Leukemia (2005) 19, 234–244
© 2005 Nature Publishing Group All rights reserved 0887-6924/05 $30.00
www.nature.com/leu

Arsenic trioxide (ATO) and MEK1 inhibition synergize to induce apoptosis in acute promyelocytic leukemia cells

P Lunghi[1], A Tabilio[2], F Lo-Coco[3], P Pelicci[4] and A Bonati[1]

[1]*Department of Clinical Sciences, Section of Hemato-Oncology, University of Parma, Parma, Italy;* [2]*Department of Clinical and Experimental Medicine, Section of Hematology and Clinical Immunology, University of Perugia, Italy;* [3]*Department of Biopathology, University of Rome 'Tor Vergata', Italy; and* [4]*Department of Experimental Oncology, Milan, Italy*

Recent studies suggest that components of the prosurvival signal transduction pathways involving the Ras-mitogen-activated protein kinase (MAPK) can confer an aggressive, apoptosis-resistant phenotype to leukemia cells. In this study, we report that acute promyelocytic leukemia (APL) cells exploit the Ras-MAPK activation pathway to phosphorylate at Ser112 and to inactivate the proapoptotic protein Bad, delaying arsenic trioxide (ATO)-induced apoptosis. Both in APL cell line NB4 and in APL primary blasts, the inhibition of extracellular signal-regulated kinases 1/2 (ERK1/2) and Bad phosphorylation by MEK1 inhibitors enhanced apoptosis in ATO-treated cells. We isolated an arsenic-resistant NB4 subline (NB4-As^R), which showed stronger ERK1/2 activity (2.7-fold increase) and Bad phosphorylation (2.4-fold increase) compared to parental NB4 cells in response to ATO treatment. Upon ATO exposure, both NB4 and NB4-As^R cell lines doubled protein levels of the death antagonist Bcl-xL, but the amount of free Bcl-xL that did not heterodimerize with Bad was 1.8-fold greater in NB4-As^R than in the parental line. MEK1 inhibitors dephosphorylated Bad and inhibited the ATO-induced increase of Bcl-xL, overcoming ATO resistance in NB4-As^R. These results may provide a rationale to develop combined or sequential MEK1 inhibitors plus ATO therapy in this clinical setting.
Leukemia (2005) **19,** 234–244. doi:10.1038/sj.leu.2403585
Published online 11 November 2004
Keywords: arsenic trioxide; MEK1 inhibition; acute promyelocytic leukemia; MEK1 sIRNA; leukemic cells apoptosis; bad phosphorylation

Introduction

It has been demonstrated that low doses (0.06–0.6 mg/km of body weight/day) of arsenic trioxide (ATO) are extremely efficacious in the treatment of relapsed acute promyelocytic leukemia (APL), inducing partial differentiation and promoting apoptosis of malignant promyelocytes.[1] This pilot trial conducted at the Memorial Sloan-Kettering Cancer Center together with a more recent multicenter clinical study[2] confirmed the impressive results obtained by Chinese investigators in early studies on ATO in the treatment of this disease.[3–5] In particular, the efficacy, safety and limited hematological toxicity of this approach was brought to light.[2] Currently, ATO is considered the treatment of choice for patients with relapsed disease, particularly in patients exposed to all-*trans* retinoic acid (ATRA) within the prior 12 months.[6]

The mechanisms of action of ATO have been investigated both *in vivo* and *in vitro*: at low doses (0.1–0.25 μM), ATO induces a partial differentiation in APL cells through degradation of PML-RARα, while at higher doses (0.5–2 μM), this agent inhibits cell growth and induces apoptosis through both PML-RARα-dependent and -independent mechanisms with caspase activation of neoplastic cells.[7–9]

In vitro and *in vivo* studies have been conducted to evaluate the effect of ATO in combination with ATRA or other biological compounds such as cAMP. The results showed that these associations are more effective than the single agents in inducing differentiation or cell apoptosis.[10–12]

According to recent laboratory studies, the blast cells of most acute myelogenous leukemias (AML) including APL show constitutive activation of extracellular signal-regulated kinases 1/2 (ERK1/2) as well as of the kinases immediately upstream of ERK, known as mitogen-activated protein (MAP)/ERK kinases (MEKs).[13–16] Furthermore, we and others have demonstrated that downmodulation of MEK1 phosphorylation inhibits proliferation and induces apoptosis of primary AML blasts[17–20] (reviewed in Platanias[21] and Lee and McCubrey[22]). In this study, we aimed at investigating whether the combination of ATO with agents that block the phosphorylation of MEK1 can potentiate the antileukemic action of ATO in APL. Moreover, blocking MEK1 function with specific inhibitors or specific double-stranded RNA oligonucleotides (siRNA) restored ATO sensitivity in an NB4 cell line rendered resistant to ATO-induced apoptosis. Finally, we demonstrated here that the downmodulation of MEK1 phosphorylation significantly enhanced ATO-induced apoptosis in primary APL blasts.

