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PROAPOPTOTIC ACTIVITY OF HETEROCYCLIC COMPOUNDS CONTAINING SUCCINIMIDE MOIETY IN THE PROMYELOCYTIC LEUKEMIA CELL LINE HL-60

BOŻENA KURAN[1]*, MARIOLA KRAWIECKA[1], JERZY KOSSAKOWSKI[1], MIROSŁAWA KORONKIEWICZ[2] and ZDZISŁAW CHILMONCZYK[2]

[1]Department of Medical Chemistry, Medical University of Warsaw, 3 Oczki St., 02-007 Warszawa, Poland
[2]National Medicines Institute, 30/34 Chełmska St., 00-750 Warszawa, Poland

Abstract : In the present paper, we describe proapoptotic activity of several heterocyclic compounds **9**, **12**, **18**, **19** and **20** possessing succinimide (as well as succinimide related) moieties. The compounds properties were examined with the aid of flow cytometry on the promyelocytic leukemia cell line HL-60. The highest proapoptotic activity exhibited compound **12** (4-{4-[4-(2-methoxyphenyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyklo[5.2.1.$0^{2,6}$]-dec-8-ene-3,5,10-trione). The synthesis of compounds **1**-**17** is also described. The structures of obtained compounds were characterized by ^{1}H NMR, ^{13}C NMR, ESI MS and/or elemental analyses.

Keywords: succinimide derivatives, flow cytometry, apoptosis

In spite of extensive research within last several years, cancer is still the cause of substantial mortality and takes the second place, after the cardiovascular illnesses, as the cause of mortality in modern societies. It also is the second leading cause of death in women of reproductive age (1). B-cell malignancies, including B-cell non-Hodgkin's lymphoma (NHL) and chronic lymphocytic leukemia (CLL), are the most common hematologic malignancies in the western world. Although excellent response rates are achieved with standard chemoimmunotherapy, patients often relapse and additional treatment is necessary. Therefore, discovery of new chemotherapeutics is still required to combat those cancers associated with dismal prognoses.

In the present paper, we examined for proapoptotic activity several succinimide and succinimide related heterocyclic compounds containing azapentacyclononadecaheksaen-16,18-dione and azatricyclodec-8-ene-3,5,10-trione moieties. Heterocyclic compounds of similar structure are the important part of medicinal chemistry and exhibit diversed pharmacological activity. They may possess antineoplastic and antiviral (2), antimalarial (3, 4), antimycobacterium tuberculosis (5), antitumor (6) and antimicrobial (7, 8) activities (Fig. 1).

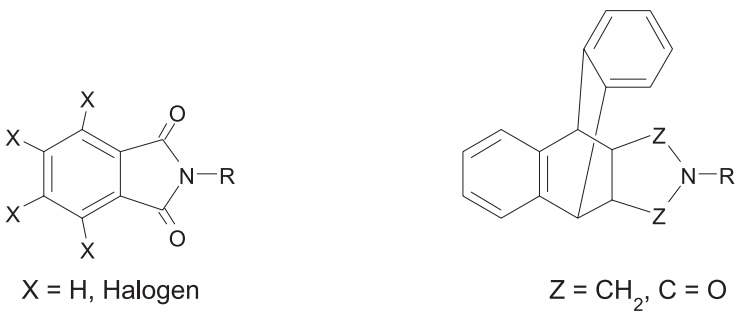


Figure 1. Structures of biologically active heterocycles containing succinimide moiety

* Corresponding author: e-mail: bozena.kuran@interia.pl

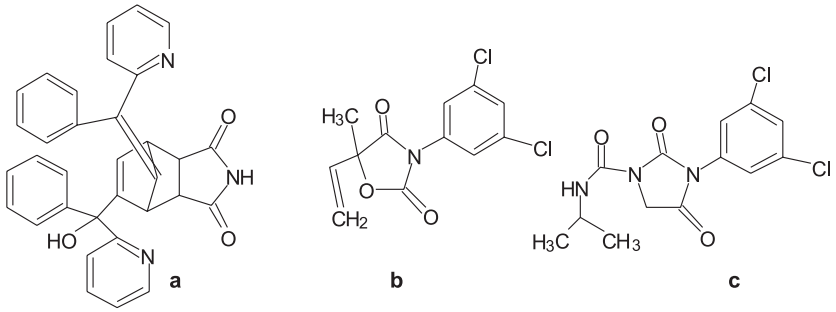


Figure 2. Structure of norbormide (**a**), vinclozolin (**b**), iprodione (**c**)

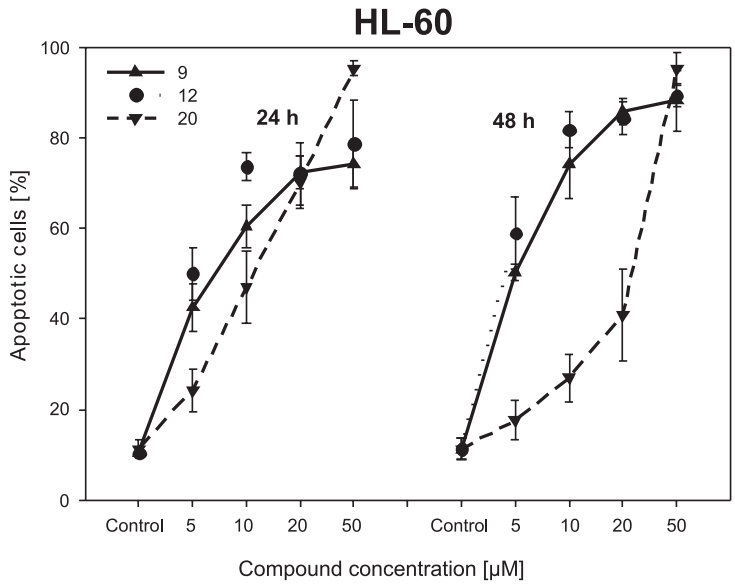
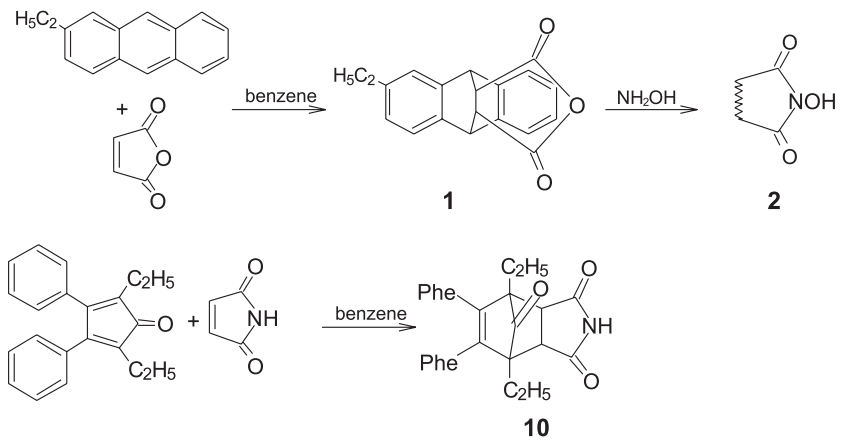


