

# A novel class of piperidones exhibit potent, selective and pro-apoptotic anti-leukemia properties

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**Abstract.** In the present pre-clinical study, a series of 1-[3-(2-methoxyethylthio)-propionyl]-3,5-bis(benzylidene)-4 piperidones and structurally-related compounds were observed to be cytotoxic *in vitro* to three human leukemia cell lines, namely Nalm-6, CEM and Jurkat. The 50% cytotoxic concentration (CC<sub>50</sub>) values of the three cell lines ranged between 0.9-126.4  $\mu$ M and 0.3-11.7  $\mu$ M at 24 and 48 h subsequent to exposure, respectively. The two lead compounds with sub-micromolar CC<sub>50</sub> concentrations, 1-(2-methoxyethylthio-propionyl)-3,5-bis(benzylidene)-4 piperidone (2a) and 3,5-bis(4-fluorobenzylidene)-1-[3-(2-methoxyethylsulfinyl)-propionyl]-4-piperidone (3e), were selected for additional analyses. Several strategies were undertaken to determine whether the above piperidones caused cell death via apoptosis or necrosis on T-lymphocyte leukemia Jurkat cells. The results revealed that the two piperidones caused phosphatidylserine externalization, mitochondrial depolarization and activation of caspase-3, which are all biochemical hallmarks of apoptosis. In addition, the selected piperidones displayed selective cytotoxicity towards leukemia cells, and were less toxic in non-cancerous control cells. Therefore, the findings of the present study revealed that the novel piperidones 2a and 3e exert a selective cytotoxic effect on

lymphocyte leukemia cells by favoring the activation of the intrinsic/mitochondrial apoptotic pathway.

## Introduction

Leukemia, lymphoma and myeloma are a diverse category of malignant hematological neoplastic diseases that are initiated primarily in the blood, bone marrow and lymphoid organs. An estimated 52,380 newly diagnosed cases and 24,090 mortalities were associated with leukemia in 2014 (1). Non-Hodgkin lymphoma (NHL), the most common type of lymphoma, was diagnosed in 70,800 individuals, with 18,990 of them succumbing to the disease, in 2014 (1). Myeloma is the second most common type of blood cancer, with 24,050 novel cases and 11,090 mortalities reported in 2014 (1). In terms of prevalence, 917,086 patients with the above blood malignancies were living in 2014 in the USA, with NHL (530,919 patients) accounting for over half of these cases (1). According to the Leukemia and Lymphoma Society, approximately every 3 min, 1 person is diagnosed with one of the aforementioned blood neoplasms in the USA (1,2). However, identifying a cure for all the types of blood cancer is extremely challenging, as they are highly heterogeneous in nature and possess great genetic variability and diverse biochemical alterations (1,3). Recently, several anti-lymphoma therapies have been developed that have improved the survival of patients with blood cancer, including novel chemotherapeutic agents and the use of combinatorial therapeutic strategies that combine cytotoxins with immune modulators such as monoclonal antibodies directed to specific receptors (4-8).

A major focus for numerous studies involves the synthesis and evaluation of novel anti-leukemic/lymphoma cytotoxins (9-16). The present study focuses on the development of conjugated unsaturated ketones or enones, which have a preferential or exclusive affinity for thiols, in contrast to amino or hydroxyl groups (17). Amino and hydroxyl substituents are present in nucleic acids. Therefore, the genotoxic problems associated with a number of current anticancer drugs may be avoided with the use of enones (18). In particular, the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore has been mounted on heterocyclic and cycloaliphatic scaffolds, and is considered to align at a primary binding site (19). The extent

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of this interaction may also be influenced by the nature of the groups placed on the piperidyl nitrogen atom (19). For example, the N-acyl group may form additional bonds with various atoms and groups in cells, thus increasing the magnitude of the interaction at the primary binding site (19). A previous study revealed that the introduction of a 3-(2-hydroxyethylthio) propionyl group ( $-\text{COCH}_2\text{CH}_2\text{SCH}_2\text{CH}_2\text{OH}$ ) on the piperidyl nitrogen atom led to a series of potent cytotoxins (20), whose general structure is represented in Fig. 1A. However, these compounds contain a terminal polar hydroxyl group that may be easily metabolized to acid analogs and impede cellular penetration (21). In the present study, the terminal polar hydroxyl group was masked by the corresponding methoxy analogue (Fig. 1B). In addition, the importance of the sulfur atom in the N-acyl side chain, in association with cytotoxic potencies, was evaluated by replacing it with related groups. The structures of the resulting compounds are presented in Fig. 1C. The aim of the present study was to evaluate the cytotoxicity of novel 1-acyl-3,5-bis(benzylidene)-4-piperidones on a limited number of leukemia cell lines. The results demonstrate that the two most active piperidones investigated in the present study are able to induce apoptosis in the cell lines tested.

