# Antitumor activity of CDA-II, a urinary preparation, on human multiple myeloma cell lines via the mitochondrial pathway

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Abstract. Cell differentiation agent II (CDA-II) is a DNA methyltransferase inhibitor isolated from healthy human urine. In the present study, the antitumor activity of CDA-II on human multiple myeloma (MM) cell lines via the mitochondrial pathway was first revealed. The human MM cell lines were exposed to CDA-II. Cytotoxicity, caspase activation, apoptosis and the effects on the mitochondrial pathway were assessed. CDA-II was capable of decreasing the depolarized mitochondrial membranes and activating caspase-3 and -9 and poly (ADP-ribose) polymerase in MM cells treated with CDA-II. CDA-II induced caspase-dependent cell death accompanied by a significant decrease in X-linked inhibitor of apoptosis protein (XIAP), survivin and Mcl-1 levels. The caspase-3 inhibitor, Z-DEVD-FMK, inhibited CDA-II-induced apoptosis. CDA-II potently increased the Bax levels, decreased the Bcl-2/Bax ratio and decreased the expression of the downstream targets of NF-κB. In conclusion, the results of the present study demonstrated that CDA-II treatment leads to the inhibition of p65 nuclear localization and potently induces caspase-dependent apoptosis in MM cells mediated through the mitochondrial pathway at low nanomolar concentrations. These results indicate that CDA-II is a novel inhibitor of NF-κB activity, with notable antimyeloma efficacy. This study provides a rationale for the clinical investigation of CDA-II in human MM.

# Introduction

Multiple myeloma (MM) is characterized by a latent accumulation of secretory plasma cells with a low proliferative

*Key words:* cell differentiation agent II, antitumor activity, multiple myeloma, mitochondrial pathway

index and an extended life span in the bone marrow. MM is the second most prevalent hematological cancer after non-Hodgkin lymphoma, accounting for 10% of all hematological cancers and ~2% of all cancer mortalities. More significantly, MM has a higher frequency in elderly individuals. Despite conventional therapies with proteasome inhibitors, thalidomide analogs, alkylating agents, anthracyclines and corticosteroids (1), as well as high-dose therapy and stem cell transplantation (2,3), MM remains incurable due to intrinsic and acquired drug resistance (4-6). The cytotoxicity of the current treatment limits the clinical effect, particularly for older patients, and the acquisition of drug resistance remains a severe problem. Therefore, novel therapeutic strategies are urgently required.

The use of urine and urine extracts for therapeutic purposes has been known for centuries (7-9). In previous years, Burzynski (7) and scientists elsewhere in the world (10) demonstrated the anticancer effects of these urine extracts. Cell differentiation agent II (CDA-II) is a mixture, isolated from healthy human urine which is produced only China. Multiple active components have been shown to act concurrently with different mechanisms of action to contribute to the anticancer effect of CDA-II (11). CDA-II has been demonstrated to act as a novel anticancer agent having multiple biological targets in the aspects of antiproliferation, apoptosis, differentiation and gene regulation in several solid tumors (12-13). CDA-II has been applied to the treatment of various cancer cells, including glioma (14) and breast cancer (15) cells. CDA-II may protect normal cells from oxidative (9) and DNA (16) damage, and also may affect skeletal myogenesis (17). However there have been no studies on the use of CDA-II antitumor activity to treat MM.

# Materials and methods

*Chemicals.* CDA-II was supplied by Everlife Pharmaceutical Co., Ltd. (Hefei, China). Briefly, human urine was acidified during collection and passed through an ultrafiltration process to remove molecules with molecular weights of >10,000 Da. The filtrate was then passed through a chromatographic column and eluted by ethanol. The colored ethanolic fraction was collected and evaporated under a vacuum. The dried extract was reconstituted with distilled water to produce a 300 mg/ml stock solution and stored

