Transcriptomic analysis of the PI3K/Akt signaling pathway reveals the dual role of the c-Jun oncogene in cytotoxicity and the development of resistance in HL-60 leukemia cells in response to arsenic trioxide

Joanna Roszak[A–D], Anna Smok-Pieniążek[B], Maciej Stępnik[A, C–F]

Nofer Institute of Occupational Medicine, Łódź, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Maciej Stępnik
E-mail: maciej.stepnik@imp.lodz.pl

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Abstract

Background. Arsenic trioxide (ATO) is a well-recognized antileukemic drug used for the treatment of newly diagnosed and relapsed acute promyelocytic leukemia (APL). A major drawback of therapy with ATO is the development of APL cell resistance, the mechanisms of which are still not clear.

Objectives. The aim of this study was to investigate the role of the PI3K/Akt signaling pathway in ATO-treated human acute myeloid leukemia (HL-60) cells and in ATO-resistant clones.

Material and methods. The cytotoxicity of ATO was assessed using Trypan blue staining or a WST-1 reduction assay. The Akt phosphorylation level was measured by immunofluorescent staining and flow cytometry. Gene expression analysis was performed using real-time polymerase chain reaction (PCR).

Results. The clones derived by culturing for 8–12 weeks in the presence of 1.75, 2.5, and 5 μM ATO were characterized by high viability but a slower growth rate compared to the parental HL-60 cells. The flow cytometry analysis showed that in the parental cells the levels of p-Akt were undetectable or very low, and that ATO had no effect on the level of p-Akt in either the ATO-treated parental cells or the clones. The gene expression analysis revealed that some of the genes involved in the Akt pathway may play a key role in the induction of resistance to ATO, e.g., genes encoding cyclin D1 (CCND1), fork head box O1 (FOXO1), Jun oncogene (JUN), protein kinase C isoform B1 (PRKCB1), because their expression profiles were predominantly changed in the clones and/or the ATO-treated parental HL-60 cells.

Conclusions. The overall results indicate that CCND1, FOXO1, and JUN may contribute to the induction of resistance to ATO, and that the C-Jun N-terminal kinase (JNK) signaling pathway may have greater significance than the phosphoinositide 3-kinase (PI3K)/Akt pathway in mediating the cytotoxic effects of ATO and the development of resistance to ATO in the HL-60 cell line.

Key words: Akt kinase, C-Jun, arsenic trioxide, HL-60 cells, arsenic resistant clones

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Arsenic trioxide (ATO) is a widely recognized antileukemic drug used for the treatment of newly diagnosed and relapsed acute promyelocytic leukemia (APL).[1] ATO effectively induces the differentiation and apoptosis of APL cells.[2,3] A major drawback of therapy with ATO is the development of APL cell resistance, the mechanisms of which are still not clear.Some data suggests that mutations in the B2 domain of the *PML/RARA* fusion gene and a mutation in the *PML* gene that was not rearranged play an important role in the development of ATO resistance.[4,5] Signal transduction studies indicate that the phosphoinositide 3-kinase (PI3K)/Akt pathway contributes to the development of resistance. Tabellini et al. reported high levels of the phosphorylated form of Akt in the leukemic NB4 clones resistant to ATO.[6] This observation was concordant with many studies confirming the important role of the Akt kinase in promoting cell survival and malignant transformation through diverse mechanisms.[7] Additionally, the PI3K/Akt pathway has been shown to be modulated by ATO in various cancer cells originating from both solid tumors and leukemia, e.g., in acute lymphoblastic leukemia cells, B-cell chronic leukemia cells, and acute promyelocytic leukemia cells.[8–11]

In a previous study, the current authors identified several target genes involved in the PI3K/Akt pathway that might mediate ATO cytotoxic effects and/or the resistance to the drug in Jurkat cells derived from human acute lymphoblastic leukemia.[12] The present study examines the effect of ATO on the growth of HL-60 cells originating from APL, but lacking the typical t(15;17) chromosomal translocation, and characterizes the contribution of the PI3K/Akt signaling pathway in the development of ATO resistance in the cells.

