we demonstrate that the 80% methanol (MeOH) extract and several solvent fractions of *C. asiaticum* decreased the survival rate of HL-60/MX2 cells by the induction of apoptosis, as well as having a chemosensitizing effect that could increase the intracellular accumulation of drug and decrease the expression of MRP-1 and BCRP.

**MATERIALS AND METHODS**

*Preparation of extract from C. asiaticum.* *C. asiaticum* var. *japonicum* was collected in March 2003 at Jeju Island, South Korea. *C. asiaticum* was washed in distilled water, dried at room temperature and ground into a fine powder. The dried plant powder (100 g) was extracted with 3 l 80% methanol (MeOH) at room temperature for 3 days and then the supernatant was concentrated under a vacuum. The resulting crude extract (20 g) was suspended in water (1 l) and successively partitioned with hexane (1 l x 3), chloroform (CHCl₃; 1 l x 3), ethyl acetate (EtOAc; 1 l x 3) and n-butanol (BuOH; 1 l x 3), to give hexane (497 mg), CHCl₃ (162 mg), EtOAc (200 mg), BuOH (1160 mg) and H₂O (4800 mg) fractions.

*Cell culture.* The HL-60/MX2 cell line is a mitoxantrone resistant derivative of the human acute promyelo- loid leukemia cell line HL-60. The clone designated HL-60/MX2 was approximately 35 fold less sensitive to MX than HL-60 parental cells. The cell line was obtained from the American Type Culture Collection (ATCC) and was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively) at 37°C in a humidified 5% CO₂ atmosphere.

**Cytotoxicity test.** The effect of the 80% MeOH extract or the solvent fractions on the growth of HL-60/ MX2 cell was determined by measuring the metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphe- nyltetrazolium bromide (MTT) assay (Carmichael et al., 1987). The MTT assays were performed as follows: HL-60/MX2 (2.5 x 10⁶ cells/ml) were treated for 4 days with 20, 50, 100 µg/ml of the 80% MeOH extract or the solvent fractions. After incubation, 0.1 mg (50 µl of a 2 mg/ml solution) MTT (Sigma, Saint Louis, MO, USA) was added to each well and the cells were then incubated at 37°C for 4 h. The plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. 150 µl dimethylsulfoxide was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader (Amersham Pharmacia Biotech., USA). All the experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of crude extract or solvent fractions compared to the untreated controls.

**Apoptosis assay.** HL-60/MX2 cells (2.5 x 10⁶ cells/ml) were treated with 100 µg/ml of the 80% MeOH extract or the solvent fractions for 24 h. For the DNA fragmentation assay, the cells were collected by centrifugation and DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The DNA fragmentation pattern was analyzed by electrophoresis on a 1.5% agarose gel containing 0.1 µg/ml ethidium bromide for 40 min at 100 V (Oberhammer et al., 1993). For the flow cytometric analysis to determine cell cycle phase distribution, the treated cells were washed twice with phosphate-buffered saline (PBS) and fixed in 70% ethanol for 30 min at 4°C. The cells were then rinsed with PBS and incubated in 50 µg/ml propidium iodide solution (PI; Sigma, Saint Louis, MO, USA) and 50 µg/ml RNase A in the dark for 30 min at 37°C. Flow cytometry analysis was performed using an EPICS-XL FACScan flow cytometer (Coulter, Miami, FL, USA). The DNA histograms obtained were analyzed
to measure the proportion of sub-G1 hypodiploid cells (Sherwood et al., 1994).

**RNA isolation and RT-PCR analysis.** Total RNA was extracted from cells using Tri-Reagent (MRC, Cincinnati, OH, USA) following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. RNA was quantified by reading the absorbance at 260 nm according to the methods described by Sambrook et al. (Sambrook et al., 1989). The reverse transcription of 1 μg RNA was carried out with M-MuLV reverse transcriptase (Promega, WI, USA), oligo (dT) 18 primer, dNTP (0.5 mM) and 1 U RNase inhibitor. The polymerase chain reaction (PCR) was performed in reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3' and 5' primer 50 μM each and 200 μM dNTP in 200 μM Tris-HCl buffer, pH 8.4, containing 500 mM KCl and 1–4 mM MgCl₂]. The following primer pairs were used for the PCR reaction: 5'-TGCAACAGTGGCCCTCGTTGC-3' as the sense primer and 5'-AGACAGCCAGAAATCAAACAG-3' as the antisense primer for Bcl-2 (Monaghan et al., 1992); 5'-ACGAAGAGCTGCGAAGGT-3' as the sense primer and 5'-CCAAGAGATGTCACGCTGTCC-3' as the antisense primer for Bax (Miyashita et al., 1995); 5'-CTGTTTGGATGTCAGATGGTG-3' as the sense primer and 5'-GCCCAAGCCTTTGACAGCTACT-3' as the antisense primer for MDR1 (Kioka et al., 1992); 5'-GACGGAGCTAGGAAATGC-3' as the sense primer and 5'-ACAACCTACTCCGTTGC-3' as the antisense primer for MRP-1 (Cole et al., 1993); 5'-GTCAAGCTGCTGAGCTGTCTGC-3' as the sense primer and 5'-GAGCTGTCTGTTGTCACGAC-3' as the antisense primer for BCRP (Zhou et al., 2002); 5'-GGTTACAACTGGACAGGAC-3' as the sense primer and 5'-AAGGAGACCTGAGAAAGAGC-3' as the antisense primer for β̂-MG (Xie et al., 2003). The PCR was performed with a DNA gene cycler (BIO-RAD, HC, USA), and the amplification was followed by 35 cycles of 94°C for 45 sec (denaturing), 60–65°C for 45 sec (annealing) and 72°C for 1 min (primer extension). The PCR products were electrophoresed on a 3.5% agarose gel.

