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Apoptotic effects of mahanine on human leukemic cells are mediated through crosstalk between Apo-1/Fas signaling and the Bid protein and via mitochondrial pathways

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ABSTRACT

Apo-1 (Fas/CD95), a cell surface receptor, triggers apoptosis after binding to its physiological ligand, Apo-1L (FasL/CD95L). This study reports that mahanine, purified from the leaves of *Murraya koenigii*, has a dose- and time-dependent anti-proliferative activity in acute lymphoid (MOLT-3) and chronic myeloid (K562) leukemic cell lines and in the primary cells of leukemic and myeloid patients, with minimal effect on normal immune cells including $CD34^+$ cells. Leukemic cells underwent phosphatidylserine externalization and DNA fragmentation, indicating mahanine-induced apoptosis. An increase in reactive oxygen species suggests that the mahanine-induced apoptosis was mediated by oxidative stress. A significant drop in the Bcl2/Bax ratio, the loss of mitochondrial transmembrane potential as well as cytochrome c release from the mitochondria to the cytosol suggested involvement of the mitochondrial pathway of apoptosis. Cytochrome c release was followed by the activation of caspase-9, caspase-3 and caspase-7, and cleavage of PARP in both MOLT-3 and K562 cells. In MOLT-3 cells, formation of the Fas-FasL-FADD-caspase-8 heterotetramer occurred, leading to the cleavage of Bid to its truncated form, which consequently resulted in formation of the mitochondrial transmembrane pore. The incubation of MOLT-3 cells with mahanine in the presence of caspase-8 inhibitor or FasL-neutralizing NOK-2 antibody resulted in the decrease of mahanine-induced cell death. Mahanine was also a potent inhibitor of K562 xenograft growth, which was evident in an athymic nude mice model. In summary, these results provide evidence for involvement of the death receptor-mediated extrinsic pathway of apoptosis in the mahanine-induced anticancer activity in MOLT-3 cells, but not in K562 cells, which are deficient in Fas/FasL.

1. Introduction

The death receptor-mediated extrinsic pathway is one of the primary apoptotic pathways operative in different cell types. The cell surface Fas receptor (Apo-1 or CD95) belongs to the family of tumor necrosis factor (TNF) and nerve growth factor (NGF) [1–3]. The mechanism of cell death induced by the Fas/Fas ligand (FasL) involves binding of FasL to Fas receptors on the cell surface that eventually results in caspase-8 activation [3,4]. Death receptor-mediated activation of caspase-8 could lead to the processing of Bid to form tBid, which mediates crosstalk between cell surface receptor-mediated apoptotic signals and the mitochondria, facilitating the release of cytochrome c into the cytosol and further amplifying relevant apoptotic signaling [5,6]. The apoptosis induced by Fas is known to regulate tumor progression and

Abbreviations: BSA, bovine serum albumin; DAB, diaminobenzidine; DISC, death-inducing signaling complex; EtOH, ethanol; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; H_2DCFDA, 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester; HPLC, high-performance liquid chromatography; JC-1, 5,5′-6,6′-tetracholoro-1,1′-3,3′-tetraethylbenzimidazolylcarbocyanine iodide; MS, mass spectrometry; NAC, N-acetyl L-cysteine; NMR, nuclear magnetic resonance; PARP, poly ADP ribose polymerase; PBMC, peripheral blood mononuclear cells; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; TDT, terminal deoxy transferase; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated dUTP end labeling; UCB, umbilical cord blood.

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chemotherapeutic drug-induced death [7,8]. The importance of the Fas/FasL pathway is evident from the aggressive disease presentation and decreased survival of patients with several cancer types in which the loss of function of Fas is reported. In addition, the disruption of Fas has been shown to result in enhanced tumor development in experimental animal models [9,10], while Fas restoration delays outgrowth of the primary tumor. Fas and FasL interactions are known to play a role in the control of distant metastases [11], as well as in the development of chemotherapeutic resistance in some cell types [12]. These observations suggest that Fas is a frequent target for inactivation during oncogenesis and that Fas-induced apoptosis plays a crucial role in the biology and response of malignant diseases.

Leukemia is the most common hemato-oncological disorder that affects different age groups. Acute type leukemia is seen in children and adults, but the chronic phase of leukemia mainly affects adults [13,14]. Due to the presence of minimal residual disease, relapse and drug resistance are the major problems in these malignancies [15–18]. Different combinational chemotherapy is available, but to improve the subset of cancer therapeutics and to upgrade the cancer prevention policy, the establishment of a novel drug is still in demand. Various cell lines derived from leukemia patients have generally been used to study the apoptotic pathways. Understanding the influence of a given drug on these pathways provides opportunities for manipulation of the pathways for better drug efficacy [19,20].

New drugs, particularly those from herbal sources, are promising modalities for the treatment of a variety of cancers [21,22]. Mahanine, a carbazole alkaloid occurring in *Micromelum minutum*, *Murraya koenigii* and related species, has been shown to exhibit antimutagenicity, antimicrobial activity and cytotoxicity [23–26]. Mahanine is also known to induce apoptosis in histiocytic lymphoma and promyelocytic leukemia [27,28], and in prostate cancer cells [29,30]. However, the involvement of various apoptotic pathways in mahanine-induced cell death is not known. Here, we demonstrate the involvement of the Fas/FasL pathway during mahanine-induced cell death in Fas/FasL-positive acute lymphoid (MOLT-3) leukemic cell line, but not in Fas/FasL-deficient chronic myeloid (K562) leukemic cell line. To our knowledge, no study has thus far shown the involvement of the Fas/FasL system specifically in lymphoid and myeloid leukemic cells as a result of mahanine treatment.

2. Materials and methods

2.1. Reagents

The primary antibodies (Bcl2, Bax, Fas, caspase-9, caspase-3, caspase-7, caspase-8, cytochrome c and PARP), secondary HRP-conjugated antibodies, all flow cytometry compatible fluorescence-conjugated antibodies, annexinV–FITC, the ApoDitect TUNEL assay kit, FasL-neutralizing NOK-2 antibody and growth factors (SCF, G-CSF, GM-CSF) were purchased from BD Bioscience (USA). The primary antibodies (FasL, FADD, Bid and β-actin) and growth factor (IL-2) were obtained from Cell Signaling Technology (USA). The apoptotic DNA laddering kit was obtained from Roche (Germany). The caspase fluoremetric assay kits, FCS and JC-1 were purchased from Invitrogen (USA). Bromophenol blue, RPMI-1640,