Material and methods

Chemicals and reagents

Arsenic trioxide was purchased from Sigma-Aldrich Co. (St. Louis, USA; PubChem CID: 24852110). Stock solutions of ATO prepared in 1M NaOH were diluted in Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich Co., St. Louis, USA) to a concentration of 10 mM, aliquoted and stored at 4–8° C. Cell Proliferation Reagent WST-1 and 0.4% solution of Trypan blue were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma-Aldrich Co. (St. Louis, USA), respectively.

Cell culture

The HL-60 (human acute myeloid leukemia; #ACC 3) cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and maintained in cRPMI (RPMI 1640 medium with Gibco® GlutaMAX™, Thermo Fisher Scientific, Inc., Waltham, USA), supplemented with Gibco® 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Co., St. Louis, USA). The cell line was screened for Mycoplasma sp. infection using MycoProbe Mycoplasma Detection Kit (R&D Systems, Minneapolis, USA).

Establishing ATO-resistant clones from HL-60 cells

HL-60 cells were cultured in the presence of ATO at selected concentrations of 1.75, 2.5 or 5 µM until resistance developed. Every 3–4 days of the culture, the cells were collected, centrifuged, and re-suspended in fresh culture medium with ATO added.

Because the developing clones grew slowly, particularly at the higher ATO concentrations, a higher initial density of HL-60 cells was used for the development of ATO resistance than is normally used for parental cells (i.e., 2×10^5 cells/mL as opposed to $0.5–1 \times 10^5$ cells/mL). The cells were changed every 3–4 days to maintain a density of $2–8 \times 10^5$ cells/mL. During the initial steps, the exposure to ATO was discontinued temporally when the Trypan blue exclusion test showed that the viability of the cells decreased below 10% (often observed at the highest concentration of ATO), and restarted when the viability attained over 80%. Usually, the cells became resistant to ATO within 8–12 weeks, which was reflected by their high viability (>85%).

WST-1 reduction assay

In brief, HL-60 cells were grown in 96-well microplates (1.5×10^3 cells/well) and exposed continuously to the test compounds for 72 h. Afterwards, 10 µL WST-1 reagent was added to all the wells for 1.5 h. The optical density (OD) of the formazan product was measured with a Multiscan RC spectrophotometer (Labsystems Diagnostics Oy, Helsinki, Finland) at 450 nm with a reference filter of 620 nm. The results were expressed as percent of cell survival, i.e., the ratio of the OD of exposed vs the OD of non-exposed control cells.

Measurements of Akt phosphorylation level by flow cytometry

The preparation of the cells and the analysis were performed following the protocol described in a previous publication.[12] In brief, for the immunofluorescent staining, rabbit monoclonal antibodies were used (anti-phospho-Akt (Ser473) and anti-Akt from Cell Signaling Technology, Inc., Danvers, USA). Alexa Fluor 488-labeled anti-rabbit IgG (Cell Signaling Technology, Inc., Danvers, USA) was used as a secondary antibody. A flow cytometry analysis was performed using FACS Canto II (BD Biosciences, San Jose, USA). Data analysis (~10,000 cells

analyzed) was performed using WinMDI v. 2.8 software. The level of activated Akt kinase (phospho-Akt or p-Akt) normalized to the level of total Akt kinase was calculated using the geometric mean intensity of fluorescence (MIF) according to the formula:

$$\text{p-Akt/Akt} = \frac{\text{MIF of cells stained with p-Akt - MIF of isotype control cells}}{\text{MIF of cells stained with Akt - MIF of isotype control cells}}$$

Real-time PCR analysis of gene expression

The preparation of the cells and the analysis were performed following the protocol described in a previous publication.[12] In brief, HL-60 cells and ATO-resistant clones (1.2×10^6 cells/6 mL) were treated with ATO for 16 h. The real-time PCR (RT-PCR) analysis was performed in 2 steps: 1) screening of genes involved in the PI3K/Akt pathway, using the RT Profiler PCR Array Human PI3K-AKT Signaling Pathway (SABiosciences, Germantown, USA); and 2) the analysis of selected genes using iQ™ SYBR Green SuperMix (Bio-Rad, Hercules, USA) and the primers presented in Table 1. The Ct values for each test gene were normalized using 3 housekeeping genes (*ACTB*, *B2M*, *GAPDH*; Table 1). For the 2nd stage of the study, genes were selected based on ≥1.7-fold up-regulation or ≤0.6-fold down-regulation compared to untreated cells. The results for gene expression are shown as geometric mean ±geometric standard error.

