Molecular mechanisms of apoptosis induced by a novel synthetic quinolinone derivative in HL-60 human leukemia cells

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ABSTRACT

The mortality rates for acute myeloid leukemia are very high, necessitating the search for novel chemotherapeutic candidates. Herein, we investigated the anticancer potential of a new synthetic compound, 2-ethyl-3-methyliden-1-tosyl-2,3-dihydroquinolin-4(1H)-one (AJ-374) against myeloid leukemia HL-60 cell line. This analog was selected from the small library of synthetic dihydroquinolinones on the basis of its strong anti-proliferative activity against HL-60 cells and 30-fold lower cytotoxicity towards healthy HUVEC cells. AJ-374 promoted the arrest of the cells in the subG0/G1 phase of the cell cycle in the first 24 h. Treatment of HL-60 cells with AJ-374 caused an increase in annexin-V positive cells, activation of caspase-8, -9 and -3, dissipation of the mitochondrial membrane potential and enhancement of FAS protein level. Apoptosis induction triggered by this quinolinone was blocked by the pre-treatment of the cells with caspase-8, -9 and -3 inhibitors. The obtained results indicated that AJ-374-induced apoptosis was executed by both, the extrinsic and intrinsic pathways. The cytotoxic activity of AJ-374 was also associated with down-regulation of the mitogen-activated protein kinase (MAPK) pathway and was independent of reactive oxygen species generation. Taken together, these results suggest that AJ-374 exerts a potent anticancer effect on leukemia cells, with a wide safety margin, which makes this analog an attractive drug candidate for further testing.

1. Introduction

The importance of natural products as a source of innumerable lead structures for drug development cannot be overestimated. Quinolinones are a large group of natural and synthetic compounds that have been investigated for almost 60 years in the search for novel treatments for various diseases [1]. Even though quinolinones are known mainly as effective antibiotics [2–4], some of them possess anti-tumor activity [5–8] and have already reached clinical trials in cancer patients [9–12].

An important subgroup of quinolinones are the quinolin-4(1H)-ones (Fig. 1) which have been used as intermediates in the synthesis of several biologically important compounds, including nonsteroidal androgen receptor agonists [13], antibacterial martellines [14], antifungal quinolone alkaloids [15] and diuretic agents [16]. Quinolin-4(1H)-ones showing anti-cancer properties have also been synthesized [17–19]. However, mechanisms of their biological activity haven’t been clarified.

In view of the efficacy reports of quinolinone derivatives, a small library of 3-methylidene-1-tosyl-2,3-dihydroquinolin-4(1H)-ones with various substituents in position 2 has recently been generated by our group [20]. One of the most promising analogs of this series was 2-ethyl-3-methyliden-1-tosyl-2,3-dihydroquinolin-4(1H)-one (AJ-374) (Fig. 1) which showed a very strong anti-proliferative effect against leukemia HL-60 cells.

In the present study we have undertaken an attempt to discover signaling pathways behind the cytotoxic activity of AJ-374.

2. Materials and methods

2.1. Chemicals and reagents

The 2-ethyl-3-methyliden-1-tosyl-2,3-dihydroquinolin-4(1H)-one, designated AJ-374, was synthesized as described in detail earlier [20], following the reaction sequence presented in Scheme 1. Reaction of diethyl methylphosphonate (1) with methyl 2-(tosylamino)benzoate (2) in the presence of three equivalents of LDA delivered diethyl 2-oxo-2-(2-N-tosylphenyl)ethylphosphonate (3). Condensation of 3 with...
propanal followed by the intramolecular cyclization yielded 3-(die-thoxyphosphoryl)-2-ethyl-1-tosyl-1,2-dihydroquinolin-4-ol (4). In the final step successful application of 4 as a Horner–Wadsworth–Emmons reagent for the olefination of formaldehyde furnished 3-methylidene-quinolin-4-one AJ-374.

For biological experiments the stock solution of AJ-374 in sterile dimethylsulfoxide (DMSO) was prepared and further diluted in the cell culture medium, so that the final DMSO concentration in the cell cultures did not exceed 0.1% DMSO.

2.2. Cell cultures

Caucasian promyelocytic leukemia (HL-60) cell line was obtained from the European Collection of Cell Cultures (ECACC) while human umbilical vein endothelial (HUVEC) cells were purchased from the American Type Culture Collection (ATCC). HL-60 cells were maintained in RPMI 1640 + Glutamax medium (Gibco/Life Technologies, American Type Culture Collection (ATCC)). HUVEC cultures did not exceed 0.1% DMSO.

