Evidence-Based Complementary and Alternative Medicine

Research Article

Enhancement of Arsenic Trioxide-Induced Apoptosis by *Mucuna macrocarpa* Stem Extract in Human Leukemic Cells via a Reactive Oxygen Species-Dependent Mechanism

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Abstract

The objective of this study was to examine the potential of enhancing antileukemic activity of arsenic trioxide (ATO) by combining with a folk remedy, crude methanolic extract of *Mucuna macrocarpa* (CMEMM). Human leukemia cells HL-60, Jurkat and Molt-3 were treated with ATO and/or CMEMM. Combination of 2.5 μM ATO and 50 μg/ml CMEMM synergistically inhibited cell proliferation in all three cell lines. Apoptosis triggered by ATO/CMEMM treatment was confirmed by accumulation of sub-G1 phase in cell cycle analyses and characteristic apoptotic nuclear fragmentation. Exposure to ATO in combination with CMEMM resulted in elevation of reactive oxygen species (ROS). Addition of *N*-acetyl cysteine prevented cells from ATO/CMEMM-induced apoptosis by inhibiting activation of caspase-3 and -9. In summary, these results suggest that CMEMM enhance ATO-provoked apoptosis through a ROS-dependent mechanism. The ATO/CMEMM-combined treatment exerts higher apoptosis-inducing effect than treatment of ATO alone and may provide a promising antileukemic approach in the future.

1. Introduction

Arsenic is one of the oldest drugs in both Western medicine and traditional Chinese medicine (TCM). More than 2,000 years ago, it was first used to treat various diseases from syphilis to cancer [1]. Due to being viewed as both a therapeutic agent and a poison, arsenic applied to only severe diseases with the principle of "taming an evil with a toxic agent" in TCM [2]. In recently years, the clinical efficacy of arsenic trioxide (ATO) has been well characterized in the treatment of newly diagnosed and relapsed acute promyelocytic leukemia (APL) [3]. The combination of ATO and all-*trans* retinoic acid is a very effective new strategy for APL patients who are unable to tolerate conventional therapy [4]. In addition, in vitro studies showed that ATO apparently affected numerous intracellular signal transduction pathways to alter cellular functions. The actions of ATO treatment could result in antiproliferation, antiangiogenesis, promotion of differentiation and induction of apoptosis in a wide variety of malignancies, including both hematologic cancer and solid tumors [5, 6]. However, clinical trials indicated that ATO as a single agent have not demonstrated significant benefit in a variety of non-APL hematological malignancies [7-9].

Mucuna macrocarpa Wallich (Leguminosae) is a large woody climber and distributed throughout Taiwan and Southeast Asia. In folk medicine, dried stems of this plant have been used to activate blood circulation for various hematologic and circulatory related ailments [10]. Previous study reported that crude methanolic extract of *M. macrocarpa* (CMEMM) exerted antileukemic effects by induction of apoptosis in HL-60 human leukemia cells in vitro and in vivo, hence the scientific support of its traditional use had been provided [11]. In addition, our preliminary study showed that the CMEMM-induced apoptosis in human leukemia cells might result from elevation of intracellular reactive oxygen species (ROS) content as a pro-oxidant agent. Since generating a moderate pro-oxidant environment may offer potential therapeutic opportunities in human leukemia cells, it will be a good strategy to enhance ATO-provoked apoptosis by combination with a pro-oxidant agent [12]. Moreover, recent reports have demonstrated that ATO combined with natural components such as dietary isothiocyanates or flavonoids could improve the efficiency of ATO as an antileukemic drug [13-15].

For these reasons, we investigated the capacity of CMEMM to sensitize to ATO-provoked apoptosis on human leukemia cells (HL-60, Jurkat and Molt-3). In the present study, antiproliferative activity was evaluated by trypan blue exclusion assay. Apoptosis induced by combination of ATO and/or CMEMM was determined by typical apoptotic morphologic changes, cell cycle analyses and Western blotting. Also,

changes in ROS production and glutathione (GSH) levels were assessed. Furthermore, the inhibition of ATO/CMEMM-induced apoptosis by addition of the free radical scavenger *N*-acetyl cysteine (NAC) or caspase inhibitor z-VAD-fmk was examined to confirm mechanisms of action of the combined treatment.

