MEK1 INHIBITION SENSITIZES PRIMARY ACUTE MYELOGENOUS LEUKEMIA TO ARSENIC TRIOXIDE-INDUCED APOPTOSIS

PAOLO LUNGHl, ANTONIO COSTANZO, LUIGI SALVATORE, NELIDA NOGUERA, LAURA MAZZERA, ANTONIO TABILIO, FRANCESCO LO-COCO, MASSIMO LEVRERO and ANTONIO BONATI

1Department of Clinical Sciences, Section of Hemato-Oncology, University of Parma; 2Department of Dermatology, University of Rome “Tor Vergata”; 3Department of Biopathology, section of Hematology, University of Rome “Tor Vergata”; 4Department of Clinical and Experimental Medicine, Section of Hematology and Clinical Immunology, University of Perugia; 5Department of Internal Medicine, and Laboratory of Gene Expression, Fondazione Andrea Cesalpino, University of Rome “La Sapienza”, 6Department of Experimental Oncology, CRS-Regina Elena Cancer Institute, Rome, 7Rome Oncogenomic Center (ROC).

Supported by grants: from Associazione Italiana per la Ricerca sul Cancro (AIRC) to A.B., A.C., M.L. and F.L.-C., from the “Ministero dell’Istruzione dell’Università e della Ricerca Scientifica” (MIUR FIN to A.B., FIL to A.B., PRIN to M.L., FIRB to M.L. and “Progetto Strategico Oncologia SP/4: Terapia preclinica molecolare in oncologia” to A.B.), from the European Community (LSHC-CT-2004-503576 to M.L.) and from Telethon (to M.L.).

Corresponding authors:
Antonio Bonati, Dipartimento di Scienze Cliniche, Via Gramsci 14, 43100 Parma, Italy. Phone: +39-0521-702702; +39-335-6000942; Fax: +39-0521-941290; e-mail: antbonny@unipr.it
Paolo Lunghi, Dipartimento di Scienze Cliniche, Via Gramsci 14, 43100 Parma, Italy. Phone: +39-330-764831; Fax: +39-0521-941290; e-mail: p.lunghi@libero.it

Words count: 1681

Scientific heading: neoplasia
ABSTRACT
We have found that MEK1 inhibitor PD184352 strikingly increases apoptosis induced by Arsenic Trioxide (ATO) in twenty-one out of twenty-five primary acute myelogenous leukemia (AML) cases. Isobologram analysis confirmed the synergistic (13/25 cases) or additive (8/25 cases) nature of this interaction. Moreover we demonstrate that the p53-related gene p73 is a molecular target of the combined treatment in AML blasts. Indeed, ATO modulates the expression of the p73 gene by inducing both the pro-apoptotic and anti-proliferative TAp73 and the anti-apoptotic and pro-proliferative ΔNp73 isoforms thereby failing to elevate TA/ΔNp73 ratio. Conversely the treatment with PD184352 reduces the level of ΔNp73 and blunts the arsenic-mediated up-regulation of ΔNp73, thus causing a raise of the TA/ΔNp73 ratio of dual-treated cells. Moreover, high doses of ATO induce p53 accumulation in 11/21 cases. Combined treatment results in the induction of the proapoptotic p53/p73-target gene p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1), and greatly enhances apoptosis of treated cells.

INTRODUCTION
Arsenic Trioxide (ATO) is considered the treatment of choice for patients with relapsed acute promyelocytic leukemia (APL) particularly in patients exposed to all-trans retinoic acid (tRA) within the prior 12 months (1).

Many trials involving ATO have been initiated testing its ability to treat hematological malignancies such as refractory AML, myelodisplastic syndromes, non Hodgkin lymphoma, chronic lymphocytic or chronic myelogenous leukemia, acute T cell leukemia and multiple myeloma (reviewed in 2, 3). Some of these trials have shown clinical effects, but nothing has come close to APL success.
We and others have suggested that a combination therapy with drugs targeting specific pro-
survival, anti-apoptotic molecules, or capable to enhance pro-apoptotic pathways may lead to an
improvement of ATO efficacy against hematological malignancies (4-7).

In this paper we tested the apoptotic activity of ATO in different subtypes of AML when combined
with an MEK1 inhibitor and we could confirm and expand in primary AML leukemia cells our
previous findings obtained in NB4 APL and in K562 Bcr/Abl + leukemic cell lines (4, 7).