2.3. Metabolic activity by MTT assay

The cytotoxicity of AJ-374 against the selected cell lines was investigated in the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay. In brief, HL-60 and HUVEC cells were seeded in 24-well plates at a density of $8 \times 10^4$/mL and left to grow for 24 h. The cells were treated with various concentrations of AJ-374 and incubated for 24 h or 48 h. Then, MTT solution (100 μL; 5 mg/mL in PBS) was added to each well. Following 1.5 h of incubation, the plates were centrifuged, washed with PBS, and resuspended in 1x binding buffer at a concentration of $1 \times 10^5$/mL. The cells were stained with FITC Annexin V and Propidium Iodide for 15 min in the dark at room temperature, in the dark. The fluorescence intensity was read at Ex/Em = 535/620 nm, 490/525 nm and 370/450 nm for caspase 3, -8, -9, respectively. Then, AJ-374 diluted in culture medium was added to the cells at 0.5 μM and 1 μM concentrations. Cells incubated without test compound were used as a control. After 24 h incubation, cells were treated with BrdU solution at a final concentration of 10 μM in culture medium and incubated for 8 h. Afterwards, HL-60 cells were collected by centrifugation. The cells were fixed, permeabilized and then incubated with DNase (300 μg/mL in PBS) for 1 h at 37 °C to expose incorporated BrdU. Subsequently, cells were stained with Anti-BrdU, Anti-H2AX (pS139) and Anti-Cleaved PARP (Asp214) fluorescent antibodies for 20 min, in the dark at room temperature. Finally, cells were resuspended in DAPI solution (1 μg/mL in staining buffer) to stain total DNA for cell cycle analysis. Cells were analyzed by flow cytometry using CytoFLEX (Beckman Coulter, Inc.). The data was analyzed using Kaluza Analysis Software v2 (Beckman Coulter, Inc.).

2.5. Apoptosis assay

Apoptosis was analyzed using FITC Annexin V Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. HL-60 cells were seeded in 6-well plates at a density of $2.0 \times 10^5$/mL in 2 mL of cell culture medium and left to grow for 24 h. Then, the cells were treated with AJ-374 diluted in cell culture medium at 0.5 μM and 1 μM concentrations for 24 h. In order to assess the involvement of caspases in AJ-374 induced apoptosis, the pretreatment of HL-60 cells with caspase 3 (Z-DEVD-FMK), caspase 8 (Z-IETD-FMK) and caspase 9 (Z-LEHD-FMK) inhibitors (R&D systems, Inc. Canada) was applied. Cells were treated with 30 μM concentration of each caspase inhibitor for 2 h and then exposed to AJ-374 (1 μM) or cell culture medium (for control) for the next 24 h. Afterwards, cells were collected by centrifugation, washed with PBS, and resuspended in 1x binding buffer at a concentration of $1 \times 10^5$/mL/mL. The cells were stained with FITC Annexin V and Propidium Iodide for 15 min in the dark at room temperature and analyzed by flow cytometry using CytoFLEX (Beckman Coulter, Inc.). Data analysis was performed using Kaluza Analysis Software v2 (Beckman Coulter, Inc.).

2.6. TUNEL assay

DNA fragmentation was detected using APO-DIRECT Kit (BD Biosciences, San Jose, CA, USA). Briefly, HL-60 cells were seeded in a 6-well plate at a density $2.0 \times 10^5$/well in 2 mL of cell culture medium and treated with AJ-374 at 0.5 μM, 0.75 μM and 1 μM concentrations or untreated for control. Following 24 h incubation cells were collected by centrifugation, washed with PBS and fixed with 1% (w/v) paraformaldehyde. The cells were rinsed with PBS, resuspended in 70% (v/v) ice cold ethanol and stored at −20 °C for at least 12 h. After washing, the cells were stained with FITC dUTP in the presence of TdT enzyme for 60 min at 37 °C and analyzed in PI/RNase Staining Buffer by flow cytometry.