Statistical analysis

Bartlett's test of homogeneity of variances was used to determine if the results had equivalent variances at the $p < 0.05$ level. The results were compared using a standard one-way analysis of variance (ANOVA) with Tukey's test for post-hoc comparisons. All the calculations, including doubling time, were performed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, USA).

Results

Characterization of the HL-60 derived ATO-resistant clones

ATO-resistant clones were generated from HL-60 cells after 8–12 weeks of continuous exposure to ATO. All the clones, in contrast to the parental cells, showed a high viability (>80%) when cultured in the presence of ATO (Fig. 1). However, the growth rate of the clones was considerably lower than that of the parental cells. For example, the doubling time of HAs5 (the clone resistant to the highest ATO concentration of 5 μM) was more than twice as long as that calculated for the parental HL-60 cells (66 h vs 25 h, respectively).

Levels of Akt kinase in HL-60 cells exposed to ATO and in ATO-resistant clones

In the flow cytometry analysis, the parental HL-60 cells showed constitutively no or very little p-Akt immunostaining. After 4 h of exposure, ATO had no effect on the p-Akt levels, either in the parental cells or in the ATO-resistant clones (Fig. 2).

Table 1. The official symbols and names of the test genes and the primer sequences used for real-time PCR in the 2nd stage of the study (the confirmation analysis of selected genes)

Accession No.	Gene symbol	Gene name	Sequence 5′ → 3′ of forward primer Sequence 5′ → 3′ of reverse primer
NM_000061	*BTK*	bruton agammaglobulinemia tyrosine kinase	5′-TGCAAGGATGTCTGTGAAGC-3′ 5′-GGACAGGCCGAAATCAGATA-3′
NM_053056	*CCND1*	cyclin D1	5′- ACAAGCTCAAGTGGAACCTG-3′ 5′-ATCTGTTTGTTCTCCTCCGC-3′
NM_002015	*FOXO1*	forkhead box O1	5′-ATGGACAACAACAGTAAATT-3′ 5′-CCAGTTATCAAAGTCATCAT-3′
NM_005311	*GRB10*	growth factor receptor-bound protein 10	5′-GTCAAATGGCAGTCAAACCC-3′ 5′-TCCATGATTTCTTTCCCAGC-3′
NM_002228	*JUN*	jun oncogene	5′-CCCCAAGATCCTGAAACAGA-3′ 5′-CCGTTGCTGGACTGGATTAT-3′
NM_002738	*PRKCB*	protein kinase C, beta 1	5′-TGAAGGGGAGGATGAAGATG-3′ 5′-GTGTTTGGTCATCAGCCCTT-3′
Housekeeping genes			
NM_001101	*ACTB*	actin beta	5′-TGACTGACTACCTCATGAAGATCC-3′ 5′-CCATCTCTTGCTCGAAGTCC-3′
NM_004048	*B2M*	beta-2-microglobulin	5′-TGCTGTCTCCATGTTTGATGTATCT-3′ 5′-TCTCTGCTCCCCACCTCTAAGT-3′
NM_002046	*GAPDH*	glyceraldehyde-3-phosphate dehydrogenase	5′-GACCTGCCGTCTAGAAAAACC-3′ 5′-GTTGAAGTCAGAGGAGACCACC-3′

Table 2. Gene expression changes in HL-60 cells exposed to 2.5 μM ATO for 16 h and in the clone resistant to the same 2.5 μM ATO concentration (H.As2.5) – the 1st screening of genes