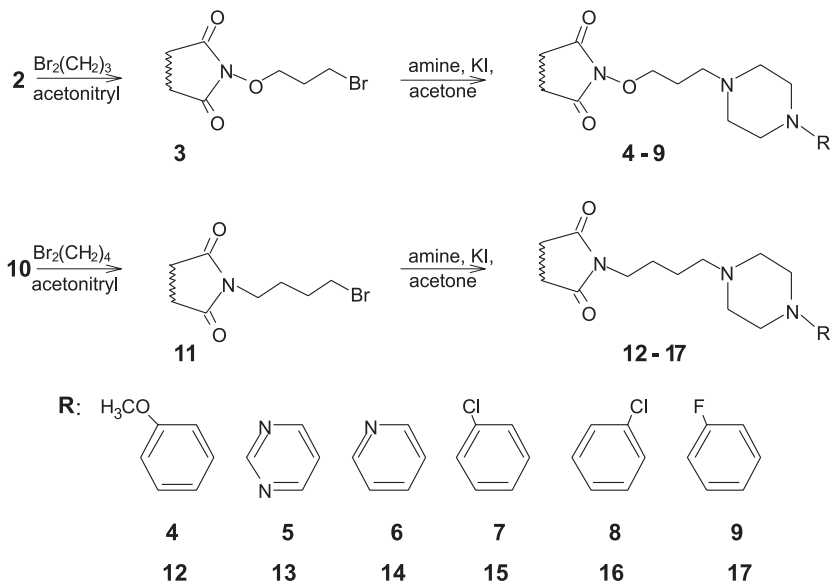
Figure 3. Induction of apoptosis (total apoptosis: early and late) by **9**, **12** and **20** in HL-60 cells. The data were determined by FACS cytometer after 24 and 48 h treatment. Cells were stained with annexin V-FITC and PI. Each point represents the mean ± S.D. (n = 3)



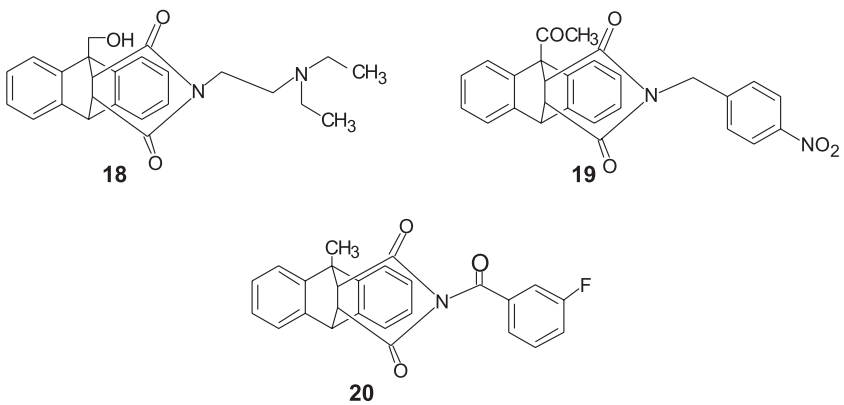
Scheme 1. Synthesis of imides **2** and **10**

Moreover, some of them are very important vasoactive compounds. Norbormide (Fig. 2), for instance, is uniquely toxic to rats but relatively harmless to other rodents and mammals (9-11). It is also known that some succinimide derivatives (12-16) as well as their 3-spirocycloalkyl analogues (17-19) can be endowed with anticonvulsant activity. Dicarboximide fungicides vinclozolin and iprodione also belong to succinimide related compounds (20, 21) (Fig. 2).

In order to prepare compounds **9** and **12** (as well as compounds **4**-**8** and **13**-**17**) imides **2** and **10** were obtained with the aid of the Diels-Alder reaction (Scheme 1). Thus 2-ethylanthracene was condensed with furan-2,5-dione in benzene to give anhydride **1**. This anhydride was subsequently condensed with hydroxylamine to give N-hydroxyimide **2**. The imide **10** was prepared using 2,5-diethyl-3,4-diphenylcyclopentadienone and 1*H*-pyrrole-2,5-dione. The standard alkylation procedure of inter-



Scheme 2. Method of preparation of derivatives **4**-**9** and **12**-**17**



Scheme 3. Structure of compounds **18**-**20**

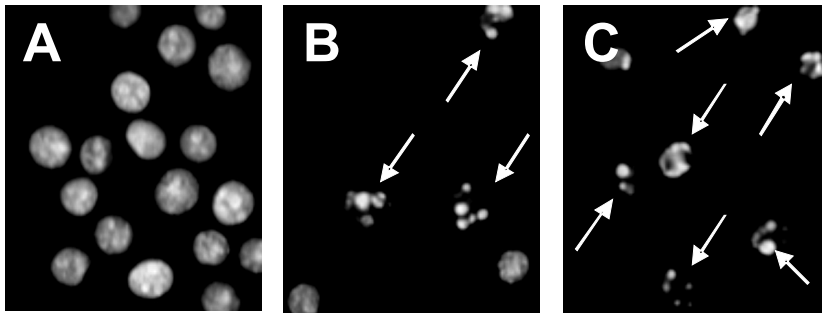


Figure 4. Morphological examination (fluorescence microscopy) of HL-60 cells stained with DAPI/sulforhodamine 101 after 48 h incubation with compound 12. **A** - control; **B** - compound **12** (20 μM); **C** - compound **12** (50 μM). Arrows indicate apoptotic bodies

