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Cyclohexyl Analogues of Ethylenediamine Dipropanoic Acid Induce Caspase-Independent Mitochondrial Apoptosis in Human Leukemic Cells

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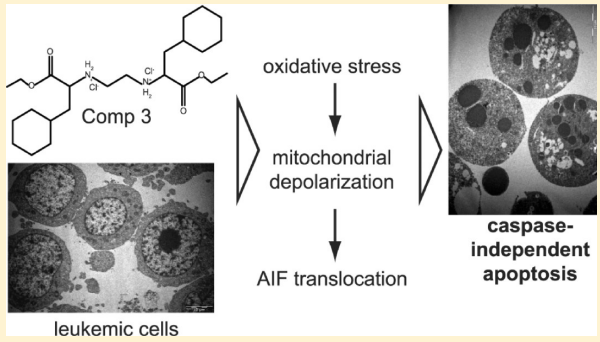
ABSTRACT: We investigated the cytotoxicity of recently synthesized (*S*,*S*)-ethylendiamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid esters toward human leukemic cell lines and healthy blood mononuclear cells. Cell viability was assessed by acid phosphatase assay, apoptosis, and differentiation were analyzed by flow cytometry and electron microscopy, while intracellular localization of apoptosis-inducing factor (AIF) was determined by immunoblotting. It was demonstrated that methyl, ethyl, and *n*-propyl esters were toxic to HL-60, REH, MOLT-4, KG-1, JVM-2, and K-562 leukemic cell lines, while the nonesterified parental compound and *n*-butyl ester were devoid of cytotoxic action. The ethyl ester exhibited the highest cytotoxic activity (IC_{50} 10.7 μM−45.4 μM), which was comparable to that of the prototypical anticancer drug cisplatin. The observed cytotoxic effect in HL-60 cells was associated with an increase in superoxide production and mitochondrial membrane depolarization, leading to apoptotic cell death characterized by phosphatidylserine externalization and DNA fragmentation in the absence of autophagic response. DNA fragmentation preceded caspase activation and followed AIF translocation from mitochondria to nucleus, which was indicative of caspase-independent apoptotic cell death. HL-60 cells treated with subtoxic concentration of the compound displayed morphological signs of granulocytic differentiation (nuclear indentations and presence of cytoplasmic primary granules), as well as an increased expression of differentiation markers CD11b and CD15. The cyclohexyl analogues of ethylenediamine dipropanoic acid were also toxic to peripheral blood mononuclear cells of both healthy controls and leukemic patients, the latter being more sensitive. Our data demonstrate that the toxicity of the investigated cyclohexyl compounds against leukemic cell lines is mediated by caspase-independent apoptosis associated with oxidative stress, mitochondrial dysfunction, and AIF translocation.



1. INTRODUCTION

The mechanisms of cytotoxic activity of metal complexes with organic ligands toward various types of cancer and normal cells have been widely investigated.[1] Most of the metal complexes, including the platinum-based compound cisplatin as a typical representative, kill cells by inducing DNA damage followed by oxidative stress, mitochondrial dysfunction, and caspase-dependent apoptosis.[2−5] Futhermore, some recent data indicate that autophagy, a process of self-digestion of intracellular components in acidified vesicles called autophagolysosomes, could have a protective role in cisplatin-triggered apoptosis.[6] We have recently reported that octahedral platinum(IV) complexes with cyclohexyl-functionalized ethylendiamine-*N*,*N*′-diacetate-type ligands killed various cancer cell lines with higher efficiency than the protypical platinum-based anticancer drug cisplatin.[7] Mechanistically, while cisplatin induced caspase-dependent apoptosis associated with a cytoprotective autophagic response, the new Pt(IV) complexes caused rapid, oxidative stress-mediated, nonapoptotic cell death characterized by massive cytoplasmic vacuolization, cell membrane damage, and the absence of protective autophagy.[7] The high efficiency and unusual mechanism of anticancer

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action of Pt(IV) complexes could be at least partly ascribed to their organic ligands designed to incorporate the ethylendiamine group, which is known for its positive contribution to the cytotoxicity of various compounds.[8] Indeed, the organic ligands alone exerted significant *in vitro* toxicity toward glioma, melanoma, and fibrosarcoma cell lines,[7] but the mechanisms of their cytotoxic activity were not investigated.

In the present study, we investigated the molecular mechanisms underlying the cytotoxic action of recently described (*S*,*S*)-ethylendiamine-*N*,*N*′-di-2-(3-cyclohexyl)-propanoic acid esters,[7] using human leukemic cell lines as a model system. The focus of our researsh was on the ability of these compounds to induce apoptotic cell death, as well as intracellular events involved in its execution, such as oxidative stress, mitochondrial dysfunction, and activation of caspases and apoptosis-inducing factor (AIF) as principal pro-apoptotic molecules. We have also compared the sensitivity of malignant and normal peripheral blood mononuclear cells to the cytotoxicity of the investigated cyclohexyl compounds.

2. MATERIALS AND METHODS

2.1. Chemicals and Elemental Analyses. The (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (Comp 1·2HCl) and its esters (methyl, ethyl, *n*-propyl, and *n*-buthyl esters) dihydrochlorides (Comp 2·2HCl−Comp 5·2HCl) were prepared as described earlier.[7]

The (*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoic acid (Comp 1·2HCl) crystallizes as a white powder and is soluble in dimethylsulfoxide (DMSO) (1.84 g, 34.52%) mp = 200 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.80 (m, C7, 4H), 1.15 (m, C5, C5′, 8H), 1.65 (m, −C***H***$_2$−Cy, 4H; C4, 2H; C6, C6′, 8H), 3.98 (m, −OOC−C***H***−NH$_2$, 2H, −NH2−C***H***$_2$C***H***$_2$−NH$_2$−, 4H), 9.90 ppm (N***H***$_2^+$, 4H, −COOH, 2H). ^{13}C NMR (50 MHz, [D6]DMSO): 25.7 (**C6**), 25.9 (**C4**), 31.9 (**C7**), 33.3 (**C5**), 36.6 (**C3**), 57.5 (**C2**,**C8**), 170.9 ppm (**C1**). IR (ATR): $\tilde{\nu}$ = 2900−2500, 1717, 1446, 1204, 888 cm^{-1}. MS (LC/MS, 4000 V): *m*/*z* (%): 367.02 (68.42%) [M−2HCl+H]$^+$. Anal. Calcd. for $C_{20}H_{38}O_4N_2C_{l2}$·1.5H_2O: C 51.28, H 8.82, N 5.98; found, C 51.34, H 8.42, N 5.93. The esters crystallize also as white powder. These compounds have good solubility in dimethylsulfoxide (DMSO), but not in water, ethanol, acetonitrile, or chloroform.