## Materials and methods

**Compounds for cytotoxic evaluations.** The structures of the 12 compounds used in the present study are presented in Fig. 1B and C. The synthesis of these compounds was conducted at the College of Pharmacy and Nutrition, University of Saskatchewan (Saskatoon, Canada), details of which have recently been published (22). In brief, a similar methodology was used as described previously (20) in which aryl-aldehydes were condensed with 4-piperidone and the resultant product was acylated with a number of acyl chlorides to give the compounds in series 2 and 3. The purified piperidones were characterized by proton nuclear magnetic resonance (NMR), carbon-13 NMR and organic elemental analysis of their carbon, hydrogen, nitrogen and sulfur content (results not shown) (22).

**Cell lines and culture conditions.** The human T-lymphocyte leukemia Jurkat (TIB-152; American Type Culture Collection, Manassas, VA, USA) (23), pre-B acute lymphoblastic leukemia Nalm-6 (ACC-128; DSMZ, Braunschweig, Germany) (24) and T lymphoblast acute lymphoblastic leukemia CCRF-CEM (American Type Culture Collection) (25) cell lines were grown in HyClone™ RPMI-1640 medium (GE Healthcare Life Sciences, Marlborough, MA, USA) supplemented with 10% heat-inactivated HyClone™ fetal bovine serum (FBS; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 µg/ml streptomycin (15140-122; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Human non-malignant epithelial mammary MCF-10A cells (CRL-10317; American Type Culture Collection) were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham media (51445C; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS, 10 µg/ml recombinant human insulin (Sigma-Aldrich), 20 ng/ml epidermal growth factor (Peprotech Inc., Rocky Hill, NJ, USA), 0.5 µg/ml hydrocortisone (Sigma-Aldrich), 2.5 mM L-glutamine (35050-061; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml

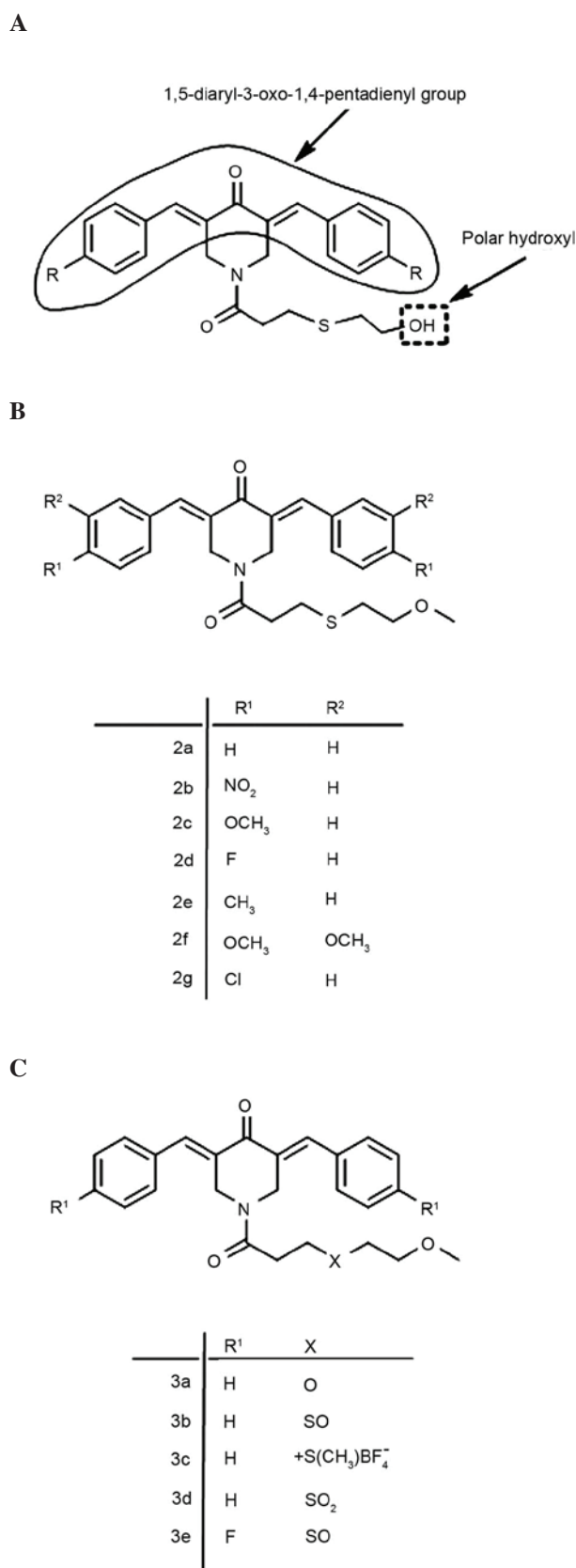


Figure 1. (A) Structure of 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. Structures of the novel 1-[3-(2-methoxyethylthio)-propionyl]-3,5-bis(benzylidene)-4-piperidones in (B) series 2 and (C) series 3 that contain conjugated unsaturated ketones or enones, in addition to the 1,5-diaryl-3-oxo-1,4-pentadienyl pharmacophore. The chemical names for compounds 2a and 3e are 1-(2-methoxyethylthio-propionyl)-3,5-bis(benzylidene)-4-piperidone and 3,5-bis(4-fluorobenzylidene)-1-[3-(2-methoxyethylsulfinyl)-propionyl]-4-piperidone, respectively.

streptomycin. Nalm-6 and Jurkat cells were derived from male donors, whereas CEM and MCF-10A cells were derived from female donors (25). Cells that were growing at 60-75% confluence in the exponential growth phase were counted and seeded into 24- and 96-well plates (cat no. 167008; Thermo Fisher Scientific, Inc.) at a density of 100,000 and 10,000 cells/well in 1,000  $\mu$ l and 200  $\mu$ l culture medium, respectively. Cell incubation was performed at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. To procure high cell viability, the cells were prepared as previously described (26).

**Differential nuclear staining (DNS) bioimaging assay.** The cytotoxicity of the experimental compounds was tested on the various cell lines via live-cell DNS bioimaging assays (27). Cells in 96-well microplates, seeded as described above, were treated with various concentrations of experimental compounds (0.25-100  $\mu$ M) for 24 and 48 h. A total of three controls were analyzed in each experimental plate, which included a compound solvent control [dimethyl sulfoxide (DMSO; D12345; Thermo Fisher Scientific, Inc.); cells treated with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; 349887; Sigma-Aldrich) as a positive control for toxicity; and untreated cells to monitor the background of dead cells. To differentially label the cells, a mixture of two DNA-intercalating fluorescent dyes, consisting of 1  $\mu$ g/ml Hoechst (H3570; Thermo Fisher Scientific, Inc.) and propidium iodide (PI; 0219545880; MP Biomedicals, Santa Ana, CA, USA), was added to each well 1 h prior to image recording (27). Hoechst stain is a blue fluorescent dye that may easily cross cell membranes of healthy and dead cells, and stains nuclear DNA, thus identifying the total number of cells (27). PI (red) only stains cells with compromised plasma membrane, thus indicating the number of dead cells (28). The fluorescence signals emitted by the stained cell nuclei were captured in two separate channels, in fulfillment with each individual fluorophore emission requirements (27). In order to capture acceptable numbers of regions of interest (equivalent to number of nuclei/cells), 2x2 image montages between four adjacent image fields were captured per well, using a 10x objective lens and a BD Pathway 855 Bioimaging System (BD Biosciences, San Jose, CA, USA). Image collection and data analysis that measured the percentage of dead cells in each individual experimental well was achieved using BD AttoVision version 1.6.2 software (BD Biosciences), and every test condition was assessed in quintuplicate. The 50% cytotoxic concentration (CC<sub>50</sub>) was calculated using a linear interpolation equation with a gradient of concentrations, as previously described (29).