2. Materials and Methods

2.1. Reagents. RPMI-1640 medium, fetal bovine serum (FBS), gentamycin, trypan blue dye solution and phosphate-buffered saline (PBS; pH 7.4) were purchased from Gibco (Grand Island, NY, USA). Arsenic trioxide, *N*-acetyl cysteine, dimethyl sulfoxide (DMSO), dihydroethidium (DHE) and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma (St. Louis, MO, USA). Methanol was purchased from Merck (Darmstadt, Germany). Cycle TEST^{TM} PLUS DNA Reagent Kit was purchased from Becton Dickinson (San Jose, CA, USA). M-PER mammalian protein extraction reagent was purchased from PIERCE (Rockford, IL, USA). Protease inhibitors cocktail and z-VAD-fmk were purchased from Calbiochem (Darmstadt, Germany). Enhanced chemiluminescent (ECL) detection reagent was purchased from PerkinElmer (Waltham, MA, USA).

2.2. Plant Material. Mucuna macrocarpa Wallich (Leguminosae) was collected in Nantou County, Taiwan. The material was identified by Professor Yuan-Shiun Chang in the Graduate Institute of Chinese Pharmaceutical Sciences, China Medical University. Crude methanolic extract of the stems of *M. macrocarpa* (CMEMM) was prepared by extraction with methanol and standardized by high-performance liquid chromatography (HPLC)-fingerprint as described in detail in the preceding works [11].

2.3. Cell Culture. Human leukemia cell lines HL-60 (acute promyelocytic leukemia), Jurkat and Molt-3 (acute T-lymphoblastic leukemia) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in RPMI-1640 medium containing 10% FBS and 0.01 mg/ml gentamycin, and incubated in a humidified atmosphere of 5% CO_2 at 37 °C.

2.4. Cell Proliferation Assay. Cell growth was determined by trypan blue exclusion assay. Cells were collected at indicated times following CMEMM and/or ATO exposure. After centrifugation, cells were resuspended in culture media and stained with 0.4% trypan blue solution. Viable cells were counted using a hemocytometer.

The percentages of cell growth were calculated by comparing the cell numbers with that of the control.

2.5. Cell Cycle Analysis. DNA staining was carried out using Cycle TEST^{TM} PLUS DNA Reagent Kit. In brief, cells (1×10^6 cells/ml) were washed and stained for DNA content according to the kit protocol. Fluorescence intensity of propidium iodide (PI) was determined using a FACScan flow cytometer and analyzed by CellQuest software (Becton Dickinson, San Jose, CA, USA).

2.6. Cytological Examination. DAPI nucleic acid stain was used to observe apoptotic morphology of individual cells. Briefly, cells (1×10^6 cells/ml) were washed once with PBS, collected on microscope slides by cytospin (Shandon Cytospin Cytocentrifuge), fixed with 10% neutral buffered formalin, and stained with 2.5 μg/ml DAPI solution at room temperature. Photographs of the slides were taken under an inverted fluorescence microscopy (Olympus AX-10). Fragmented nuclei were suggestive of apoptosis.

2.7. ROS Production Measurement. The probe DHE was used for detection of cytosolic superoxide anion (O^{2-}). Briefly, cells (1×10^6 cells/ml) were washed once with PBS and stained with DHE-containing RPMI-1640 medium (without phenol red) at a final concentration of 10 μM. After 30 minutes incubation in a water bath at 37 °C, samples were placed on ice for 10 minutes. Then, intracellular ROS levels were immediately examined by a FACScan flow cytometer and analyzed by CellQuest software. Data are expressed as mean fluorescence of ethidium.