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dimethyl ester dihydrochloride, Comp 2·2HCl (0.68 g, 63.00%) mp = 204 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.88 (m, C7, 4H), 1.16 (m, C5, 5′, 8H), 1.70 (m, C***H***$_2$−Cy, 4H; C4, 2H; C6, 6′ 8H), 3.41 (m, −NH$_2$−C***H***$_2$C***H***$_2$−NH$_2$−, 4H), 3.78 (m, C***H***$_3$−OOC−, 6H), 4.15 (m, −OOC−C***H***−NH$_2$−, 2H), 10.00 ppm (m, N***H***$_2^+$, 4H). ^{13}C NMR (50 MHz, [D6]DMSO): 25.7 (**C7**), 26.0 (**C6**), 32.0 (**C5**), 33.2 (**C4**), 36.9 (**C3**), 53.3 (**C8**), 57.5 (**C2**, −O**C**H$_3$), 170.2 ppm (**C1**). IR (ATR): $\tilde{\nu}$ = 2900−2500, 1739, 1491, 1224, 802 cm^{-1}. MS (LC/MS, 4000 V): *m*/*z* (%): 397.31 (40.00) [M−2HCl+H]$^+$. Anal. Calcd. for $C_{22}H_{42}O_4N_2Cl_2$·
0.5H_2O: C 55.22, H 9.06, N 5.85; found, C 55.14, H 8.64, N 5.89.

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate diethyl ester dihydrochloride, Comp 3·2HCl (0.56 g, 41.42%) mp = 208 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.89 (m, C7, 4H), 1.26 (m, C5, 5′, 8H; C***H***$_3$CH$_2$OOC−, 6H), 1.72 (m, C***H***$_2$−Cy; C4, 2H; C6, 6′, 4H), 3.46 (m, CH$_3$−C***H***$_2$−OOC−), 4.25 (m, −OOC−C***H***−NH$_2$−, 2H, −NH$_2$−C***H***$_2$C***H***$_2$−NH$_2$−, 4H), 10.09 ppm (m, N***H***$_2^+$, 4H). ^{13}C NMR (50 MHz, [D6]DMSO): 14.1 (**C**H$_3$CH$_2$OOC−), 25.6 (**C7**), 25.6 (**C4**), 31.8 (**C6**), 33.4 (**C5**), 36.9 (**C3**), 57.3 (**C8**), 62.3 (**C2**; CH$_3$**C**H$_2$OOC−), 169.3 ppm (**C1**). IR (ATR): $\tilde{\nu}$ = 2900−2500, 1739, 1450, 1215, 802 cm^{-1}. MS (LC/MS, 4000 V): *m*/*z* (%): 425 (45.00) [M−2HCl+H]$^+$. Anal. Calcd. for $C_{24}H_{46}O_4N_2Cl_2$·0.5H_2O: C 56.90, H 9.35, N 5.53; found, C 56.40, H 8.92, N 5.50.

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dipropyl ester dihydrochloride, Comp 4·2HCl (0.76 g, 53.85%) mp =216 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.93 (m, C***H***$_3$CH$_2$−, 6H; C7, 4H), 1.16 (m, C5, C5′, 8H), 1.43 (m, C6, C6′, 8H), 1.63 (m, C***H***$_2$−Cy; C4, 6H; CH$_3$C***H***$_2$−, 4H), 3.58 (m, −C***H***$_2$−OOC−, 4H), 4.16 (m, 4.0 and 3.2, −OOC−C***H***−NH$_2$−, 2H, −NH$_2$−C***H***$_2$C***H***$_2$−NH$_2$−, 4H), 9.78 ppm (m, N***H***$_2^+$, 4H). ^{13}C NMR (50 MHz, [D6]DMSO): 10.5 (**C**H$_3$CH$_2$CH$_2$-OOC−), 21.6 (CH$_3$**C**H$_2$CH$_2$OOC−), 26.0 (**C6**), 26.0 (**C4**), 32.0 (**C7**), 33.5 (**C5**), 37.0 (**C3**), 57.5 (**C8**), 67.7 (**C2**, CH$_3$CH$_2$**C**H$_2$OOC−), 169.9 ppm (**C1**); IR (ATR): $\tilde{\nu}$ = 2900−2500, 1735, 1451, 1215, 829. ESI/MS/TOF: *m*/*z* = 453.37 [M−2HCl+H]$^+$. Anal. Calcd for $C_{26}H_{50}O_4N_2Cl_2$·0.5H_2O: C, 58.91; H, 9.62; N, 5.24; found, C, 58.91; H, 9.89; N, 5.19.

(*S*,*S*)-ethylenediamine-*N*,*N*′-di-2-(3-cyclohexyl)propanoate dibuthyl ester dihydrochloride, Comp 5·2HCl (0.77 g, 51.13%) mp = 218 °C. ^{1}H NMR (200 MHz, [D6]DMSO): 0.91 (m, C***H***$_3$CH$_2$−, 6H; C7, 4H), 1.16 (m, CH$_3$C***H***$_2$−, 4H), 1.38 (m, C5, 5′, 8H), 1.62 (m, −C***H***$_2$−Cy, 4H; C4, 2H; C6, 6′, 8H, CH$_3$CH$_2$C***H***$_2$CH$_2$−, 4H), 3.50 (m, −C***H***$_2$−OOC−, 4H), 4.20 (m, −OOC−C***H***−NH$_2$−, 2H; −NH$_2$−C***H***$_2$C***H***$_2$−NH$_2$−, 4H), 9.85 ppm (m, N***H***$_2^+$, 4H). ^{13}CNMR (50 MHz, [D6]DMSO): 13.7 (**C**H$_3$CH$_2$**C**H$_2$CH$_2$OOC−), 18.8 (CH$_3$**C**H$_2$CH$_2$-CH$_2$OOC−), 25.6 (**C6**), 25.8 (**C4**), 30.2 (CH$_3$CH$_2$**C**H$_2$CH$_2$OOC−), 32.0 (**C7**), 33.5 (**C5**), 37.0 (**C3**), 57.6 (**C8**), 65.9 (**C2**, CH$_3$CH$_2$CH$_2$-**C**H$_2$OOC−), 169.9 ppm (**C1**). IR (ATR): $\tilde{\nu}$ = 2900−2500, 1738, 1451, 1215, 810 cm^{-1}. MS (LC/MS, 4000 V): *m*/*z* (%): 481.41 (62.40) [M−2HCl+H]$^+$. Anal. Calcd. for $C_{28}H_{54}O_4N_2Cl_2$·0.5H_2O: C 59.77, H 9.85, N 4.98; found, C 59.50, H 9.61, N 4.88.