STUDY DESIGN

Twenty-five cases of non-APL AML were investigated (Table 1). Leukemia cells were isolated and
enriched as described (7).

ATO was purchased from Sigma (St Louis, MO); PD184352, a potent and highly selective MEK1/2
inhibitor (8), was kindly provided by Dr J.S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer
Global Research & Development, Ann Arbor, MI).

Cell lysis, in vitro treatment, apoptosis assays, immunoblotting, transfection and statistical analysis
were carried out as previously described (4, 7).

Approval for the study was obtained from the Department of Clinical Sciences, University of
Parma, institutional review board for these studies. Informed consent for blood samples was
provided according to Declaration of Helsinki.

RESULTS AND DISCUSSION

Twenty-five primary non-APL AML samples with different FAB classification were used in this
study (Table 1). We first analyzed the pharmacologic interactions between ATO and PD184352
using a fixed-ratio experimental design and found that combined treatment with PD plus ATO
resulted in the synergistic (CI \leq 0.85) , additive (0.85<CI<1.1) or antagonistic (CI\geq 1.1 ) induction of
apoptosis in 13, 8 or 4 cases of primary AML blasts, respectively (Table 1).

We recently demonstrated the involvement of the proapoptotic p73-p53AIP1 pathway in NB4 and
K562 cell lines treated with PD+ATO (4). p73 is a p53 paralogue (9-11), able to transactivate the promoters of several p53-responsive genes involved in apoptosis and cell cycle regulation (9-11). Indeed, p73 is sufficient to trigger cell death independently of the status of p53 (12-14) and, conversely, p53 requires p63 and p73 to induce apoptosis in response to DNA damaging drugs (15). However, p73 exists as multiple TA (transactivation competent, pro-apoptotic and anti-proliferative) p73 COOH-terminal splicing isoforms (α,β,γ,δ,ε,ζ) of which the two major forms are p73α and p73β (16). 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 (17-20).

In order to evaluate whether the combined treatment modulates p73 isoforms in AML blasts, the protein expression of TA-p73α, TA-p73β and ΔN-p73 were evaluated before and after the treatment with PD and/or ATO in 21/25 AML cases and the TA/ΔN-p73 ratio was calculated (Table 1 and representative cases in Figure 1A and B).

The basal expression of TA-p73α and TA-p73β were clearly evident in 19/21 and 20/21 cases respectively (Table 1 and representative cases in Figure 1A). In addition ΔN-p73 expression was detectable in 19/21 cases. These latter observations are in good agreement with a recent report showing the expression of ΔN-p73 in over 90 percent of non-APL AML cases analyzed (21).

In the responsive cases (with the exception of case no. 16 lacking both TA-p73α and ΔN-p73 expression, see Table 1) we found that MEK1 inhibitor reduced the levels of dominant negative ΔNp73 proteins and promoted the accumulation of endogenous TA-p73α and/or TA-p73β elevating the TA/ΔN ratio (see representative cases in figure 1A, 1B and Table 1). ATO alone promoted the increase of both TA and ΔN-p73 proteins expression failing, or resulting less efficient than PD or PD+ATO in elevating TA/ΔN ratio (see representative cases in figure 1A, 1B and Table 1). The expression of p73 isoforms in ATO-treated blasts was quite different from that we observed in NB4 and K562 cell lines where the ATO treatment did not affect TA-p73 and partially reduced ΔN-p73
in both cell lines (4). Moreover in the responsive case no. 12 presenting a strong basal expression of ΔN-p73 the treatment with ATO did not modulate its expression, conversely PD strongly inhibited the ΔN expression (Table 1 and data not shown). Interestingly, the lack of p73 proteins expression (case no. 5) or the inability of PD in elevating TA/ΔN-p73 ratio (case no. 18) resulted in loss of efficacy by PD+ATO treatment (Figure 1A, B and Table 1).

Since in AML, p53 mutations have been detected only in about 5% of patients, mostly with 17p monosomy (22-25), we also studied the expression of p53 protein in 21/25 cases. Interestingly, in 11/21 (52%) cases p53 was strongly induced (more than two fold increase compared to control, see Table 1) after the treatment with ATO 2μM or PD+ATO 2μM whereas, in the majority (20/21) of the cases analyzed, neither PD nor ATO 1μM mono-treatment (nor their combination) were able to significantly promote p53 accumulation (see representative cases in figure 1A and Table 1). Taken together these data indicate that in AML blasts an high concentration of ATO (2μM) promotes the accumulation of both p73 and p53 protein levels whereas at low concentration (1μM) ATO only induces p73.

