TREATMENT WITH ARSENIC TRIOXIDE (ATO) AND MEK1 INHIBITOR ACTIVATES THE P73-P53AIP1 APOPTOTIC PATHWAY IN LEUKEMIA CELLS

P73-P53AIP1 apoptotic pathway in leukemia

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ABSTRACT

Arsenic trioxide (ATO) induces differentiation and apoptosis of malignant cells *in vitro* and *in vivo* and has been used in the treatment of a variety of hematological malignancies. We found that in NB4 acute promyelocytic and in K562 erythroleukemia cell lines treatment with the MEK1 inhibitors PD98059 and PD184352 greatly enhances apoptotic cell death induced by ATO alone. Combined treatment results in the induction of the p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1) gene in both cell lines. Since NB4 and K562 cell lines carry an inactive p53 we investigated the possible role of p73, a p53 paralog that has been shown to regulate several p53-target genes including p21, Bax and p53AIP1. We found that MEK1 inhibitors reduce the levels of dominant negative ΔN-p73 proteins and promote the accumulation of endogenous p73α through its transcriptional activation and its tyrosine phosphorylation, resulting in p21 up-regulation and significant cell growth inhibition. ATO reduces ΔN-p73 levels and promotes a p300-mediated acetylation of endogenous p73, thus favouring cell cycle arrest and apoptosis. Finally, the combined treatment with MEK1 inhibitors and ATO enhances the affinity of phospho-acetylated p73 for the p53AIP1 promoter *in vivo*, as determined by chromatin immunoprecipitation experiments, leading to p53AIP1 up-regulation and increased apoptosis.
INTRODUCTION

Arsenic trioxide (ATO) suppresses neoplastic cells growth *in vitro* and *in vivo* by inducing both apoptosis and cell cycle arrest. Under certain conditions, ATO also induces differentiation of leukemia cells (1). Based on the results of multicenter clinical trials (2, 3), ATO is considered the treatment of choice for patients with relapsed acute promyelocytic leukemia (APL), particularly in patients exposed to all-trans retinoic acid (ATRA) within the prior 12 months (4).

Despite the well documented clinical efficacy of ATO the precise mechanisms regulating arsenic-dependent induction of apoptosis have not been elucidated and several molecular targets have been proposed (reviewed in 5), including PML and other nuclear bodies proteins (6, 7), NFkB (8), glucocorticoid nuclear receptors (9) as well as components of the MAPKs signalling cascade (10). The p53-mdm2 pathway has also been shown to be targeted by ATO (6). Whereas the role of p53 in stress responses is well established, recent advances strongly support a pivotal role for the p53 paralogues p73 and p63 (11, 12) in the execution of drug-induced cell death and chemosensitivity of cancer cells in both p53 wild type and p53 null tumors (reviewed in 13). Indeed, p73 is sufficient to trigger cell death independently of the status of p53 (14-16) and, conversely, p53 requires p63 and p73 to induce apoptosis (17).

Multiple TA (transactivation competent, pro-apoptotic and anti-proliferative) p73 COOH-terminal splicing isoforms (α,β,γ,δ,ε,ζ) exist (18). In addition, dominant negative (ΔN) variants are expressed from a second promoter, which lack the amino-terminal transactivation domain, act as trans-repressors of p53 and p73-dependent transcription and possess anti-apoptotic and pro-proliferative potential (13, 19-23). ΔNp73 inhibits both TAp73 and p53-induced apoptosis (13, 19-23). ΔNp73 is induced by TAp73 and p53, thus
creating a dominant-negative feedback loop that regulates p53 and p73 function (24, 25). Enhanced expression of ΔNp73, rather than inactivating mutations within the TP73 gene, has been associated with tumor development (26). The complex expression strategy of the p53 family of tumor suppressors is consistent with the failure to correlate p53 status alone with prognosis and response to anticancer treatments.