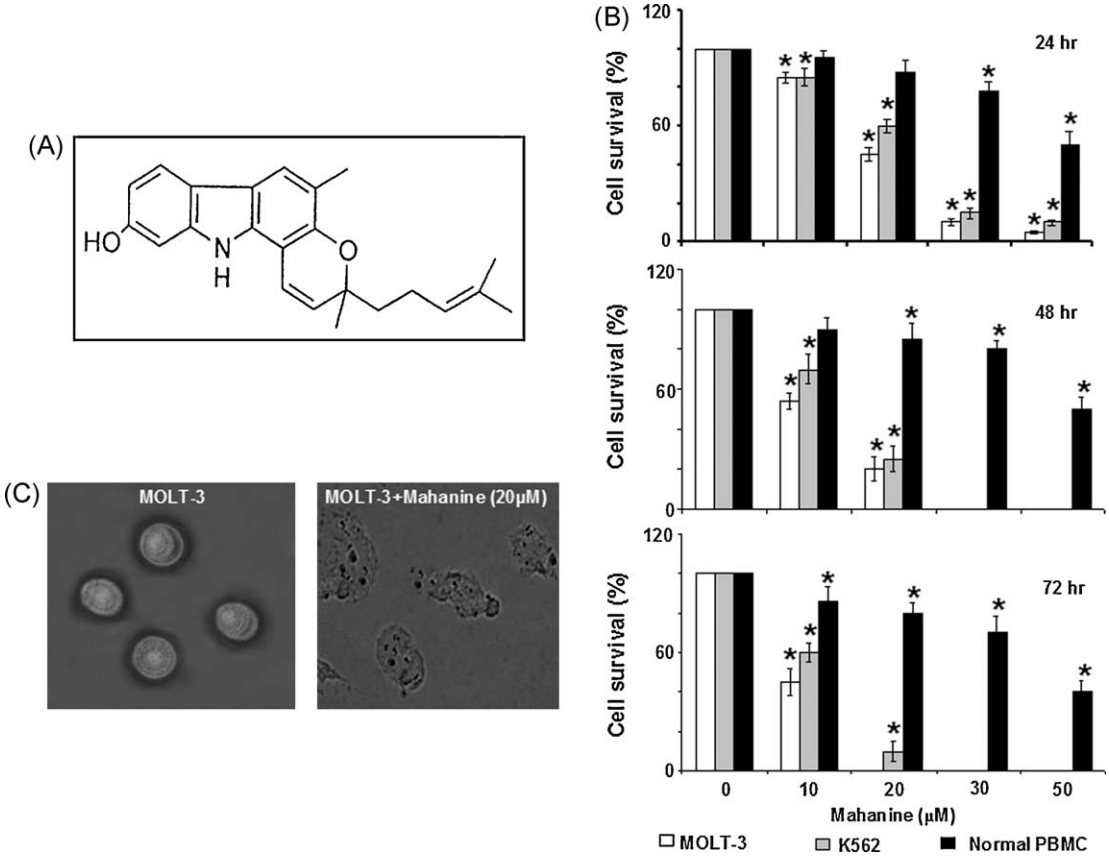

Fig. 1. Anti-proliferative activity of mahanine as determined by the trypan blue assay. (A) Chemical structure of mahanine isolated from leaves of *Murraya koenigii*. (B) Effects of mahanine (0–50 μM) on the inhibition of cell proliferation in the MOLT-3 (□) and K562 (■) cell lines and normal PBMCs (■) at 24, 48 and 72 h as determined by the trypan blue exclusion assay. (C) Mahanine (20 μM)-induced morphological changes in MOLT-3 cells after 24 h of treatment as determined by phase contrast microscopy. Each value is the mean ± SD of three independent experiments. $^{*}P < 0.05$, significant difference between two test groups.

antibiotic–antimycotic, PI, ficoll, H_2O_2, diaminobenzidine, H_2DCFDA, NAC, molecular grade BSA, Tween-20, Tris–HCl, DMSO, collagenase type II and DNase I were from Sigma–Aldrich (USA). Caspase inhibitors Z-VAD-fmk (pan-caspase inhibitor), Z-IETD-fmk (caspase-8 inhibitor) and the Fas/FasL antagonist (KP 7-6) were purchased from Calbiochem (USA).

2.2. Purification and characterization of mahanine

Mahanine was purified as previously described [29] from fresh leaves of a native Indian plant, *M. koenigii*, which belongs to the family Rutaceae. The purity was confirmed by HPLC (Fig. S1). LC–MS, ^{1}[H] and ^{13}[C] NMR spectral data analyses established its structure as mahanine (Fig. 1A).

2.3. Cell lines, patients and umbilical cord blood sample

Human T-(MOLT-3) acute lymphoblastic and chronic myelogenous (K562) leukemic cells were purchased from ATCC (USA) and grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS and 1% antibiotic–antimycotic solution in a humidified atmosphere at 37 °C and with 5% CO_2.

Blood samples (~2–3 ml) from clinically and cytogenetically confirmed lymphoid (n = 12) and chronic myeloid (n = 5) leukemic patients were collected at a hospital before any treatment was started and transferred to the laboratory of the Indian Institute of Chemical Biology. PBMCs were isolated by ficoll density gradient centrifugation. The diagnosis was confirmed by immunophenotyping using known CD cell surface markers and intracellular markers (CD10, CD19, CD3, CD7, CD8, CD4, CD34, CD33, CD13, CD14, CD45, CD11a, CD11b, CD11c and MPO) by flow cytometry.

Umbilical cord blood (UCB, ~15–20 ml) from six individuals was collected just after birth of the baby by gravitation. UCB mononuclear cells were isolated by ficoll gradient centrifugation. The $CD34^+$ population was enriched by using a human primitive progenitor enrichment kit (Stem cell technology, Canada), and cultured in RPMI-1640 in the presence of growth factors SCF, G-CSF, GM-CSF and IL-2 [31].

2.4. Cell viability analyses by ^{3}[H] thymidine uptake and the trypan blue exclusion assay

Cells (1×10^4) were exposed to mahanine (0–50 μM) in 96-well tissue culture plates in duplicate. After 24–48 h of exposure, ^{3}[H] thymidine (0.2 μCi) was added to the culture media and further incubated for 10–12 h for cancer cell lines and 16–18 h for human primary cells and the $CD34^+$ hematopoietic progenitor cells. The cells were harvested and the radioactivity was measured by a liquid scintillation counter. Cell viability was also measured under a light microscope using the trypan blue exclusion assay after 24–72 h of mahanine treatment [32]. In each treatment experiment, control cells were exposed to the highest amount of the vehicle, 0.15% absolute ethanol.

2.5. Flow cytometric detection of exposed surface phosphatidylserine (PS)

Cells (1×10^6) were exposed to mahanine (0–20 μM) for 24 h. Evaluation of apoptosis was performed using annexinV–FITC and propidium iodide (5 μg/ml) according to the manufacturer's instructions. At least 1×10^4 cells were examined and the data were analyzed by CellQuest Pro software (BD FACSCalibur) [32–34].

2.6. Apoptosis assay

Cells (1×10^6) were treated with mahanine (0–20 μM) for 24 h. Then, the DNA was extracted using an apoptotic DNA laddering kit, separated in a 2% agarose gel containing ethidium bromide and visualized by a UV illuminator. Quantitative flow cytometric assessment of the apoptotic cells was performed by the TdT-mediated TUNEL assay using a BD ApoDirect kit. At least 2×10^4 cells were analyzed [32].

2.7. Measurement of intracellular ROS

Cells (1×10^6) were treated with mahanine (0–20 μM) for 1 h and the levels of intracellular H_2O_2 were assessed flow cytometrically by incubating with H_2DCFDA (20 μM) for 30 min at 37 °C. For the inhibition of ROS generation, cells were pretreated with N-acetyl cysteine (10 mM) for 30 min before mahanine treatment. Ten thousand cells were analyzed [27,28,35].

2.8. Mitochondrial membrane potential assay

Cells (1×10^6) were incubated with or without mahanine for 12 h, washed with PBS and stained with JC-1 (a potentiometric probe for mitochondria, 25 μM) in the dark for 30 min at 37 °C. Cells (1×10^4) were then analyzed by a flow cytometer to determine the mitochondrial membrane depolarization [35].