2.7. Caspase 3, caspase 8 and caspase 9 activity

The activity of caspase 3, -8, -9 was assessed fluorometrically using Caspase 3, Caspase 8 and Caspase 9 Multiplex Activity Assay Kit (Abcam, Cambridge, UK) according to the manufacturer protocol. In brief, HL-60 cells were seeded in a 96-well plate at a density of $2.0 \times 10^5$/well in 90 μL of cell culture medium. The cells were exposed to AJ-374 at 0.5 μM and 1 μM concentrations. Untreated cells served as a control. After 24 h incubation the fluorogenic indicators DEVD-ProRed, IETD-R110, LEHD-AMC (for caspase 3, -8 and -9 activity, respectively) were added to each well followed by 50 min incubation at room temperature, in the dark. The fluorescence intensity was read at Ex/Em = 355/620 nm, 490/525 nm and 370/450 nm for caspase 3, -8, -9, respectively, using FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, LLC). The results were expressed as fold increase of caspase-3,-8 and -9 activity relative to the result of the control.
condition.

2.8. Quantitative real-time PCR assay

Expression of genes involved in apoptosis was assessed using real-time PCR. Briefly, HL-60 cells were seeded in a 6-well plate at a density $2.5 \times 10^5$/mL in 2 mL of cell culture medium and left to grow for 24 h. Then, the cells were treated with AJ-374 at 0.5 μM concentration. Untreated cells were used as a control. The cells were collected by centrifugation (300 × g, 5 min). Total RNA extraction was performed using Total RNA Mini Kit (A&A Biotechnology, Poland), cDNA synthesis was carried out using Transcriba Kit (A&A Biotechnology, Poland) according to the manufacturer’s instructions. Quantitative RT-PCR was performed using the Real-Time 2xPCR Master Mix SYBR A (A&A Biotechnology, Poland) and gene specific primers in Stratagene MX3005P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA) in 25 μL of total reaction volume, accordingly to the manufacturer’s protocol. A melting curve was produced to ensure product specificity. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The primer sequences used for QRT-PCR are listed in Table 1. The $2^{-\Delta\Delta CT}$ method was used to determine the expression levels of the tested genes.

2.9. MAPK pathway levels

Phosphorylated MAPK pathway protein levels were detected using Human MAPK Phosphorylation Antibody Array (Abcam, Cambridge, UK). Briefly, HL-60 cells were seeded in 6-well plates at a density $5.0 \times 10^5$/mL in 2 mL of cell culture medium. After 24 h incubation, AJ-374 diluted in culture medium was added to the cells at 0.5 μM and 1 μM concentrations. The cells incubated without the tested compound were used as a control. After 24 h the cells were washed with PBS twice and resuspended in Lysis Buffer (2–8 °C, 30 min). Cell lysates were centrifuged (14,000 × g for 10 min), supernatants were collected and stored at −70 °C until use. Protein concentration was determined using Bradford protein assay (from Bio-Rad Laboratories Inc., CA, USA). Samples were analyzed using a membrane spotted with Akt (pS473), CREB (pS133), ERK1 (pT202/Y204), ERK2 (pT185/Y187), GSK3α (pS21), GSK3b (pS9), HSP27 (pS2), JNK (pT183), MEK (pS217/221), MKK3 (pS189), MKK6 (pS207), MSK2 (pS360), mTOR (pS2448), p38 (pT180/Y182), p53 (pS15), P70S6K (pT421/S424), RSK1 (pS380), RSK2 (pS386) capture antibodies (Table 2). Phosphorylated proteins from cell lysates were bound specifically with antibodies arrayed on the membrane. Then, they were detected with paired detector antibodies and HRP-Anti-Rabbit IgG. The array was analyzed using a chemiluminescence imaging system (ChemiDoc™ XRS + System, Bio-Rad Laboratories, Inc.) and the samples were semiquantitatively compared using densitometry software (Image Lab™, Bio-Rad Laboratories, Inc.).

2.10. Determination of reactive oxygen species (ROS)

Reactive oxygen species (ROS) were measured using CellROX Oxidative Stress Green Reagent (Life Technologies, Carlsbad, CA, USA). HL-60 cells were seeded in a 24-well plate at a density $3.5 \times 10^5$/mL in