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at 4°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) was dissolved in RPMI-1640 media to produce a 5 mg/ ml solution. The caspase-3 inhibitor, Z-DEVD-FMK (40 µmol/l; BioVision, Milpitas, CA, USA), was added 1 h prior to treatment with CDA-II. Primary antibodies included caspase-3, poly (ADP-ribose) polymerase (PARP), caspase-9, X-linked inhibitor of apoptosis protein (XIAP) (all rabbit; all BioVision, Mountain View, CA, USA), actin (goat; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Bcl-2 (rabbit), Bax (mouse), Mcl-1 (rabbit) and survivin (rabbit) antibodies (Cell Signaling, Danvers, MA, USA). Horseradish peroxidase-conjugated secondary anti-mouse and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology, Inc. Enhanced chemiluminescence western blotting detection reagents were purchased from Amersham Biosciences (Little Chalfont, UK). The mitochondrial fluorescent probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodide (JC-1), was purchased from Molecular Probes (Eugene, OR, USA).

Cell culture. The U266, RPMI8226, MM.1R and MM.1S cell lines were maintained in RPMI-1640 (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL), 0.2 mg/ml streptomycin/penicillin and 0.1% (w/v) L-glutamine (Gibco-BRL) in a 5% humidified CO<sub>2</sub> atmosphere at 37°C. The MM cell lines, RPMI8226 and U266, were obtained from the American Type Culture Collection (Rockville, MD, USA). MM.1R and MM.1S cells were provided by Professor Steven Rosen (Northwestern University, Chicago, IL, USA). Hypaque gradients were run to obtain normal peripheral blood mononuclear cells (PBMCs). All the healthy volunteers approved use of their samples.

*Cytotoxicity assay.* The cells were seeded at a density of 10,000 per well in 96-well microtiter plates. The cells were treated with various concentrations of CDA-II and incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 48 h. Subsequently, 20  $\mu$ l MTT (Sigma) stock solution was added to each well (final concentration: 0.5 mg/ml) for another 4-h incubation (37°C and 5% CO<sub>2</sub>). Following 4 h of incubation, 200  $\mu$ l dimethylsulfoxide was added to each well and the optical density was read at 570 nm. The sensitivity of cells to CDA-II was measured by the IC<sub>50</sub> (50% inhibitory concentration). The experimental conditions were analyzed in sextuplicate (six wells of the 96-well plate per experimental condition). All the experiments were performed in triplicate.

Flow cytometric analysis for apoptosis. Human MM cell lines in the exponential growth phase were incubated at  $5x10^5$  cells/ml on six-well flat-bottomed microplates in RPMI-1640 medium supplemented with 10% fetal calf serum in the presence of 4 mg/ml CDA-II for 0-24 h. Apoptosis was measured by Annexin V and propidium iodide (PI) staining. Briefly, the cells were harvested, washed with PBS [10 mmol/l *N*-2-hydroxyl piperazine-*N*'-2-ethane sulfonic acid/NaOH (pH 7.4), 140 mmol/l NaCl and 2.5 mmol/l CaCl<sub>2</sub>], incubated with 10  $\mu$ l Annexin V-fluorescein isothiocyanate (Pharmingen, Immunocytometry System, San Jose, CA, USA) and 10  $\mu$ l PI (10  $\mu$ g/ml in binding buffer) in the dark for 15 min, and assayed following the addition of 300  $\mu$ l binding buffer to each sample. The data acquisition and analysis were conducted on a BD FACS caliber (Becton-Dickinson, Franklin Lakes, NJ, USA) using CellQuest software (Becton-Dickinson). Annexin V bound to those cells that expressed phosphatidylserine on the outer layer of the cell membrane, and PI stained the cellular DNA of those cells with a compromised cell membrane. All the experiments were performed in triplicate.