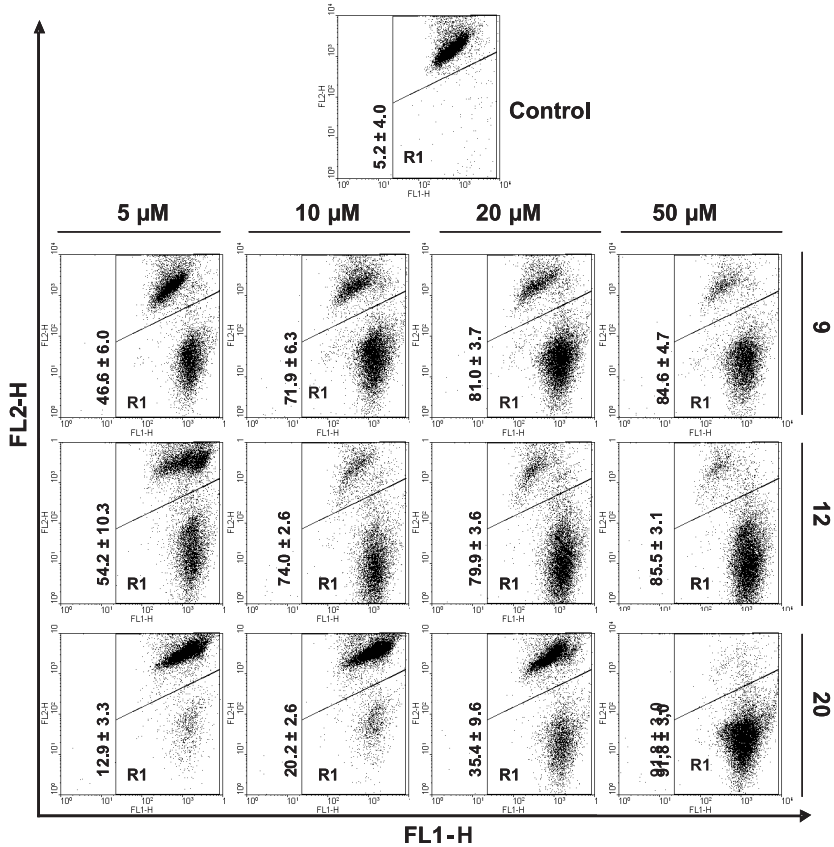


Figure 5. Representative flow cytograms demonstrating changes in mitochondrial membrane potential ($\Delta\Psi_m$) of HL-60 cells induced by 48 h culturing with compounds**.** The cells were stained with JC-1 dye. The cells in the lower right region (R1) showed green fluorescence (percentage of apoptotic cells)

mediates **2** and **10** with 1,4-dibromobutane or 1,3-dibromopropane led to 4-bromobutyl (**11**) and 3-bromopropoxy (**3**) derivatives which were then condensed with appropriate phenylpiperazines to yield the final compounds **4**-**9** and **12**-**17** (Scheme 2). All synthesized compounds possess the same parts: succinimide moiety, aromatic rings and phenylpiperazine. Additionally, in our research we used several compounds **18**-**20** (Scheme 3) which we obtained in our lab earlier (22). We chose them because they

also contain large imide complex with aromatic rings in the structure.

Finally, five selected compounds (**9**, **12** and **18**-**20**) were tested for cytotoxic properties on HL-60 cell line. The results are presented in Table 1.

Table 1. IC_{50} values of compounds in HL-60 cell line.

Compound	IC_{50} (μM)
9	8.74 ± 1.85
12	3.98 ± 1.06
18	90.16 ± 15.05
19	16.93 ± 0.27
20	38.33 ± 5.56

Data are presented as the means ± S.D. (n ≥ 3).

RESULTS AND DISCUSSION

Cytotoxicity study

Determination of IC_{50} values (concentration required to reduce the viability of cells by 50% as compared with the control cells) were used to compare the cytotoxicity of compounds in HL-60 cell line. Regression analysis was performed with SigmaPlot software (San Jose**,** CA**,** USA**).** The highest cytotoxic activity was exhibited by compound **12** (Tab. 1). Cytotoxicity study allowed to select compounds (**9**, **12**, **20**) for further biological examination.

Induction of apoptosis by tested compounds in HL-60 cell line

All of the tested compounds induced apoptotic death in HL-60 cell line (Fig. 3). The observed apoptotic effect was dose- and time-dependent for compounds **9** and **12**, but only dose-dependent for compound **20**. The highest apoptotic activity was exhibited by compound **12** in HL-60 cell line (Fig. 3). Additionally, the compounds induced typical morphological changes in cells such as apoptotic bodies formation (Fig. 4).

Changes in mitochondrial membrane potential ($\Delta\Psi m$)

Cells, previously treated for 48 h with the compounds, were stained with the JC-1 dye and then examined by flow cytometer. The analysis of cytograms showed that the tested compounds increased mitochondrial membrane depolarization (green fluorescence) in cell line studied. The effect was dose-dependent (Fig. 5).

In summary, the study has shown that some of the synthesized compounds possess significant proapoptotic activity in HL-60 line but unfortunately, only one exhibited the highest effect. Because of promising results of proapoptotic activity against cell line HL-60, the compounds will be testing on other cell lines.

Generally, the compounds were prepared easily, what is important if we think about future drugs. On the other hand, they possess a complex molecule of high molecular weight but despite this fact, they penetrate the cell membranes and induce typical morphological changes in cells such as apoptotic bodies formation. Therefore, it can be suspected that size is not a factor in weakening their proapoptotic activity.

EXPERIMENTAL

Chemistry

Melting points were determined by the capillary method using Electrothermal 9100 apparatus and were uncorrected.

Nuclear magnetic resonance spectra ^{1}H NMR and ^{13}C NMR were recorded in DMSO-d_6 on a Bruker VMNRS300 operating at 300 MHz (^{1}H NMR) and 75 MHz (^{13}C NMR). Chemical shift values were expressed in ppm (parts per million) in relation to tetramethylsilane as an internal standard and coupling constants *J* are given in Hz.

Mass spectral ESI (electrospray ionization) measurements were carried out on a Mariner Perspective – Biosystem instrument with TOF detector. The spectra were obtained in the positive ion mode with a declustering potential of 140-300 V.

Elemental analyses were recorded with CHN model 2400 Perkin-Elmer analyzer.