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### Table 1

| Gene     | Primer sequence           | Primer sequence           |
|----------|---------------------------|---------------------------|
| Caspase 3| GAAGCTACCTACAAACTCCTC     | GAGAGCTCCTGAACTTGCCTC     |
| Caspase 8| AAGAAGTGGGATCACCTCTGAAA   | GGGAAGCTCCTGAGATTGTCCA   |
| Caspase 9| GTCATGTTCTTGTGTCTAC       | CTTTTTACACACCTTGCTCA     |
| MAPK 14 | GTCATGTTCTTGTGTCTAC       | CTTTTTACACACCTTGCTCA     |
| MAPK 11 | GTCATGTTCTTGTGTCTAC       | CTTTTTACACACCTTGCTCA     |
| MAPK 12 | GTCATGTTCTTGTGTCTAC       | CTTTTTACACACCTTGCTCA     |
| MAPK 13 | GTCATGTTCTTGTGTCTAC       | CTTTTTACACACCTTGCTCA     |
| JNK 1   | GAGAGCCTGGAAGAGGTTT       | TTTCCTTGCACTTCTGCTC      |
| GRB2    | ATTTGGGATGCTAGGAGG       | GGGGCTACATATGCTGGGAAAA   |
| ERK 1   | TCGAGCTCCTGAGAGGTTT       | GGGGCTACATATGCTGGGAAAA   |
| ERK 2   | AAGAGCTGAGGAGGAGGAGG     | GGGGCTACATATGCTGGGAAAA   |
| Ras     | GAAGCTGAGGAGGAGGAGG     | GGGGCTACATATGCTGGGAAAA   |
| Raf     | GAAGCTGAGGAGGAGGAGG     | GGGGCTACATATGCTGGGAAAA   |
| GAPDH   | GTCGAGGAGGAGGAGGAGG     | GGGGCTACATATGCTGGGAAAA   |
1 mL of cell culture medium and left to grow for 24 h. Cells were treated with AJ-374 at 0.5 μM and 1 μM concentrations for 24 h. Then, CellROX Green Reagent at 5 μM final concentration was added to the cells, followed by 30 min incubation at 37 °C. The cells were washed with PBS and analyzed by flow cytometry using CytoFLEX (Beckman Coulter, Inc.). Data analysis was performed using Kaluza Analysis Software v2 (Beckman Coulter, Inc.). Cells treated with menadione (100 μM) for 1 h at 37 °C were used as a positive control. Some of the menadione and AJ-374 treated cells were preincubated for 1 h with 1 mM N-acetylcysteine.

2.11. Mitochondrial membrane potential (ΔΨm) assay

Mitochondrial membrane potential was evaluated with Flow Cytometry Mitochondrial Potential Detection Kit (BD Biosciences, San Jose, CA, USA). HL-60 cells were seeded in 6-well plates at a density 4 × 10^5/mL in 2 mL of cell culture medium and left to grow for 24 h. Then, the cells were treated with AJ-374 at 0.5 μM and 1 μM concentrations or not treated for control and incubated for 24 h. FCCP (carbonilcyanide p-trifluoromethoxyphenylhydrazone, 30 μM), a mitochondrial oxidative phosphorylation uncoupler, was used as a positive control and incubated with the cells for 30 min at 37 °C. After treatment, cells were collected by centrifugation, resuspended in JC-1 Solution (1st J-aggregate-forming cationic dye) and incubated for 15 min. Afterwards the cells were washed twice with Assay Buffer, resuspended in Assay Buffer and analyzed by flow cytometry using CytoFLEX (Beckman Coulter, Inc.). Data analysis was performed using Kaluza Analysis Software v2 (Beckman Coulter, Inc.).

2.12. Human Factor-related apoptosis (FAS) detection by ELISA assay

The level of FAS protein was detected using Human Factor-related Apoptosis ELISA Kit (Bioassay Technology Laboratory, Shanghai, China) according to the manufacturer’s protocol. Briefly, HL-60 cells were seeded in culture flasks at a density 7 × 10^5/mL in 10 mL of cell culture medium and left to grow for 24 h. Then, cells were treated with 0.5 μM, 0.75 μM and 1 μM AJ-374 or not treated for control and incubated for 24 h. The cells were collected by centrifugation, suspended in PBS and damaged by sonication (2 cycles: 10s and 20s, 1 min resting time on ice in between). The cell lysates were centrifuged (5000 × g for 10 min at 4 °C), the supernatants were aliquoted and stored at −20 °C. The protein concentration was estimated using Bradford protein assay (from Bio-Rad Laboratories Inc., CA, USA). The samples were added to a FAS antibody pre-coated plate and biotinylated detection Human FAS Antibody and Streptavidin-HRP were added. After incubation followed by addition of a substrate solution and a stop solution, a color developed proportionally to the amount of FAS. Absorbance was measured at 450 nm using FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices, LLC).