*JC-1 stain for mitochondrial membrane potential* ( $\Delta \psi m$ ). Alterations in the  $\Delta \psi m$  were analyzed by flow cytometry using the  $\Delta \psi$ m-sensitive dye, JC-1. Briefly, following treatment, 2x10<sup>6</sup> cells were harvested, washed once and then resuspended in phosphate-buffered saline (PBS), prior to incubation with 1  $\mu$ mol/l JC-1 at 37°C for 10 min. The stained cells were then washed once in PBS and analyzed by flow cytometry. A BD FACS caliber (Becton-Dickinson) was used to analyze a minimum of  $1 \times 10^4$  cells per sample. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 $\pm$ 10 nm) to red (610 $\pm$ 10 nm). The data were evaluated using a CellQuest software package (Becton-Dickinson). The forward and side scatter were used to gate the viable populations of cells. JC-1 monomers emit at 527 nm (FL-1 channel) and 'J-aggregates' emit at 590 nm (FL-2 channel). All the experiments were performed in triplicate.

Western blotting. The human MM cells were incubated with 4 mg/ml CDA-II for 12 h. The cells ( $5x10^6$ ) were harvested and lysed in 200  $\mu$ l lysis buffer [0.5 M Tris-HCl (pH 6.8), 2 mM EDTA, 10% glycerol, 2% SDS and 5%  $\beta$ -mercaptoethanol]. In total, 40  $\mu$ g per lane of the extracted total protein was loaded on 12% Tris-glycine gels and then transferred to a polyvinylidine difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% skimmed milk dissolved in Tris-buffered saline with 0.1% Tween-20, and subsequently probed with the primary antibody and horseradish peroxidase-labeled secondary antibody. The bands were visualized using enhanced chemiluminescence western blotting detection reagents. All the experiments were performed in triplicate and the proteins were normalized against actin prior to analysis.

Statistical analysis. All values are presented as the mean  $\pm$  standard deviation. The differences between the two groups were analyzed by unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

# Results

Effect of CDA-II on the growth of MM cell lines independent of IL-6. To investigate the effects of CDA-II on the growth and survival of human MM cells, four human MM cell lines, RPMI8226, U266, MM.1R and MM.1S, and tumor cells from MM patients were treated. The dose-response curves are shown in Fig. 1A. All the cells exhibited a dose-dependent sensitivity to CDA-II (0-16 mg/ml) at 24 h. The CDA-II IC<sub>50</sub> for the RPMI8226, U266, MM.1R and MM.1S cell lines were ~2.90, 3.53, 4.23 and 2.68 mg/ml, respectively. CDA-II also induced dose-dependent cytotoxicity in the tumor cells from three *de novo* MM patients, with an IC<sub>50</sub> at 24 h of 4.97,



Figure 1. (A) Four types of human multiple myeloma (MM) cell lines were treated with CDA-II (2-16 g/l) for 24 h and the cytotoxicity was analyzed by an MTT assay. Cell survival was expressed as the optical density ratio of the treatment to the control. CDA-II exhibited cytotoxicity in a dose-dependent manner in RPMI8226, U266, MM.1R and MM.1S cells. The data represent the mean ± SD of three independent experiments. (B) RPMI8226, U266, MM.1R and MM.1S cells. The data represent the mean ± SD of three independent experiments. (B) RPMI8226, U266, MM.1R and MM.1S cells were treated with CDA-II (4 mg/ml) in the presence or absence of IL-6 (50 ng/ml). At 24 h, the cells were harvested and the viability was analyzed by MTT assay. The median viability of the RPMI8226 cells treated with CDA-II and IL-6 was 28.28 and 117.30%, respectively, at 24 h, whereas that of cells treated with CDA-II + IL-6 (4 mg/ml, 50 ng/ml) was 61.19% (P<0.01). The median viability of the U266 cells treated with CDA-II and IL-6 was 36.20 and 118.67%, respectively. IL-6 enhances the growth of cells at 24 h. The median viability of MM.1R cells treated with CDA-II and IL-6 was 38.49 and 108.67%, respectively, at 24 h, that of cells treated with CDA-II + IL-6 (4 mg/ml, 50 ng/ml) was 33.94% (P<0.01). The median viability of the mean ± standard error of the mean (n=3, \*P<0.01 vs. control). CDA-II, cell differentiation agent II; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; IL-6, interleukin-6.