2.9. Caspase activity assay

Cells (1×10^6) were treated with mahanine (0–20 μM) for 18 h, washed and labeled with the FLICA assay solution by incubating for

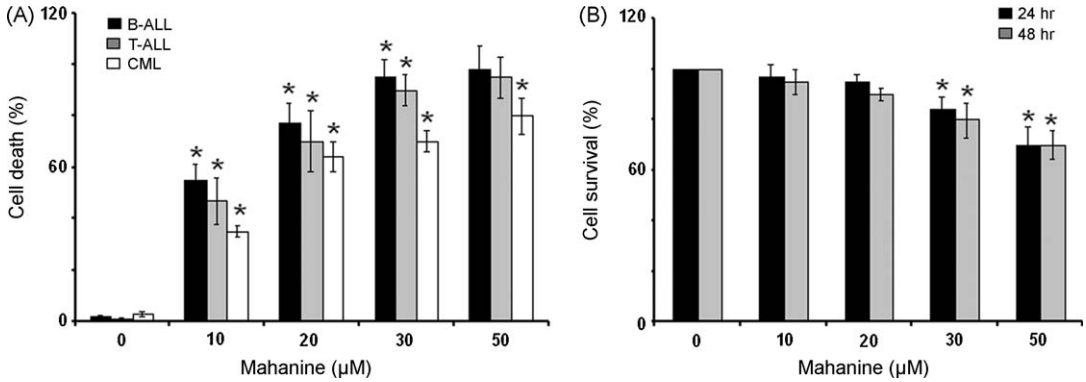

Fig. 2. Effects of mahanine in an *ex vivo* model by the trypan blue exclusion assay. (A) Mahanine (0–50 μM) showed growth inhibition on the primary cells of leukemic patients of lymphoid [B-ALL (■) and T-ALL (■)] and myeloid [CML (□)] origins at 48 h. (B) Dose-dependent effects of mahanine (0–50 μM) on umbilical cord blood-derived $CD34^+$ hematopoietic progenitor cells at 24 h (■) and 48 h (■) signify the non-toxic effect of the compound. Each value is the mean ± SD of three independent experiments. $^*P < 0.05$, significant difference between two test groups.

2 h at 37 °C. Samples were spectrofluorimetrically analyzed at 400 nm (excitation)/505 nm (emission). The results were represented as the fold change in the activity of caspase-3, caspase-8 and caspase-9 compared to the untreated control.

2.10. Immunoblot and immunoprecipitation

Cells (1.5×10^6) were exposed to mahanine (0–20 μM) and an equivalent amount of protein (50 μg) from each sample was resolved by SDS-PAGE (10%), then electrotransferred into nitrocellulose membrane. The membrane was blocked by 2% PBS-BSA, probed with primary antibody overnight at 4 °C, washed with PBS containing 0.1% Tween-20 and incubated with the appropriate HRP-conjugated secondary antibody. Subsequently, the membrane was washed and immunoreactive protein was identified by the DAB–H_2O_2 system.

For cytochrome c release from the mitochondria to the cytosol, mahanine-treated cells (1×10^7) were homogenized with a glass homogenizer and subjected to differential centrifugation [36]. The proteins were resolved by SDS-PAGE (15%).

For the detection of association of the Fas-FasL-FADD-caspase-8 heterotetramer, total cell lysate (400 μg) was treated with

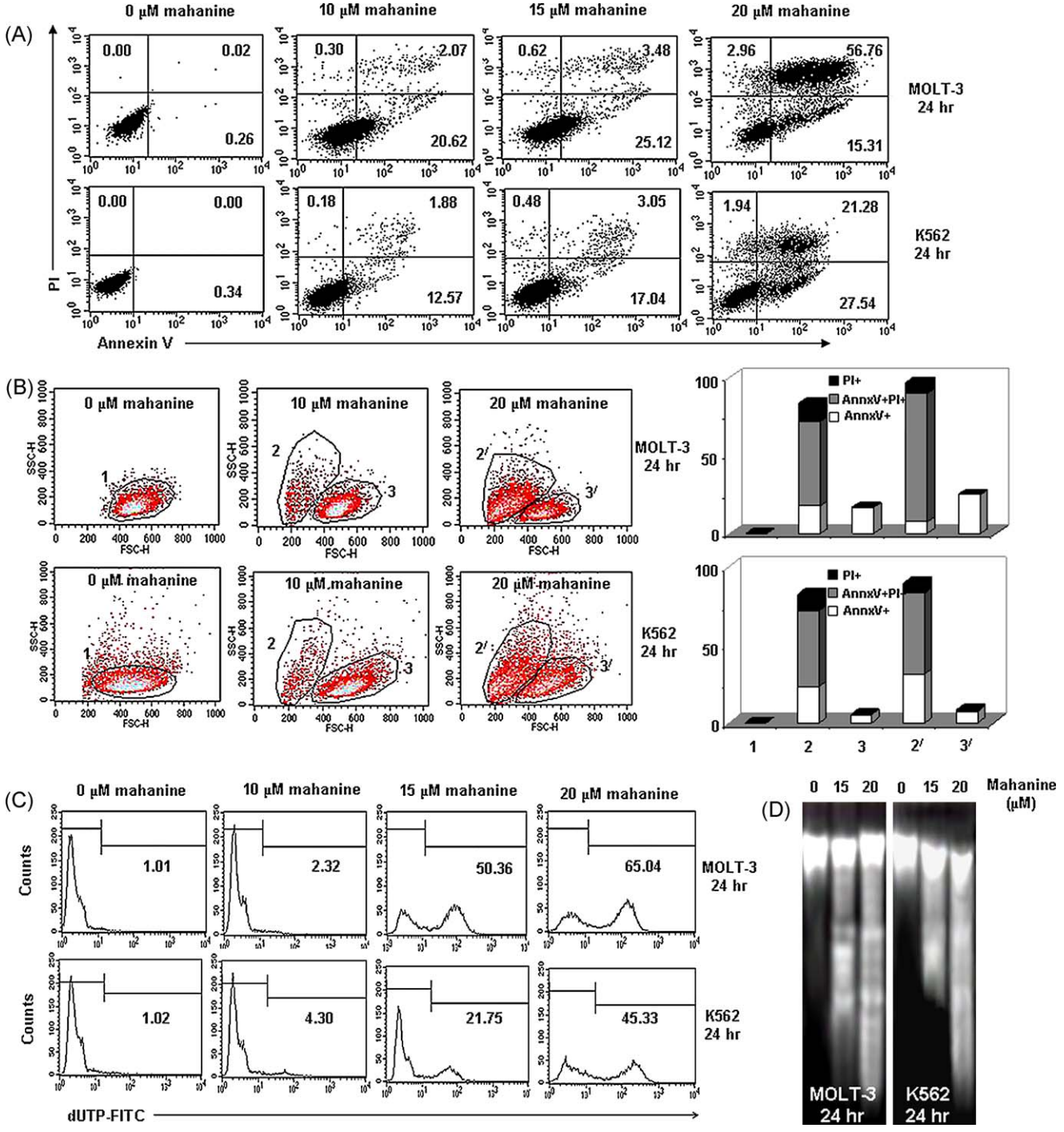

Fig. 3. Mahanine induces apoptosis in the MOLT-3 and K562 leukemic cell lines. (A) Mahanine (0–20 μM)-induced dose-dependent phosphatidylserine externalization and nuclear staining with PI in MOLT-3 and K562 cells at 24 h. (B) Effects of mahanine (0–20 μM) on the morphology of MOLT-3 and K562 cell populations as determined by flow cytometry using annexinV–PI staining at 24 h. 1, 1′, 2, 2′, 3 and 3′ indicated the number of the populations. (C and D) Mahanine (0–20 μM)-induced apoptosis in MOLT-3 and K562 cells at 24 h as measured by the TUNEL assay and DNA laddering, respectively.