Elemental analyses were carried out with an Elemental Vario EL III microanalyser (Elementar Analysensysteme GmbH, Hanau-Germany). Infrared spectra were recorded on a Nicolet 6700 FT-IR spectrometer using the ATR technique (Thermo Electron Scientific Instruments Corp., Madison, USA). The NMR spectra were recorded on a Varian Gemini 200 instrument (Varian, Santa Clara, USA). Chemical shifts for ^{1}H and ^{13}C spectra were referenced to residual ^{1}H and ^{13}C presented in deuterated DMSO ([D6]DMSO). Mass spectra were carried out on a MS system consisted of 6210 Time-of-Flight LC/MS (G1969 A, Agilent Technologies, Santa Clara, USA) in DMSO/water solution, in both positive and negative ion modes. Electronic spectra were carried out on a GBC UV−visible Cintra 6 spectrometer (GBS, Melbourne, Australia), in DMSO, 0.2 mmol L^{-1} solution of complexes. Melting points were determined on an Electrothermal melting point apparatus (Keison Products, Chelmsford, Essex CM1 3UP, England).

2.2. Cell Culture and Reagents. Human leukemia cell lines, promyelocytic (HL-60), lymphocytic (REH), myelogenous-erythroleukemia (K562), acute myelogenous (KG-1), acute lymphoblastic (MOLT-4), and B-prolymphocytic (JVM-2), were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). Peripheral blood mononuclear cells (PBMCs) containing high percentage of blasts were obtained from venous blood of 9 patients with leukemia (4 with *de novo* diagnosed chronic myeloid leukemia, 2 in acute myeloid transformation of chronic myeloid leukemia, and in 3 with acute myeloid leukemia; mean age 41 ± 12) from the Outpatient Clinic of the Outpatient & Diagnostic Department, Clinic of Hematology, Clinical Centre of Serbia, Serbia, Belgrade. The diagnosis was established according to the diagnostic criteria for classification of tumors of hematopoietic and lymphoid tissue.[9] Control PBMCs were obtained from 11 healthy volunteers (mean age 32 ± 6.7). The study was approved by the Ethical Committee of the Clinical Centre of Serbia and the Ethical Committee of the Faculty of Medicine, University of Belgrade. All patients and volunteers provided their informed consent before obtaining blood samples. PBMCs were isolated from heparinized blood by density gradient centrifugation using LymphoPrep (Axis Shield, Norway). The cell lines and PBMC were maintained at 37 °C in a humidified atmosphere with 5% CO_2, in a HEPES (20 mM)-buffered RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 1% of antibiotic/antimycotic mixture (all from PAA, Austria). Cells were incubated in 96-well flat-bottom plates (3 × 10^4 or 1.5 × 10^5 cells/well for the cell lines or PBMC, respectively) for the viability assessment or 24-well plates (2.5 × 10^5 or 5 × 10^5 cells/well for the cell lines or PBMC, respectively) for the flow cytometry analysis (plates were from Sarstedt, Nümbrecht, Germany). Cells were treated 2 h after seeding as described in Results and Discussion and Figure legends. Cyclohexyl analogues of ethylenediamine dipropanoic acid (Figure 1) were synthesized as previously described[7] and kept as stock solutions (10 mM) in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO). Cyclosporin A was obtained from Novartis (Basel, Switzerland), all-*trans* retinoic acid was purchased from Sigma and

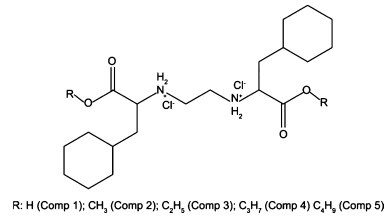

Figure 1. Structure of cyclohexyl analogues of ethylenediamine dipropanoic acid (Comp 1−Comp 5).

pan-caspase inhibitor zVAD-fmk from R&D Systems (Minneapolis, MN). Each experiment contained two controls: untreated cells and cells treated with maximal concentration of dimethyl sulfoxide used in that experiment. No significant difference was observed between the two controls (data not shown), so only untreated controls were presented for clarity.

2.3. Cell Viability. The acid phosphatase assay was used to measure the number of viable cells as previously described.[10] The assay is based on the hydrolysis of the *p*-nitrophenyl phosphate by intracellular acid phosphatases in viable cells and subsequent production of *p*-nitrophenol. In brief, *p*-nitrophenyl phosphate solution (10 mM *p*-nitrophenyl phosphate in 0.1 M sodium acetate buffer, pH 5.5, with 0.3% Triton X-100) was added to each well, and the samples were incubated for 2 h at 37 °C. Reaction was stopped by the addition of 1.3 M NaOH, and the absorbance, which is directly proportional to the cell number, was measured in an automated microplate reader at 405 nm (Sunrise; Tecan, Dorset, UK). The results were presented as % absorbance relative to untreated control cultures. The IC_{50} values were calculated using GraphPad Prism software.

2.4. Flow Cytometric Analysis of Apoptotic Parameters and Superoxide Production. Flow cytometry analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, Heidelberg, Germany), using CellQuest Pro software for acquisition and analysis. The type of cell death (apoptotic or necrotic) was analyzed by double