2.13. Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was assessed using one-way ANOVA followed by a post-hoc multiple comparison Student-Newman-Keuls test (for comparisons of three or more groups) or Student's t-test (for comparisons of two groups). *p < 0.05, **p < 0.01, and ***p < 0.001, ****p < 0.0001 were considered significant.

3. Results

3.1. Inhibition of cell metabolic activity and proliferation

To assess the effect of AJ-374 on metabolic activity of HL-60 and HUVEC cell lines, cells were exposed to various concentrations of this analog for 24 and 48 h and investigated using conventional MTT assay. AJ-374 was found to effectively inhibit metabolic activity of HL-60 cells in a dose-dependent manner, with the half maximal inhibitory concentration (IC_{50}) of 0.30 ± 0.01 μM (Fig. 2a) and 0.48 ± 0.01 μM (Fig. 2b) after 48 and 24 h, respectively. The compound exhibited almost 30-fold higher inhibitory activity in malignant HL-60 cells than in healthy HUVEC cells (IC_{50} = 8.9 ± 0.64 μM) (Fig. 2a).

To establish actively cycling cell populations, HL-60 cells were also exposed to bromodeoxyuridine (BrdU), an analog of thymidine. The cells treated with 0.5 μM and 1 μM concentrations of AJ-374 showed 21- and 22.1-fold decrease in BrdU incorporation, compared to control, respectively, indicating dose-dependent inhibition of proliferation (Fig. 2c).

The antiproliferative effect of AJ-374 was further assessed by the analysis of the cell cycle progression. The tested compound caused subG0/G1 cell cycle arrest. The percentage of the cells in subG0/G1 phase increased from 3.7% in untreated cells (used as a control) to 12.8% and 43.5% in cells exposed to 0.5 μM and 1 μM concentrations of AJ-374, respectively. The increase was significant only at the higher concentration. These changes corresponded with decreases of the cells in G0/G1 and G2/M phases and were dose-dependent (Fig. 2d).

3.2. Apoptosis induction

To check whether the cytotoxic effect of AJ-374 in HL-60 cells was associated with apoptosis induction, the morphological changes of cells incubated with increasing concentrations of the tested compound for 24 h were investigated. As shown in Fig. 3a, the occurrence of the characteristic morphological features of apoptosis, including cell rounding and shrinking, membrane blebbing and formation of apoptotic bodies were observed.

Other typical features of apoptotic cell death such as phosphatidylserine (PS) translocation to extracellular membrane, caspase-3 activation and DNA damage [21] were then investigated.

Translocation of PS was assessed using double-staining with Annexin V and propidium iodide (PI). AJ-374 slightly increased the population of annexin-V positive cells (early apoptotic cells), whereas it strongly enhanced the number of Annexin-V and PI-positive cells (late apoptotic/death cells), whose population raised from 3.6% for control to 42.7% and 92.3% for AJ-374 used at 0.5 μM and 1 μM, respectively (Fig. 3b and c). Apoptosis can be initiated by two main apoptotic pathways: the extrinsic and the intrinsic, which both lead to activation of effector caspase-3. Caspase-3 inactivates PARP by its cleavage, disabling DNA damage repair [22]. Therefore, detection of cleaved PARP

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Table 2

| A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|
| 1 | Positive | Positive | Negative | Negative | Akt (pS473) | CREB (pS133) | ERK1 (pT202/Y204)/ERK2 (pT185/Y187) | GSK3α (pS21) |
| 2 |  |  |  |  |  |  |  |  |
| 3 | GSK3b (pS9) | HSP27 (pS82) | JNK (pT183) | MEK (pS217/221) | MKK3 (pS189) | MKK6 (pS207) | MSK2 (pS360) | mTOR (pS2448) |
| 4 |  |  |  |  |  |  |  |  |
| 5 | p38 (pT180/182) | p53 (pS15) | P70S6K (pT421/424) | RSK1 (pS380) | RSK2 (pS386) | Negative | Negative | Positive |

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fragments can be used to investigate programmed cell death. Indeed, the incubation of HL-60 cells with AJ-374 significantly and dose-dependently induced PARP cleavage. Treatment with AJ-374 at 0.5 μM and 1 μM concentrations enhanced the number of 89 kDa-cleaved PARP positive cells up to 35.7% and 84.4% of cell population, respectively (Fig. 3d).

DNA double strand breaks (DSBs) induce phosphorylation of the histone variant H2AX on serine 139 leading to structural modifications at the site of damage. Phosphorylated H2AX was shown to promote DNA repair and play a role in genomic stability, cell cycle checkpoint responses and tumor suppression [23]. Thus, analysis of H2AX expression can be used to detect the genotoxic effect of potential anticancer agents.