Next we evaluated whether the changes in TA/ΔN-p73 ratio observed in PD+ATO vs ATO treated cells resulted in an increased expression of the proapoptotic p53/p73 target genes Bax, PUMA (p53 up-regulated Modulator of Apoptosis) (26, 27), and P53AIP1 (p53-regulated Apoptosis-Inducing Protein 1). P53AIP1, a primary effector gene of wild type p53 and TAp73-induced apoptosis (28, 29), is located in mitochondria and its overexpression induces massive apoptotic cell death through dissipation of mitochondrial ΔΨm (28). Interestingly, after a 48 hours treatment we found that Bax and PUMA proteins accumulated to a greater extent when cells were challenged with ATO treatment than with PD+ATO in 13/16 AML. p53AIP1 expression was greatly enhanced after PD+ATO treatment compared to ATO alone (2 or more fold increase, see representative cases in Figure 1C), thereby confirming our previous data that PD and ATO cooperate to induce and activate the p73-p53AIP1 pathway (4). In the responsive case no. 23 the combined treatment did not
upregulate P53AIP1 but the amount of Bax expression was 2.8 fold more compared to ATO treatment (data not shown). Conversely no differences in p53AIP1, Bax and PUMA expression between ATO and PD+ATO treatment were observed in non responsive cases (representative case no. 18 shown in Figure 1C). In the responsive cases the combined treatment also led to an increased poly (ADP-ribose) polymerase (PARP) fragmentation that reflected the increased apoptosis (Figure 1C). In agreement with the biochemical findings indicating the activation of the mitochondrial apoptotic pathway, we could also demonstrate that the combined treatment with PD184352 and ATO strikingly potentiates the loss of ΔΨ\textsubscript{m} induced by ATO alone in the responsive AML blasts (representative cases in Figure 1D). Indeed, isobologram analysis confirmed that the proapoptotic interaction between PD and ATO was synergistic (CI≤0.85; Figure 1D inset). Loss of ΔΨ\textsubscript{m} and decrease in the DNA content to sub-G1 levels were paralleled by the exposure of phosphatidylserine on the outer leaflet of the plasma membrane (Annexin V assay) (see representative cases in figure 1E).

It is noteworthy that treatment with ATO at 1μM, a dose at which p73 but not p53 is accumulated, either alone or in combination with PD, led to increased levels of PUMA, BAX or p53AIP1, thus suggesting that p73 indeed plays an important role in the transcriptional activation of p53/p73 proapoptotic target genes and apoptosis induction in most AML cases (see representative cases 3, 6 and 22 in Figure 1C). At higher concentrations of ATO the accumulation of both p73 and p53 indicates that both pathways could contribute to ATO or PD+ATO induced apoptosis.

Recently, we demonstrated that MEK1 inhibition sensitizes both parental and arsenic resistant NB4 cell lines and APL primary blasts to ATO-induced apoptosis through the MEK1 inhibition-mediated de-phosphorylation of Bad at Ser112, leading to an increased capacity to heterodimerize with Bcl-xL and Bcl-2 thereby blocking theirs antiapoptotic functions (7, 31, 32). Since Bcl-2 interacts with P53AIP1 in the mitochondrion and the overexpression of Bcl-2 blocks both the ΔΨ\textsubscript{m} down-regulation and p53AIP1 proapoptotic activity (28, 33) we also investigated Ser112 phosphorylation and Bad protein levels after ATO treatment, both with and without MEK1 inhibitor.
in primary AML blasts (13 cases). Pre-treatment with MEK1 inhibitor strongly increased the expression of de-phosphorylated Bad and blunted the ATO-mediated phosphorylation of Bad at Ser112 in all the AML cases analyzed, thus suggesting that this pathway might contribute, together with the p73-P53AIP1 pathway, to the induction of apoptosis in dual treated cells (see representative cases in Figure 2).

To better understand the effect of ATO and MEK inhibitor on cell proliferation in AML blasts, we have analyzed cell cycle (25 cases) and p21 modulation (14 cases). We found that treatment with the MEK1 inhibitor and/or ATO significantly decreased cell cycle progression (Figure 3A) and up-regulated p21^Waf1/CIP1 (see representative cases in Figure 3B) in the responsive cases, but not in non-responsive cases.