2.8. Western Blotting Analysis. To obtain total cellular protein extracts, cells (2×10^6) were washed once with PBS and prepared by M-PER mammalian protein extraction reagent supplemented with protease inhibitors cocktail following the manufacturer's protocol. Cell lysates containing equal protein amounts were analyzed by Western blotting as previously described [16]. In brief, samples were subjected to electrophoretic separation in 12% SDS-polyacrylamide gels, blotted onto nitrocellulose membranes and probed with primary antibodies against poly-ADP ribose polymerase (PARP), caspase-3, caspase-9 and β-actin. Following wash cycles with TBS-T buffer (Tris-Buffer Saline with Tween 20), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using ECL detection reagent and X-OMAT (Kodak) processing.

2.9. Drug Combination and Statistical Analysis. To assess the interaction of two drugs

such as synergy, additivity or antagonism, the data of cell proliferation assay were analyzed by CalcuSyn V2 for Windows software (Biosoft, Cambridge, United Kingdom). The combination index (CI) were calculated based upon the multiple drug effect equation of Chou and Talalay, where CI < 1, = 1, and > 1 indicate synergism, additive effect, and antagonism, respectively [17]. Each CI value represents the mean of three independent experiments which were performed in duplicate. Statistical significant differences between the control and ATO/CMEMM-treated groups were estimated by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test using the SPSS for Windows 10.0 version software. Differences were defined as significant when $P < 0.05$.

3. Results

3.1. ATO and CMEMM Synergistically Inhibit Cell Growth on HL-60 Cells. To evaluate whether ATO and CMEMM present additive, synergistic or antagonistic effect, HL-60, Jurkat and Molt-3 cells were treated with 2.5 or 5 μM ATO alone or in combination with increasing doses of CMEMM from 25 to 75 μg/ml. As shown in Figure 1, CMEMM was found to sensitize HL-60, Jurkat and Molt-3 cells to the growth inhibitory effects of ATO up to 48 h treatment. CI values for the combination of ATO and CMEMM after 24 h treatment were calculated and shown in Table 1. Combinatorial treatment of ATO and CMEMM synergistically inhibit cell growth on HL-60 cells. However, when combined 2.5 μM ATO with 75 μg/ml CMEMM or 5 μM ATO with 25 μg/ml CMEMM, the drug interaction showed antagonism in Jurkat or Molt-3 cells, respectively.

3.2. CMEMM Enhances Induction of Apoptosis by ATO on Leukemic Cells. Since combinatorial treatment of ATO and CMEMM resulted in reduced cell growth, cell cycle of HL-60, Jurkat and Molt-3 cells was analyzed using flow cytometry. As shown in Figure 2, treatments with 2.5 or 5 μM ATO alone had negligible or very low apoptosis in leukemic cells, as measured by the accumulation of cells in sub-G1 phase (or hypodiploid apoptotic cells). However, co-treatment of ATO and CMEMM up to 48 h induced cells staying in sub-G1 phase in more than additive manner, with maximum effects at 5 μM ATO plus 50 μg/ml CMEMM in Jurkat cells (Figure 2(b)) or 5 μM ATO plus 75 μg/ml CMEMM in HL-60 and Molt-3 cells (Figure 2(a) and 2(c)). Additionally, nuclear morphological changes in 5 μM ATO and/or 50 μg/ml CMEMM treated cells are shown in Figure 3. ATO/CMEMM-treated cells presented with fragmented chromatin and formation of apoptotic bodies, which were in clear

contrast to the intact nuclei of control groups. The apoptotic morphology results together with the flow cytometry data suggested that co-treatment exerted higher apoptosis-inducing effect than treatment of ATO or CMEMM alone.