**Apoptosis and necrosis assays.** Disruption of phosphatidylserine (PS) membrane asymmetry, which is an early event of apoptotic cell death, was detected via flow cytometry upon treatment of cells with 1-(2-methoxyethylthio-propionyl)-3,5-bis(benzylidene)-4 piperidone (2a) and 3,5-bis(4-fluorobenzylidene)-1-[3-(2-methoxyethylsulfinyl)-propionyl]-4 piperidone (3e). Following incubation with the above compounds, cells were stained with fluorescein isothiocyanate (FITC)-labeled annexin V and PI (IM2375; Beckman Coulter, Inc., Miami, FL, USA), and monitored using flow cytometry (Cytomics FC 500 Flow Cytometer; Beckman Coulter, Inc.). Flow cytometry readily detects PS exposure on the external leaflet of the plasma membrane due to the high affinity of annexin for binding to PS

on the cellular membrane (15). Jurkat cells seeded in 24-well plates were subsequently exposed to piperidones at their CC<sub>50</sub> concentration (1.4  $\mu$ M) for 24 h, and the treated cells were then harvested from each well in a pre-chilled ice-water cytometric tube (60818-437; VWR International, Houston, TX, USA), washed and processed as previously described (13). In each experimental plate, the following controls were included: Untreated cells; cells treated with compound solvent (0.2% v/v DMSO); and cells exposed to 1 mM H<sub>2</sub>O<sub>2</sub> as a positive control for cytotoxicity. The total percentage of apoptotic cells was determined by the addition of the annexin V-FITC<sup>+</sup> early and late phases of apoptosis. Cells that stained only with PI, but not with annexin V-FITC, were considered to be the necrotic population. A total of ~5,000 individual cells/sample were collected and analyzed with CXP software version 2.2 (Beckman Coulter, Inc.).

**Detection of mitochondrial membrane potential ( $\Delta\Psi_m$ ) disruption.** Jurkat cells, seeded in a 24-well plates as described earlier, were exposed to CC<sub>50</sub> concentrations of 2a and 3e for 6 h, and labeled with 2  $\mu$ M 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) fluorophore, following the manufacturer's protocol (MitoProbe™; Thermo Fisher Scientific, Inc.). Upon labeling with JC-1, cells with polarized mitochondria emitted a red fluorescence signal, while cells with depolarized mitochondria emitted a green fluorescence signal (11,13). A flow cytometry protocol was used to monitor the percentage distribution profile of cells emitting red or green signals (11,13). As a control for the dissipation of  $\Delta\Psi_m$ , 1 mM H<sub>2</sub>O<sub>2</sub> was utilized. In addition, a solvent control (1% v/v DMSO) and untreated control cells were analyzed. Data collection and analysis was performed using CXP software.

**Live-cell detection of caspase-3 activation.** Jurkat cells were incubated for 6 h with CC<sub>50</sub> concentrations of 2a and 3e. Subsequently, caspase-3 activation was measured using the fluorogenic NucView™488 Caspase-3/7 substrate for live cells, following the manufacturer's protocol (Biotium, Inc., Hayward, CA, USA). This caspase-3/7 substrate penetrates live cells with undamaged plasma membranes, and permits the identification of living cells with active caspase-3 (9). Cells exhibiting a green fluorescence signal, indicative of caspase-3 activation, were examined via flow cytometry (Cytomics FC 500 Flow Cytometer). The same positive and negative controls used in the preceding experiments were included and analyzed in the present series of experiments. CXP software was used for data acquisition and analysis.

**Statistical analysis.** Each data point was derived from  $\leq 3$  replicates. Data are presented as the mean  $\pm$  standard deviation to determine experimental variability. Two-tailed paired Student's *t*-tests were performed to determine the statistical significance of differences among experimental samples and controls. *P* < 0.01 was considered to indicate a statistically significant difference for comparisons of two treatments.

## Results and Discussion

**Cytotoxicity examined by DNS assay.** A well-established criterion that is used to define cell death is the irreversible

Table I. CC<sub>50</sub><sup>a</sup> values of experimental compounds upon exposure to human leukemia/lymphoma cells for 24 or 48 h.