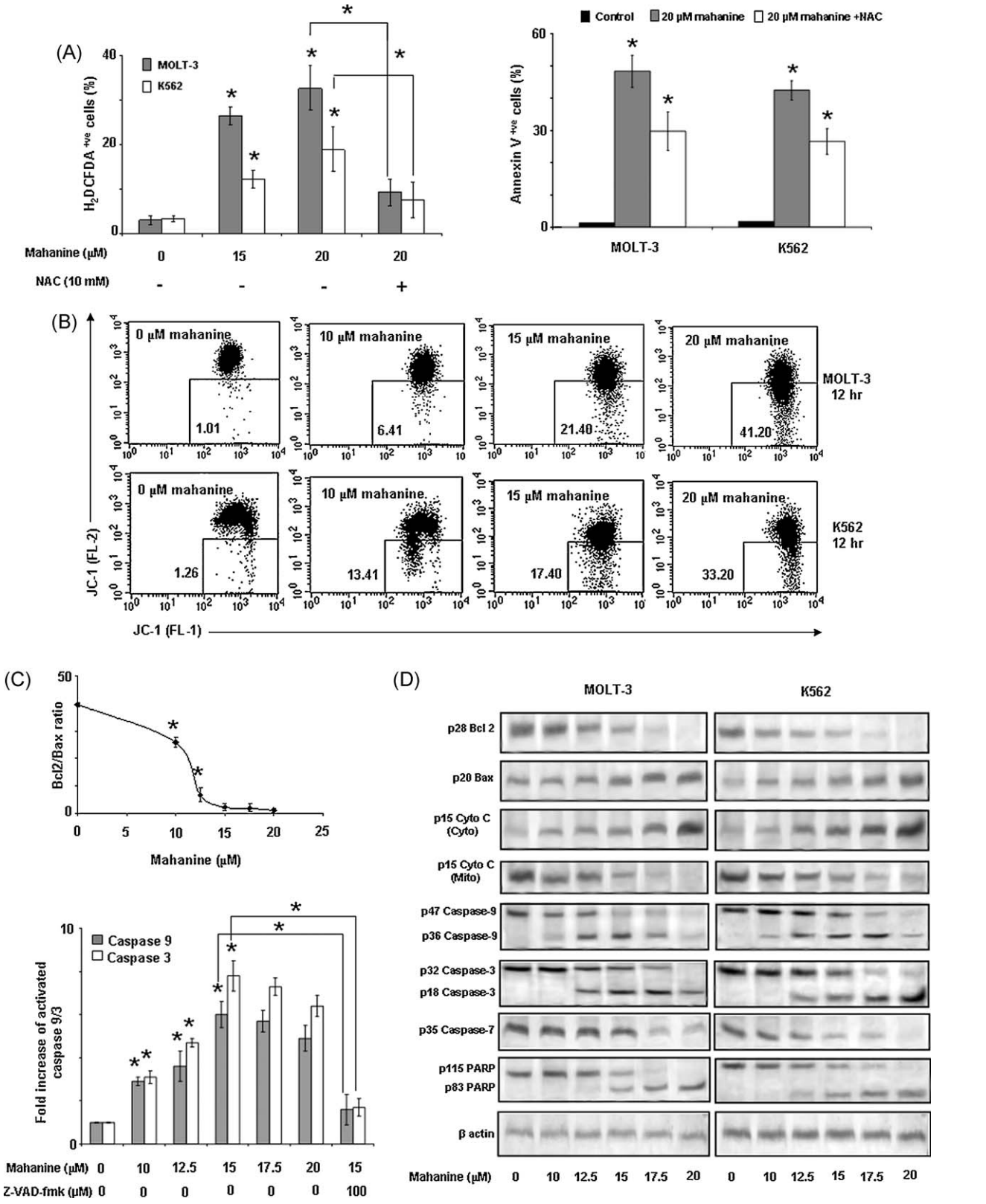

Fig. 4. Mahanine induces ROS production and mitochondria-mediated death cascade activation in MOLT-3 and K562 cells. (A) Effect of mahanine (0–20 μM) on the generation of intracellular H_2O_2 after 1 h measured by H_2DCFDA in MOLT-3 (■) and K562 (□) cells. Mahanine (20 μM)-induced annexinV$^+$ apoptotic cells decreased at 24 h when ROS were scavenged by NAC (□). Untreated cells were considered as a control (■). (B) Loss of mitochondrial transmembrane potential measured by flow cytometry analysis was revealed by the shift from red to green fluorescence in dose-dependent manner within 12 h of treatment. (C) Decrease in the Bcl2/Bax ratio and activation of caspase-9 (■) and caspase-3 (□) in MOLT-3 cells in dose-dependent manner at 18 h. The activation of caspases by mahanine was suppressed by pretreatment with the pancaspase inhibitor, Z-VAD-fmk. (D) Mahanine treatment led to the downregulation of Bcl2, upregulation of Bax, translocation of cytochrome c from the mitochondria to the cytosol, activation of caspase-9, caspase-3 and caspase-7 and the cleavage of PARP as determined by immunoblot analysis after 18 h in MOLT-3 and K562 cells. In each Western blot, β-actin served as the loading control. Each value is the mean ± SD of three independent experiments. $^*P < 0.05$, significant difference between two test groups.