The ability of TAp73 proteins to induce cell cycle arrest and apoptosis in cells exposed to anti-cancer drugs rely on their ability to activate a number of p53-responsive elements (p53-RE) containing target genes. p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1), whose expression is induced by p53 and p73 under apoptotic conditions (27, 28), has been recognized as a primary effector gene of wild type p53 and TAp73-induced apoptosis (29). We show here that the p73-p53AIP1 pathway plays an important role in the apoptotic response of NB4 and K562 leukemic cells to ATO and that inhibition of MEK1 activity greatly enhances this response by acting on the same pathway.
MATERIALS AND METHODS

Reagents

ATO was purchased from Sigma (St Louis, MO). A 1 mmol/L stock solution was obtained by dissolving ATO in phosphate-buffered saline (PBS): the solution was diluted to working concentration immediately before use. 100 mM stock solutions of the MEK-1 inhibitors PD98059 (2’-amino-3’-methoxyflavone; Cell Signaling Technology, Beverly, MA) and PD184352 (2-[chloro-4-iodo-phenylamino]-N-cyclopropylmethoxy-3,4-difluoro-benzamide), kindly provided to us by Dr J. S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI), were prepared in dimethyl sulfoxide (DMSO). These reagents are highly selective inhibitors of MEK-1 phosphorylation and activation (30-32). We used 1 µM PD184352 and 10-40 µM PD98059 concentrations that were proved to be effective in vitro in leukemic cells as documented by ourselves and other authors (33-34). The specific phosphatidylinositol 3-kinase (PI3-K) inhibitor Wortmannin was purchased from Sigma and prepared in DMSO.

Apoptosis detection, immunoprecipitation and immunoblotting

To evaluate apoptosis, after in vitro treatment with PD98059 and/or ATO, cells were collected by centrifugation, washed twice in cold PBS, and permeabilized in 90% ethanol, 10% PBS prior to DNA staining. The permeabilized cells were incubated with 50 µg/ml propidium iodide, 100 units/ml RNase A (Sigma), 0.1% Nonidet P-40, and 0.1% trisodium citrate for 30 min prior to analysis using a Becton Dickinson FACSort analyzer. Cells with a hypodiploid DNA content (< 2n, > 0.2n) were counted as apoptotic. Flow cytometry was performed with a FACSCalibur apparatus (Becton Dickinson). Data were analyzed using FlowJo 3.4 software (Tree Star, San Carlos, California). Immunoblotting and immunoprecipitations assays were performed essentially as described (33, 34).
Immunoprecipitations were carried out by incubating 2000-3000 µg of total cell lysate as described (28, 33, 35).

**RT-PCR assay**

After 24 hours of treatment, total RNAs were extracted (Trizol Reagent, Gibco-BRL LifeTechnologies, Burlington, Ontario, Canada) and reverse transcribed using the SuperScript One-Step RT-PCR with Platinum Taq kit (Gibco-BRL Life Technologies) and the resulting cDNA were amplified with primers specific for ΔN-p73, TA-p73 and β-Actin. The primer sequences were as follows p73TA forward: TTG CTA TGG ACG TCT TCC ACC TGG, p73TA reverse: AGA GCT GGG TTG TGC GAA GGG CGA GTG GGT GG-, p73ΔN forward: AGT TGA CAG AAC TAA GGG AGA TGG G-, p73ΔN reverse: TGC TCA GCA GAT TGA ACT GGG.