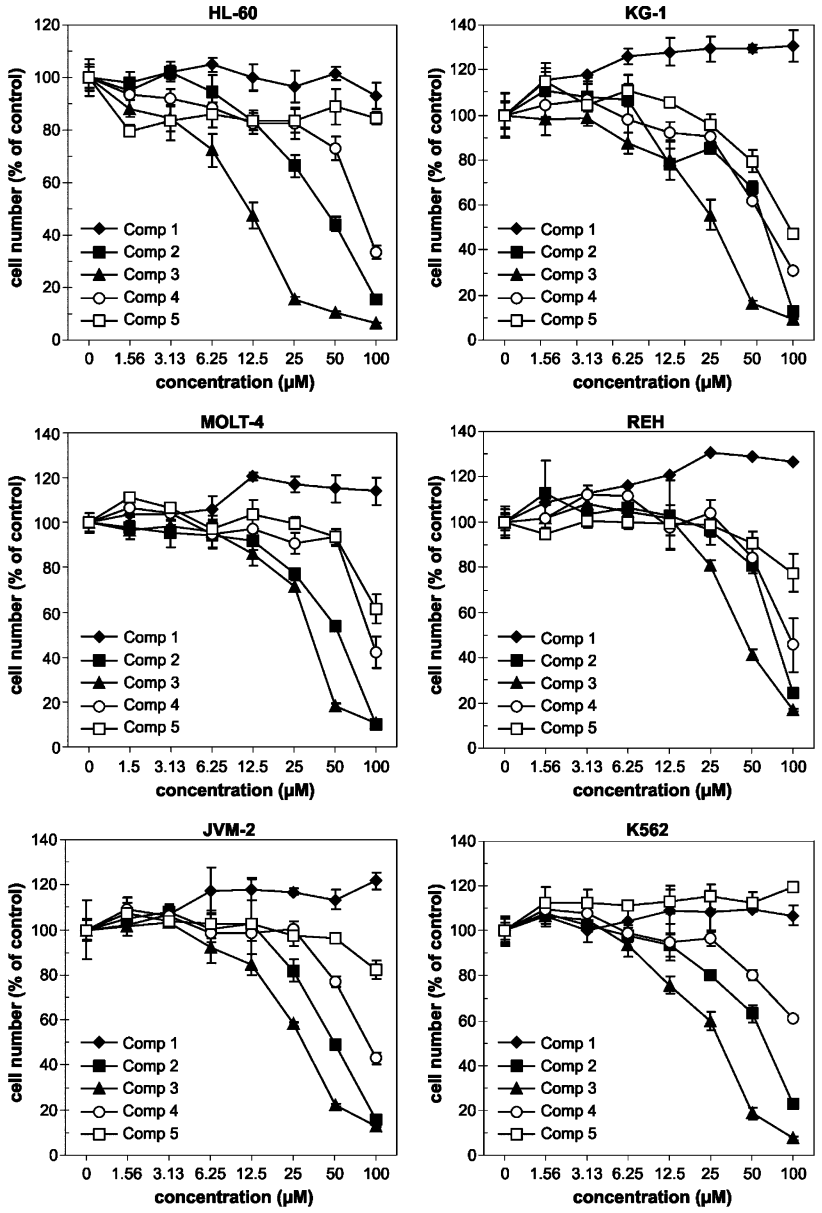

Figure 2. Cytotoxicity of Comp 1−Comp 5 toward human leukemic cell lines. Human leukemic cell lines were incubated with different concentrations of Comp 1−Comp 5 for 24 h, and the number of viable cells was determined by the acid phosphatase assay. The results from a representative of three experiments are presented as mean ± SD values of triplicate observations.

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Table 1. *In Vitro* Antileukemic Activity of Propanoic Acid Cyclohexyl Analogues[a]

	IC_{50} (μM)					
	cell line					
compd	HL-60	MOLT-4	JVM2	KG-1	REH	K562
Comp 1	>100	>100	>100	>100	>100	>100
Comp 2	19.8 ± 1.3	47.4 ± 1.6	48.4 ± 2.6	58.0 ± 3.0	73.6 ± 2.0	58.8 ± 1.6
Comp 3	10.7 ± 1.4*	32.1 ± 1.6*	28.9 ± 0.5*	25.4 ± 3.3*	45.4 ± 1.0*	26.8 ± 2.0*
Comp 4	80.8 ± 9.1	93.1 ± 5.6	88.1 ± 3.2	70.3 ± 7.9	93.7 ± 13.3	>100
Comp 5	>100	>100	>100	>100	>100	>100
cisplatin	17.5 ± 2.4	26.6 ± 4.5	n.a.	37.7 ± 6.2	17.1 ± 1.6	24.0 ± 3.7

[a]The data are the mean ± SD values (IC_{50}, 24 h) from three independent experiments (*$p < 0.05$ denotes lower IC_{50} value compared to Comp 1, Comp 2, Comp 4, and Comp 5; n.a., not assessed).

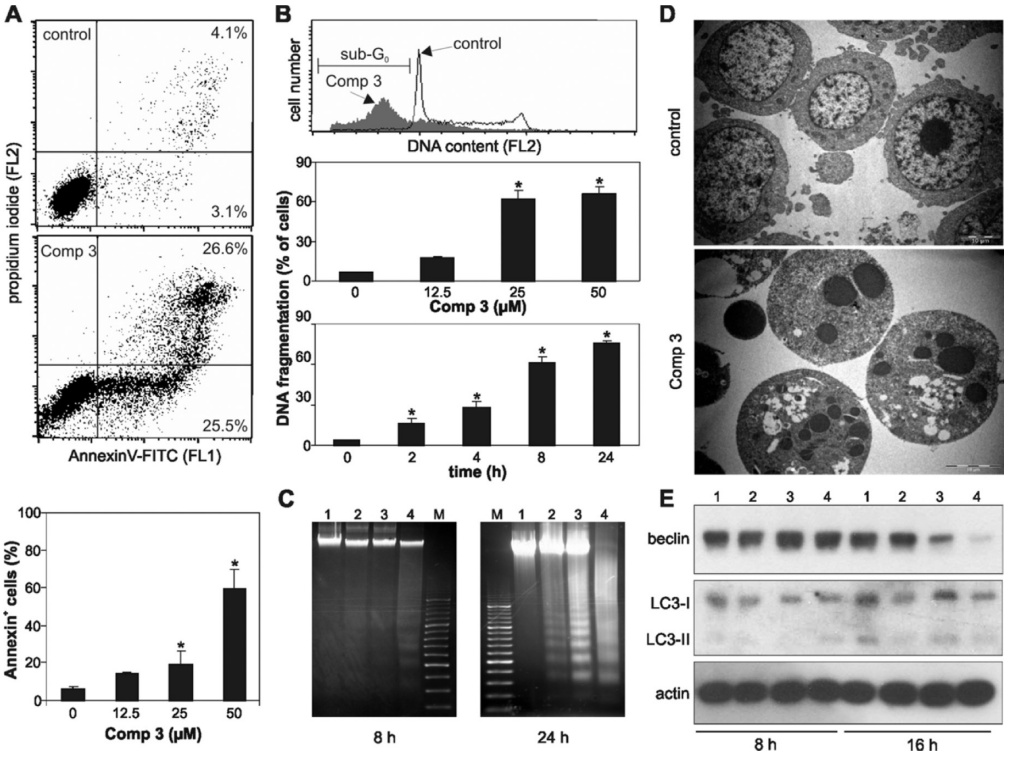

Figure 3. Comp 3 induces apoptotic death of HL-60 cells. HL-60 cells were incubated with 50 μM or different concentrations of Comp 3 for 8 h (B, upper panel, D, and E), 24 h (A), or as indicated (B,C,E). Phosphatidylserine externalization and membrane damage were assessed by flow cytometry (A). DNA fragmentation was determined by flow cytometry (B) or DNA ladder (C; line 1, control; line 2 - 12.5 μM Comp 3, line 3 - 25 μM Comp 3, line 4 - 50 μM Comp 3, M - marker). Ultrastructural morphology was analyzed by electron microscopy (D; scale bar 10 μm) and the levels of beclin-1 and LC3-II were determined by immunoblotting (E; lines 1−4 as in C). Representative dot plots (A), histograms (B), gel electrophoresis (C), electron micrographs (D) and blots (E) are presented. The data in (A) and (B) are mean ± SD values from three independent experiments (*$p < 0.05$).