Chromatographic columns were filled with Merck 0.05-0.2 mm (70-325 mesh ASTM) silica gel. Reactions were monitored by TLC on silica gel G (plates with 254 nm fluorescent indicator, layer thickness 0.2 mm, Merck), eluted with 9.8 : 0.2 or 9.5 : 0.5, v/v chloroform : methanol solvent system.

Synthesis of imide 2

A mixture of 2-ethylanthracene (0.02 mol) and furan-2,5-dione (0.022 mol) in benzene (15 mL) was refluxed for 8 h. During the proceeding reaction, a solid precipitated. The crude product (**1**) was recrystallized from benzene. The obtained product was dissolved in methanol, next, an aqueous solution of hydroxylamine (6 mL) was added. The reaction mixture was heated in a water bath at 70^{o}C for 4 h. When the reaction was completed, the mixture was

cooled and left in a fridge. The residue was separated by column chromatography on silica gel; developing system: chloroform/methanol from 100 : 0.2 to 100 : 5, v/v. Finally, the N-hydroxyimide **2** was obtained.

4-Ethyl-17-oxapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-heksaen-16,18-dione (**1**)

$C_{20}H_{16}O_3$; M = 304.34; yield 89%; m.p. 256-257°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 7.46 (m, 2H, Ar-H), 7.18 (m, 4H, Ar-H), 7.03 (m, 1H, Ar-H), 4.84 (s, 2H, C1-H, C8-H), 3.64 (s, 2H, C15-H, C19-H), 2.53 (m, 2H, -CH_2-), 1.10 (t, 3H, *J* = 7.6 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.61 (1C), 27.90 (1C), 44.02 (1C), 44.44 (1C), 47.96 (1C), 48.04 (1C), 124.33 (1C), 124.37 (1C), 124.45 (1C), 124.71 (1C), 126.27 (1C), 126.45 (1C), 126.49 (1C), 136.40 (1C), 139.15 (1C), 141.25 (1C), 141.41 (1C), 142.70 (1C), 171.60 (1C), 171.63 (1C). Analysis: calcd.: C 78.93, H 5.30%; found: C 78.91, H 5.35%.

4-Ethyl-17-hydroxy-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-heksaen-16,18-dione (**2**)

$C_{20}H_{17}NO_3$; M = 319.35; yield 89%; m.p. 104-106°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 10.50 (s, 1H, OH), 7.43 (m, 1H, Ar-H), 7.33 (m, 1H, Ar-H), 7.13 (m, 3H, Ar-H), 6.97 (m, 1H, Ar-H), 4.71 (s, 2H, C1-H, C8-H), 3.17 (s, 2H, C15-H, C19-H), 2.54 (m, 2H, -CH_2-), 1.12 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.82 (1C), 28.04 (1C), 43.21 (1C), 43.34 (1C), 43.81 (1C), 44.22 (1C), 124.06 (1C), 124.65 (1C), 124.72 (1C), 125.48 (1C), 126.26 (1C), 126.58 (1C), 126.62 (1C), 136.38 (1C), 139.12 (1C), 139.29 (1C), 141.81 (1C), 141.98 (1C), 172.10 (2C). Analysis: calcd.: C 75.22, H 5.37, N 4.39%; found: C 75.20, H 5.35, N 4.40%.

Synthesis of imide 10

A mixture of 2,5-diethyl-3,4-diphenylcyclopentadienone (0.01 mol) and 1*H*-pyrrole-2,5-dione (0.012 mol) in benzene (15 mL) was refluxed for 14 h. When reaction was finished, the solvent was evaporated. The residue was crystallized from benzene.

1,7-Dietyl-8,9-diphenyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5,10-trione (**10**)

$C_{25}H_{23}NO_3$; M = 385; yield 92%; m.p. 206.8-209°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.70 (s, 1H, NH), 7.18 (m, 6H, Ar-H), 6.97 (m, 4H, Ar-H), 3.58 (s, 2H, C2-H, C6-H), 2.05 (m, 2H, -CH_2-), 0.87 (t, 6H, *J* = 7.5 Hz, -CH_3). Analysis: calcd.: C 77.93, H 5.98, N 3.63%; found: C 77.94, H 5.97, N 3.62%.

Synthesis of alkyl derivatives 3 and 11

An appropriate imide (0.01 mol) was dissolved in acetonitrile (30 mL) and anhydrous K_2CO_3 (0.01 mol) and 1,4-dibromobutane (0.03 mol) or 1,3-dibromopropane (0.05 mol) were added, respectively. The mixture was refluxed for 7-15 h. When the reaction was completed, the mixture was filtered and the solvent was evaporated. The residue was purified by column chromatography, eluent: chloroform.

17-(3-Bromopropoxy)-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**3**)

$C_{23}H_{22}BrNO_3$; M = 440; yield 90%; oil, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 7.37 (m, 1H, Ar-H), 7.30 (m, 2H, Ar-H), 7.22 (m, 1H, Ar-H), 7.17 (m, 2H, Ar-H), 7.00 (m, 1H, Ar-H), 4.77 (s, 2H, C1-H, C8-H), 3.37 (m, 4H, C1'-H, C3'-H), 3.12 (m, 2H, C15-H, C19-H), 2.59 (m, 2H, -CH_2-), 1.70 (m, 2H, C2'-H), 1.20 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.78 (1C), 28.04 (1C), 30.26 (1C), 43.34 (1C), 43.47 (1C), 43.90 (1C), 44.30 (1C), 44.34 (1C), 74.15 (1C), 124.31 (1C), 124.75 (1C), 124.84 (1C), 125.63 (1C), 126.38 (1C), 126.74 (1C), 126.78 (1C), 136.35 (1C), 139.12 (1C), 139.29 (1C), 141.37 (1C), 141.59 (1C), 171.38 (2C). Analysis: for $C_{23}H_{22}BrNO_3 \times 3^1/_2H_2O$ calcd.: C 54.87, H 5.17, N 2.78%; found: C 54.65, H 4.65, N 2.79%.