Gene symbol	HL-60 cells exposed to 2.5 μM ATO	H.As2.5 clone resistant to 2.5 μM ATO
ADAR	1.4	1.4
AKT1	1.5	1.4
AKT2	1.4	1.6
APC	1.6	1.3
BAD	1.3	1.4
BTK	0.9	2.1
CASP9	1.0	1.3
CCND1	2.4	0.2
CD14	0.9	0.7
CDC42	1.1	1.2
CDKN1B	1.0	0.8
CHUK	1.1	1.1
CSNK2A1	0.8	1.1
CTNNB1	1.1	1.0
EIF2AK2	0.9	1.1
EIF4E	0.9	1.0
EIF4EBP1	0.7	0.9
ELK1	1.0	1.0
FASLG	0.9	1.8
FKBP1A	0.6	0.9
FOS	0.7	1.4
FOXO1	2.0	0.6
FOXO3	1.0	1.3
FRAP1	0.7	1.2
GRB10	0.5	0.9
GRB2	0.9	1.5
HSPB1	1.0	1.2
IGF1	1.2	0.9
IGF1R	0.6	0.7
IRAK1	0.8	1.1
IRS1	1.2	0.6
ITGB1	1.0	1.0
JUN	0.8	0.4
MAP2K1	1.2	1.2
MAPK1	0.9	1.2
MAPK14	1.0	1.2
MAPK3	1.0	1.5
MAPK8	1.0	1.0
MYD88	1.1	1.2
NFKBIA	0.9	0.9
PABPC1	0.7	1.3
PAK1	1.0	0.8
PDGFRA	0.8	0.9
PDK1	0.8	1.0
PDPK1	0.9	1.2
PRKCA	1.0	1.1
PRKCB1	2.9	2.7
PRKCZ	1.1	1.0
PTEN	1.0	1.0
PTPN11	0.8	0.7
RAC1	0.6	0.8
RASA1	0.7	1.0
RBL2	1.3	1.2
RHOA	0.7	0.9
RPS6KA1	1.0	1.6
RPS6KB1	0.9	1.3
SHC1	1.2	0.9
SOS1	0.8	0.7
SRF	0.7	0.9
TIRAP	1.4	1.4
TLR4	1.1	0.7
TOLLIP	0.9	0.8
TSC1	1.2	1.4
TSC2	0.8	1.2
WASL	0.7	1.1
YWHAH	0.8	0.8

The results are presented as the 2^{-ddCt} values from 1 experiment; light gray shadow – mean values ≤0.5; dark gray shadow – mean values ≥2.

Expression of genes involved in the PI3K/Akt signaling pathway in ATO-treated HL-60 cells and in the corresponding ATO-resistant clones

Based on the expression analysis of 66 genes involved in the PI3K/Akt signaling pathway in the parental cells exposed for 16 h to 2.5 μM ATO and in the corresponding resistant clone (HAs2.5), 6 genes whose expression was changed compared to non-exposed HL-60 cells were selected: *BTK, CCDN1, FOXO1, GRB10, JUN* and *PRKCB1* (Table 2).

In the subsequent analysis, these 6 genes were verified in the parental HL-60 cells exposed for 16 h to 1.75 μM or 5 μM ATO, and in the clone resistant to 1.75 μM ATO (HAs1.75). The results of the analysis (Table 3) indicated that the expression of *FOXO1* was up-regulated in a concentration-dependent manner in the ATO-treated parental cells, and down-regulated at least 2-fold ($2 \geq 2^{-\Delta\Delta Ct} \leq 0.5$, where Ct is the threshold cycle; according to the delta-delta Ct method) in the ATO-resistant cells. Strong down-regulation of *CCND1* and *JUN* was shown only in the ATO-resistant cells. In the ATO-exposed