Finally, in order to confirm the biological relevance of ΔN-p73 modulation in response to ATO treatment, we silenced the expression of endogenous p73 transcripts by using specific siRNA. The selective downregulation of ΔNp73 sensitized both K562 and NB4 leukemic cell lines to ATO-induced apoptosis (Figure 4-i and 4-ii) suggesting that it may contribute to ATO-resistance in leukemia cells. The importance of ΔN-p73 in regulating apoptosis and cell transformation is supported by the observation that ΔN-p73 indeed cooperates with oncogenic Ras in in vivo transformation assays (30) and that Ras-MAPK pathway activation impairs ATO-induced apoptosis in leukemic cells (7).

Taken together, our data indicate that the disruption of the MEK/MAPK pathway potentiates the antileukemic activity of ATO in AML blasts through the activation of p73 and Bad proapoptotic pathways with the possible contribution, at high doses of ATO, of the p53 pathway.

These findings provide a rationale for an effective and relatively specific therapeutic strategy for AML.
REFERENCES


26) Pierre-François Cartron, Tristan Gallenne, Gwenola Bougras, et al. First α Helix of Bax Plays a Necessary Role in Its Ligand-Induced Activation by the BH3-Only Proteins Bid and PUMA Mol Cell 2004: 16: 807-818


<table>
<thead>
<tr>
<th>Case</th>
<th>FAB</th>
<th>Age (yrs)</th>
<th>Cytogenetics abnormalities</th>
<th>PD+ATO Combination Index</th>
<th>TA-p73α</th>
<th>TA-p73β</th>
<th>ΔN-p73</th>
<th>TA-p73/ΔN-p73 ratio</th>
<th>P53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PD+ATO 1 μM vs ATO 1 μM (fold increase)</td>
<td>PD+ATO 2 μM vs ATO 2 μM (fold increase)</td>
</tr>
<tr>
<td>1</td>
<td>M0</td>
<td>50</td>
<td>45XX,-7</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>2</td>
<td>M0</td>
<td>36</td>
<td>46,XY</td>
<td>++</td>
<td>0.5</td>
<td>0.9</td>
<td>0.4</td>
<td>7.3</td>
<td>5.6</td>
</tr>
<tr>
<td>3</td>
<td>M0</td>
<td>42</td>
<td>45XY,-7</td>
<td>++</td>
<td>0.1</td>
<td>0.7</td>
<td>0.3</td>
<td>2.2</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>M1</td>
<td>35</td>
<td>46,XX</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>M1</td>
<td>49</td>
<td>t(6;9)(p23;q34)</td>
<td>-</td>
<td>-</td>
<td>.</td>
<td>.</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>M1</td>
<td>52</td>
<td>t(3;3)(q21;q26)</td>
<td>++</td>
<td>0.8</td>
<td>1.0</td>
<td>0.5</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>7</td>
<td>M2</td>
<td>63</td>
<td>46,XY</td>
<td>++</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>8</td>
<td>M2</td>
<td>61</td>
<td>t(8;21)(q22;q22)</td>
<td>++</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>8.1</td>
<td>9.3</td>
</tr>
<tr>
<td>9</td>
<td>M2</td>
<td>54</td>
<td>t(8;21)(q22;q22)</td>
<td>++</td>
<td>0.6</td>
<td>1.3</td>
<td>0.1</td>
<td>24.1</td>
<td>27.8</td>
</tr>
<tr>
<td>10</td>
<td>M2</td>
<td>48</td>
<td>t(8;21)(q22;q22)</td>
<td>+</td>
<td>0.7</td>
<td>0.5</td>
<td>0.8</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>11</td>
<td>M2</td>
<td>65</td>
<td>47,XX,+8</td>
<td>-</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>M2</td>
<td>51</td>
<td>t(8;21)(q22;q22)</td>
<td>+</td>
<td>0.1</td>
<td>0.7</td>
<td>1.6</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>13</td>
<td>M2</td>
<td>28</td>
<td>t(8;21)(q22;q22)</td>
<td>+</td>
<td>0.1</td>
<td>0.4</td>
<td>0.7</td>
<td>4.2</td>
<td>4.7</td>
</tr>
<tr>
<td>14</td>
<td>M2</td>
<td>50</td>
<td>t(8;21)(q22;q22)</td>
<td>+</td>
<td>0.4</td>
<td>0.9</td>
<td>0.5</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>15</td>
<td>M4</td>
<td>52</td>
<td>46,XY</td>
<td>++</td>
<td>0.7</td>
<td>1.4</td>