A, 24 h treatment												
Cell line	2a	2b	2c	2d	2e	2f	2g	3a	3b	3c	3d	3e
Nalm-6	1.80	23.80	79.07	5.20	- <sup>b</sup>	46.60	- <sup>b</sup>	29.90	24.30	126.40	27.00	3.40
CEM	0.90	2.70	45.20	25.10	99.40	52.50	5.80	5.20	3.10	21.50	3.10	2.90
Jurkat	1.40	4.60	12.10	7.30	4.60	34.00	0.70	5.30	65.60	23.10	3.00	1.40
MCF-10A	- <sup>c</sup>	-	-	-	-	-	-	-	-	-	-	14.80
B, 48 h treatment												
Cell line	2a	2b	2c	2d	2e	2f	2g	3a	3b	3c	3d	3e
Nalm-6	0.90	1.50	6.20	0.90	3.60	10.70	0.50	10.60	7.00	10.50	11.70	0.50
CEM	0.60	0.60	6.30	2.00	3.20	5.00	1.30	1.40	1.50	6.30	1.30	0.30
Jurkat	0.50	0.30	5.60	1.50	1.70	8.00	0.80	1.50	2.00	3.40	0.40	0.60
MCF-10A	- <sup>c</sup>	-	-	-	-	-	-	-	-	-	-	3.90

<sup>a</sup>CC<sub>50</sub> values are presented in  $\mu\text{M}$  units. Missing data are marked by -. The CC<sub>50</sub> is defined as the concentration of drug required to disrupt the plasma membrane integrity of 50% of the cell population following 24 h and 48 h of incubation, as compared with DMSO-treated cells. Cells with a compromised plasma membrane were quantified using propidium iodide via differential nuclear staining assay. All compounds were dissolved in DMSO. <sup>b</sup>The CC<sub>50</sub> was not calculated for 2e and 2g in Nalm-6 cells at 24 h, since their toxicity was <30% at the maximum soluble concentration of the compounds tested. <sup>c</sup>In MCF-10A cells, <15% cytotoxicity was detected at the maximum concentration of soluble compound tested (8  $\mu\text{M}$  for 2a and 3e). CC<sub>50</sub>, 50% cytotoxic concentration; DMSO, dimethyl sulfoxide.

disruption of the plasma membrane, which may be easily visualized with PI fluorescent dye that specifically enters dead or dying cells (11). In the present study, PI was employed to quantify the cytotoxicity of all the experimental compounds tested on various cell lines using the live-cell DNS assay (27). As indicated in Table I, a wide disparity in the cytotoxic potency of the compounds was observed in series 2 and 3 at the two time points tested. At 24 h, the 2a and 3e CC<sub>50</sub> values for the three malignant cell lines ranged between 0.9 and 1.8  $\mu\text{M}$ , and between 1.4 and 3.4  $\mu\text{M}$ , respectively. In addition, 48 h subsequent to treatment, only 2a and 3e exhibited consistent sub-micromolar CC<sub>50</sub> values for all the malignant cells tested, with values ranging between 0.5 and 0.9  $\mu\text{M}$ , and between 0.3 and 0.6  $\mu\text{M}$ , respectively. The most potent compound in series 2 was the unsubstituted analogue 2a. Therefore, in series 2, substituents in the aryl rings may decrease the cytotoxic potencies. Analysis of the toxicity of compounds in series 3a-d revealed that replacement of the sulfur atom of 2a with other groups led to an increase in CC<sub>50</sub> values, and resulted in less toxic compounds. Subsequent to incubation of the various cell lines with piperidones for 48 h, the unsubstituted compound in series 2 and the 2 analogues that contained strongly-withdrawing substituents (2b and 2g), exhibited CC<sub>50</sub> values in the sub-micromolar range. In series 3, the most potent piperidone was 3e, with a CC<sub>50</sub> value that ranged between 0.3 and 0.6  $\mu\text{M}$  subsequent to 48 h incubation. A comparison of the various sensitivities of the cell lines to the compounds in series 2 and 3 was performed. The combined CC<sub>50</sub> values of series 2 and 3 compounds subsequent to 24 h incubation on Nalm-6, CEM and Jurkat cells ranged between 1.8-126.4, 0.9-99.4 and 0.7-65.6  $\mu\text{M}$ , respectively. Subsequent