**Chromatin immunoprecipitation assay**

Protein complexes were cross-linked to DNA in living nuclei by adding formaldehyde (Merk, Inc.) directly to tissue culture medium to a final concentration of 1%. Cross-linking was allowed to proceed for 10 min at room temperature and was then stopped by the addition of glycine to a final concentration of 0.125 M. Cross-linked cells were washed with phosphate-buffered saline, and swelled in RSB buffer (3 mM MgCl2, 10 mM NaCl, 10 mM Tris-chloride (pH 7.4), and 0.1% IGEPAL CA-330 (Sigma)). Nuclei were pelleted by microcentrifugation and lysed by incubation in nuclear lysis buffer (1% sodium dodecyl sulfate, 10 mM EDTA, 50 mM Tris-chloride (pH 8.1), 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin per ml, and 100 ng of aprotinin per ml). The resulting chromatin solution was sonicated for 10 pulses of 20 seconds at 80% power to generate 300-2000 bp DNA fragments. After microcentrifugation, the supernatant was precleared with
blocked protein A-positive Staph cells (Boehringer Mannheim), diluted 1:5 with dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-chloride (pH 8.1), 167 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 100 ng of leupeptin per ml, and 100 ng of aprotinin per ml), and divided into aliquots. Five micrograms of anti-p73 (H79) antibody was added to each aliquot of chromatin and incubated on a rotating platform for 12 to 16 h at 4°C. Antibody-protein-DNA complexes were isolated by immunoprecipitation with blocked protein A-positive Staph A cells. Following extensive washing, bound DNA fragments were eluted and analyzed by subsequent PCR using primers specific for the bax, p21 and p53AIP1 promoters. Primers sequences are available upon request.

**siRNA transfections**

Prior to electroporation, NB4 and K562 cells were washed twice with serum-free Opti-MEM (Gibco BRL Paisley, United Kingdom) and resuspended to a final concentration of 5.10^6 cells/mL in Optimem-MEM (Gibco BRL). Subsequently, 0.5 mL of cells suspension was mixed with 10 µg of Fluorescein-labeled double-stranded RNA oligonucleotides (siRNA) specific for either GFP and TA-p73 and electroporated in a 0.4-cm cuvette using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories Inc., Hercules, California, USA) using a single-pulse protocol (voltage 260 V and Capacitance 1050 µF). Under these conditions we consistently reached a transfection efficiency of 80% or more without significant reduction of viability in both cell lines (data not shown and 36). 24 hours after transfection cells were treated with the different compounds for 72 hours and FITC-positive cells were analyzed for sub-G1 DNA content. To check for siRNA efficiency (Figure 4B rows a) we transfected NB4 cells with an expression vector encoding for an HA-tagged version of TAp73 (28) alone or in the presence of siRNAp73 or
siRNAGFP. Cells were lysed 24 hours after transfection and TA-p73 expression was assessed by anti-HA immunoblot. The sequences of GFP and TA-p73 specific fluorescein-labeled siRNAs is available upon request.

**Statistical analysis**

The statistical analysis was performed using the Dunnet test.
RESULTS AND DISCUSSION

Recent studies suggest that components of the pro-survival signal transduction pathways involving Ras and the mitogen-activated protein kinases (MAPKs), can confer an aggressive, apoptosis-resistant phenotype to leukemia cells. The use of small molecule MEK1 inhibitors like PD98059 or PD184352, can induce apoptosis \textit{in vitro} and \textit{in vivo} and could sensitize leukemia cells to drug-induced apoptosis (33, 34, 37, and reviewed in 38, 39).

We observed that the combined treatment with MEK1 inhibitors and Arsenic Trioxide (ATO) significantly increased the amount of apoptotic cells, as compared to ATO alone, in both the NB4 promyelocytic leukemia and K562 erythroleukemia cell lines. The percentage of sub-G1 apoptotic cells after 72 hours of treatment with MEK1 inhibitor PD98059 (40 \(\mu\)M) and ATO (1 \(\mu\)M in NB4 cells, 2 \(\mu\)M in K562 cells) was 2.7 or 3.2 fold higher than in ATO alone treated cells (Figure 1A). Similar results were obtained with PD184352 (1 \(\mu\)M) (Figure 4A).

Apoptosis and cell cycle arrest are the major tumor suppression functions of p53 but in K562 and NB4 leukemic cells this protein has lost the ability to bind and activate its target genes (11, 40). Given the well recognized ability of p73 to transactivate p53 target genes (11, 13) irrespective to p53 status we investigated p73’s role in ATO-induced apoptosis.