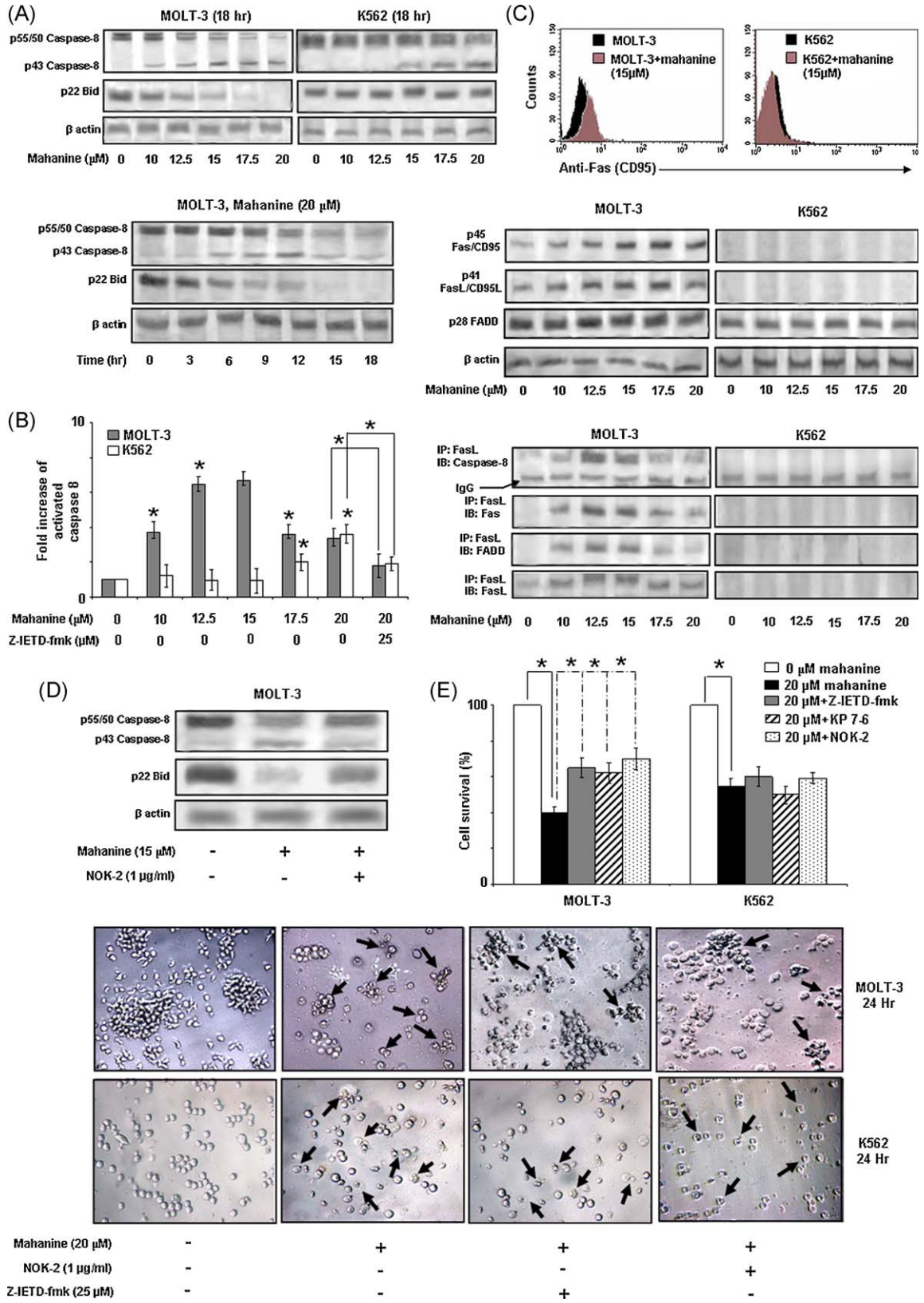

Fig. 5. Mahanine induces differential activation of the death receptor and associated proteins. (A) Comparative immunoblot analysis of the dose- and time-dependent manner of caspase-8 activation and Bid cleavage in mahanine-treated MOLT-3 and K562 cells. (B) Fluorometric estimation of the dose-dependent activated caspase-8 in MOLT-3 (■) and K562 (□) cells at 18 h. The activation was downregulated in the presence of the caspase-8-specific inhibitor Z-IETD-fmk. (C) Flow cytometric identification of overexpressed cell surface Fas/CD95 in mahanine (15 μM)-treated MOLT-3 cells, but not in K562 cells. Comparative immunoblot analysis of the endogenous expression levels of Fas, FasL, FADD in MOLT-3 and K562 cells after treatment with mahanine (0–20 μM) for 18 h. Identification of the association between Fas-FasL-FADD-caspase-8 and DISC formation in MOLT-3 and K562 cells measured by an immunoprecipitation assay using the anti-FasL antibody, and the corresponding identification of other proteins by immunoblot analysis at 18 h. (D) FasL-neutralizing antibody NOK-2-mediated inhibition of caspase-8 activation and Bid cleavage in mahanine (15 μM)-treated MOLT-3 cells at 18 h. (E) Measurement of cell survival by ^{3}[H] thymidine uptake assay of mahanine only (20 μM, ■)-treated and Z-IETD-fmk- (■), KP 7-6- (▨) and NOK-2 (⬚)

immunoprecipitation-compatible anti-FasL antibody (1:100 dilutions) and incubated overnight at 4 °C. The immune complex was resolved by SDS-PAGE (10%), transferred and probed separately with anti-Fas, anti-caspase-8, anti-FADD and anti-FasL antibodies.

2.11. In vivo toxicity testing and tumor xenograft study

Female nude mice (NIH(s) nu/nu, 6–7 weeks old) were maintained under pathogen free conditions. K562 cells (1×10^7, 200 μl) suspended in RPMI-matrigel (BD Bioscience, 1:1) were injected s.c. into the right hind limb [33]. The tumor was allowed to develop for 20–25 days until it reached 100–150 mm^3, after which mahanine (100 mg/kg, 250 μl) in 10% DMSO containing NaCl (0.15 M) was administrated intraperitoneally per day for 8 days in one group of tumor-containing mice ($n = 10$). The control group of nude mice was treated with only the same volume of vehicle ($n = 10$). Tumor volume was measured with an external Vernier caliper and the volume of tumor was calculated by the formula: $L \times W^2/2$ (mm^3), where L = length and W = width. On the 9th day after the initiation of treatment, all the mice were killed and the tumor xenografts were excised. The tumor cells were isolated from tumor tissue by collagenase type II-DNase I treatment [37]. Cells from the control and treated mice were subjected to PI exposure and analyzed by flow cytometry. For toxicity testing, 150 mg/kg/day mahanine was injected into the peritoneal cavity of normal healthy Balb/c mice for 20 consecutive days and body weight was measured on each day.

2.12. Statistical analysis

The data were from at least three independent experiments. Statistical analysis of data was performed using a two-tail student t-test. Error bars represent the standard deviation of the mean (±SD). Significant differences ($P < 0.05$) between the means of the control and the mahanine-treated cells, or two test groups were analyzed by Microsoft Excel and Graph pad Prism.

3. Results

3.1. Mahanine inhibits the proliferation of leukemic cells of myeloid (K562) and lymphoid (MOLT-3) origin

Leukemic cells (K562 and MOLT-3) were subjected to mahanine treatment to evaluate its cytotoxic effect. Mahanine showed significant anti-proliferative activity in both a dose- and time-dependent manner as assessed by the trypan blue (Fig. 1B) and thymidine uptake assays. The IC_{50} values of mahanine after 48 h of treatment were 10.6 μM and 13.0 μM for MOLT-3 and K562 cells, respectively. The radioactive thymidine uptake assay also showed the same trend of viability. Complete inhibition of proliferation was observed with 20 μM and 15 μM mahanine after 48 h and 72 h, respectively. Morphological changes in mahanine-treated cells were reflected in the complete collapse of their shape and density (Fig. 1C).

Mahanine also induced significant cell death in primary leukemic cells of myeloid and lymphoid origin isolated from leukemic patients (Fig. 2A), and 10–15 μM of the compound was required to inhibit 50% of the primary leukemic cells after 48 h of treatment. In contrast, mahanine had very little cytotoxic effect on normal PBMCs isolated from the blood of healthy individuals (Fig. 1B) and the cord blood-derived $CD34^+$ hematopoietic precursor cells (Fig. 2B). At the IC_{50} dose at 48 h, only a 5% reduction in viability occurred in normal PBMCs (Fig. 1B). The normal PBMCs and MOLT-3 spiked model experiment shows that mahanine is not toxic to normal PBMCs (Fig. S2).

3.2. Apoptosis occurs in MOLT-3 and K562 cells in response to mahanine

Since significant cell death was observed in mahanine-treated leukemic cells, the type of cell death was explored in MOLT-3 and K562 cell lines. Mahanine treatment-induced surface phosphatidylserine (PS) externalization in MOLT-3 and K562 cells at 10 and 15 μM, respectively, after 24 h, illustrating the presence of cells with features of early apoptosis (Fig. 3A).

After 24 h of treatment with 20 μM mahanine, membrane integrity was lost and a substantial proportion of MOLT-3 and K562 cells formed late apoptotic bodies as evidenced by staining with both annexinV and PI. Under these conditions, very few cells were only PI-positive, implying minimal formation of necrotic bodies (Fig. 3A).