4-(4-Bromobutyl)-1,7-diethyl-8,9-diphenyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5,10-trione (**11**)

$C_{29}H_{30}NO_3Br$; M = 520.46; yield 89%; m.p. 116.8-118.8°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 7.16 (m, 6H, Ar-H), 6.90 (m, 4H, Ar-H), 3.55 (t, 2H, *J* = 7.0 Hz, C4'-H), 3.49 (s, 2H, C2-H, C6-H), 3.36 (t, *J* = 6.3 Hz, 2H, C1'-H), 2.18 (m, 2H, -CH_2-), 2.03 (m, 2H, -CH_2-), 1.79 (m, 4H,C2'-H, C3'-H), 1.00 (t, *J* = 7.5 Hz, 6H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.03 (2C), 18.96 (2C), 25.97 (1C), 29.37 (1C), 34.48 (1C), 37.81 (1C), 43.52 (2C), 59.31 (2C), 127.49 (2C), 128.06 (4C), 129.15 (4C), 133.48 (2C), 141.61 (2C), 176.23 (2C), 198.94 (1C); ESI MS (m/z): 100% = 542.1 [L + Na^+].

Synthesis of 4-arylpiperazinyl derivatives of N-substituted imides (**4–9**, **12– 7**)

To a mixture of N-bromoalkanyl/-alkanolimide (0.01 mol), a powdered anhydrous K_2CO_3 (0.01 mol), a catalytic amount of KI in acetone (30 mL)

and an appropriate amine (0.01 mol) were added. The reaction mixture was heated for 10-20 h. Then, an inorganic residue was filtered off and the solvent was evaporated. The obtained compound was purified by column chromatography, eluent: chloroform, chloroform/methanol 50 : 0.2, v/v .

All new derivatives were converted to their hydrochlorides and crystallized from methanol/diethyl ether mixtures.

17-{3-[4-(2-Methoxyphenyl)piperazin-1-yl]-propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}. 0^{9,14}.0^{15,19}] nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**4**)

$C_{34}H_{37}N_3O_4 \times HCl$; M = 551.67 × HCl; yield 84%; m.p. 149-150°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.10 (s, 1H, HCl), 7.38 (m, 2H, Ar-H), 7.22 (m, 4H, Ar-H), 7.00 (m, 5H, Ar-H), 4.77 (s, 2H, C1-H, C8-H), 3.80 (s, 3H, -OCH_3), 3.47 (m, 4H, C1'-H, C3'-H), 3.35 (m, 2H, H-piperazine), 3.21 (m, 2H, C15-H, C19-H), 3.10 (m, 6H, H-piperazine), 2.55 (m, 2H, -CH_2-), 1.71 (m, 2H, C2'-H), 1.12 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.79 (1C), 27.92 (1C), 28.04 (1C), 44.33 (1C), 43.45 (1C), 43.91 (1C), 44.31 (1C), 46.85 (1C), 51.13 (1C), 52.43 (1C), 55.41 (1C), 73.58 (1C), 111.93 (1C), 118.28 (1C), 120.86 (1C), 124.37 (1C), 124.77 (1C), 124.85 (1C), 125.66 (1C), 126.44 (1C), 126.73 (1C), 126.77 (1C), 136.39 (1C), 139.03 (1C), 139.14 (1C), 139.30 (1C), 141.30 (1C), 141.50 (1C), 151.81 (1C), 171.58 (2C). Analysis: for $C_{34}H_{37}N_3O_4 \times HCl \times 2^1/_4 H_2O$ calcd.: C 64.96, H 6.67, N 6.69%; found: C 64.63, H 6.41, N 6.63%.

17-{3-[4-(2-Pyrimidyl)piperazin-1-yl]propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**5**)

$C_{31}H_{33}N_5O_3 \times HCl$; M = 523.62 × HCl; yield 78%; m.p. 166.4-167.6°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.52 (s, 1H, HCl), 8.46 (d, 2H, *J* = 4.8 Hz, Ar-H), 7.46 (m, 1H, Ar-H), 7.36 (m, 1H, Ar-H), 7.28 (m, 1H, Ar-H), 7.16 (m, 3H, Ar-H), 7.00 (m, 1H, Ar-H), 6.78 (t, 1H, *J* = 4.8 Hz, Ar-H), 4.77 (s, 2H, C1-H, C8-H), 4.69 (d, 2H, *J* = 12.9 Hz, H-piperazine), 3.37 (m, 6H, C1'-H, C3'-H, H-piperazine), 3.21 (m, 2H, C15-H, C19-H), 3.00 (m, 4H, H-piperazine), 2.55 (m, 2H, -CH_2-), 1.70 (m, 2H, C2'-H), 1.08 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.80 (1C), 27.92 (1C), 28.05 (1C), 44.34 (1C), 43.46 (1C), 44.30 (1C), 44.34 (1C), 50.42 (1C), 52.52 (2C), 64.95 (2C), 73.57 (1C), 111.33 (1C), 124.20 (1C), 124.77 (1C), 124.85 (1C), 125.66 (1C), 126.00 (1C), 126.44 (1C), 126.73 (1C), 136.38 (1C), 138.63 (1C), 141.52 (1C), 142.15 (1C), 142.60 (1C), 158.20 (1C), 160.52 (1C), 171.58 (2C). Analysis for $C_{31}H_{33}N_5O_3 \times HCl \times 4^1/_4H_2O$: calcd. 58.44% C, 6.60% H, 11.00% N, found 58.11% C, 6.22% H, 10.73% N.

17-{3-[4-(2-Pyridyl)piperazin-1-yl]propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**6**)

$C_{32}H_{34}N_4O_3 \times HCl$; M = 522.64 × HCl; yield 78%; m.p. 177-177.8°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.42 (s, 1H, HCl), 8.13 (m, 1H, Ar-H), 7.90 (m, 1H, Ar-H), 7.47 (m, 1H, Ar-H), 7.29 (m, 3H, Ar-H), 7.17 (m, 2H, Ar-H), 7.01 (m, 1H, Ar-H), 6.93 (m, 1H, Ar-H), 4.76 (s, 2H, C1-H, C8-H), 4.45 (m, 2H, H-piperazine), 3.52 (m, 3H, H-piperazine), 3.37 (m, 4H, C1'-H, C3'-H), 3.21 (m, 2H, C15-H, C19-H), 3.10 (m, 3H, H-piperazine), 2.57 (m, 2H, -CH_2-), 1.70 (m, 2H, C2'-H), 1.09 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.80 (1C), 27.92 (1C), 28.04 (1C), 42.81 (1C), 43.35 (1C), 43.47 (1C), 43.91 (1C), 44.31 (1C), 49.95 (3C), 52.46 (1C), 73.56 (1C), 113.94 (1C), 124.33 (1C), 124.77 (1C), 124.84 (1C), 125.66 (1C), 126.00 (1C), 126.42 (1C), 126.78 (1C), 136.38 (1C), 138.62 (1C), 139.12 (1C), 1391.29 (1C), 141.29 (1C), 141.40 (1C), 141.51 (1C), 142.16 (1C), 142.61 (1C), 171.59 (1C), 171.64 (1C). Analysis: for $C_{32}H_{34}N_4O_3 \times HCl \times 4^1/_2H_2O$: calcd.: C 60.00, H 6.25, N 8.75%; found: C 60.41, H 6.66, N 8.44%.