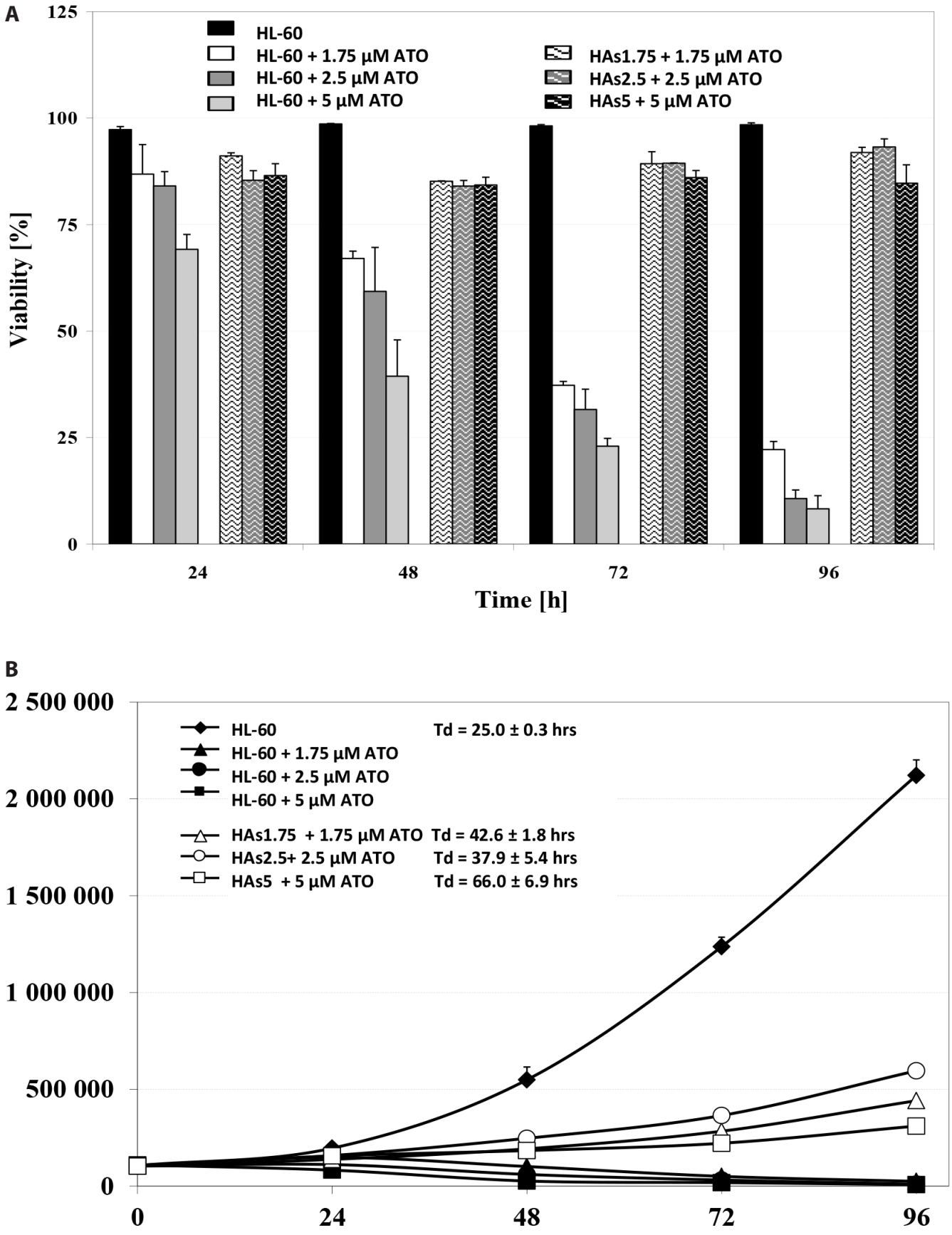

Fig. 1. The viability and growth of HL-60 cells exposed to the indicated concentrations of ATO

The viability (A) and number (B) of the parental HL-60 cells and THE ATO-resistant clones derived from this cell line (HAs1.75, HAs2.5 and HAs5) were determined using the Trypan blue exclusion test after 96 h of incubation with or without ATO. Based on the results doubling time (Td) was calculated for untreated HL-60 cells as well as their ATO-resistant clones.

HL-60 parental cells, the expression of *CCND1* and *JUN* was increased only after treatment with the highest concentration of the drug (5 μM), which was in contrast to concentration-dependent decreased expression observed in the resistant clones. The expression of *PRKCB1* was strongly up-regulated both in the ATO-treated parental cells and the ATO-resistant clones. *BTK* and *GRB10* showed a tendency toward increased expression in the ATO-resistant clones, but the increase was less than 2-fold.