First, we assessed the effect of MEK1 inhibitors and/or ATO treatment on \(\Delta Np73\) and TAp73 expression. We found that \(\Delta Np73\) expression was decreased, both at the mRNA (Figure 1B) and the protein level (Figure 1C and 1D, rows a), after PD98059 40 \(\mu\)M or PD184352 1\(\mu\)M treatment in NB4 and in K562 cells. Under ATO exposure NB4 showed only a slight decrement in \(\Delta Np73\) expression, whereas a more marked decrement in \(\Delta Np73\)
protein levels was observed in ATO-treated K562 cells (Figure 1C and 1D, rows a). When
NB4 and K562 cells were exposed to a combination of PDs and ATO a striking
cooperative effect was observed (Figures 1C and 1D, rows a). Endogenous TAp73α
protein expression was sharply increased after MEK1 inhibition, whereas only a slight
increment of TAp73α was observed in both cell lines after ATO treatment (Figures 1C and
1D, rows b). Although both cell lines showed a MEK1 inhibition-mediated increment of
TAp73α protein, a significant TAp73 transcriptional activation after PD treatment was
observed only in NB4 cell line (Figure 1B).

Altogether, our analysis indicates that the treatment with PD98059 or PD184352 promotes
the accumulation of endogenous TAp73α and the reduction of ΔN-p73, both events
contributing to the observed p73-dependent cell cycle arrest (Figure 1A). Since ATO, that
is able to induce NB4 and K562 apoptotic cell death (Figure 1A), was relatively less
efficient than MEK1 inhibitors in elevating TA/ΔN p73 ratio, we sought to compare ATO
and MEK1 inhibitors ability to induce p73 acetylation, a post-translational modifications
that is known to boost TA-p73 proapoptotic activity (28). Specific phosphorylation and
acetylation events contribute to the activation of p73 gene products in response to DNA
damage and bolster p73 apoptotic functions by potentiating the selective recruitment of
p73 onto the promoters of apoptotic target genes versus gene involved in cell cycle arrest
and re-entry (14-16, 28). Since ATO has been shown to induce DNA strand breaks and
DNA-protein cross-links in a variety of cell lines (41-45) we asked whether apoptotic
concentrations of ATO were able to induce TAp73α acetylation and/or tyrosine
phosphorylation. We found that endogenous TA-p73α was strongly acetylated in response
to apoptotic concentrations of ATO, but not after PD98059 treatment, in both NB4 and
K562 cell lines (Figure 2A). Similar results were observed with the MEK1 inhibitor
PD184352 (1 μM) (data not shown). To investigate the mechanisms underlying the
increased levels of p73 acetylation in response to ATO treatment we first evaluated if ATO treatment influenced the physical interaction between p73 and the acetyltransferase p300, which is known to acetylate p73 in response to doxorubicin and cisplatin treatment (28). As shown in figure 2B (rows a), treatment of both NB4 and K562 cells with ATO or ATO+PD increases the levels of p73-p300 interaction as detected by anti p73 immunoprecipitation experiments followed by anti-p300 immunoblotting. Endogenous p73 is both activated and stabilized in response to γ-irradiation, doxorubicin and cisplatin through a c-abl-dependent pathway, participating in the apoptotic response to DNA damage (14-16). To investigate whether the accumulation of p73 correlated with its tyrosine phosphorylation, we immunoblotted the anti-p73 immunoprecipitates from ATO and/or PD treated cells with anti-phospho-tyrosine antibodies. A very slight increment in TAp73α tyrosine phosphorylation was observed in NB4 and K562 cells after ATO treatment (Figure 2B, rows b). By contrast, a strong tyrosine phosphorylation of p73α was observed after PD treatment in both cell lines (Figure 2B, rows b). The stronger tyrosine-phosphorylation of p73α observed after PD compared to ATO treatment well correlated with the accumulation of p73α observed in PD treated cells (Figures 1C and 1D, rows b and 2B, rows c). Altogether, these results strongly suggest that the high levels of apoptosis we observed in ATO + PD treated cells is the result of the increased levels of phospho-acetylated p73 species. It is noteworthy to underline that the kinetics of p73 phosphorylation/acetylation in NB4 and K562 cells are quite different. The phosphorylation of p73 as well as its interaction with acetyltransferase p300 are earlier events in NB4 (2 hours) as compared to K562 (24 hours) (Figure 2B, rows a and b). It is tempting to speculate that this differential behaviour might be the consequence of an altered DNA damage-cAbl-p300-p73 pathway in the K562 cells due to the presence of p210 BCR-Abl (46).
To evaluate whether ATO-induced acetylation/phosphorylation of p73 modulates its affinity to cell cycle arrest and apoptotic target genes, we performed a chromatin immunoprecipitation (ChIP) assay using anti-p73-specific antibodies to immunoprecipitate crosslinked chromatin from untreated or PD98059, ATO and PD98059+ATO treated cells. As shown in figure 3A, after 24 hours of PD98059 treatment p73 was readily recruited to the p21 promoter whereas after either ATO or PD98059+ATO treatment p73 was strongly recruited to the promoters of proapoptotic genes Bax and p53AIP1 and, conversely, its affinity to the p21 was reduced.