17-{3-[4-(2-Chlorophenyl)piperazin-1-yl]propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}. 0^{9,14}.0^{15,19}] nonadeca-2,4,6,9,11,13-heksaen-16,18-dione (**7**)

$C_{33}H_{34}ClN_3O_3 \times HCl$; M = 556.09 × HCl; yield 79%; m.p. 244.3-246.6°C, ^{1}H NMR (300 DMSO-d_6, δ, ppm): 10.79 (s, 1H, HCl), 7.40 (m, 2H, Ar-H), 7.32 (m, 4H, Ar-H), 7.17 (m, 4H, Ar-H), 7.00 (m, 1H, Ar-H), 4.77 (s, 2H, C1-H, C8-H), 3.44 (m, 4H, H-piperazine), 3.33 (m, 4H, C1'-H, C3'-H), 3.21 (m, 2H, C15-H, C19-H), 3.14 (m, 4H, H-piperazine), 2.57 (m, 2H, -CH_2-), 1.69 (m, 2H, C2'-H), 1.13 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.77 (1C), 27.93 (1C), 28.03 (1C), 43.33 (1C), 43.45 (1C), 43.89 (1C), 44.29 (1C), 47.63 (1C), 51.20 (2C), 52.78 (2C), 73.56 (1C), 121.03 (1C), 123.90 (1C), 124.19 (1C), 124.76 (1C), 124.84 (1C), 125.65 (1C), 126.44 (1C), 126.76 (1C), 127.54 (1C), 128.30 (1C), 130.48 (1C), 136.39 (1C), 138.62 (1C), 139.13 (1C), 141.29 (1C), 141.50 (1C), 142.14 (1C), 147.38 (1C), 171.59 (1C), 171.63 (1C). Analysis: for $C_{33}H_{34}ClN_3O_3 \times HCl$ calcd.: C 66.89, H 5.95, N 7.09%; found: C 66.60, H 5.98, N 7.02.

17-{3-[4-(3-Chlorophenyl)piperazin-1-yl]propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**8**)

$C_{33}H_{34}ClN_3O_3 \times HCl$; M = 556.09 × HCl; yield 85%; m.p. 230-233°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 10.87 (s, 1H, HCl), 7.45 (m, 2H, Ar-H), 7.23 (m, 5H, Ar-H), 7.00 (m, 3H, Ar-H), 6.86 (m, 1H, Ar-H), 4.76 (s, 2H, C1-H, C8-H), 3.89 (m, 2H, H-piperazine), 3.45 (m, 2H, H-piperazine), 3.31 (m, 4H, C1'-H, C3'-H), 3.20 (m, 2H, C15-H, C19-H), 3.12 (m, 4H, H-piperazine), 2.54 (m, 2H, -CH_2-), 1.66 (m, 2H, C2'-H), 1.08 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.96 (1C), 27.91 (1C), 27.95 (1C), 43.30 (1C), 43.90 (1C), 44.35 (1C), 44.82 (1C), 50.41 (2C), 52.41 (2C), 56.52 (1C), 73.75 (1C), 114.15 (1C), 115.28 (1C), 124.20 (1C), 124.27 (1C), 124.33 (1C), 124.35 (1C), 124.73 (1C), 125.99 (1C), 126.41 (1C), 130.66 (1C), 133.37 (1C), 136.39 (1C), 139.03(1C), 140.56 (1C), 141.39 (1C), 141.39 (1C), 141.49 (1C), 142.60 (1C), 150.77 (1C), 171.57 (1C), 171.63 (1C). Analysis: for $C_{33}H_{34}ClN_3O_3 \times HCl$ calcd.: C 66.89, H 5.95, N 7.09%; found: C 66.70, H 5.95, N 7.03%.

17-{3-[4-(2-Fluorophenyl)piperazin-1-yl]propoxy}-4-ethyl-17-azapentacyclo[6.6.5.0^{2,7}.0^{9,14}.0^{15,19}]nonadeca-2,4,6,9,11,13-hexaen-16,18-dione (**9**)

$C_{33}H_{34}FN_3O_3 \times HCl$; M = 539.64 × HCl; yield 86%; m.p. 222.6-225.8°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.06 (s, 1H, HCl), 7.46 (m, 1H, Ar-H), 7.33 (m, 2H, Ar-H), 7.18 (m, 6H, Ar-H), 7.04 (m, 2H, Ar-H), 4.77 (s, 2H, C1-H, C8-H), 3.47 (m, 4H, H-piperazine), 3.34 (m, 4H, C1'-H, C3'-H), 3.21 (m, 2H, C15-H, C19-H), 3.16 (m, 4H, H-piperazine), 2.55 (m, 2H, -CH_2-), 1.70 (m, 2H, C2'-H), 1.09 (m, 3H, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 15.97 (1C), 27.94 (1C), 28.06 (1C), 43.34 (1C), 43.45 (1C), 43.92 (1C), 44.37 (1C), 46.92 (1C), 50.90 (3C), 52.49 (1C), 73.56 (1C), 116.06 (1C), 116.33 (1C), 119.60 (1C), 124.33 (1C), 124.77 (1C), 125.01 (1C), 125.06 (1C), 125.67 (1C), 126.03 (1C), 126.45 (1C), 126.76 (1C), 136.39 (1C), 138.63 (1C), 139.14 (1C), 141.32 (1C), 141.53 (1C), 142.16 (1C), 156.48 (1C), 171.59 (1C), 171.65 (1C). Analysis: for $C_{33}H_{34}FN_3O_3 \times HCl$ calcd.: C 68.74, H 6.07, N 7.29%; found: C 68.61, H 6.18, N 7.28%.