**Chemosensitizing effect of C. asiaticum.** To determine MX uptake, HL-60/MX2 cells (1.0 × 10⁶ cells/ml) were treated with 20, 50 and 100 μg/ml of the 80% MeOH extract or the solvent fractions of C. asiaticum in culture media for 30 min at 37°C. Then 5 μmol/l MX was added for a further 40 min incubation at 37°C, after which the cells were washed twice with ice-cold PBS and kept on ice until analysis. The measurement of cellular MX fluorescence was made using flow cytometry in which a focused argon laser beam (485 nm) excited the cells in a laminar sheath flow and their fluorescence emissions (547 nm) were collected to generate a histogram. Cellular fluorescence (x-axis) from intracellular accumulation of MX is plotted versus cell number (y-axis). In addition, HL-60/MX2 cells were treated with the solvent fractions of C. asiaticum in the presence of cyclosporin A or verapamil which are known to be chemosensitizers (List, 1996; Loor et al., 2002; Garrigues et al., 2002; Choi et al., 1998).

**Statistical analyses.** The student's t-test and one-way ANOVA were used to determine the statistical significance of differences between values for a variety of experimental and control groups. Data are expressed as a mean ± standard deviation (SD) from three independent experiments performed in triplicate. P-values of 0.05 or less were considered statistically significant.

**RESULTS**

**The effect of C. asiaticum on the growth of HL-60/MX2 cells.** When HL-60/MX2 (2.5 × 10⁶ cells/ml) were treated with 20, 50, 100 μg/ml of the 80% MeOH extract or the solvent fractions for 4 days, we found that 100 μg/ml of the MeOH extract, the CHCl₃ fraction and

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>80% MeOH</th>
<th>hexane</th>
<th>CHCl₃</th>
<th>EtOAc</th>
<th>BuOH</th>
<th>H₂O</th>
</tr>
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<tr>
<td>20</td>
<td>9.4 ± 4.7</td>
<td>5.6 ± 4.6</td>
<td>15.7 ± 0.7</td>
<td>9.9 ± 1.4</td>
<td>25.4 ± 3.8</td>
<td>8.5 ± 3.9</td>
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<tr>
<td>50</td>
<td>23.0 ± 1.2</td>
<td>16.9 ± 3.7</td>
<td>38.3 ± 1.9</td>
<td>23.4 ± 2.2</td>
<td>52.5 ± 1.8</td>
<td>18.2 ± 3.5</td>
</tr>
<tr>
<td>100</td>
<td>39.7 ± 3.4</td>
<td>22.6 ± 2.0</td>
<td>53.4 ± 1.9</td>
<td>32.3 ± 3.1</td>
<td>73.2 ± 1.4</td>
<td>23.2 ± 3.2</td>
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</table>