The recruitment of p73 to the apoptotic target genes Bax and p53AIP1 (Figure 3A) correlates with p73 acetylation status (Figure 2A). Moreover, after ATO treatment p73 was acetylated and recruited somehow more efficiently on Bax as compared to p53AIP1 promoter. When ATO was associated with PD98059 the further increase of p73 recruitment to the p53AIP1 well correlates with the striking increase of the sub-G1 population observed after PD98059+ATO combined treatment (Figure 1A and 4A). The combined treatment also led to an increased poly (ADP-ribose) polymerase (PARP) fragmentation that reflects increased apoptosis (Figure 3B). Interestingly, we found that Bax protein accumulated to a greater extent after ATO treatment than after PD98059+ATO treatment in both cell lines (Figure 3B) whereas p53AIP1 expression was greatly enhanced after PD98059+ATO treatment compared to ATO alone (2.4 and 4.0 fold increase in K562 and NB4 cells, respectively). Similar results were obtained with PD184352+ATO (data not shown). Interestingly, also in these experiments we observed a delay in Bax and p53AIP1 up-regulation in ATO and PD+ATO treated K562 (72 hour) versus NB4 (48 hours) cell line (Figure 3B). Since the Bcr-Abl kinase is known to exert resistance against apoptosis (46, 47) and given the capability of ATO to downregulate Bcr-Abl protein levels (48, 49), we postulate that the delay occurred in K562 was Bcr-Abl expression dependent. As shown
in figure 3D, an exposure interval of 24 hours of ATO treatment was necessary to sensibly decrease the level of Bcr-Abl protein in K562. In addiction, the longer interval in PD-mediate p73 tyrosine phosphorylation and its interaction with p300 occurred in ATO treated K562 (24 hours) versus NB4 (2 hours), as shown in figure 2B, might be explained by the same mechanism.