to 48 h incubation, the CC<sub>50</sub> values ranged between 0.9-11.7, 0.6-6.3 and 0.3-5.6  $\mu\text{M}$ , respectively. These assays revealed that the most potent and promising compounds were 2a and 3e. These piperidones were additionally tested on the human non-cancerous MCF-10A cell line. As shown in Table I, 2a possessed decreased cytotoxic activity (<15%) at the highest concentration used (8  $\mu\text{M}$ ), following 24 and 48 h incubation with the non-cancerous cells. Notably, the CC<sub>50</sub> values were not determined for cells with decreased cytotoxicity, as 2a was not soluble at high concentrations. The cytotoxicity of 3e was 10.6 (24 h) and 6.5 (48 h)-fold higher in the T-lymphocyte leukemia cells than in the non-malignant cells (Table I). Varying degrees of 3e selective cytotoxicity were also observed on Nalm-6 and CEM cells (Table I). However, 2a exhibited <15% cytotoxicity towards the non-cancerous cell line at 24 and 48 h at the highest soluble concentration tested (8  $\mu\text{M}$ ). Furthermore, 2a also demonstrated selective toxicity towards leukemia cells (Table I). These findings demonstrate that the piperidone analogues 2a and 3e possessed selective cytotoxicity towards the three leukemia cell lines tested at sub-micromolar concentrations.

**Cell death analysis by PS externalization.** Apoptosis and necrosis are the two major modes of cell death, and PS externalization is a biochemical event that distinguishes between the two (30). PS is an aminophospholipid that is predominantly confined in the inner leaflet of the plasma membrane of resting cells (31). The presence of PS at the surface of cells is indicative of the loss of phospholipid membrane asymmetry, and is also a distinctive event of apoptosis (32). Assays that detect PS externalization are centered on the high-affinity

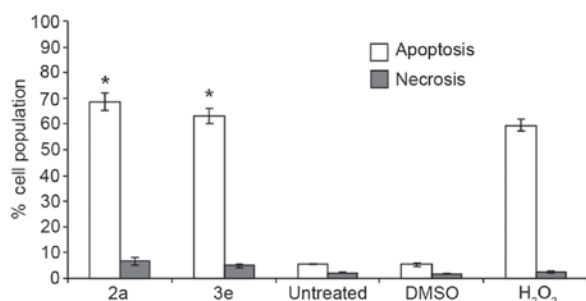


Figure 2. Exposure of Jurkat cells to compounds 2a and 3e results in phosphatidylserine externalization. The cell death mechanism (apoptosis or necrosis) was monitored via flow cytometry upon co-staining the cells with annexin V-FITC and PI. Cells were exposed for 48 h to 2a and 3e at their 50% cytotoxic concentration (1.4  $\mu$ M, as determined at 24 h). The total percentage of apoptotic (annexin V-FITC<sup>+</sup>) Jurkat cells is expressed as the sum of percentages of cells at the early and late stages of apoptosis (open bars). Cells that were stained only with PI due to their compromised plasma membrane integrity, but which did not exhibit FITC signal, are considered to be necrotic cells (grey bars). Each bar represents the mean  $\pm$  standard deviation of 3 independent replicates. The following controls were included: Untreated cells, as a negative control; cells treated with 0.2% v/v dimethyl sulfoxide, as a control for solvent effects; and cells exposed to 1 mM H<sub>2</sub>O<sub>2</sub>, as a positive control. Statistically significant differences between cells treated with compounds and solvent-treated control cells are denoted by asterisks (\* $P$ <0.0001), and were analyzed using the two-tailed Student's *t*-test. FITC, fluorescein isothiocyanate; PI, propidium iodide; DMSO, dimethyl sulfoxide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