Morphological analysis established a distinct subpopulation (FSC < 400) separate from the main population of cells (FSC 400–600) after treatment with mahanine (Fig. 3B). There was a dose-dependent increase in the FSC < 400 population, which was strongly stained by annexinV–PI, suggesting the enrichment of secondary apoptotic bodies. The FSC 400–600 population also showed a minimal number of annexinV-positive cells, indicating the induction of a minimal number of primary apoptotic cells. In contrast, the FSC 400–600 population of control cells in the absence of mahanine did not show any annexinV–PI^+ cells (Fig. S3).

The substantial amount of TUNEL-positive cells (Fig. 3C) and genomic DNA fragmentation (Fig. 3D) of both MOLT-3 and K562 cells due to exposure to mahanine (15–20 μM, after 24 h) confirmed that growth inhibition was due to apoptosis.

3.3. Mahanine induces the generation of ROS

Since there was significant apoptosis of the cells, it was possible that mahanine was able to induce biochemical changes in the cells, such as the generation of reactive oxygen species (ROS), which are known as the initial mediators of apoptosis that would lead to the dysfunction of intracellular macromolecules like DNA and proteins [38–42]. We found that mahanine (15–20 μM) was capable of producing ROS within an hour (Fig. 4A). Pretreatment with NAC inhibited ROS production and reduced the apoptotic body formation in mahanine-treated MOLT-3 and K562 cells. Therefore, ROS may play an initial and important role in the mahanine-induced apoptosis of these leukemic cells.

3.4. Mahanine leads to the induction of the mitochondrial pathway of apoptosis, caspase activation and PARP cleavage

The increased ROS production raised the possibility that mitochondrial function may be altered. Therefore, the mitochondrial potential was measured. A significant dose-dependent change of red to green fluorescence after 12 h of mahanine treatment was observed in MOLT-3 and K562 cells (Fig. 4B), which implied mitochondrial transmembrane depolarization.

Flow cytometric analysis revealed a dose-dependent drop in the Bcl2/Bax ratio after 18 h of treatment with mahanine in MOLT-3 cells (Fig. 4C). Caspase-9 and caspase-3 were also activated within 18 h, and maximum activation was seen in MOLT-3 cells after treatment with 15 μM mahanine (Fig. 4C).

antibody-pretreated mahanine-treated MOLT-3 and K562 cells. Untreated cells were designated as the control (□-). Morphological study by phase contrast microscopy of mahanine-treated MOLT-3 and K562 cells in the presence or absence of Z-IETD-fmk and NOK-2 at 24 h. The arrow (→) indicated the apoptotic body. Each value is the mean ± SD of three independent experiments. *$P < 0.05$, significant difference between two test groups.

Western blot analysis also revealed the same observation of Bax upregulation and Bcl2 downregulation, followed by cytochrome c release from the mitochondria to the cytosol within 18 h of treatment with mahanine in both MOLT-3 and K562 cells (Fig. 4D). Subsequently, caspase-9 was activated, followed by activation of caspase-3, which is an effector caspase. Caspase-7, another effector caspase, was also activated in MOLT-3 and K562 cells at a higher dose of mahanine (17.5 μM) after 18 h of treatment. Thus, it can be suggested that caspase-7 activation occurred at the end of the caspase cascade activation.

The DNA repairing enzyme PARP was cleaved by mahanine (15–17.5 μM) treatment after 18 h (Fig. 4D), which is known to induce nucleosomal condensation and apoptosis. Taking these results together, it is clear that both cell lines undergo apoptosis through the mitochondrial pathway after mahanine treatment.

3.5. Caspase-8 and Bid are differentially activated during mahanine-mediated apoptosis in MOLT-3 and K562 cells

Interestingly, a significant difference in the protein profile of activated caspase-8 and Bid was observed between MOLT-3 and K562 cells (Fig. 5A). In MOLT-3 cells, both caspase-8 and Bid were activated in a dose-dependent manner as measured by both fluorometric and Western blot analyses after 18 h of treatment. Maximum caspase-8 was activated by treatment with 12.5 μM mahanine, which was inhibited by the specific caspase-8 inhibitor, Z-IETD-fmk (Fig. 5B). Significant Bid cleavage was observed after 18 h of treatment with $\geq$12.5 μM mahanine. This cleavage signifies the formation of truncated Bid (tBid), which could translocate to the mitochondrial membrane and open channels for the release of cytochrome c from the mitochondrial intermembrane space to the cytosol.

In contrast, caspase-8 was not activated and Bid was not cleaved at the lower dose of mahanine in K562 cells (Fig. 5A and B). However, at higher dose of mahanine (17.5 μM) induced active caspase-8, but Bid was not truncated. As Bid is considered to be the molecule responsible for crosstalk between the cell surface-mediated apoptotic signal and the mitochondria, its activation is very important. Accordingly, we wanted to investigate the time point of activation of Bid and its upstream activator caspase-8 in MOLT-3 cells (Fig. 5A). Activation of caspase-8 was initiated within 6 h of treatment with mahanine (20 μM), and reached its optimum after 12 h. Comparable significant cleavage of Bid occurred after 6 h. Taken together, these results could suggest that caspase-8 and Bid activation in MOLT-3 cells may be induced by activation of the death receptor because truncation of Bid is a death receptor-mediated event that occurs via active caspase-8 [6]. This particular mechanism might not work properly in the K562 cells under these circumstances.

3.6. Differential expression of Fas and FasL and DISC formation occurs in mahanine-treated MOLT-3 and K562 cells

Since caspase-8 and Bid were activated in MOLT-3 cells, a search for the death receptor mechanism was carried out. Using flow cytometric analysis, we observed a 5-fold upregulation of Fas/CD95 after 18 h of treatment with mahanine (15 μM) in MOLT-3 cells (Fig. 5C). Interestingly, under identical conditions, K562 cells did not show any overexpression of Fas/CD95. Western blot analysis of mahanine-treated MOLT-3 cells also suggested a significant overexpression of Fas/CD95 and its ligand molecule FasL/CD95L, but expression of the adaptor protein FADD remained almost unchanged. In contrast, we did not find any significant level of expression of either Fas/CD95 or FasL/CD95L in mahanine-treated K562 cells, and the expression of FADD was not altered from the control. Therefore, it may be envisioned that due to the deficiency of Fas and FasL, this extrinsic apoptotic machinery was not active in mahanine-treated K562 cells.

Next, to establish the mechanism of death receptor-mediated apoptosis, we wanted to identify DISC formation by Fas-FasL-FADD-caspase-8 heterotetramerization. Accordingly, we performed the immunoprecipitation assay using anti-FasL antibody and separately probed the immunoblot with immunoreactive anti-Fas, anti-FADD, anti-caspase-8 and anti-FasL antibodies (Fig. 5C). Maximum DISC formation was observed after 18 h in 15 μM mahanine-treated cells as evidenced by the immunoblot analysis; however, at higher doses (17.5 and 20 μM), DISC formation was somewhat downregulated. Therefore, it may be suggested that at higher concentrations of mahanine, cells were in the process of undergoing apoptosis and therefore little less DISC formation occurred than at the lower dose. Accordingly, shorter time point might be suitable for the detection of a higher amount of DISC at the higher doses of mahanine. In contrast, when we assessed the same DISC formation in K562 cells, we did not identify any association between Fas-FasL-FADD-caspase-8.