Finally, we have performed a combined analysis of cell growth inhibition and induction of apoptosis in NB4 and K562 cells treated with the different drug combinations. PD98059 (40 or 10 µM) or PD184352 (1 µM) alone triggers significant cell growth inhibition and only slight induction of apoptosis, that is more evident in NB4 cells (Figure 4A). This biological effects well correlate with PD’s ability to promote both the accumulation of endogenous p73α, through its transcriptional up-regulation and tyrosine phosphorylation, and the reduction of ΔN-p73 (Figures 1B, 1C, and 1D); with p21 gene upregulation (Figure 3C) and with PD’s inability to induce p73 acetylation (Figure 2A) and p73 recruitment onto the promoters of p53/p73 apoptotic target genes (Figure 3A). ATO alone induces both growth inhibition and apoptosis (Figure 4A) as a result of an increased expression of both p21 and Bax (Figures 3B and 3C), the down-regulation of ΔN-p73 expression, more relevant in K562 (Figure 1C and 1D), and the acetylation of endogenous p73 (Figure 2A). When PD98059 and ATO were combined p73 acetylation was strongly induced (Figure 2A), the affinity of p73 for the p53AIP1 promoter was boosted (Figure 3A) resulting in the up-regulation of the p53AIP1 expression (Figure 3B) and a greatly enhanced apoptosis of treated cells (Figures 1A and 4A). As shown in figure 4A, the percentage of sub-G1 apoptotic cells after 72 hours of treatment with MEK1 inhibitors, PD98059 (40 µM and 10 µM) or PD 184352 (1 µM), in combination with ATO (1 µM in NB4 cells, 2 µM in K562 cells) was significantly higher than in ATO alone treated cells: means % ± SD of apoptotic cells in PD plus ATO vs ATO treated cells were 85.4% ± 8.2 (PD98059 40 µM + ATO),
72.6% ± 7.7 (PD98059 10 μM + ATO), 83.2% ± 7.0 (PD 184352 1 μM + ATO) vs 41.3% ± 9.8 (ATO 1 μM) (P <0.001) and 47.6% ± 4.8 (PD98059 40 μM + ATO), 38.2% ± 3.9 (PD98059 10 μM + ATO), 44.5% ± 6.7 (PD 184352 1 μM + ATO) vs 19.3% ± 4.8 (ATO 2 μM) (P <0.001) respectively in NB4 and K562 cells.

To test the specificity of the pathways involved in the activation of p73-dependent pathways in our system, we also inhibited the phosphatidylinositol-3-kinase (PI3K) pathway with the specific inhibitor wortmannin (30). No relevant effects on induction of apoptosis in NB4 and K562 cells were observed when ATO was associated with the PI3K inhibitor Wortmannin (0,2 and 1 μM) (Figure 4A).

Collectively, our results support a model in which an elevated TA/ΔN p73 ratio, together with an increased recruitment of TAp73 onto its apoptotic target genes promoters due to TAp73 acetylation and its tyrosine phosphorylation both contribute to ATO induced apoptosis and its enhancement by PDs co-treatment. Further investigation will be necessary to determine whether additional posttranslational modification(s) and the kinase(s) are involved in these mechanisms. Given the homology between p53 and p73, is not excluded that HIPK2 (Homeodomain-interacting protein kinase-2) could play an important role in these processes. This serine/threonine kinase binds to and phosphorylates at Ser-46 p53 (50) inducing a subtle change of p53 conformation that enhances the affinity to the promoters of apoptosis related genes such as P53AIP1 (27); moreover HIPK2 interacts with p73 in vivo (51).

Finally, to determine the contribution of p73-p53AIP1 pathway activation in mediating PD+ATO-induced apoptosis, the TA-p73 mRNA was selectively knocked-down by means of specific fluorescein-labeled double-stranded RNA oligonucleotides (siRNA). Recently, siRNA have been shown to achieve a high degree of specificity with low toxicity also in mammalian cells (52, 53) acting through a degradative chain reaction catalyzed by the
activation of a cellular RNA-dependent RNA polymerase (54-56). The efficiency of siRNA mediated TA-p73 downregulation was evaluated by transient transfection experiments. Transfection of TA-p73 siRNA, but not the unrelated GFP siRNA, led to decrease TA-p73 in NB4 cells (Figure 4B rows a) without affecting the levels of the unrelated protein actin (Figure 4B, rows a). The percentage of sub-G1 apoptotic NB4 and K562 cells after 72 hours of treatment with MEK1 inhibitor PD184352 (1 μM) and ATO (1 μM in NB4 cells, 2 μM in K562 cells) was significantly diminished in cells transfected with TA-p73 siRNA relative to cells transfected with control siRNA (Figure 4B, rows b). These findings indicate that p73 is a major determinant of PD+ATO efficacy in leukemia cells carrying an inactive p53. A number of recent reports have shown the importance of a functional p73 for tumor cells chemosensitivity (57, 58). Our observations further confirm this concept and suggest that modulation of p73 proteins expression and/or function might represent in the future a new molecular target for leukemia treatment.
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FIGURE LEGENDS