staining with annexinV-fluorescein isothiocyanate (FITC), which binds to early apoptotic cells with exposed phosphatidylserine, and propidium iodide (PI), which labels the late apoptotic/necrotic cells with membrane damage (staining kit from BD Pharmingen, San Diego, CA). DNA fragmentation was assessed by flow cytometric analysis of ethanol-fixed cells stained with DNA-binding dye PI as previously described.[11] The hypodiploid cells (sub-G compartment) were considered apoptotic. Activation of caspases was assessed by measuring the increase in green fluorescence (FL1) of the cells stained with FITC-conjugated pan-caspase inhibitor (ApoStat; R&D Systems) according to the manufacturer's instructions. Mitochondrial membrane potential was assessed using DePsipher (R&D Systems), a lipophilic cation which has the property of aggregating upon membrane polarization forming an orange-red fluorescent compound. If the potential is disturbed, the dye cannot access the transmembrane space and remains in or reverts to its green monomeric form. The cells were stained with DePsipher as described by the manufacturer, and the green monomer and the red aggregates were detected by flow cytometry. The results are presented as a green/red (FL1/FL2) fluorescence ratio, which corresponds to the extent of mitochondrial depolarization. The mitochondrial production of superoxide was analyzed using flow cytometry by measuring the intensity of red fluorescence (FL2) emitted by a superoxide-specific fluorochrome dihydroethidium, DHE (Sigma-Aldrich), which was incubated with cells (20 μM) for 30 min at the end of the treatment.

2.5. DNA ladder. Internucleosomal fragmentation of nuclear DNA was determined by the DNA ladder. DNA was isolated using DNAzol (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions, and DNA content was quantified spectrophotometrically. Agarose gel (1.8%) was used for the separation of 5 μg of isolated DNA from each treatment. A 100 bp ladder standard (Fermentas, Vilnius, Lithuania) was used as the marker. Gels were photographed using a Gel Logic 200 imaging system (Eastman Kodak, Rochester, NY).

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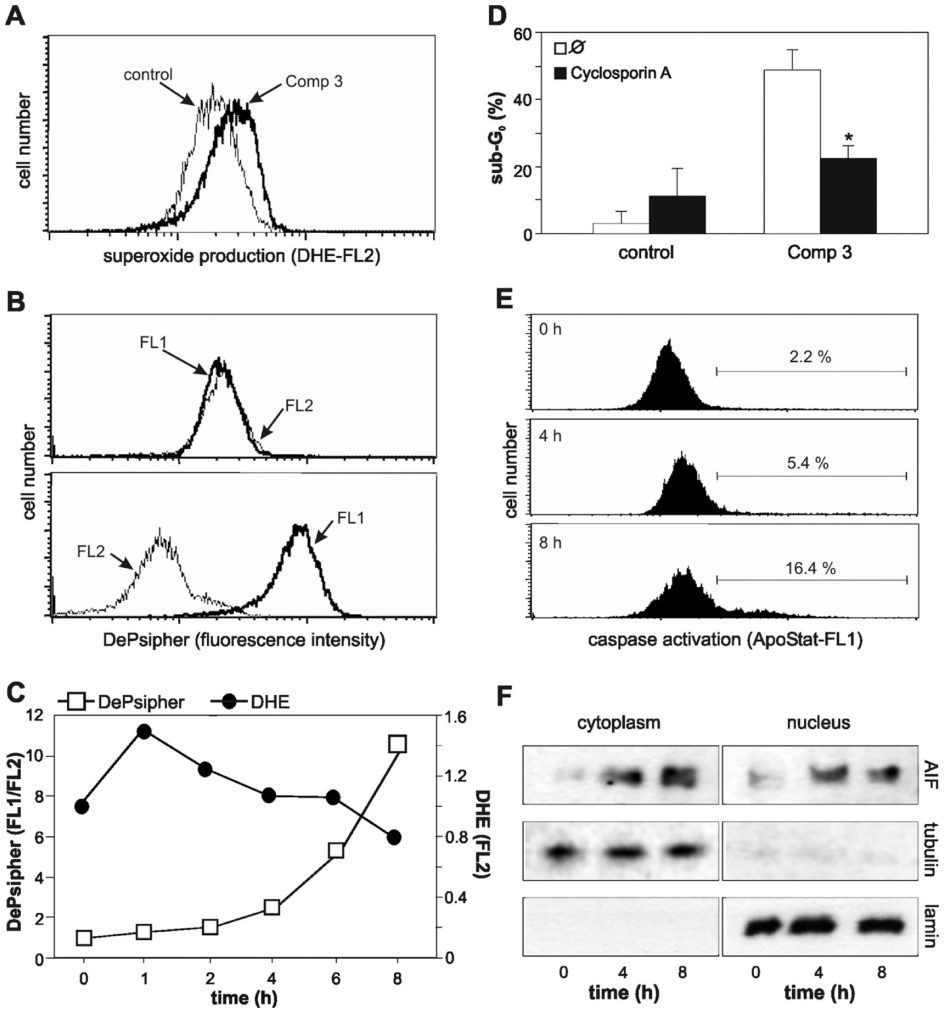

Figure 4. Role of mitochondria and AIF in Comp 3-induced apoptosis. HL-60 cells were incubated with Comp 3 (50 μM) for 1 h (A), 8 h (B,D), or as indicated (C,E,F). Superoxide production (A), mitochondrial membrane potential (B,C), DNA fragmentation (D), and caspase activation (E) were analyzed by flow cytometry, while nuclear and cytoplasmic levels of AIF were determined by immunoblotting (F). Representative histograms (A,B,E) and blots (F) are presented. The data in C are from one of two experiments with similar results. The data in D are the mean ± SD values from two independent experiments (*$p < 0.05$).