HL-60/MX2 cells (2.5 × 10⁶ cells/ml) were treated with 20, 50 and 100 μg/ml of the 80% MeOH extract or the solvent fractions from C. asiaticum for 4 days and measured for viability by MTT assay. The results are expressed as the percentage of inhibition that produced a reduction in the absorbance by the treatment of crude extract or solvent fractions compared to the untreated controls. All experiments were performed in triplicate. Data are presented as a mean ± SD from three separate experiments. *p < 0.05, **p < 0.01 compared with the control.
The effect of *C. asiaticum* on induction of apoptosis in HL-60/MX2 cells. DNA fragmentation, a hallmark of apoptosis, was detected by the DNA agarose gel electrophoresis method (Fig. 1). DNA ladder bands were clearly detectable in HL-60/MX2 cells treated with the CHCl₃ fraction and the BuOH fraction. Furthermore, we quantified the cells in the sub-G1 population after treatment with the CHCl₃ fraction and the BuOH fraction by flow cytometry analysis. When treated with 100 μg/ml of the fractions for 24 h, the percentage of cells in the sub-G1 fraction increased (Fig. 2). To investigate the possible mechanism underlying the induction of apoptosis by *C. asiaticum*, we examined the expression of Bcl-2 and Bax in HL-60/MX2 cells after treatment with the MeOH extract or the solvent fractions of *C. asiaticum*. The expression of Bax increased markedly, while the expression of Bcl-2 decreased in the cells treated with 100 mg/ml of the CHCl₃ fraction or the BuOH fraction (Fig. 3).

**Chemosensitizing effect of *C. asiaticum* in HL-60/MX2 cells.** To explore for the chemosensitizing effect of *C. asiaticum* in HL-60/MX2 cells, we examined whether *C. asiaticum* affected the intracellular accumulation of MX and mRNA expression levels of MDR-related genes. Flow cytometry revealed that the 80% MeOH extract, the hexane fraction, the CHCl₃ fraction and the BuOH fraction inhibited cell growth by 40%, 53% and 73% respectively (Table 1).
Table 2. The effect of the 80% MeOH extract or several solvent fractions from C. asiaticum on MX accumulation in HL-60/MX2 cells

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Control</th>
<th>80% MeOH</th>
<th>hexane</th>
<th>CHCl₃</th>
<th>EtOAc</th>
<th>BuOH</th>
<th>H₂O</th>
</tr>
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<tbody>
<tr>
<td>20</td>
<td>14.3 ± 1.6</td>
<td>19.3 ± 0.5**</td>
<td>24.0 ± 0.3**</td>
<td>45.8 ± 1.4**</td>
<td>15.4 ± 0.3**</td>
<td>15.7 ± 0.1**</td>
<td>14.5 ± 0.2</td>
</tr>
<tr>
<td>50</td>
<td>14.3 ± 1.6</td>
<td>20.4 ± 0.3**</td>
<td>24.4 ± 0.9**</td>
<td>44.7 ± 0.2**</td>
<td>14.8 ± 0.1**</td>
<td>24.0 ± 0.2**</td>
<td>13.8 ± 0.1</td>
</tr>
<tr>
<td>100</td>
<td>14.3 ± 1.6</td>
<td>33.2 ± 0.2**</td>
<td>30.1 ± 0.2**</td>
<td>46.1 ± 0.8**</td>
<td>16.6 ± 0.3**</td>
<td>42.7 ± 0.3**</td>
<td>14.5 ± 0.1</td>
</tr>
</tbody>
</table>

HL-60/MX2 cells (1.0 x 10⁶ cells/ml) were treated with 100 μg/ml of the 80% MeOH extract or several solvent fractions from C. asiaticum and flow cytometry analysis of MX accumulation was performed as described in the materials and methods. The results are expressed as the percentage of cell population that produced cellular fluorescence by intracellular accumulation of MX. All experiments were performed in triplicate. Data are presented as a mean ± SD from three separate experiments. *p < 0.05, **p < 0.01 compared with the untreated control.

Fig. 4. The effect of the CHCl₃ fraction and the hexane fraction from C. asiaticum on the MX accumulation in HL-60/MX2 cells. HL-60/MX2 cells (1.0 x 10⁶ cells/ml) were treated with 100 μg/ml of the CHCl₃ fraction or the hexane fraction from C. asiaticum and flow cytometry analysis of MX accumulation was performed as described in the materials and methods.

Fig. 5. The combined effect of cyclosporine A (CsA) or verapamil (Vrp) with the CHCl₃ fraction and the hexane fraction from C. asiaticum on MX accumulation in HL-60/MX2 cells. HL-60/MX2 cells (1.0 x 10⁶ cells/ml) were treated with 100 μg/ml of the CHCl₃ fraction or the hexane fraction from C. asiaticum in the presence of CsA or Vrp and flow cytometry analysis of MX accumulation was performed as described in the materials and methods.