3.3. CMEMM Enhances ATO-Provoked Apoptosis on Leukemic Cells Through a ROS-Dependent Mechanism. Since ATO or other DNA damaging agents may turn on the apoptotic pathway through the production of ROS, we sought to assess the role of ROS generation in ATO/CMEMM-induced apoptosis using the oxidant sensitive probe DHE. As shown in Figure 4, 50 μg/ml CMEMM increased intracellular ROS content in HL-60 and Jurkat cells when combined with 2.5 μM ATO after 48 h treatment. To further demonstrate that the enhancement of ATO-provoked apoptosis by CMEMM is mediated by ROS, the influence of ATO/CMEMM-induced apoptosis in the presence of a free radical scavenger, *N*-acetyl cysteine (NAC) was evaluated. As shown in Figure 5, addition of 5 mM NAC treatment decreased the accumulation of cells in sub-G1 phase as compared to those observed in ATO/CMEMM-treated cells (Figure 2). These results indicated that the apoptosis-inducing effect of ATO/CMEMM combination on leukemia cells was mediated by oxidative stress.

3.4. ATO/CMEMM Combination Induces Apoptosis via the Intrinsic Pathway. Induction of apoptosis through the intrinsic, mitochondrial pathway was further investigated in leukemia cells via assessment of cleavage/activation of caspase 3, caspase 9 and PARP. As shown in Figure 6, higher levels of cleaved caspase 3, caspase 9 and PARP were present in HL-60 and Jurkat cells exposed to the combination of ATO/CMEMM than with ATO alone. In addition, addition of NAC in the combinatorial treatment obviously inhibited the action of cleavage in these apoptosis-regulatory factors. To determine whether ATO/CMEMM combination induces caspase-dependant cell death, we further evaluated ATO/CMEMM-induced apoptosis in the presences of a pan-caspase inhibitor, z-VAD-fmk by flow cytometry. After 24 h combinatorial treatment of ATO and CMEMM, the frequency of apoptosis was greatly reduced by the addition of 25 μM z-VAD-fmk in Jurkat cells, however, not in HL-60 cells (data not shown).

4. Discussion and Conclusions

Since ATO has remarkable success in the treatment of APL, an increasing number of preclinical studies to broaden the therapeutic potential have been reported for decades. Unfortunately, ATO as a single agent has demonstrated only limited benefit in

non-APL hematological malignancies. Recent studies reported that combinatorial treatment will be a good strategy to enhance ATO-provoked apoptosis by combination with a pro-oxidant agent or natural components in vitro. In this present study, the results revealed that antileukemic activity of ATO was possibly potentiated by combining with a folk remedy, CMEMM through enhancing growth inhibition (Figure 1) and increasing the induction of apoptosis via a ROS-dependent mechanism (Figure 2, 3, 4 and 5) and intrinsic, mitochondria-mediated pathway (Figure 6).

Drug interaction of ATO and CMEMM was evaluated by CI values (Table 1). Interestingly, ATO and CMEMM synergistically inhibit cell growth on HL-60 cells, whereas variable effects were exerted by combining ATO with different dose of CMEMM on Jurkat and Molt-3 cells. As combined 2.5 μM ATO with 75 μg/ml CMEMM or 5 μM ATO with 25 μg/ml CMEMM, the drug interaction indicated antagonism in Jurkat or Molt-3 cells, respectively. The discrepancy in drug interaction between promyelocytic and lymphoid leukemia cells could be attributed to differences in cell-type specific molecular or genetic factors [18]. For instance, the degradation of PML-RARalpha protein, an APL marker protein resulted from chromosomal t(15;17) translocation, or the reduction of genomic methylation level may involve in drug interaction of antileukemic agents, and hence would need a deeper investigation at the molecular level [19-21]. Nevertheless, when combined ATO at a clinically achievable concentration (2.5 μM) with 50 μg/ml CMEMM, the synergistic effect of drug interaction was observed on growth inhibition in all three cell lines in the present study. For future therapeutic application, the determination of optimum dose of ATO/CMEMM-combined treatment will be a worthwhile task.