2.6. Electron Microscopy. For the assessment of morphological features by electron microscopy, cells were fixed in 3% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and then embedded in Epon (Agar Scientific, Stansted, UK). Thin sections were mounted on copper grids, stained with uranyl acetate/lead citrate, and subsequently examined using a Morgani 268D electron microscope (FEI, Hillsboro, OR).

2.7. Immunoblot Analysis. Analysis of apoptosis-inducing factor (AIF) and autophagy markers (beclin-1 and LC3-II) was performed by immunoblotting. For AIF localization assessment, nuclear and cytoplasmic cellular fractions were extracted using ProteoJet Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas) according to the manufacturer's instructions. For evaluation of beclin-1 and microtubule-associated protein 1 light-chain 3 (LC3)-II levels, cells were lysed in lysis buffer (30 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonylfluoride, and protease inhibitor cocktail) on ice for 30 min, centrifuged at 14000*g* for 15 min at 4 °C, and the supernatants were collected. Equal amounts of protein from each sample were separated by SDS−PAGE and transferred to nitrocellulose membranes (Bio-Rad, Marnes-la-Coquette, France). After incubation with primary rabbit antibodies against human tubulin (1:200; Invitrogen, Carlsbad, CA), AIF (1:900), lamin A/C (1:2000), beclin-1 (1:3000), LC3 (1:900), and β-actin (1:5000), followed by incubation with HRP-linked antirabbit IgG (1:1000; all from Cell Signaling Technology, Beverly, MA), protein bands were visualized using enhanced chemiluminiscence reagent (GE Healthcare, Little Chalfont, UK).

2.8. Differentiation of HL-60 Cells. Differentiation of the leukemic HL-60 cell line was analyzed by examining morphological features, as well as the expression of granulocyte differentiation markers CD11b and CD15. Nuclear morphology and the presence of primary granules were examined on 1 μM thick sections and thin sections embedded in Epon (Agar Scientific). Thick sections were stained with toluidine blue and examined under a 100× oil immersion objective on an Olympus BX41TF light microscope. Thin sections were mounted on copper grids, stained with uranyl acetate/lead citrate and examined using a Morgagni 268D electron microscope (FEI). The expression of CD11b and CD15 was analyzed by flow cytometry. Briefly, HL-60 cells were stained with FITC-conjugated antihuman CD15 or PE-conjugated CD11b following the manufacturer's instructions, using antihuman IgG_1/IgG_2 (FITC/PE) as an isotype control (all from BD Pharmingen). Changes in the expression of CD15 and CD11b were expressed as a fold increase of mean fluorescence intensity.

2.9. Statistical Analysis. Statistical analyses were performed using Student's *t* test or Wilcoxon signed ranks test where appropriate. A *p* value of less than 0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Cyclohexyl Analogues of Ethylenediamine Dipropanoic Acid Are Toxic to Leukemic Cell Lines. The basic

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structure of cyclohexyl analogues of ethylenediamine dipropanoic acid used in the present study is presented in Figure 1, showing that all compounds have two cyclohexyl rings and variable length side chains (Comp 1 = H; Comp 2 = CH_3; Comp 3 = C_2H_5; Comp 4 = C_3H_7; Comp 5 = C_4H_9). According to a cell number analysis using acid phosphatase assay (Figure 2), the order of cytotoxic efficiency of the investigated compounds toward leukemic cell lines was as follows: Comp 3 > Comp 2 > Comp 4 > Comp 5 = Comp 1 (Table 1). The cytotoxicity of Comp 3 was comparable to that of the prototypical anticancer drug cisplatin (Table 1). Regarding the structure–activity relationship of the investigated compounds, the absence of the effect of Comp 1 indicates that esterification was required for the cytotoxicity. Although no direct correlation was observed between the cytotoxic potency and the alkyl side-chain length of the compounds, the increase in alkyl side-chain length was apparently associated with the loss of activity of *n*-butyl ester (Comp 5). Also, it should be noted that Comp 3, which was the most toxic, had higher solubility in DMSO (~10 mg/mL at 25 °C) in comparison with other compounds (~5–6 mg/mL at 35–45 °C). It is therefore possible that the high cytotoxicity of Comp 3 was partly due to the formation of smaller particles with higher surface-to-volume ratio in the cell culture medium. This possibility, as well as the extent of intracellular internalization of the investigated compounds, is currently under investigation in our laboratory. Comp 3 as the most potent compound and the HL-60 cell line as the most sensitive to its cytotoxic action were selected for further characterization of the antileukemic action of cyclohexyl analogues of ethylenediamine dipropanoic acid.

3.2. Comp 3 Induces Apoptotic Death of HL-60 Cells.

We next investigated the type of death (apoptotic or necrotic) and the presence of autophagy in Comp 3-treated leukemic cells. While necrosis is typified by vacuolization of the cytoplasm and plasma membrane breakdown, apoptosis is characterized by phosphatidylserine exposure on the outer side of cell membrane, chromatin condensation, and internucleosomal DNA fragmentation in the absence of plasma membrane damage.[12] In our experiments, flow cytometric analysis demonstrated that 24 h treatment caused a dose-dependent increase in phosphatidylserine externalization (Figure 3A), as well as dose- and time-dependent increase in DNA fragmentation (Figure 3B) in HL-60 cells exposed to Comp 3. Furthermore, apoptosis-specific internucleosomal DNA cleavage was confirmed by DNA ladder analysis, which demonstrated a time- and dose-dependent appearance of the characteristic oligonucleosome sized fragments in HL-60 cells after incubation with Comp 3 (Figure 3C). Ultrastructural morphological analysis using electron microscopy confirmed that untreated cells had intact cellular, nuclear, and mitochondrial membranes with euchromatin in the nucleus and a large nucleolus typical for HL-60 cells (Figure 3D). However, after 8 h of treatment with Comp 3 (50 μM), typical apoptotic changes were clearly visible: cells became smaller and more spherical, nuclear content was fragmented, but the cell membrane was still intact. The lack of cell membrane damage was also confirmed by the lactate dehydrogenase release assay (data not shown). We have also investigated the ability of Comp 3 to induce autophagy, which can serve either as a salvage mechanism against apoptosis/necrosis or as an alternative cell death pathway.[13] Immunoblot analysis showed that the expression of pro-autophagic protein beclin-1 and conversion of LC3-I to autophagosome-associated LC3-II isoform was not increased in HL-60 cells exposed to different concentrations of Comp 3 for 8 and 16 h (Figure 3E). Moreover, the levels of beclin-1 and LC3-II were reduced in HL-60 cells after 16 h of treatment with Comp 3, indicating that basal autophagy was inhibited. We have recently reported that Pt(IV) complexes containing the ligands investigated here cause oxidative stress-mediated necrotic death of glioma, melanoma, and fibrosarcoma cells.[7] It therefore appears that cyclohexyl analogues of ethylenediamine dipropanoic acid and the corresponding metal complexes cause different types of cancer cell death (apoptosis and necrosis, respectively). However, the possibility that the observed difference could be due to some specific properties of HL-60 or leukemic cells in general remains to be investigated. Also, a possible role of