Figure 2. (A) RPMI8226 cells incubated with the indicated concentrations of CDA-II for 12 h were stained for flow cytometric analysis. The early apoptotic cells increased in a dose-dependent manner. (B) The human MM ROMI8226, U266, MM.1R and MM.IS cells were all incubated with 4 mg/ml CDA-II for 12 h. The CDA-II-induced apoptotic percentage, particularly in the early apoptotic cells, was increased in all these human MM cell lines. \*P<0.01 and \*\*P<0.05 vs. the untreated control. The data represent the mean ± standard deviation of three independent experiments. MM, multiple myeloma; CDA-II, cell differentiation agent II.

5.64 and 3.54 mg/ml, respectively. By contrast, CDA-II did not induce cytotoxicity in PBMCs from three normal volunteers (18). The effect of CDA-II on the RPMI8226, U266 and MM.1R cell lines was evaluated in the presence of exogenous IL-6, which is an important growth factor in MM. Fig. 1B demonstrates that IL-6 (50 ng/ml) did not provide protection against CDA-II-induced growth inhibition and apoptosis. *CDA-II induces apoptosis of human MM cell lines.* To analyze whether CDA-II induced apoptosis of the human MM cell line, the translocation of phosphatidylserine was examined. Non-apoptotic cells revealed neither Annexin V nor PI fluorescence. Annexin V<sup>+</sup>/PI<sup>-</sup> and Annexin V<sup>+</sup>/PI<sup>+</sup> cells represented an early and a late phase of apoptosis, respectively. The percentage of early apoptosis in the RPMI8226



Figure 3. Apoptosis triggered by CDA-II was mediated via the caspase cascades. CDA-II induced dose-dependent activation of caspases-3 and -9 and PARP. Human multiple myeloma RPMI8226, U266, MM.1R, MM.1S cells treated with CDA-II 2-8mg/ml. CDA-II, cell differentiation agent II; PARP, poly (ADP-ribose) polymerase.



Figure 4. RPMI8226 and MM.1R cells were preincubated with 40  $\mu$ mol/l Z-DEVD-fmk for 1 h prior to the treatment with 4mg/ml CDA-II for 24 h. Z-DEVD-fmk completely inhibited CDA-II-induced caspase-3 activation and PARP cleavage. CDA-II, cell differentiation agent II; MM, multiple myeloma; PARP, poly (ADP-ribose) polymerase.

cell lines following treatment by 2-8 mg/ml CDA-II for 12 h was increased (Fig. 2A). CDA-II-induced apoptosis was also verified in other human MM cell lines, including the U266, MM.1R and MM.1S cells (Fig. 2B). The findings also indicated that the increase in CDA-II-mediated apoptotic cells occurred in a dose-dependent manner.

Apoptosis triggered by CDA-II is mediated via caspase-3 and -9 and by PARP cleavage, through decreasing the Bcl-2/Bax ratio and Mcl-1 expression. On the basis of the results of MTT and Annexin V/PI staining, the present study attempted to identify the mechanisms of CDA-II-induced cell death in MM cells, and furthermore, the activation of caspase-3 and -9 and the cleavage of PARP in the RPMI8226, U266, MM.1R and MM.1S cell lines was examined. CDA-II induced activation of caspase-3 and -9, and the cleavage of PARP. The activation of caspase-3 is important in the induction of apoptosis by a variety of stimuli. As demonstrated in Fig. 3, caspase-3 and -9 were noted to be activated in a dose-dependent manner.

An attempt was made to determine whether caspase-3 is important in CDA-II-induced apoptosis by treating the cells with the specific caspase-3 inhibitor, Z-DEVD-FMK (19). The inhibition of caspase-3 activity by pretreatment with 40  $\mu$ mol/l Z-DEVD-FMK significantly decreased the apoptotic cells of the RPMI8226 and MM.1R cell lines following CDA-II treatment (Fig. 4). These results indicated that CDA-II-induced apoptosis may be caspase-3-dependent.