4-{4-[4-(2-Methoxyphenyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5,10-trione (**12**)

$C_{40}H_{45}N_3O_4 \times HCl$; M = 631.80 × HCl; yield 79%; m.p. 162.8-165°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.14 (s, 1H, HCl), 7.21 (m, 6H, Ar-H), 7.00 (m, 2H, Ar-H), 6.91 (m, 6H, Ar-H), 3.79 (s, 3H, -OCH_3), 3.49 (s, 2H, C2-H, C6-H), 3.40 (m, 6H, C1'-H, C4'-H, H-piperazine), 3.08 (m, 6H, H-piperazine), 2.05 (m, 2H, -CH_2-), 1.84 (m, 2H, -CH_2-), 1.74 (m, 2H, C3'-H), 1.54 (m, 2H, C2'-H), 0.89 (t, 6H, *J* = 7.5, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.05 (2C), 19.00 (2C), 20.49 (1C), 24.38 (1C), 38.12 (2C), 43.64 (2C), 46.83 (1C), 50.94 (2C), 55.40 (2C), 59.38 (2C), 111.94 (1C), 118.28 (1C), 120.87 (1C), 123.59 (1C), 127.55 (2C), 128.13 (4C), 129.17 (4C), 133.50 (2C), 139.25 (1C), 141.65 (2C), 151.80 (1C), 176.30 (2C), 198.86 (1C); ESI MS (m/z): 100% = 632.5, 18% = 633.5 [L + H$^+$].

4-{4-[4-(2-Pyrimidyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5,10-trione (**13**)

$C_{37}H_{41}N_5O_3 \times HCl$; M = 603.75 × HCl; yield 71%; m.p. 196.7-199°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.57 (s, 1H, HCl), 8.45 (d, 2H, *J* = 4.8 Hz, Ar-H), 7.18 (m, 6H, Ar-H), 6.90 (m, 4H, Ar-H), 6.77 (t, 1H, *J* = 4.8 Hz, Ar-H), 4.62 (m, 2H, H-piperazine), 3.69 (s, 2H, C2-H, C6-H), 3.42 (m, 6H, C1'-H, C4'-H, H-piperazine), 3.02 (m, 2H, H-piperazine), 2.89 (m, 2H, H-piperazine), 2.03 (m, 2H, -CH_2-), 1.85 (m, 2H, -CH_2-), 1.73 (m, 2H, C3'-H), 1.53 (m, 2H, C2'-H), 0.87 (t, 6H, *J* = 7.3 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.05 (2C), 18.99 (2C), 20.43 (1C), 24.37 (1C), 38.10 (2C), 43.65 (2C), 50.22 (2C), 54.82 (2C), 59.37 (2C), 111.30 (1C), 127.54 (2C), 128.13 (4C), 129.17 (4C), 133.49 (2C), 141.66 (2C), 158.17 (2C), 160.33 (1C), 176.29 (2C), 198.86 (1C); ESI MS (m/z): 100% = 604.4, 28% = 605.5 [L + H$^+$].

4-{4-[4-(2-Pyridyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyclo[5.2.1.0^{2,6}]dec-8-ene-3,5,10-trione (**14**)

$C_{38}H_{42}N_4O_3 \times HCl$; M = 602.76 × HCl; yield 67%; m.p. 216.6-219.2°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.47 (s, 1H, HCl), 8.12 (dd, 1H, *J* = 5.4 Hz, *J* = 5.7 Hz, Ar-H), 7.91 (m, 1H, Ar-H), 7.20 (m, 7H, Ar-H), 6.91 (m, 5H, Ar-H), 4.43 (m, 2H, H-piperazine), 3.69 (s, 2H, C2-H, C6-H), 3.52 (m, 6H, C1'-H, C4'-H, H-piperazine), 3.07 (m, 4H, H-piperazine), 2.05 (m, 2H, -CH_2-), 1.86 (m, 2H, -CH_2-), 1.74 (m, 2H, C3'-H), 1.54 (m, 2H, C2'-H), 0.88 (t, 6H, *J* = 7.5 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.05 (2C), 18.98 (2C), 20.49 (1C), 24.39 (1C), 38.05 (1C), 42.83 (1C), 43.62 (2C), 49.83 (2C), 54.74 (2C), 59.35 (2C), 113.93 (2C), 127.54 (3C), 128.13 (4C), 129.16 (4C),

133.49 (3C), 141.64 (3C), 176.26 (2C), 198.90 (1C); ESI MS (m/z): 100% = 603.3, 41% = 604.3 [L + H^+].

4-{4-[4-(2-Chlorophenyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenylo-4-azatricyklo[5.2.1.$0^{2,6}$] dec-8-ene-3,5,10-trione (15)

$C_{39}H_{42}ClN_3O_3 \times HCl$; M = 636.22 × HCl; yield 78%; m.p. 233-235.3°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 10.78 (s, 1H, HCl), 7.46 (m, 1H, Ar-H), 7.34 (m, 1H, Ar-H), 7.20 (m, 7H, Ar-H), 6.91 (m, 4H, Ar-H), 3.69 (s, 2H, C2-H, C6-H), 3.43 (m, 6H, C1'-H, C4'-H, H-piperazine), 3.10 (m, 6H, H-piperazine), 2.05 (m, 2H, -CH_2-), 1.89 (m, 2H, -CH_2-), 1.71 (m, 2H, C3'-H), 1.55 (m, 2H, C2'-H), 0.88 (t, 6H, *J* = 7.5 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.05 (2C), 18.99 (2C), 20.55 (1C), 24.36 (1C), 38.15 (1C), 43.64 (2C), 47.63 (2C), 51.06 (2C), 54.83 (1C), 59.38 (2C), 121.04 (2C), 124.86 (1C), 127.53 (2C), 128.13 (4C), 128.31 (1C), 129.17 (4C), 130.49 (1C), 133.49 (2C), 141.65 (2C), 147.39 (1C), 176.30 (2C), 198.86 (1C); ESI MS (m/z): 100% = 636.3, 30% = 637.3, 29% = 638.3 [L + H^+].