Discussion

A previous study by the current authors identified several genes involved in the PI3K/Akt pathway associated with ATO cytotoxic effects and/or the resistance to the drug in Jurkat cells.[12] This concurred with other data indicating that dysregulation of PI3K/Akt signaling pathway may be the reason for apoptosis resistance in leukemic cells.[6,8,13–15] In the present study, the focus was on the effect of ATO on the PI3K/Akt signaling pathway in HL-60 acute promyelocytic leukemia cells, which – similarly to NB-4 cells but in contrast to other leukemic cell lines (e.g., Jurkat, CEM, and MOLT-4) – do not display phosphorylated (activated) Akt (p-Akt).[6,12]

The present study has shown that prolonged incubation of HL-60 cells in the presence of clinically relevant ATO concentrations of 1–5 μM induced resistance to the drug.[16,17] In contrast to the NB-4-derived ATO-resistant clones, the HL-60-derived clones in the present study did not display elevated levels of p-Akt.[6] Similarly, a short (4-hour) exposure of the parental HL-60 cells to ATO did not induce any detectable changes in the level of activated Akt. This contrasts with the findings of Choi et al., who observed a decrease in p-Akt levels associated with apoptosis induction in ATO-treated U937 cells.[18]

The present study identified 4 gene targets for ATO that might mediate its cytotoxic effect and the development of ATO resistance in HL-60 cells, i.e., *CCND1, FOXO1, JUN*, and *PRKCB1*. The expression of these genes changed at least 2-fold after 16 h of exposure to clinically relevant ATO concentrations, but only *PRKCB1* (the beta isoform of protein kinase C, or PKC beta) was up-reg-