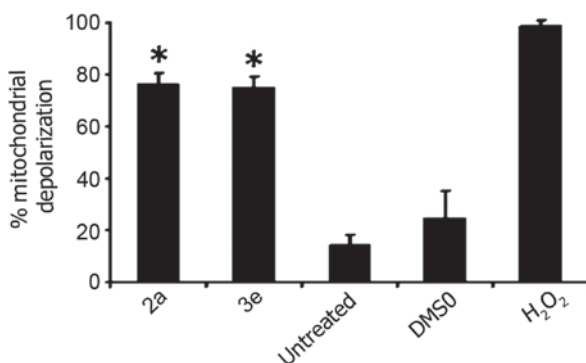


Figure 3. Cytotoxicity mediated by 2a and 3e is dependent of  $\Delta\Psi_m$  disruption in Jurkat cells. Cells were exposed for 6 h to 2a and 3e at their 50% cytotoxic concentration (1.4  $\mu$ M, as determined at 24 h). Variations in the  $\Delta\Psi_m$  were detected by staining the cells with the aggregate-forming lipophilic cationic fluorophore 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (2  $\mu$ M), and monitored via flow cytometry. Percentages of cells emitting green fluorescence signal (indicative of mitochondrial depolarization) are depicted on the y axis. Each bar represents the mean  $\pm$  standard deviation of 4 independent replicates. For the assays, three controls were included: Untreated cells; cells treated with 0.1% v/v dimethyl sulfoxide; and cells exposed to 1 mM H<sub>2</sub>O<sub>2</sub>. Statistically significant differences between cells treated with compounds and solvent-treated control cells are denoted by asterisks (\* $P$ <0.0001), and were analyzed using the two-tailed Student's *t*-test. For each sample, ~10,000 cells were acquired and analyzed using CXP software.  $\Delta\Psi_m$ , mitochondrial membrane potential; DMSO, dimethyl sulfoxide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

binding of annexin V to PS (11). FITC-labeled annexin V, a 36 kDa Ca<sup>2+</sup>-dependent PS-binding protein, may readily identify the event (33). The compounds with the lowest CC<sub>50</sub> across all cell lines were 2a and 3e (Table I). Therefore, the 2a and 3e piperidones were used to elucidate the mechanism of cell death induction on T-lymphocyte leukemia cells. The

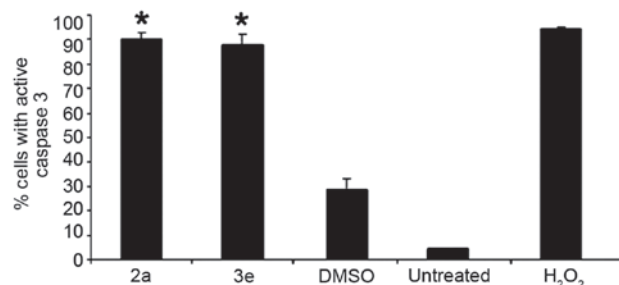


Figure 4. Piperidones 2a and 3e elicited caspase-3 activation in T-lymphocyte leukemia Jurkat cells. Cells were exposed for 6 h to 50% cytotoxic concentration of 2a and 3e (1.4  $\mu$ M, as determined at 24 h). Percentages of cells with active caspase-3, which exhibit a green fluorescence signal, are indicated on the y axis. Cells were subjected to different treatments, as indicated on the x axis. Each bar represents the mean value of 3 independent measurements, and the error bars represent the standard deviation. Statistically significant differences between cells treated with compounds and solvent-treated control cells are denoted by asterisks (\* $P$ <0.0001), and were analyzed using the two-tailed Student's *t*-test. In total, ~5,000 cells/sample were collected and analyzed using CXP software. DMSO, dimethyl sulfoxide; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

detection of PS exposure on the external leaflet of the cellular membrane was examined by flow cytometry using a live-cells mode protocol. Jurkat cells were individually exposed for 48 h to the CC<sub>50</sub> values of 2a and 3e determined at 24 h (1.4  $\mu$ M; Table I). As shown in Fig. 2, PS externalization was clearly detected subsequent to 48 h of exposure to 2a and 3e (29,34). Cells treated with 2a or 3e exhibited significantly increased (>60%) numbers of annexin V-FITC<sup>+</sup> cells, compared with the solvent controls ( $P$ <0.0001; Fig. 2). Notably, 2a and 3e induced increased PS externalization, similarly to the positive control used in the present study (1 mM H<sub>2</sub>O<sub>2</sub>; Fig. 2). These findings suggest that compounds 2a and 3e are likely to induce cell death via apoptosis rather than necrosis.