Since the Bcl-2 family is associated with the activation of caspase-9 and the mitochondrial pathway, the expression of Bcl-2, Bax and Mcl-1 in CDA-II-induced apoptosis was investigated further. Following treatment with 4 mg/ml CDA-II,



Figure 5. RPMI8226, U266, MM.1R and MM.1S cells were cultured with CDA-II (2, 4 and 8 mg/ml) for 12 h. The total cell lysates were analyzed by western blotting using Bcl-2 (28 kDa), Bax (20 kDa), Mcl-1 (40 kDa), XIAP (53 kDa), survivin (16 kDa) and actin (46 kDa) antibodies. CDA-II, cell differentiation agent II; XIAP, X-linked inhibitor of apoptosis protein.



Figure 6. CDA-II induced the change of mitochondrial membrane potential ( $\Delta \psi m$ ) in four human MM cell lines. Cells were treated with 4 mg/ml CDA-II for 12 h and stained with the mitochondria-selective JC-1 dye. The percentage of cells with fluorescence show the corresponding tendency of apoptotic cells. (A) The left image represents the control, the right image represents cells treated with 4 mg/ml CDA-II. (B) The data represent the mean  $\pm$  standard deviation of three independent experiments. \*P<0.01 and \*\*P<0.05 vs. the untreated control. CDA-II, cell differentiation agent II; MM, multiple myeloma.

there was a dose-dependent decrease in the level of Bcl-2 and Mcl-1 protein and a dose-dependent increase in the level of Bax protein (P<0.01; Fig. 5), which activates caspase-9 by the decrease of the Bcl-2/Bax ratio (Fig. 5) and the downregulation of Mcl-1.

CDA-II inhibits expression of the IAP family, triggering caspase-dependent cell death in human MM cell lines. The Bcl-2 and IAP family proteins regulate mitochondria-mediated apoptosis, and the present study attempted to directly demonstrate that mitochondria are involved in CDA-II-induced apoptosis. Following treatment of the cells with 4 mg/ml CDA-II for 12 h, an increased ratio of cells with depolarized mitochondrial membranes was observed in all the human MM cell lines (P<0.01; Fig. 6), which indicated that the mitochondria were involved in CDA-II-induced apoptosis. CDA-II-induced cell death appears to be caspase dependent, while XIAP is the most potent natural cellular inhibitor of caspases (20), therefore, the effect of CDA-II on XIAP and the survivin protein was examined. Following treatment of

CDA-II for 12 h, the XIAP and survivin protein levels were significantly decreased in the human MM cell lines in a dose-dependent manner (P<0.01; Fig. 5).

# Discussion

MM is currently an incurable hematological malignancy, and novel biologically-based treatment strategies for elderly patients and for overcoming conventional drug resistance are urgently required. Previously the medical use of urine preparations in cancer prevention has been studied and various mechanisms have been proposed to explain their effects. Clinically, antineoplastones were prepared from urine by Burzynki (21) and shown to produce objective responses in cancer patients, such as tumor reduction. Antitumor urinary proteins were identified and revealed to possess anticancer activity (22-23). The anticancer activity of CDA-II on acute myeloid leukemia cell lines was also analyzed (18). The components of this urine extract, including endostatin and angiostatin, have been described as potent inhibitors of angiogenesis and malignant growth (24). In the present study, CDA-II, a urinary preparation, was first demonstrated to be capable of inducing apoptosis in the human MM cell lines, RPMI8226, U266, MM.1R and MM.1S, however, it had exhibited no cytotoxicity in the PBMCs from normal volunteers in previous studies (8,18,25). The results of in vitro experiments indicated the potent antitumor activity of CDA-II via the nuclear translocation of the NF-KB p65 subunit. In the present study, the results indicated that CDA-II induced apoptotic death in a dose-dependent manner in human MM cell lines. Following the incubation of 4 mg/ml CDA-II for 12 h, Annexin V<sup>+</sup> cells were revealed to be associated with cytoplasmic membrane damage, as evaluated by PI staining. Apoptosis was also confirmed by the activation of the caspase cascade, which is a crucial gateway involved in the execution of apoptosis in a variety of cellular systems. It was shown that when the caspase cascade was activated, caspase-3 disassembled PARP into cleaved fragments. Observations in the present study demonstrated that cleaved caspase-3 and PARP appeared following incubation of CDA-II 4 mg/ml for 12 h, which indicated that the activation of the caspase family was correlated with CDA-II-induced apoptosis. The caspase-3 inhibitor, Z-DEVD-FMK (40  $\mu$ mol/l), was able to significantly block CDA-II-induced apoptosis in the MM cell lines, which indicated that the activation of caspase-3 was closely correlated with CDA-II-induced apoptosis.