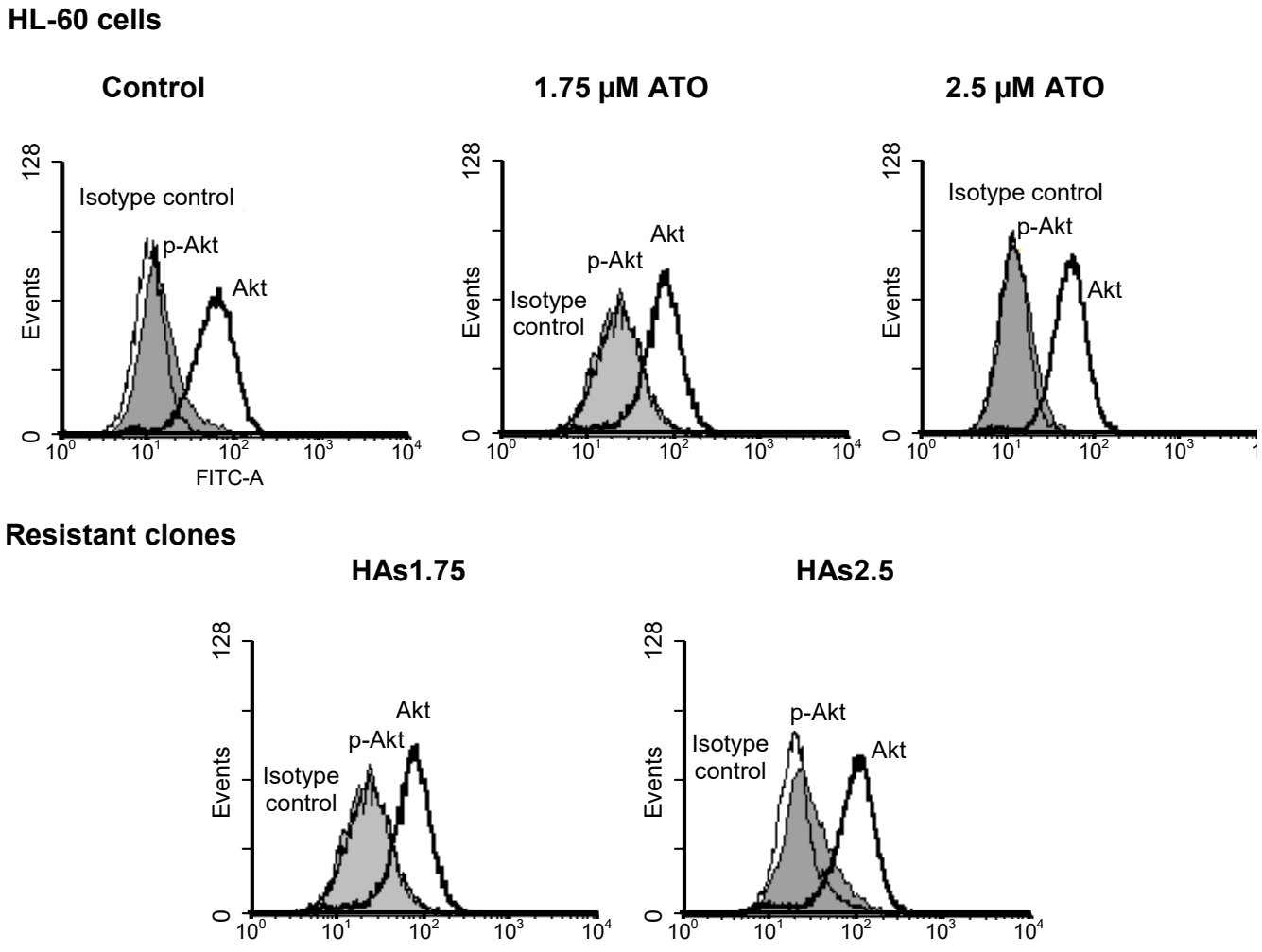

Fig. 2. The effect of ATO on Akt kinase in parental cells and HAs1.75 and HAs2.5 clones

The graph presents the mean level of p-Akt kinase normalized to the value of the total Akt kinase level (p-Akt/Akt) ±SD (representative histograms for 2–3 independent experiments).

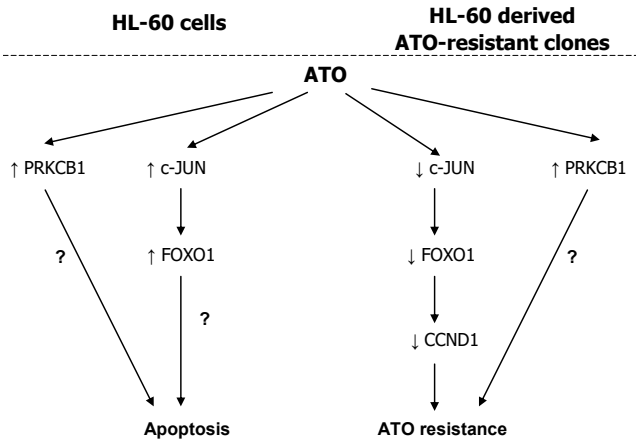

Fig. 3. The possible signaling pathways activated in HL-60 cells in response to arsenic trioxide (ATO)

ulated in both ATO-treated and ATO-resistant cells. Although there exists data that demonstrates the role of PKC beta in the induction of apoptosis, the results of the present study show that the role of this kinase is far-reaching and cell-dependent.[19] The results in HL-60 cells implicate elevated levels of the kinase in the development of ATO resistance, while the authors' previous work showed that in Jurkat cells down-regulation of *PRKCB1* expression was responsible for the development of ATO resistance.[12] Other data links PKC beta with increased invasion and proliferation of cancer cells.[20]

JUN, the gene encoding the c-Jun oncogene, seems to play a dual role in cell response to ATO. In the present study, it was up-regulated in ATO-treated HL-60 cells and down-regulated in ATO-resistant clones. Elevated levels of mRNA for c-Jun leading to cell death were also observed in Jurkat cells in the authors' previous study.[12] In addition, other authors, e.g., Redondo-Munoz et al. and Wu et al., have demonstrated that ATO induced the activation of c-Jun N-terminal kinase (JNK, an upstream activator of c-Jun), leading to Akt inactivation and induction of apoptosis in various cell systems.[9,21] The c-Jun protein, along with other members of the Jun family (JunB and JunD) and Fos family members (c-Fos, FosB, Fra1, and Fra2) form dimerized transcription factors: activator proteins 1 (AP-1). The AP-1 proteins have been implicated in the regulation of many important biological responses, including opposing cellular responses such as cell cycle progression, transformation, differentiation, and apoptosis. The activity of the AP-1 dimers is defined by their composition.[22] In addition, the activity of c-Jun may be regulated at both the transcriptional level (changes in *JUN* expression) and post-transcriptional level (phosphorylation of c-Jun protein mediated by JNK). The molecular mechanism by which c-Jun triggers apoptosis remains unclear. It is speculated that c-Jun may activate cell death by acting as a transcriptional regulator leading to changes in the ratio of death-inhibiting vs death-enhancing Bcl-2 proteins.[23] On the other hand, c-Jun may also activate members of the IL-1 beta converting enzyme (ICE)-related proteases, known to be overexpressed in apoptotic cells and required in Fas- or TNFR1-mediated apoptosis.[24]