We observed that treatment of HL-60 cells with AJ-374 increased the levels of cellular phosphorylated H2AX by 2.6- and 3.9-fold (for 5 μM and 1 μM, respectively) in comparison to control. The obtained results indicated modest but significant and dose-dependent induction of DNA damage (Fig. 3e).

Apoptotic DNA fragmentation was further detected by the observed increased number of TUNEL positive cells indicative for the late stage of apoptosis (Fig. 3f and g).

3.3. Caspase expression and activity

To investigate which of the two key apoptotic pathways was involved in AJ-374-initiated apoptosis in HL-60 cells, the expression levels of caspase-8, -9 and -3 genes were measured using real-time PCR. After 24 h treatment of the cells with AJ-374 (0.5 μM), the mRNA levels of executioner caspase-3 and initiator caspase-8 were up-regulated (11.4- and 7.7-fold, respectively), whereas caspase-9 gene expression decreased (5.88-fold) (Fig. 4a). To further study the implication of AJ-374 in caspases induction, the activities of caspase-8, -9 and -3 were assessed. At 0.5 μM concentration AJ-374 increased the activity of caspase-8 and -9 (4.18- and 2.33-fold, respectively) but not caspase-9. At higher concentration (1 μM), the effect was much stronger and enhancement of activity for all three caspases was observed. AJ-374 elevated the activity of caspase-3, -8 and -9 by 11.77-, 8.24- and 3.83-fold, respectively (Fig. 4b). Furthermore, 2 h pretreatment of the cells with specific caspase-3, -8 and -9 inhibitors (30 μM) significantly inhibited AJ-374-induced apoptosis (Fig. 4c). The use of higher caspase inhibitor concentrations, in order to obtain complete inhibition, was not possible as it affected the growth of the control cells.

3.4. Mitochondrial membrane potential (ΔΨm) changes and reactive oxygen species (ROS) production

The upstream signaling of apoptosis induced by AJ-374 in HL-60 cells was further investigated. To assess the status of ΔΨm in response to AJ-374 treatment (at 0.5 μM and 1 μM, 24 h), the cells were stained with JC-1 fluorochrome and analyzed using flow cytometry. Low fluorescence in the FL-2 channel is indicative of depolarized ΔΨm (Fig. 5a). In the control (untreated) cells, only 3.3% of population showed decreased fluorescence, while exposure to 0.5 μM and 1 μM AJ-374 and 30 μM FCCP (positive control) increased the number of cells with low fluorescence up to 13.2%, 68.1% and 90.3%, respectively (Fig. 5b). This result suggests that induction of apoptosis by AJ-374 was associated with mitochondrial membrane depolarization.

To study whether AJ-374-mediated mitochondrial membrane potential disruption was related to the presence of ROS, the intracellular ROS generation was examined using the fluorescent probe CellROX Green Reagent. Menadione, a strong inducer of ROS generation (100 μM, 3 h incubation), served as a positive control. The effect of menadione was reversed by 2 h pretreatment of the cells with a ROS scavenger, N-acetylcysteine (NAC) (1 mM). As shown in Fig. 5c, AJ-374 (0.5 μM and 1 μM) did not significantly change the level of ROS, neither did the pretreatment of the cells with NAC. The obtained results suggest that ROS production was not involved in AJ-374 induced apoptosis.

3.5. Human Factor-related apoptosis (FAS) levels

FAS is a membrane protein engaged in triggering a caspase cascade and extrinsic pathway of programmed cell death. Its involvement in apoptosis induction in HL-60 cells treated with AJ-374 (0.5, 0.75 and 1 μM) was assessed. Cell lysates were analyzed by ELISA assay. Significantly increased concentration of FAS was detected in the cells treated with 1 μM AJ-374 (Fig. 6).

3.6. MAPK pathway regulation

To investigate the role of mitogen-activated protein kinase (MAPK) pathway in the induction of apoptosis by AJ-374, the gene expression levels of the members of three major MAPK subfamilies (p38, JNK,
ERK1/2) were examined. The expression of the p38α, p38β and p38γ isomers was significantly downregulated (16.7-, 4.8- and 1.3-fold, respectively). The expression of JNK1/2 was decreased, however the change was not significant. ERK1, ERK2 and their upstream genes GRB2, Ras and Raf were all significantly downregulated (2.3-, 1.35-, 2.8-, 3.3- and 1.4-fold, respectively) (Fig. 7a).