3.7. The intrinsic mitochondrial and extrinsic Fas/FasL-mediated pathways of apoptosis occurs simultaneously in MOLT-3 cells, but not in K562 cells

To establish the Fas/FasL-mediated apoptotic pathway in MOLT-3 cells, we initially blocked the surface FasL with the FasL-neutralizing NOK-2 antibody and treated with mahanine (15 μM) for 18 h. We found that FasL-blocked cells showed reduced caspase-8 and Bid activation (Fig. 5D). Therefore, the above experiment confirmed that in MOLT-3 cells, caspase-8 and Bid were downstream substrates of the Fas/FasL interaction. To confirm the role and importance of the extrinsic Fas/FasL-mediated apoptotic signal in both cell lines, cells pretreated with Z-IETD-fmk, a Fas/FasL antagonist (KP 7-6) or NOK-2 were exposed to 20 μM mahanine and the cell survival rate was measured by the ^{3}[H] thymidine uptake assay after 24 h of incubation (Fig. 5E). Caspase-8 and Fas/FasL-inhibited mahanine-treated MOLT-3 cells were significantly less susceptible to apoptosis. An almost 25–30% increase in cell survival in the treated MOLT-3 cells established that the extrinsic Fas/FasL-mediated pathway was an important apoptotic route, along with the intrinsic mitochondrial apoptotic signals. However, the inhibitor- and antibody-pretreated K562 cells did not show any improvement in the cell survival rate in the presence of mahanine, confirming that the Fas/FasL pathway was absent. However, both the Fas/FasL and mitochondrial pathways of apoptosis were on in the MOLT-3 cells at the same time, and Bid was the crosstalking molecule between the cell surface and the mitochondria. This observation was corroborated by the morphological improvement seen in the caspase-8 inhibitor- and NOK-2-treated MOLT-3 cells, but not in the K562 cells as shown by bright field phase contrast microscopy (Fig. 5E). In K562 cells, minimal Fas/FasL expression, no DISC formation, weak caspase-8 activation and no Bid truncation occurred, suggesting that only the mitochondrial pathway was activated and there was no involvement of the Fas/FasL-mediated extrinsic pathway of apoptosis.

3.8. Mahanine was a non-toxic and potent compound that inhibited tumors in the nude mice model

MOLT-3 cells are a poor tumor xenograft-producing cell line [43]. We therefore implanted a K562 xenograft into nude mice to establish the antitumor activity of mahanine *in vivo*. A dose of 100 mg/kg/day of mahanine comprehensively inhibited tumor growth and reduced tumor volume after 8 days of consecutive treatment (Fig. 6A). Additionally, it was observed that a greater number of PI-positive tumor cells, were isolated from the

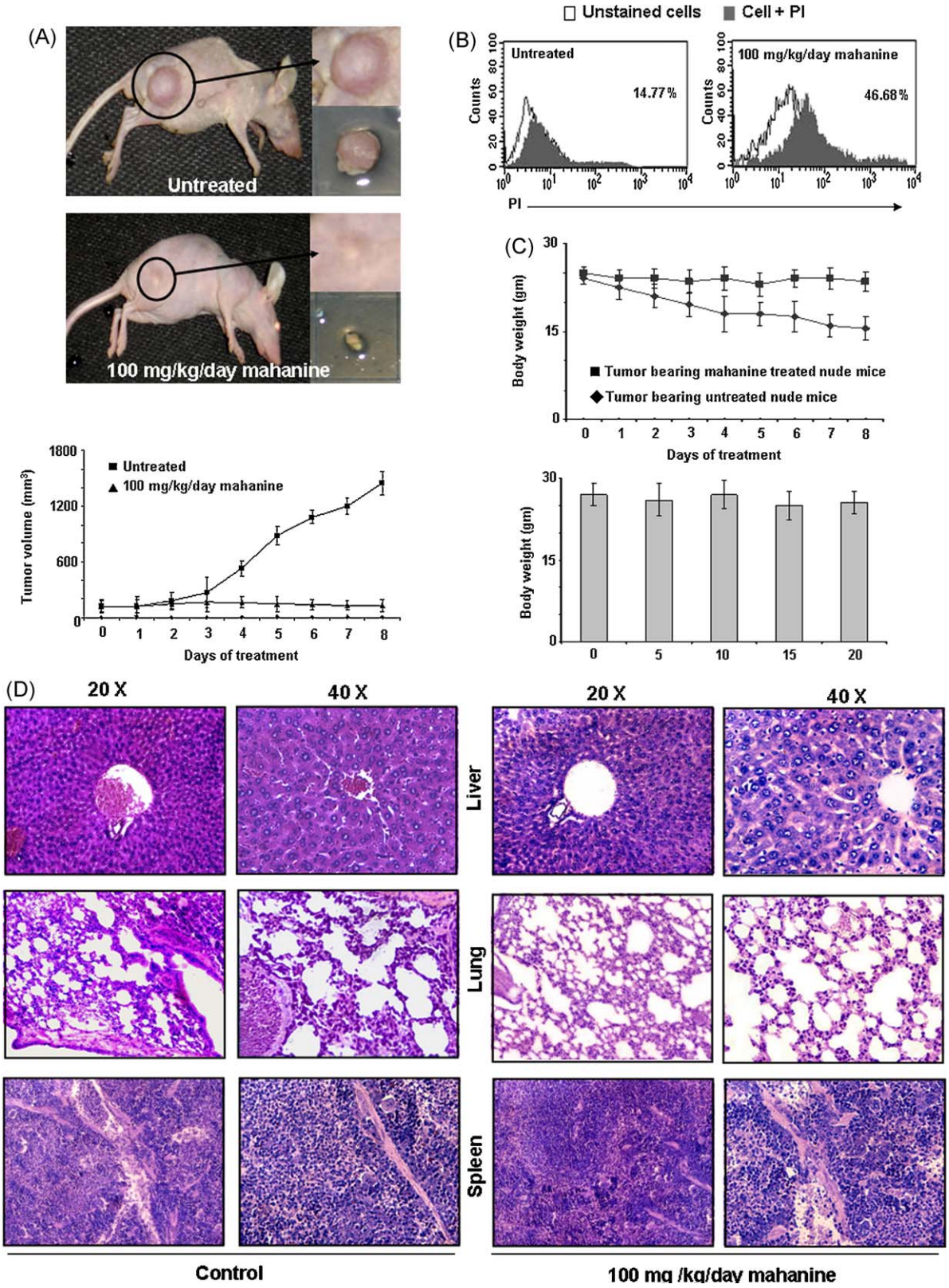

Fig. 6. Mahanine inhibits growth and induces apoptosis in the K562 xenograft in an athymic nude mice model. (A) Mean of tumor volume in mm^3 measured at the indicated number of days after initiation of treatment with mahanine. The treated mice group ($n = 10$, ▲) was exposed to 100 mg/kg/day of mahanine, while untreated mice group ($n = 10$, ■) was exposed to the vehicle only. (B) Status of apoptotic cells in the tumor measured by PI positivity in mahanine-treated and untreated mice as determined by flow cytometric analysis. PI-positive cells (■) were determined with respect to PI unstained cells (□). (C) Effect of mahanine on the body weight of mahanine-treated (■) and untreated (◆) tumor-bearing nude mice. Effect of mahanine (150 mg/kg/day) on the body weight of wt Balb/c mice for 20 days (■). (D) Tissue sections of the livers, lungs, and spleen of untreated and mahanine-treated nude mice analyzed by H and E staining.