Overproduction or accumulation of ROS, such as superoxide anion and hydrogen peroxide, have been considered resulting in nonspecific damage to proteins, nuclear acids and other cellular components as toxic products of cellular metabolism. Oxidative stress may come out due to dysfunction of antioxidants or antioxidant enzymes although ROS at moderate concentration are not toxic but rather act as signaling molecules [22]. ATO-stimulated ROS generation in cultured cells is well recognized. In vitro studies have indicated that the pathway responsible for ATO-induced apoptosis on leukemic cells was through NADPH oxidase [23], mitochondrial electron transport chain [24] or the inhibition of antioxidant enzymes such as thioredoxin reductase (TrxR) [25] and glutathione peroxidase (GPx) [26], thereby inducing ROS-dependent apoptosis. For these reasons, in the current study we demonstrate the important role of ROS generation to enhancement of ATO-mediated apoptosis by combining with CMEMM. Similarly, recent studies reported that ATO-mediated cytotoxicity could be augmented by natural polyphenols, such as quercetin, genistein and curcumin, in human leukemia cells through generating a

pro-oxidant environment [14, 15, 27].

Moreover, changes in the intracellular milieu of the cells, such as alterations in the redox environment, have been indicated as important regulators of the progression to apoptosis [28]. Depletion of glutathione (GSH) or superoxide dismutase (SOD) has been shown to play an important role in cell survival and act as a target for the selective killing of cancer cells [29, 30]. However, our preliminary results showed that relative levels of total intracellular GSH or SOD did not significantly decrease in ATO/CMEMM-treated HL-60 or Jurkat cells as compared to controls (data not shown). Since the exact mechanisms involved in the regulation of apoptosis by GSH or SOD remain elusive, other regulators of apoptosis such as glutathionylation (protein-SSG) and nitrosylation (protein-SNO) may involve in redox-directed cytotoxicity and require a further investigation [31].

Dried stems of *M. macrocarpa* are used as blood-activating and stasis-resolving herbs to relieve symptoms related to leukemia in folk medicine [10]. Phytochemical components of CMEMM were analyzed by chromatography and spectroscopy in our previous study. Several bioactive isoflavones including calycosin, afrormosin and genistein were identified in CMEMM with pure marker components using HPLC [11]. Calycosin and afrormosin have been reported to exhibit growth inhibition on U937 lymphoma cells [32] and inhibitory effects on TPA-induced skin tumor promotion in mouse [33], respectively. Moreover, genistein has been found to be an antileukemic agent [34] and also a pro-oxidant to potentiate ATO-induced apoptosis in human leukemia cells via reactive oxygen species generation [25]. This implied that the antileukemic activity of ATO/CMEMM combination may have resulted from not only the interaction of different flavonoids but also generating a pro-oxidant environment by genistein.

In conclusion, we presented evidence that treating human leukemia cells with CMEMM led to the potentiation of ATO-provoked apoptosis via a ROS-dependent mechanism. The synergistic drug interaction correlates with the pro-oxidant action of CMEMM, as measured by ROS overproduction, and the activation of apoptosis-regulatory proteins, caspase-3 and -9. Therefore, our results suggested that combinatorial treatment of ATO and CMEMM may provide a beneficial therapeutic approach in the future. For future therapeutic application, further studies of optimum dose in comedications of ATO and CMEMM are now in progress in our laboratory.

Acknowledgments

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TABLE 1: CI values for the combination of ATO and CMEMM in leukemia cellines after 24 h treatment.

Drug combination		Cell line					
ATO	CMEMM	HL-60		Jurkat		Molt-3	
(μM)	(μg/ml)	CI	Interpretation	CI	Interpretation	CI	Interpretation
2.5	25	0.644	Synergistic	1.043	Additive	0.772	Synergistic
2.5	50	0.683	Synergistic	0.802	Synergistic	0.935	Synergistic
2.5	75	0.888	Synergistic	0.820	Synergistic	1.155	Antagonistic
5	25	0.887	Synergistic	1.446	Antagonistic	0.852	Synergistic
5	50	0.610	Synergistic	0.868	Synergistic	0.850	Synergistic
5	75	0.843	Synergistic	0.899	Synergistic	1.021	Additive