Next, antibody-pair-based assay was used to examine the phosphorylation status of several major MAPK pathway proteins. As shown in Fig. 7b and c the decreased phosphorylation was observed for all investigated proteins, but the effect was the most prominent for ERK1/2, p38, JNK, CERB, MEK, MKK3, MSK2, P70S6K, RSK2 (1.5–1.9-fold).

4. Discussion

The treatment options for acute myeloid leukemia (AML) have been largely unchanged for many years, highlighting the importance of developing novel efficacious agents which are often based on natural product structures [24].

In the present report, we tried to disclose the molecular mechanisms of action of a novel synthetic quinolinone analog AJ-374 which showed potent anti-proliferative activity, dose-dependently inhibiting the growth of leukemic HL-60 cells with sub-micromolar IC50 value, while exhibiting 30-fold lower cytotoxic effect against healthy HUVEC cells.

It is well known that many cancer therapeutic drugs act through...
induction of apoptosis in cancer cells [25] and thus apoptosis represents a popular target in the studies on new cancer treatments [26,27]. Morphological changes indicative of apoptosis include rounding and shrinking of cells, retracting of pseudopods, condensation of chromatin, and later in the process, membrane blebbing and formation of apoptotic bodies [21,28]. Such morphological changes were observed in HL-60 cells exposed to AJ-374, demonstrating the ability of this compound to induce late stages of apoptosis.

There are also several biochemical changes observed in apoptotic cells, that enable quantification of the number of cells undergoing apoptosis [29]. Apoptosis may be executed by two major routes: intrinsic, also called mitochondrial or extrinsic (receptor-mediated) [30,31]. The intrinsic pathway is mediated by activation of the pro-apoptotic proteins of Bcl-2 family, followed by mitochondrial outer membrane permeabilization and subsequent release of cytochrome c from mitochondria to cytoplasm. Binding of cytochrome c with Apaf-1 and procaspase-9 leads to apoptosome formation and activation of caspase-9 [32,33]. In the extrinsic pathway, initiator caspase-8 is activated, following binding of ligands to the death receptors, such as FAS, tumor necrosis factor (TNF) or TNF-related apoptosis-inducing ligand (TRAIL) receptors, located on the cell membrane. Active caspase-8 and -9 in turn cleave and activate effector caspase-3, which executes apoptotic cell death [34–36].

AJ-374 up-regulated expression of caspase-8 and -3 genes, increased the levels of caspase-8 and -3 proteins and increased FAS concentration in HL-60 cells. FAS is a part of a complex activating caspase-8 which
involvement of caspase-3, -8 but also higher concentration mitochondrial pathway was also activated. The induced apoptosis mainly through the extrinsic pathway but at the drial membrane potential. Thus, the obtained results indicate that AJ-creased also caspase-9 activity and induced the loss of the mitochon-
pathway. However, at the higher concentration (1 μM), AJ-374 suggests that AJ-374 carried out apoptosis through the extrinsic pathway. However, at the higher concentration (1 μM), AJ-374 increased also caspase-9 activity and induced the loss of the mitochondrial membrane potential. Thus, the obtained results indicate that AJ-374 induced apoptosis mainly through the extrinsic pathway but at the higher concentration mitochondrial pathway was also activated. The involvement of caspase-3, -8 but also -9 was further confirmed when HL-60 cells were simultaneously incubated with AJ-374 and caspase-3, -8 or -9 inhibitors.

The cell cycle arrest is yet another mechanism by which anticancer agents can exert their effects [37]. Herein, we found that AJ-374 induced accumulation of the cells in sub-G0/G1 phase which is indicative of DNA fragmentation and cell death but not solely apoptosis [38]. To substantiate the apoptotic death of HL-60 cells exposed to AJ-374, staining with Annexin V and PI and TUNEL assay were performed. Treatment with AJ-374 led to an increase in Annexin V positive and TUNEL positive cells.

ROS generation can be associated with activation of both, the intrinsic and extrinsic pathways of apoptosis [39] and poses an important mechanism of anticancer activity of numerous compounds [40]. On the other hand, ROS production has been linked with chemotherapy-induced cardiotoxicity, limiting the clinical benefits of ROS-generating agents [41]. AJ-374 did not significantly induce ROS production. Moreover, pretreatment with a NAC did not influence ROS level in AJ-374 treated cells, indicating ROS-independent mitochondrial membrane potential loss and subsequent apoptosis. ROS have been implicated in the induction of apoptosis triggered by several known anticancer drugs, including doxorubicin and taxol [42, 43]. However, in some other studies the ROS-independent mechanism of cell death in HL-60 cells was reported [44, 45]. This contradicting results indicate that ROS is not always necessary for apoptosis to occur.