Figure 1. MEK-1 inhibition sensitizes leukemic cells to ATO-induced apoptosis. (A) NB4 and K562 cell lines were seeded at 1x10^5 in the presence of DMSO (vehicle) or PD98059 for 3 hours, and then incubated for 72 hours with the indicated concentration of ATO. Apoptosis was then measured as percentage of cells with hypodiploid DNA content. Results are representative of one of three indipendent experiments. (B) Total RNAs extracted from leukemic cells treated for 24 hours, were reverse transcribed using the specific primers for ΔN-p73 and TA-p73. NB4 (C) and K562 (D) cell lines were seeded at 1x10^5 in the presence of DMSO (vehicle), PD98059 40 μM or PD184352 1 μM for 3 hours, and then incubated for 24 hours with the indicated concentration of ATO. Endogenous p73α and ΔNp73 proteins were revealed by immunoblotting analysis using a mouse monoclonal anti-p73 (clone 1288 Imgenex, Inc., San Diego, CA), or a mouse monoclonal anti-ΔNp73 (clone 38C674 Imgenex, Inc). Anti-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) immunoblotting was performed as loading control. β-actin, ΔN-p73 and TA-p73 bands were subjected to densitometric scanning using the TINA 2 software (Raytest Isotopenmessgerate GmbH, Germany) and the ΔN-p73: β-actin or TA-p73: β-actin ratio was calculated .

Figure 2. Apoptotic doses of ATO induce TA-p73α acetylation and MEK1 inhibition promotes TA-p73α tyrosine phosphorylation in leukemic cells. (A) The leukemic cells were either pre-treated with DMSO or PD98059 40 μM for 3 hours and then treated with ATO 1 μM (NB4) or 2 μM (K562) for 24 hours. Lysates form treated cells were
subsequently immunoprecipitated with a rabbit polyclonal anti-p73 (H-79 Santa Cruz Biotechnology) or with a control antibody from lysates of NB4 and K562 treated with ATO. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using a rabbit polyclonal anti-acetylated lysines (Upstate Biotechnology, Inc., Lake Placid, NY). For immunoprecipitation a rabbit polyclonal anti-p73 antibody was used (H-79 Santa Cruz Biotechnology). To evaluate the relative levels of p73 acetylation, bands were subjected to densitometric scanning using the TINA 2 software (Raytest Isotopenmessgerate GmbH). (B) The leukemic cells were either pre-treated with DMSO or PD184352 1 µM for 3 hours and then treated with ATO 1 µM (NB4) for 2 hours or 2 µM (K562) for 24 hours. Extracts from mock and treated NB4 and K562 cells were immunoprecipitated with a rabbit polyclonal anti-p73 (H-79 Santa Cruz Biotechnology) or with a control antibody. The immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using a rabbit polyclonal anti-p300 antibodies (N-15 Santa Cruz Biotechnology) (rows a) or a mouse monoclonal anti-phosphotyrosine antibody (clone 4G10 Upstate Biotechnology, Inc) (rows b). An 1/5 aliquot of the immunoprecipitated material was immunoblotted with the anti-p73 monoclonal antibody (clone 1288 Imgenex, Inc) (panel c).