<td>0.6</td>
<td>3.3</td>
<td>3.2</td>
</tr>
<tr>
<td>16</td>
<td>M4</td>
<td>44</td>
<td>inv(16)(p13;q22)</td>
<td>+</td>
<td>1.2</td>
<td>1.2</td>
<td>0.9</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td>17</td>
<td>M4</td>
<td>77</td>
<td>46,XX</td>
<td>+</td>
<td>1.0</td>
<td>1.2</td>
<td>0.9</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td>18</td>
<td>M4</td>
<td>60</td>
<td>46,XY</td>
<td>-</td>
<td>0.5</td>
<td>0.6</td>
<td>1.3</td>
<td>0.7</td>
<td>1.1</td>
</tr>
</tbody>
</table>
Table 1. Synergistic, additive or antagonistic effect of PD184352 with Arsenic Trioxide on primary AML blasts. Correlation with p73 isoforms expression, relative TA/ΔN p73 ratio and p53 protein levels in ATO and PD+ATO treated cells.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Cytogenetics</th>
<th>CFU-C Response</th>
<th>TA/ΔN p73 Ratio</th>
<th>p53 Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>47</td>
<td>47,XY,+8 inv(16)(p13;q22)</td>
<td>+</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>M4</td>
<td>73</td>
<td>46,XX t(16;16)(p13;q22)</td>
<td>++</td>
<td>0.5</td>
<td>1.1</td>
</tr>
<tr>
<td>M4</td>
<td>49</td>
<td>47,XY,+8 inv(16)(p13;q22)</td>
<td>++</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>M4</td>
<td>43</td>
<td>46,XY</td>
<td>++</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>M5</td>
<td>32</td>
<td>46,XX</td>
<td>++</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>M5</td>
<td>65</td>
<td>46,XX t(8;16)(p11;p13)</td>
<td>+</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>M5b</td>
<td>1</td>
<td>46,XX</td>
<td>++</td>
<td>0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Fresh bone-marrow, from twenty-five patients, were taken at diagnosis and before therapy. After obtaining the informed consent, marrow was extracted by aspiration from the posterior iliac crest. All patients studied had more than 90% blast cells. Patients’ age, FAB classification and cytogenetics a re reported. FAB: French-American-British classification. PD+ATO Combination Index: to determine combination index (CI), primary AML blasts from twenty-five patients were cultured [2.5x10^5/mL in RPMI 1640 medium containing 10% FBS, 2 mmol/l L-glutamine, penicillin G (100 U/ml), streptomycin (100 mg/ml)] in presence of escalating doses of PD184352 (0.1-10μM), ATO (0.125-10μM) or combinations of the 2 agents at a 1:1 ratio (0.25/0.25, 0.5/0.5, 1/1, 1.5/1.5 and 2/2). After 48 h of treatment the cells were harvested for sub-G1 DNA content, annexin V and mitochondrial transmembrane potential detection. Combination Index (CI) plots were then generated using the Chou-Talalay method and Calcosyn software (Biosoft, Ferguson, MO). ++ = CI ≤0.85 indicates synergism; + = 0.85<CI<1.10 indicates additive effect; - = CI ≥1.10 indicates antagonistic effect. TA-p73α, TA-p73β, ΔN-p73: basal endogenous proteins expression, detectable by Western blotting of whole cell lysates (100μg), of p73 isoforms in AML blasts from twenty-one patients. The relative amount of TA-p73α, TA-p73β and ΔN-p73 isoforms in AML blasts were normalized to a positive control K562 cell line. = undetectable levels. TA-p73/ΔN-p73 ratio: TA-(p73α+p73β)/ΔN-p73 ratio expressed as fold increase in PD+ATO vs ATO treated cells. p53 protein expression: - = fold increase respect to control ≤1.3; -/+ = fold increase respect to control comprised between 1.4 and 1.9; + = fold increase respect to control comprised between 2.0 and 4.0; ++ = fold increase respect to control comprised between 4.1 and 6.0; +++ = fold increase respect to control ≥6.1. PD= PD184352, ATO= arsenic trioxide, ND: not done.
FIGURE LEGENDS