CI: combination index

ATO: arsenic trioxide

CMEMM: crude methanolic extract of *Mucuna macrocarpa*

Figure legends

FIGURE 1: Antiproliferative effects of combined application of arsenic trioxide (ATO) and crude methanolic extract of *Mucuna macrocarpa* (CMEMM) on human leukemia cells. HL-60, Jurkat or Molt-3 cells (1×10^5 cells/ml) were seeded into 6-well plates and exposed to 0, 2.5 or 5 μM ATO alone or together with 0, 25, 50 or 75 μg/ml CMEMM up to 48 h. Control cells were treated with 0.1% DMSO in medium. The percentages of cell growth were measured by trypan blue exclusion assay and calculated by comparing the cells numbers with that of the controls. Each value represents the mean ± S.E. of duplicate cultures from three independent experiments. * $P < 0.05$, ** $P < 0.01$ indicate significant difference from the control value.

FIGURE 2: Cell cycle progression in leukemia cells exposed to arsenic trioxide (ATO) and/or crude methanolic extract of *Mucuna macrocarpa* (CMEMM). HL-60 (a), Jurkat (b) or Molt-3 (c) cells (1×10^5 cells/ml) were treated with 0, 2.5 and 5 μM ATO combined with 0, 25, 50 and 75 μg/ml CMEMM, respectively. After 24 or 48 h treatment, cells were collected and stained with propidium iodide, and determined for DNA content using flow cytometry. The percentages of sub-G1 or hypodiploid cells were analyzed by CellQuest software. The representative cell cycle progressions in ATO and/or CMEMM-treated or control cells were from one of three independent experiments.

FIGURE 3: Nuclear morphological changes induced by arsenic trioxide (ATO) and/or crude methanolic extract of *Mucuna macrocarpa* (CMEMM). HL-60, Jurkat or Molt-3 cells (1×10^5 cells/ml) were treated with 0.1% DMSO (control), 2.5 μM ATO, 50 μg/ml CMEMM or 2.5 μM ATO plus 50 μg/ml CMEMM. After 24 or 48 h incubation, cells were washed with PBS and collected on microscope slides by cytospin. The nuclei were stained with 2.5 μg/ml DAPI. Arrows indicate apoptotic bodies of nuclear fragmentation. Magnification × 200.

FIGURE 4: Changes in the level of intracellular reactive oxygen species (ROS) in leukemia cells exposed to arsenic trioxide (ATO) and/or crude methanolic extract of *Mucuna macrocarpa* (CMEMM). HL-60 or Jurkat cells (1×10^5 cells/ml) were treated with 0.1% DMSO (control), 2.5 μM ATO, 50 μg/ml

CMEMM or 2.5 μM ATO plus 50 μg/ml CMEMM up to 48 h. Then, cells were washed with PBS, incubated with dihydroethidium for 30 min and analyzed for red fluorescence by flow cytometry. The mean fluororescence intensity was used as read-out for intracellular ROS levels.

FIGURE 5: Effect of addition of *N*-acetyl cysteine (NAC) on hypodiploid cells induced by arsenic trioxide (ATO) and/or crude methanolic extract of *Mucuna macrocarpa* (CMEMM). HL-60 (a) or Jurkat (b) cells (1×10^5 cells/ml) were first treated with 5 mM NAC, following 0, 2.5 and 5 μM ATO combined with 0, 25, 50 and 75 μg/ml CMEMM, respectively. After 24 or 48 h treatment, cells were collected and stained with propidium iodide, and determined for DNA content using flow cytometry. Hypodiploid cells (percentages of sub-G1) were analyzed by CellQuest software. The representative cell cycle progressions in combination-treated or control cells were from one of three independent experiments.

FIGURE 6: Expressions of apoptosis-related proteins in leukemia cells treated with arsenic trioxide (ATO), crude methanolic extract of *Mucuna macrocarpa* (CMEMM) and/or *N*-acetyl cysteine (NAC). Whole cell lysates were prepared from HL-60 or Jurkat cells treated with 2.5 μM ATO, 50 μg/ml CMEMM, 5 mM NAC, or indicated combinations for 24 h. Proteins as indicated were analyzed by Western blotting with β-actin as loading control. Representative blots were shown from one of three independent experiments.