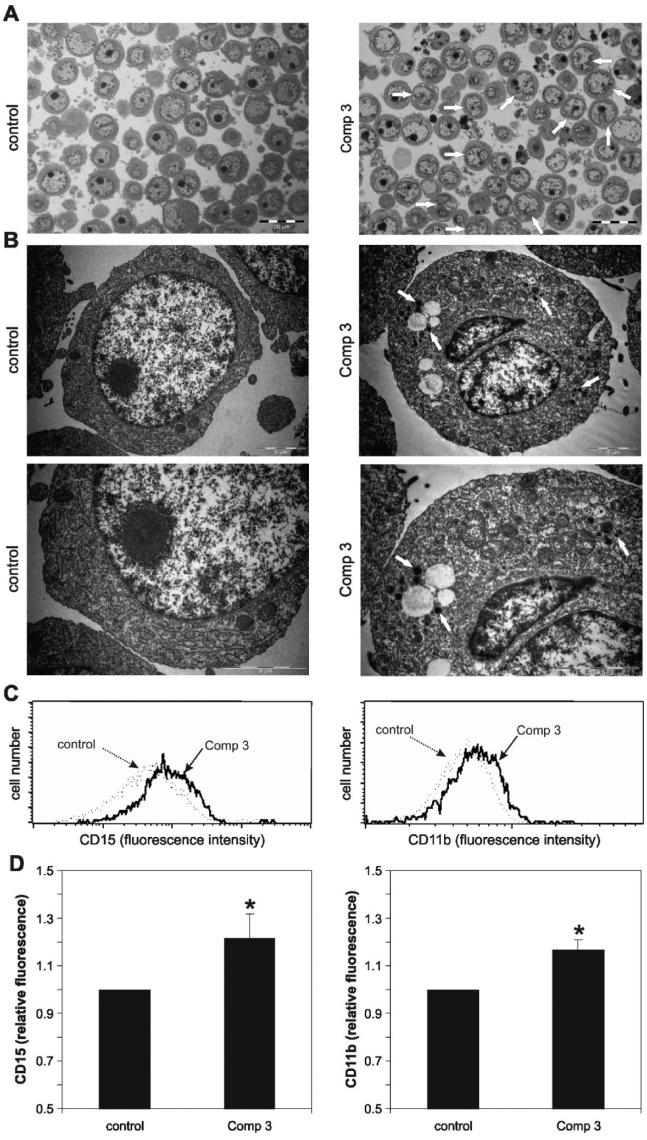

Figure 5. Comp 3 induces granulocytic differentiation of HL-60 cells. HL-60 were incubated with Comp 3 (12.5 μM) for 48 h. Granulocytic differentiation was analyzed by light microscopy of toluidine blue-stained thick sections (A; arrows point to nuclei with indentations; scale bar 20 μm), electron microscopy (B; arrows point to primary granules; scale bar 5 μm), or flow cytometry measurement of CD11b and CD15 expression (C,D). Representative light or electron micrographs (A,B) and histograms are presented (C). The data in D are the mean ± SD values of triplicate observations from a representative of three experiments (*$p < 0.05$).

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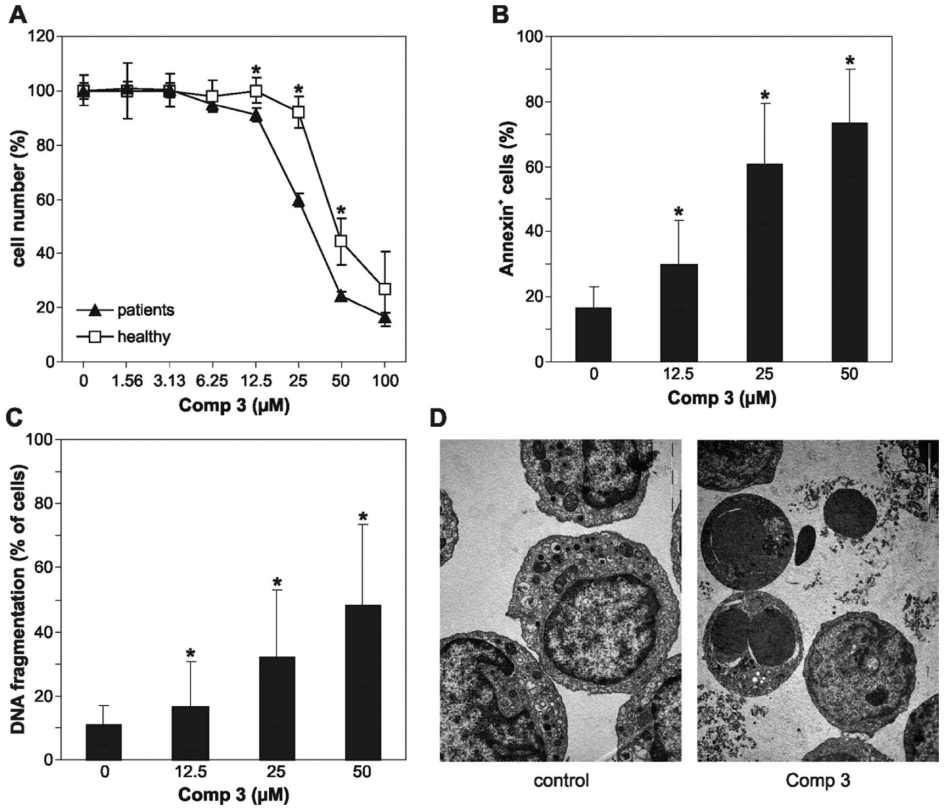

Figure 6. Comp 3 induces apoptotic death of primary leukemic cells. PBMC obtained from leukemic patients and healthy volunteers were incubated with different concentrations (A–C) or 25 μM of Comp 3 (D) for 24 h. Cell numbers were assessed by acid phosphatase assay (A), while phosphatidylserine externalization (B) and DNA fragmentation (C) were analyzed by flow cytometry (the data are the mean ± SD values obtained from 9 patients and 11 healthy controls; *$p < 0.05$). (D) A representative electron micrograph showing nuclear morphology (scale bar 10 μm).

autophagy inhibition in antileukemic action of the investigated compounds seems worthy of further exploration.