Figure 1. MEK-1 inhibition sensitizes AML blasts to ATO-induced apoptosis. (A) Primary AML blasts were seeded at $2.5 \times 10^5$ in the presence of DMSO (vehicle) or PD184352 (1μM) for 3 hours, and then incubated for 18 hours with the indicated concentration of ATO. Endogenous TA-p73α, TA-p73β and ΔN-p73 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). Endogenous p53 was revealed by immunoblotting using a mouse monoclonal anti-p53 (DO-1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Anti-actin immunoblotting was performed as loading control (Santa Cruz Biotechnology). (B) TA-p73α, TA-p73β, ΔN-p73 and β-actin bands were subjected to densitometric scanning using the TINA 2 software (Raytest Isotopenmessgerate GmbH, Germany) and the TA-(p73α+p73β)/ΔN-p73 ratio was calculated. (C) Expression of PARP cleavage, Bax, PUMA and p53AIP1 were revealed after 48 hours of treatment. Cell lysates were analyzed by immunoblotting analysis using a mouse monoclonal anti-PARP (F2) (Santa Cruz Biotechnology), rabbit polyclonal anti-Bax (Cell Signaling Technology Inc. Beverly, MA), rabbit polyclonal anti-PUMA (Cell Signaling Technology), rabbit polyclonal anti-p53AIP1 (CT) (AnaSpec, Inc. San Josè, CA) and goat polyclonal anti-human actin (Santa Cruz Biotechnology). Anti-actin immunoblotting (Santa Cruz Biotechnology) was performed as loading control. (D) Primary AML blasts were cultured as described above. After 48 h of treatment the cells were harvested for ΔΨm detection by flow cytometry. Values are expressed as percentage of cells with low ΔΨm. The inset shows the CI plot for the combination of escalating doses of PD184352 and ATO at a 1:1 ratio, obtained as described in the legend of Table 1; the dashed line indicates CI =1. (E) Primary AML blasts were cultured as described above and stained for annexin V binding.

Figure 2. Effects of the combined MEK inhibition and ATO treatment on serine 112 Bad phosphorylation and Bad protein levels in AML blasts. For the analysis of Bad phosphorylation lysates were subsequently immunoprecipitated with rabbit polyclonal anti-Bad or with a control
antibody, and the immunoprecipitated samples were subjected to 15% SDS-PAGE followed by Western blotting with the anti-phospho-Ser-112-Bad antibody both provided by Cell Signaling Technology Beverly, MA. The filters were then stripped and re-probed with the anti-Bad antibody (Cell Signaling Technology).

**Figure 3.** MEK blockade causes growth inhibition of leukemic cells and modulates p21\textsuperscript{Waf1/CIP1} expression in AML blasts. (A) Leukemic blasts from patients were cultured in presence of PD 184352 and/or ATO for 48 hours and stained for DNA content (*P<.05, **P<.001 vs control vehicle, Dunnett's test). (B) AML blasts were cultured for 48 hours in the presence of PD 184352 and/or ATO and subjected to Western blot analysis with mouse monoclonal anti-p21\textsuperscript{Waf1/Cip1} (DCS60) provided by Cell Signaling Technology.

**Figure 4.** ΔN-p73 downregulation potentiates ATO-induced apoptosis in K562 and NB4 cells. (i) Transfection of ΔN-p73 siRNA, but not the unrelated GFP siRNA, led to decrease ΔN-p73 in K562 cells without affecting the levels of the unrelated protein actin; K562 cells were transfected with an expression vector encoding for an HA-tagged version of ΔNp73 alone or in the presence of siRNAΔNp73 or siRNAGFP. Cells were lysed 24 hours after transfection and ΔN-p73 expression was assessed by anti-HA immunoblot (Santa Cruz Biotechnology). The sequences of the siRNA ΔNp73 are: siRNA ΔNp73 sense 5’-CGUCGGUGACCACCACGGUU-3’, siRNA ΔNp73 antisense 5’-CCGUCCGGGGUCACCACCGUU-3’. (ii) The percentage of sub-G1 apoptotic K562 and NB4 cells treated with ATO was significantly increased in cells transfected with ΔN-p73 siRNA relative to cells transfected with control siRNA (*P<.01 Dunnett’s test); K562 and NB4 cells were transfected with the indicated siRNAs and subsequently treated with ATO (2 μM for K562 and 1 μM for NB4) for 72 hours prior to apoptosis analysis. Values are the mean ± SD of four independent experiments.
MEK1 inhibition sensitizes primary acute myelogenous leukemia to arsenic trioxide-induced apoptosis

Paolo Lunghi, Antonio Costanzo, Luigi Salvatore, Nelida Noguera, Laura Mazzer, Antonio Tabilio, Francesco Lo-Coco, Massimo Levrero and Antonio Bonati