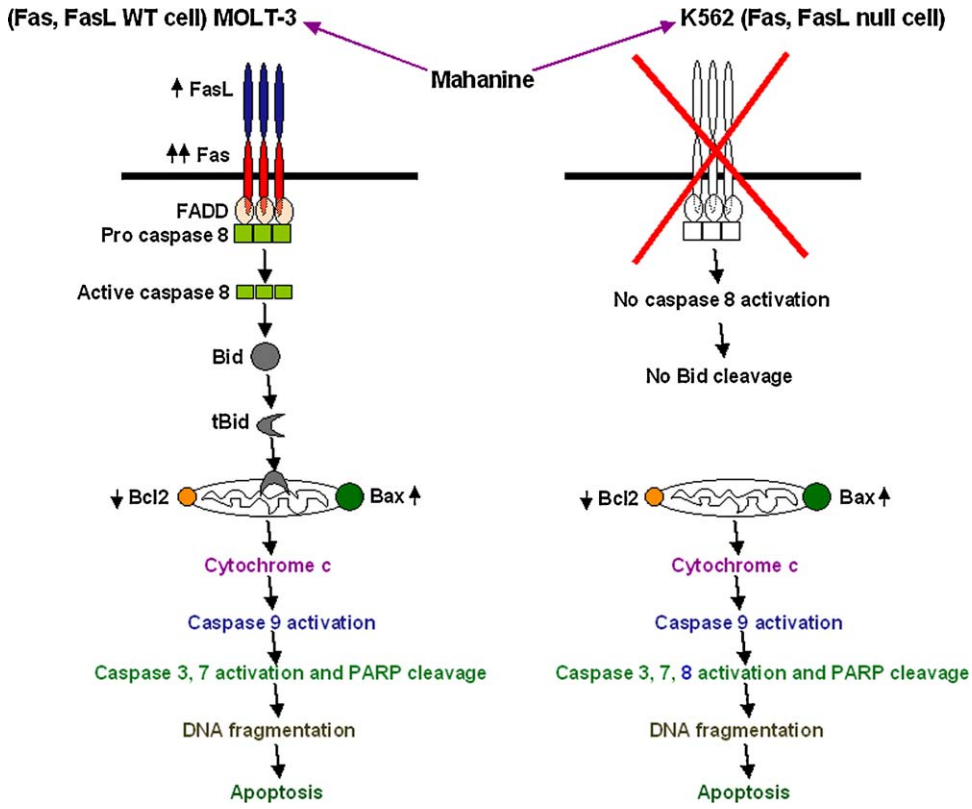

Fig. 7. Schematic representation of the probable apoptotic pathways in the mahanine-induced death of MOLT-3 and K562 cells.

mahanine-treated mouse than the control, which signified the accumulation of more apoptotic cells (Fig. 6B). Mahanine-mediated toxicity was not seen in the treated nude mice group as judged by body weight measurements (Fig. 6C) and tissue sections of liver, lung and spleen with respect to the controls (Fig. 6D). But due to the increase of the tumor load, mahanine-untreated nude mice drastically lost body weight. Interestingly, an even higher dose of mahanine administered to normal healthy Balb/c mice for 20 days showed no toxic effect at that indicated amount, as the body weight remained approximately the same (Fig. 6C).

4. Discussion

The development of novel herbal remedies for the treatment of leukemia is important because of the side effects of existing synthetic pharmaceutical medications [44,45], which target a variety of target molecules [46–48]. We have recently demonstrated two key enzymes responsible for induction of leukemia-associated antigens, which may also be considered as drug targets in leukemia [49,50]. Treatments can be further improved if the mechanisms of induction of cell death associated with the compounds are known. In this manuscript, we demonstrate the anti-proliferative effect of mahanine, an herbal compound, on acute lymphoblastic and chronic myelogenous leukemic cells and delineate the underlying involvement of the various apoptotic pathways using both *in vitro* and *in vivo* models for the first time. For both leukemic cell lines, multiple apoptotic pathways were induced, which demonstrates the effectiveness of mahanine as an anti-proliferative agent (Fig. 7).

The activation of the mitochondrial death cascade is one of the primary apoptotic pathways [27,28]. The data presented in this manuscript established that mahanine induced early ROS production, mitochondrial membrane depolarization, a decrease in the Bcl2/Bax ratio, the release of cytochrome c from the mitochondria to the cytosol and the subsequent activation of the caspase cascade and PARP cleavage. All these findings indicated that the mitochondria-dependent intrinsic apoptotic pathway was activated by mahanine.

Most strikingly, we identified some differences in the activation patterns of caspase-8, which was activated at lower doses of mahanine in MOLT-3 cells, but at higher doses in K562 cells. Bid activation was also associated with activated caspase-8, predominantly in MOLT-3, but not in K562 cells. Bid, a BH3 domain-containing protein, is the substrate of activated caspase-8 when activated caspase-8 is regulated by the death receptor, mainly through the Fas/FasL interaction [6]. While processed or truncated Bid (tBid) can migrate to the mitochondria and alter membrane integrity directly, it can also change the Bcl2/Bax ratio in the mitochondrial membrane and induce cytochrome c release into the cytosol. Thus, Bid mediates the relay of the apoptotic signal from the cell surface to the mitochondria, and our results indicated that Bid acts as a molecule assisting in the crosstalk between the two apoptotic pathways in MOLT-3 cells, but not in K562 cells.

We also showed that the activation of caspase-8 and Bid was associated with the Fas/FasL interaction in MOLT-3 cells, since the Fas-FasL-FADD-caspase-8 heterotetramer, also known as DISC, was detected in this cell type. In K562 cells, DISC was not formed, as they are Fas-null and FasL-deficient cells [51,52]. Therefore, one may speculate that caspase-8 activation in K562 cells was regulated primarily by mitochondria.

We also described mahanine-mediated Fas and FasL upregulation in MOLT-3 cells, which could facilitate the particular DISC formation. Since anticancer agents are generally pro-oxidant, ROS-induced alterations of macromolecules occurred in the cell [35]. Oxidative stress induces the DNA damage response and upregulates tumor suppressor proteins, like p53 and $p21^{Cip1/waf1}$, which transmit the apoptotic signal to their downstream substrates

[41,42]. As MOLT-3 is a wild type p53 cell line, one might postulate that the oxidative stress-induced DNA damage response first activated p53, which might have transactivated Fas and FasL as reported elsewhere [3,41]. As K562 is a p53 null cell line, p53-dependent transactivation was not possible. Therefore, an upstream apoptotic signaling pathway needs to be investigated.

Mahanine has been shown to be effective against some forms of malignancy and reported to induce the mitochondrial death cascade in U937, HL-60, PC-3 and LNCap cell lines [27–29], and inhibit AKT and PDK phosphorylation in PC-3 and LNCap. RASSF1A, one of the epigenetically suppressed tumor suppressor genes, is also the target of mahanine in different cell lines [30]. Our findings suggest for the first time that mahanine is a potent molecule to induce the death receptor. It mainly induced Fas-mediated extrinsic pathway-dependent apoptosis in MOLT-3 cells, while it induced Fas-independent apoptosis in K562 cells.

In conclusion, the evidence provided here suggests that mahanine is a multi-targeted or multi-functional compound that works on an array of different cancer types and has the potential to inhibit tumor growth *in vivo*. Therefore, we propose the introduction of mahanine as a broad-spectrum potential anticancer candidate. There is also the opportunity to use this herbal remedy as a combinational chemotherapeutic agent along with traditional anti-leukemic drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.09.007.

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