3.3. Comp 3-Induced Apoptosis Is Associated with Oxidative Stress, Mitochondrial Depolarization, and Nuclear Translocation of AIF. In order to get further insight into the mechanisms of Comp 3 antileukemic action, we measured the production of superoxide anion (O_2^{-}) and mitochondrial membrane potential in Comp 3-treated HL-60 cells. The excessive mitochondrial generation of superoxide causes mitochondrial permeability transition pore (MPTP) opening, depolarization of the inner mitochondrial membrane, and subsequent release of small molecules such as cytochrome *c* or AIF, leading to activation of caspase-dependent or caspase-independent apoptosis.[14−16] In our experiments, superoxide production in HL-60 cells was observed after 1 h of treatment with Comp 3 (50 μM), as demonstrated by an increase in intracellular DHE fluorescence (Figure 4A). This was associated with a strong shift of DePsipher fluorescence from red (FL2) to green (FL1) after 8 h of treatment, indicating the ability of Comp 3 to cause depolarization of the inner mitochondrial membrane (Figure 4B). The time-kinetics study revealed that the peak production of superoxide occurred at 1 h, thus preceding mitochondrial depolarization which began after 2 h and continuously increased up to 8 h of treatment with Comp 3 (Figure 4C). Cyclosporin A (10 μM), a potent inhibitor of MPTP opening,[17] significantly reduced Comp 3-mediated DNA fragmentation in HL-60 cells (Figure 4D), indicating the involvement of mitochondrial depolarization in Comp 3-mediated apoptosis. However, only a marginal activation of apoptosis-executing enzymes of the caspase family[18] was observed after 4 h of treatment with Comp 3, and a discernible increase in caspase activation (from 2 to 16% after 8 h) clearly lagged behind DNA fragmentation (Figure 4E). Moreover, the pan-caspase inhibitor zVAD-fmk (20 μM) completely failed to prevent Comp 3 toxicity toward HL-60 cells (cell viability 33.5 ± 7.2% vs 35.1 ± 6.9% in Comp 3- and Comp 3 + zVADfmk-treated cells; $p > 0.05$), confirming that it did not require caspase activation. However, an increase in the amount of the mitochondrial intermembrane space-localized flavoprotein AIF[19] was readily detected by immunoblotting in cytoplasmic and nuclear fractions of HL-60 cells treated with Comp 3 for 4 h (Figure 4F), correlating with the initiation of DNA fragmentation (Figure 3B). AIF causes DNA fragmentation and phosphatidylserine exposure which are prevented by MPTP blocker cyclosporine A but not by pan-caspase inhibitor zVAD-fmk, thus providing a direct, caspase-independent molecular link between mitochondria and nuclei.[14,19] Therefore, our data suggest that nuclear translocation of AIF, rather than caspase activation, was involved in Comp 3-mediated apoptosis of HL-60 cells.

3.4. Comp 3 Induces Differentiation of HL-60 Cells. In addition to the induction of apoptosis, promoting differentiation of leukemic cells is a valid therapeutic strategy, successfully implemented in acute promyelocytic leukemia treatment with all-*trans* retinoic acid.[20] Because of their sensitivity to all-*trans* retinoic acid-induced differentiation,[21] the promyelocytic HL-60 cell line has been widely used as a model for studying granulocyte differentiation. Therefore, we next examined if Comp 3 at a lower concentration (12.5 μM) might cause granulocytic differentiation of HL-60 cells after 48 h of treatment. Morphological examination of toluidine blue-stained thick sections by light microscopy (Figure 5A) and thin sections by electron microscopy (Figure 5B) revealed changes

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consistent with differentiation toward the granulocytic phenotype.[22] Namely, cells treated with Comp 3 were observed to have indented nuclei (Figure 5A), as well as primary granules in their cytoplasm (Figure 5B). Additionally, flow cytometry demonstrated that the treatment with Comp 3 caused a significant increase in the expression of granulocyte markers CD15 and CD11b (Figure 5C), thus further confirming the ability of Comp 3 to cause granulocytic differentiation of HL-60 cells. Although the differentiation-inducing potential of Comp 3 was somewhat lower than that of all-*trans* retinoic acid (data not shown), our data indicate that it might represent additional mechanisms for Comp 3 antileukemic action.

3.5. Comp 3 Induces Apoptosis of Primary Leukemic Cells. Finally, we investigated the cytotoxicity of the cyclohexyl analogue Comp 3 toward PBMC of leukemic patients. The acid phosphatase assay revealed that Comp 3 was significantly more toxic to PBMC of leukemic patients compared to those of healthy individuals (IC_{50} values 31.5 ± 8.3 μM and 54.7 ± 23.6 μM, respectively) (Figure 6A). The proportion of cells displaying phosphatydilserine exposure and DNA fragmentation increased in a dose-dependent manner upon treatment with Comp 3 (Figure 6 B,C). Electron microscopy analysis demonstrated that patients' PBMC treated with Comp 3 (25 μM; 24 h) became smaller, more spherical, with intact cell membrane and fragmented nuclei in comparison with those of untreated cells (Figure 6D), confirming the ability of Comp 3 to trigger apoptotic death of primary leukemic cells.

4. CONCLUSIONS

The present study demonstrates the ability of esterified cyclohexyl analogues of ethylenediamine dipropanoic acid to cause caspase-independent apoptosis of leukemic cells by inducing superoxide production, mitochondrial membrane depolarization, nuclear translocation of AIF, and consecutive DNA fragmentation. The observed proapoptotic action was fairly selective toward leukemic cells, in comparison with normal blood mononuclear cells, and was associated with granulocytic differentiation of leukemic cells. By providing mechanistic insight into the cytotoxic effects of these novel cyclohexyl compounds, these data could contribute to further research of their biological effects alone or as a part of different organo-metallic compounds.

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Notes

Ethical Standards: We declare that all experiments were performed in compliance with all laws of the Republic of Serbia. The authors declare no competing financial interest.

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ABBREVIATIONS

AIF, apoptosis-inducing factor; FITC, fluorescein isothiocyanate; PI, propidium iodide; PBMC, peripheral blood mononuclear cells; DHE, dihydroethidium; MPTP, mitochondrial permeability transition pore; PE, phycoerythrin; Comp, compound

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