fraction of C. asiaticum increased accumulation of MX in HL-60/MX2 cells (Table 2). Moreover, 100 μg/ml of the CHCl₃ fraction or the hexane fraction caused a right shift in the fluorescence peak associated with MX (Fig. 4). When HL-60/MX2 cells were treated with the CHCl₃ fraction or the hexane fraction of C. asiaticum in the presence of cyclosporine A or verapamil, known to be chemotherapy sensitizing compounds, no additive or synergistic effects on MX accumulation were observed (Fig. 5). We also investigated the expression of MDR1, MRP-1 and BCRP in HL-60/MX2 cells. The 295 bp mRNA of MDR1 was not detected in HL-60/MX2 cells. When HL-60/MX2 cells (2.5 x 10⁶ cells/ml) were treated with 100 μg/ml of the 80% MeOH extract or the solvent fractions
ers compete with Bax and other pro-apoptosis proteins to regulate the release of proteins and cytochrome c from mitochondria, which in turn activate 'initiator' caspases (Hanahan et al., 2000). High levels of Bcl-2 expression have been found in several human tumors, and levels of Bcl-2 expression have been correlated with the aggressiveness of the malignancies (Peterson et al., 1992). Bcl-2 has been shown to block cytotoxic agents, whereas the inhibition of Bcl-2 function, by Bcl-2 antisense oligonucleotides (ASO) for example, precipitates apoptosis (Heere-Ress et al., 2002). Since Bcl-2 functions by forming a heterodimer with its pro-apoptotic partner, Bax, the Bcl-2:Bax ratio is proportional to the relative sensitivity or resistance of the cells to various apoptotic stimuli (Oltvai et al., 1993). Here, we showed that the CHCl₃ fraction and the BuOH fraction from C. asiaticum fraction reduced Bcl-2 mRNA levels, whereas Bax mRNA level increased. These results suggest that the inhibitory effects of C. asiaticum on the growth of HL-60/MX2 cells may arise from the induction of apoptosis via down-regulation of Bcl-2 levels. Crinamine was identified from BuOH fraction of C. asiaticum (Park, 2001) and reported to induce apoptosis in hepatoma cancer cells (McNulty et al., 2007). In further study, it remains to be identified whether crinamine could induce apoptosis of HL-60/MX2.

Another approach that has been the subject of many studies is the use of chemosensitizers to modulate MDR chemo-resistant cells so that they become sensitive to chemotherapeutic agents. These chemosensitizers have a broad spectrum of chemical structures, which cause great difficulty in identifying the chemosensitizing properties of their structures. Several reviews have illustrated the required chemical structures of MRP-1 modulators (Boumendjel et al., 2005). In this study, we examined the chemosensitizing properties of C. asiaticum. MX accumulation was increased in HL-60/MX2 cells when treated with the CHCl₃ fraction and the hexane fraction of C. asiaticum. The addition of cyclosporine A or verapamil, which are competitive inhibitors of MDR1 (List, 1996; Loor et al., 2002; Garigues et al., 2002; Choi et al., 1998), failed to show additive or synergistic effects on the accumulation of MX due to the CHCl₃ fraction and the hexane fraction of C. asiaticum. We found the expression of MRP-1 and BCRP, but not MDR1 in HL-60/MX2 cells as previously reported (Harker et al., 1989). Active components from the CHCl₃ fraction and the hexane fraction of C. asiaticum might cause accumulation of MX via the inhibition of MRP-1 or BCRP. Furthermore, treatment of the HL-60/MX2 cells with the CHCl₃ fraction and the BuOH fraction resulted in marked decreases of MRP-1 and
BCRP mRNA levels. From these results, an active compound (or compounds) from the CHCl₃ fraction of C. asiaticum seems to play a pivotal role as a chemosensitizing mediator. The C. asiaticum has four alkaloids from BuOH fraction and two flavonoids from CH₂Cl₂ fraction (Park, 2001). The alkaloids were identified as (+)-cinammine, (5S,16S)-N-demethylgalanthamine, (5S,16R)-N-demethylgalanthamine and lycorine. The two flavonoids were identified as 4’,7’-dihydroxy flavan and 4’,7’-dihydroxy-4’-methoxy chalcone. In particular, cin- 
ammin from C. asiaticum has been reported to induce apoptosis in hepatoma cancer cells (McNulty et al., 2007) and inhibit HIF-1 activity (Kim et al., 2006). Comerford et al. have shown that inhibition of HIF-1 expression resulted in significant inhibition of hypoxia-inducible MDR1 expression (Comerford et al., 2002). The purified chemosensitizing principle from the CHCl₃ fraction of C. asiaticum remains to be identified and further studied for its mechanism of action in relation to HIF-1.

In conclusion, the CHCl₃ fraction of C. asiaticum might have cytotoxic effects on HL-60/MX2 cells by induction of apoptosis and chemosensitizing effects via the increase of MX accumulation and the down-regulation of MRP-1 and BCRP expression. The components of C. asiaticum should be studied in more detail in order to explain the molecular mechanism involved in apoptosis and modulation of efflux transporters connected with HIF-1.

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selected HL-60 leukemia cells in the absence of P-glyco- 