**$\Delta\Psi_m$  status of treated cells.** Apoptosis may be initiated via the intrinsic or the extrinsic pathway (34-36). The intrinsic pathway is generally characterized by a perturbation of the mitochondrial function, whereas the extrinsic pathway is activated by membrane death receptor signaling (35). In order to gain additional insight into the succession of events induced by 2a and 3e,  $\Delta\Psi_m$  integrity was examined at an early incubation time point (6 h). The mitochondrion is the principal energy-producing organelle in animals, and is considered to be important in cell survival and death (36). The stimulus that elicits the dissipation of the  $\Delta\Psi_m$  is an initial distinctive biochemical feature of the intrinsic apoptosis pathway (37). The loss of  $\Delta\Psi_m$  provokes the release of downstream pro-apoptotic proteins into the cytosol, consequently activating several apoptotic effectors such as caspase-3 (37). Additionally, impaired mitochondrial function may negatively impact cellular energy levels leading to ATP depletion and cell death (36). At present, there are several approved anticancer agents that induce apoptotic cell death by disrupting the  $\Delta\Psi_m$ , including etoposide, doxorubicin and 1- $\beta$ -D-arabinofuranosyl cytosine (38). To determine the initial signal utilized by 2a and 3e to activate cytotoxicity in T-lymphocyte leukemia Jurkat cells, the JC-1 reagent was utilized to detect changes in the  $\Delta\Psi_m$  (39). As shown in Fig. 3, the majority of Jurkat cells

(>75%) exhibited a green fluorescence signal following 6 h of exposure to 2a and 3e, which is indicative of the dissipation of the  $\Delta\Psi_m$ . The strong  $\Delta\Psi_m$  perturbation mediated by 2a and 3e indicates that these compounds induced the intrinsic apoptotic pathway as a mechanism that elicits cell death (Fig. 3).

**Live-cell detection of intracellular caspase-3 activation.** Caspase-3, the major downstream apoptotic executioner enzyme, is involved in the most destructive phases of apoptosis (40,41). Also, caspase-3 activation is a well-known biochemical marker of apoptosis (40). In the present study, the activation of caspase-3 in living cells was analyzed via flow cytometry, using the fluorogenic enzyme substrate NucView™ 488 Caspase-3/7. This substrate contains the tetrapeptide sequence DEVD, which corresponds to the cleavage site by active caspase-3 present in poly(adenosine diphosphate-ribose) polymerase-1. Using flow cytometry, 2a and 3e were observed to induce the conversion of pro-caspase-3 to active caspase-3, following 6 h of treatment in Jurkat cells (Fig. 4). A significant (>85%;  $P<0.0001$ ) induction of caspase-3 activity was detected at  $CC_{50}$  concentrations of 2a and 3e, compared with DMSO-treated and untreated cells (Fig. 4). These results indicate that 2a and 3e induced apoptosis via caspase-3 activation, and are in agreement with the findings of the present study, which indicated that 2a and 3e induced PS externalization and mitochondrial depolarization. Therefore, 2a and 3e appear to activate early and middle biochemical facets of the apoptosis cascade.

**Conclusion.** The present study revealed that two novel piperidones, 2a and 3e, exert potent and selective cytotoxicity towards human leukemia Nalm-6, CEM and Jurkat cells. Additionally, the T-lymphocyte leukemia Jurkat cell line exhibited increased sensitivity to the two compounds subsequent to 24 and 48 h of exposure. Examination of the cell death mechanism that was involved in the observed cytotoxicity revealed that 2a and 3e elicited PS externalization, depolarization of the mitochondrial membrane potential and activation of caspase-3 on Jurkat cells. A key result of the present study is that the novel 2a and 3e piperidones are promising experimental anti-leukemia cytotoxins. The piperidones primarily inflict programmed cell death in human T-lymphocyte leukemic cells via the intrinsic/mitochondrial/caspase-3 apoptotic pathway, which indicates that additional studies regarding these compounds are required. Future studies on the drugs discussed in the present study may include the development of novel piperidone-derived analogues based on the structure of the aforementioned two lead compounds, with the aim of improving potency and selective cytotoxicity against malignant cells in anti-cancer drug design strategies.

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