While the primary cause of treatment failures in MM is the emergence of resistant disease and early relapse, among the most frequent causes of these phenomena are the defects in the mitochondrial-mediated apoptotic pathway (26-27). The expression pattern of the Bcl-2 family of pro-apoptotic and anti-apoptotic genes in MM have been the subject of multiple studies in which it was demonstrated that increased levels of Bcl-2, Bcl-xL, and Mcl-1 expression are linked to MM cell survival and resistance to chemotherapeutic agents. This pathway was regulated by the Bcl-2 family of anti-apoptotic (Bcl-2 and Mcl-1) and pro-apoptotic (Bax and Bak) proteins. Bcl-2 functioned as an inhibitor of mitochondrial permeabilization, by changing its conformation on the mitochondrial membrane to affect membrane insertion (28). Overexpression of the anti-apoptotic members has been linked to resistance to various chemotherapeutic agents. Following exposure to chemotherapeutic agents, increased expression of these proteins of the MM cell lines indicated that these agents may contribute to acquired chemoresistance. Thus, we suggest that regulation of anti-apoptotic proteins may be a significant strategy for sensitizing MM cells to various therapeutic agents. In the present study, CDA-II determined a strong and rapid downregulation of Bcl-2 and an upregulation of Bax, decreasing the Bcl-2/Bax ratio, thus indicating that this may present a novel therapeutic regimen, capable of inducing apoptosis at an early treatment state. CDA-II also overcame the multidrug resistance *in vivo*. These results indicated that CDA-II induced apoptosis in the human MM cell lines through the mitochondria-mediated pathway.

The findings of this study confirmed that CDA-II downregulated XIAP and Mcl-1 expression, potently inhibited cell growth and promoted cell death through the mitochondrial pathway in various MM cells, including the dexamethasone-resistant MM cell line. Mcl-1 is a member of the anti-apoptotic Bcl-2 family of proteins that inhibit cell death at the mitochondrial level. Mcl-1 downregulation and cleavage has been shown to induce apoptosis of tumor cells (29-30). Furthermore, Mcl-1 acts not only as an anti-apoptotic protein that opposes drug-induced apoptosis, but also as a pro-apoptotic cleaved protein enhancing mitochondrial/caspase activation and thereby leading to apoptosis (31). Furthermore, XIAP may be involved in worsening the prognosis of MM patients in association with the chemotherapy-induced overexpression of multidrug- or lung-resistance proteins (32). Survivin is also a notable member of the IAP family, with dual roles in mitosis and apoptosis. The data of the present study demonstrated that the expression of XIAP and survivin was decreased during CDA-II-induced apoptosis. Since Mcl-1, Bcl-2 and XIAP are frequently overexpressed in MM cells (33), the ability of CDA-II to reduce the levels of Mcl-1 and XIAP rendered it a powerful inducer of apoptosis and overcoming the multidrug resistance. By decreasing XIAP and Mcl-1, CDA-II may also lower the apoptotic threshold and thereby enhance cell death induced by chemotherapeutic agents.

In conclusion, this study provided evidence that CDA-II induced the significant cytotoxicity of MM cells, particularly drug resistant MM cells, *in vitro*. The results of this study provide evidence supporting the use of CDA-II as a novel NF- $\kappa$ B inhibitor with marked anti-MM efficacy *in vitro*. The potent effects of CDA-II in MM cells and its moderate effect reported in this study provided a rational method, particularly for elderly patients.

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