Apoptosis-associated signaling may also be regulated by MAPK pathway [46, 47]. This pathway regulates the expression of many proteins involved in the control of cell proliferation and apoptosis. Activation of the MAPK signaling can cause the transformation of normal cells into cancer cells [48]. Targeting any of the proteins of MAPK pathway has the potential to block this process. The MAPKs are serine-threonine kinases comprising three major subfamilies: c-Jun N-terminal kinase (JNK1, JNK2, JNK3), extracellular signal-regulated kinase (ERK1, ERK2) and p38 in four isoforms p38-α, -β, -γ, -δ. The MAPK signaling pathways have been shown to transduce extracellular signals through a cascade of kinases, including MAPK kinase kinases (MAP3K), MAPK kinases (MAP2K) and MAPK, that in turn phosphorylate various target proteins, such as transcription factors, to induce cellular response. In such a manner MAPKs may modulate cell proliferation, development, differentiation or affect cell death or survival [49, 50].

The early studies of JNK activation and inhibition suggested its pro-apoptotic activity. However, further investigation revealed also anti-apoptotic function of JNK, also in HL-60 cells [51]. The discrepancies in the JNK activity may be associated with the different cell types and various agents used in the studies. Following AJ-374 treatment, the expression of the major MAPK pathway genes, p38 and ERK1/2, was down-regulated, while JNK1/2 expression was not significantly changed in HL-60 cells. Moreover, exposure of the cells to AJ-374 led to a decrease in the phosphorylation level of proteins involved in MEK/ERK1/2, MKK3/p38 and JNK pathways and their downstream proteins, including RSK2, MSK2, p53, p70S6K and CREB. The cytotoxic activity of AJ-374 may be therefore associated with down-regulation of MAPK pathway. Altogether, the obtained results indicate that AJ-374 induced arrest of the cell cycle in the subG0/G1 phase and executed apoptosis through the extrinsic and intrinsic route, independently of ROS generation. Additionally, MAPK pathway down-regulation could be involved in AJ-374 mediated cytotoxicity.

5. Conclusions

This study demonstrated that a novel quinolinone derivative, AJ-374, exerted potent anti-proliferative activity against HL-60 cells, while...
being 30-fold less cytotoxic for normal cells. The compound’s cytotoxicity was associated with its ability to induce subG0/G1 cell cycle arrest and apoptosis. Apoptotic cell death induced by AJ-374 was mediated through both, extrinsic and intrinsic pathways and involved activation of the initiator and executioner caspases (8, 9 and 3, respectively). Interestingly, apoptosis occurred through a ROS-independent mechanism. Efficacy of AJ-374 may also be associated with MAPK signaling pathway down-regulation. The presented findings

**Fig. 7.** Effect of AJ-374 on the MAPK pathway in HL-60 cells after 24 h treatment. **a** Gene expression changes analyzed by real-time PCR. Data represent mean ± SEM of two independent experiments performed in triplicate; *p < 0.05, **p < 0.01, ns = not statistically significant. *p < 0.05, **p < 0.01. **b** Image of MAPK phosphorylation signals form HL-60 cell lysates analyzed by Human MAPK Phosphorylation Antibody Array. **c** Levels of the phosphorylated MAPK pathway proteins assessed by antibody array membrane. Data are expressed as mean ± SEM pixel density of two results.

**Fig. 8.** Illustration of the effect of quinolinone AJ-374 on HL-60 cells.
increase our understanding of the molecular mechanisms by which compounds with quinolinone motif can exert their anticancer activity and indicate that AJ-374 or its derivatives have a potential in the development of novel therapeutics against leukemia cells. Fig. 8 illustrates the effect of quinolinone AJ-374 on leukemia HL-60 cells.

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**Author contributions**

J.D-S., A.J. conceived and designed research, J.D-S., K.G.-J. carried out all biological experiments, A.J synthesized the analog, J.D-S., K.G-J drafted the manuscript. A.J. and T.J. made critical contributions and prepared the final version. All authors read and approved the final manuscript.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Appendix A. Supplementary data**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.cej.2020.100905.

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