Figure 3. Endogenous p73α is recruited onto apoptotic target genes in vivo in response to ATO. (A) Chromatin immunoprecipitation assay was performed from untreated or PD98059, ATO and PD98059+ATO treated NB4 and K562 leukemic cells. Genomic DNA obtained from untreated or treated cells was employed to normalize the DNA to immunoprecipitation. An anti-p73 (H79 polyclonal antiserum from Santa Cruz Biotechnology) or control unrelated antibodies were used. Immunoprecipitated material was amplified using primers specific for p21 or p53AIP1 or bax promoters. (B) NB4 or K562 cells, after 3 hours pretreatment with PD98059, were incubated with the indicated
concentration of ATO. Expression of PARP, p53AIP1 and Bax were revealed after 24 and 48 hours in NB4 and after 24, 48 and 72 hours of treatment in K562 cells. (C) p21 protein levels were assessed by immunoblotting after 24 hours in NB4 and after 48 hours treatments in K562 cells. (D) Expression of Bcr-Abl after 24 hours treatments. Cell lysates were analyzed by immunoblotting analysis using a mouse monoclonal anti-PARP (F2) (Santa Cruz Biotechnology), rabbit polyclonal anti-p53AIP1 (CT) (AnaSpec, Inc. San Jose, CA), rabbit polyclonal, anti-Bax (Cell Signaling Technology Inc. Beverly, MA), HRP-coniugate anti-p21WAF1/CIP1 (Santa Cruz Biotechnology), mouse monoclonal anti-c-Abl (24-11 Santa Cruz Biotechnology) and goat polyclonal anti-human actin (Santa Cruz Biotechnology). Anti-actin immunoblotting (Santa Cruz Biotechnology) was performed as loading control.

Figure 4. Abrogation of TA-p73 expression inhibits PD+ATO-induced apoptosis in leukemia cells

(A) NB4 and K562 cells seeded at 1x10^5 cells/mL were pre-treated for 3 hours with MEK1 inhibitors PD98059 (40 and 10 μM) or PD184352 1 μM or with the PI3K inhibitor Wortmannin (0.2 and 1 μM) and then incubated for 72 hours with the indicated concentration of ATO. Viable cells were counted by the trypan blue dye exclusion method and apoptosis was measured as the percentage of cells with hypodiploid DNA content. Each value represents the mean ± SD of four independent experiments. (B rows a) NB4 cells were transfected with an expression vector encoding for an HA-tagged version of TA\(^{p73}\) alone or in the presence of siRNA\(^{p73}\) or siRNA\(^{GFP}\). Cells were lysed 24 hours after transfection and TA-p73 expression was assessed by anti-HA immunoblot.
K562) or PD+ATO for 72 hours prior to apoptosis analysis. Values are the mean ± SD of three independent experiments.
Figure 1

A

B

C

D

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Figure 2

**A**

**NB4 24h**

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**Relative acetylation**

**Ac-p73**

**IB**

anti Lys Acetylated

**K562 24h**

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**Relative acetylation**

**Ac-p73**

**IB**

anti Lys Acetylated

**B**

**NB4 2h**

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**p300**

**IB**

anti-p300

anti-p300

p-Tyr p73c

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**p-Tyr p73c**

**IB**

anti-p300

anti-p73

anti-Tyr Phosphorylated

**p73c**

**IB**

anti-p300

anti-p73

**K562 24h**

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**p300**

**IB**

anti-p300

anti-p73

p-Tyr p73c

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**p-Tyr p73c**

**IB**

anti-p300

anti-p73

anti-Tyr Phosphorylated

**p73c**

**IB**

anti-p300

anti-p73


Figure 4

A

**NB4 72h**

- QSOx
- BSO 0.0100 60 μM
- BSO 0.0100 10 μM
- BSO 0.0100 1μM

- QSOx
- WORTMANNIN 0.2 μM
- WORTMANNIN 1 μM

**K562 72h**

- QSOx
- BSO 0.0100 40 μM
- BSO 0.0100 10 μM
- BSO 0.0100 1 μM

- QSOx
- WORTMANNIN 0.2 μM
- WORTMANNIN 1 μM

B

**siRNA GFP**

- HA-p73α
- siRNA TAp73

- IB anti-HA
- IB anti-actin

**NB4 72h**

- CTR
- PD
- AS
- PD+AS

**K562 72h**

- CTR
- PD
- AS
- PD+AS
Treatment with arsenic trioxide (ATO) and MEK1 inhibitor activates the P73-P53AIP1 apoptotic pathway in leukemia cells

Paolo Lunghi, Antonio Costanzo, Massimo Levrero and Antonio Bonati