4-{4-[4-(3-Chlorophenyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyclo[5.2.1.$0^{2,6}$] dec-8-ene-3,5,10-trione (16)

$C_{39}H_{42}ClN_3O_3 \times HCl$; M = 636.22 × HCl; yield 80%; m.p. 146.4-148.5°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.15 (s, 1H, HCl), 7.21 (m, 7H, Ar-H), 7.04 (m, 1H, Ar-H), 6.89 (m, 6H, Ar-H), 3.85 (m, 2H, H-piperazine), 3.68 (s, 2H, C2-H, C6-H), 3.44 (m, 4H, C1'-H, C4'-H), 3.20 (m, 2H, H-piperazine), 3.01 (m, 4H, H-piperazine), 2.06 (m, 2H, -CH_2-), 1.86 (m, 2H, -CH_2-), 1.74 (m, 2H, C3'-H), 1.53 (m, 2H, C2'-H), 0.88 (t, 6H, *J* = 7.4 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.04 (2C), 18.96 (2C), 20.50 (1C), 24.38 (1C), 39.06 (1C), 43.61 (2C), 44.82 (2C), 50.28 (2C), 54.68 (1C), 59.35 (2C), 114.18 (2C), 115.28 (2C), 119.24 (1C), 127.54 (2C), 128.11 (3C), 129.16 (3C), 130.66 (1C), 133.48 (2C), 133.95 (1C), 141.62 (2C), 150.77 (1C), 176.25 (2C), 198.68 (1C); ESI MS (m/z): 100% = 636.3, 49% = 637.3, 45% = 638.3 [L + H^+].

4-{4-[4-(2-Flurophenyl)piperazin-1-yl]butyl}-1,7-diethyl-8,9-diphenyl-4-azatricyklo[5.2.1.$0^{2,6}$]dec-8-ene-3,5,10-trione (17)

$C_{39}H_{42}FN_3O_3 \times HCl$; M = 619.77×HCl; yield 82%; m.p. 126.6-128.5°C, ^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm): 11.02 (s, 1H, HCl), 7.12 (m, 10H, Ar-H), 6.89 (m, 4H, Ar-H), 3.69 (s, 2H, C2-H, C6-H), 3.44 (m, 6H, C1'-H, C4'-H, H-piperazine), 3.10 (m, 6H, H-piperazine), 2.05 (m, 2H, -CH_2-), 1.87 (m, 2H, -CH_2-), 1.72 (m, 2H, C3'-H), 1.54 (m, 2H, C2'-H), 0.88 (t, 6H, *J* = 7.4 Hz, -CH_3); ^{13}C NMR (75 MHz, DMSO-d_6, δ, ppm): 9.04 (2C), 18.98 (2C), 20.51 (1C), 24.36 (1C), 38.09 (1C), 43.62 (2C), 46.92 (1C), 50.77 (2C), 54.80 (1C), 59.37 (2C), 116.05 (1C), 116.31 (1C), 119.60 (1C), 123.52 (1C), 125.04 (1C), 127.54 (2C), 128.12 (3C), 129.16 (3C), 130.56 (1C), 133.48 (3C), 138.31 (1C), 141.64 (2C), 142.57 (1C), 176.28 (2C), 198.84 (1C); ESI MS (m/z): 100% = 620.4, 31% = 621.4 [L + H^+].

Cell culture and compounds treatments

HL-60 (human promyelocytic leukemia) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with heat-inactivated fetal bovine serum (FBS, Gibco; 10% v/v) and antibiotic–antimycotic solution (Gibco, Grand Island, NY, USA; 1%, v/v), at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed in exponentially growing cultures. For experiments, 3 mL per well aliquots of cell suspension in the same medium, containing 2.5 × 10^5 cells/mL, were seeded onto 6-well plates (Nunc, Denmark). The compounds studied were added to the cultures as solutions in dimethyl sulfoxide (DMSO; Sigma), and control cultures were treated with the same volume of the solvent. The concentration of the compounds were in the range from 5 to 50 μM. Cells were incubated with compounds for 24 and 48 h. Cells were subsequently collected, rinsed with PBS and prepared for labeling.

Cell viability (MTT - colorimetric assay)

Cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT; Sigma Aldrich). Cells were cultured in 96-well plates with the addition of compounds, incubation was performed for 48 h. MTT stock solution was added to each well to a final concentration of 0.5 mg/mL. After 4 h of incubation at 37°C formazan crystals were dissolved by the addition of SDS-HCl solution (10% SDS in 0.001 M HCl, final concentration). MTT and SDS were added directly to the cell culture. The solubilized formazan product was spectrophotometrically quantified in microplate reader, Power Wave XS (Bio Tek, Winooski, VT, USA) at 570 nm wavelength. The MTT data were analyzed to determinate the IC_{50} values (concentration required to reduce the viability of cells by 50% as compared with the control cells) of each compound. Regression analysis was performed with SigmaPlot software (San Jose, CA, USA).

Apoptosis assay by annexin V/propidium iodide (PI) labeling

Apoptosis was measured using the Annexin-V FITC Apoptosis Kit (Invitrogen). The cells were harvested 24 h and 48 h post-treatment. Cells were subsequently collected by centrifugation, rinsed twice with cold PBS and prepared for labeling. After washing with PBS, 2×10^6 cells per mL were suspended in binding buffer. One hundred µL aliquots of the cell suspension were labeled according to manufacturer's instructions. Flow cytometry measurements were performed within 1 h after staining.

Morphological evaluation

After incubation with compounds for 48 h, the cells were collected, washed with cold PBS and fixed at -20°C in 70% ethanol for at least 24 h. Next, ethanol was washed out and the cells were stained with 1.0 µg/mL DAPI and 20 µg/mL sulforhodamine 101. Cell morphology was evaluated using a BX60 fluorescence microscope equipped with a DP50 digital camera (Olympus, Japan).

Analysis of mitochondrial membrane potential ($\Delta\Psi$)

The mitochondrial membrane potential was assessed by flow cytometry using JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide; Sigma). Cells were harvested 48 h post-treatment, suspended in 1 mL of complete culture medium at approximately 1×10^6 cells/mL and incubated with 2.5 µL JC-1 (1 mg/mL DMSO) for 15 min at 37°C in the dark. Stained cells were washed with cold PBS, suspended in 400 µL of PBS and then examined with a FACSCalibur flow cytometer. JC-1 dye shows potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift, red (FL-2 channel), and green fluorescence (FL-1 channel).

Flow cytometry

Cytometric data were measured using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose CA, USA), analyzed by CellQuest software (BD Biosciences, San Jose, CA, USA) and WinMIDI 2.9 (Joseph Trotter).

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