The role of c-Jun in promoting cell growth has been well documented in several studies showing that it participates in at least 2 signaling pathways.[25] The c-Jun proteins may activate cyclin D1 transcription, leading to a progression from G1 to S phase, or may be involved in the negative regulation of p53 transcription and its downstream target, the cyclin-dependent kinase inhibitor 1A (p21). In the present study, the proliferation of ATO-resistant clones was accompanied by strong down-regulation of *JUN*; in addition, changes in *CCND1* (the gene encoding cyclin D1) expression seemed to be c-Jun-dependent. ATO up-regulated the level of *CCND1* mRNA (although to a much lower degree than *JUN*) in HL-60 cells exposed to the highest ATO concentration, and strongly down-regulated it in ATO-resistant clones. Since cyclin D1 is an important regulator of G1/S phase transition, and has been reported to be a direct target of c-Jun transcriptional activation, this observation suggests an important role of cyclin D1 in the development of resistance to ATO. The role, however, is not clear at the moment. The results of the current authors' previous study, in which Jurkat cells showed a strongly elevated level of mRNA for cyclin D1 in the clones resistant to higher ATO concentrations (2.5–5 μM), suggest that different cell type-dependent mechanisms may be induced in the development of resistance to ATO.[12]

In the present study, the transcription of *FOXO1* (the gene encoding forkhead box O1) showed strong up-regulation in ATO-treated HL-60 cells and strong down-regulation in HL-60-derived ATO-resistant clones. FOXO proteins are activators of transcription. They interact with the DNA binding to the insulin response sequence (IRS) that was identified in promoters of genes encoding important proteins involved in the regulation of apoptosis (e.g., Fas ligand, Bim, Bcl-XL) and cell cycle progression (e.g., cyclin-dependent kinase inhibitor 1B, also referred to as p27Kip1, cyclin D1).[26] The involvement of proteins belonging to the FOXO family in apoptosis induction has been clearly demonstrated by others.[27] In addition, FOXO proteins can be regulated at the post-transcriptional level through phosphorylation in 2 signaling pathways, leading to opposite effects. Akt-mediated phosphorylation has been shown to inactivate FOXO proteins, in contrast to c-Jun N-terminal kinase (JNK)-mediated phosphorylation, which activates FOXO.[28] In the present study, the strong up-regulation of *FOXO1* and *JUN* expression detected in ATO-treated HL-60 cells suggests that the JNK pathway might play a role in the induction of apoptosis in these cells. The observation that prolonged exposure to ATO led to down-regulation of *FOXO* in HL-60-derived clones is also noteworthy, although no Akt kinase activation was detected in ATO resistant cells. These results

are consistent with the data published by Grabiec et al., who observed the Akt-independent, but JNK-dependent, reduction of FOXO1 mRNA that was required for the survival of fibroblast-like synoviocytes in rheumatoid arthritis.[29]

This study demonstrated that prolonged incubation of HL-60 cells in the presence of ATO at clinically relevant concentrations induced resistance to the drug. Apparently, this effect was not dependent on Akt activation. Although ATO modified the expression of some genes involved in the PI3K/Akt signaling pathway in both parental HL-60 cells and resistant clones (Fig. 3), the results suggest that the JNK signaling pathway has a stronger impact on the induction of cytotoxicity and resistance to ATO in HL-60 cells than the PI3K/Akt signaling pathway.

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