Materials and methods

Reagents

ATO was purchased from Sigma (St Louis, MO, USA). 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. A 100 mM stock solution of the MEK1 inhibitors PD98059 (2′-amino-3′-methoxyflavone; Cell Signaling Technology, Beverly, MA, USA) or PD184352 (2-[chloro-4-iodo-phenylamino]-*N*-cyclopropylmethoxy-3,4-di-fluoro-benzamide) kindly provided to us by Dr JS Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI, USA) was prepared in dimethyl sulfoxide (DMSO). These reagents are highly selective inhibitors of MEK1 phosphorylation and activation.[23,24] The doses used by us of 1 or 2 μM for PD184352 and of 10, 20 or 40 μM for PD98059 were those that proved effective *in vitro* in leukemic cells as documented both by ourselves and other authors.[17–19] The doses of ATO hereby employed were selected based on the evidence that ATO, from concentrations of 0.5 to 2 μM upwards, has the capacity to induce apoptosis without differentiation in NB4 cells. These *in vitro* concentrations are within the range found in the plasma of patients receiving ATO treatment for APL

Correspondence: Professor A Bonati and Dr P Lunghi, Dipartimento di Scienze Cliniche, Via Gramsci 14, Parma 43100, Italy; Fax: +39 0521 941290; E-mails: antbonny@unipr.it and p.lunghi@libero.it
Received 4 March 2004; accepted 28 September 2004; Published online 11 November 2004

and entering complete hematologic remission after such therapy.[25]

Cell lines and patient primary blasts

Selection of NB4 arsenic-resistant cell line (NB4-As^R). An arsenic-resistant NB4 subline named NB4-As^R Paolo Lunghi 1 (PL1), hereafter referred to as NB4-As^R, was derived from the NB4 cell line following the method described by Gianni et al.[10] NB4-As^R was obtained by treating parental cells with ATO 1 μM weakly and was maintained with the same dose. In the presence of high doses of ATO (1 μM), the NB4-As^R grew, although at a reduced rate, while the parental cell line died. NB4-As^R cells were also constantly grown in the presence of 1 μM ATO. In experiments examining the response of NB4-As^R to ATO, the cells were first washed thoroughly to remove ATO from the media, and then cultured for 24 h in the media alone prior to initiating the experiment.

Human leukemia cell lines NB4 or NB4-As^R in logarithmic growth were seeded at 1×10^5 cells/ml of fresh RPMI 1640 medium (Gibco BRL,Grand Island, NY, USA), supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, penicillin G (100 U/ml), streptomycin (100 mg/ml) (Gibco BRL) and with or without the compounds described above, in a humidified atmosphere of 95% air/5% CO_2.

The patients studied were affected by typical hypergranular APL (M3). Patients # 1 and # 2 were investigated at diagnosism and patient # 3 at the time of relapse. Patient # 3 relapsed after receiving ATRA + idarubicin (AIDA regimen)[26] as front-line treatment. In all analyzed samples, the percentage of leukemic infiltration exceeded 80%. The disease-specific PML/RARα fusion transcript was detected in all cases by RT-PCR as described previously.[26]

Peripheral blood was obtained after informed consent. Leukemia cells were isolated and enriched on Ficoll–Hypaque density gradients (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), washed twice with calcium and magnesium-free PBS and cultured as described above. Cell number was determined by trypan blue dye exclusion. The presence of cell differentiation after treatment was evaluated by means of microscopic examination of May–Grunwald-stained cells and nitroblue tetrazolium (NBT, Sigma-Aldrich) reduction assay as described.[27] CD11b-positive cells were detected by flow cytometry as described,[27] using the corresponding FITC-conjugated antibodies that were purchased from Becton Dickinson, San José, CA, USA. All the experiments performed utilizing the NB4 and NB4-As^R cell lines were carried out in duplicate and repeated at least three times.

Flow cytometry assay

Flow cytometry assay to evaluate cell cycle and apoptosis after *in vitro* treatment with PD98059, PD184352 and ATO was performed as described previously.[17] A FACSCalibur apparatus (Becton Dickinson) was utilized. Data were analyzed using FlowJo 3.4 software (Tree Star, San Carlos, CA, USA).

Mitochondrial transmembrane potential ($\Delta\Psi_m$)

To investigate the changes in mitochondrial transmembrane potential that occur during apoptosis, the MitoLight apoptosis detection kit (Chemicon International Inc., Temecula, CA, USA) was used. Briefly, after 48 h of treatment, cells were incubated with MitoLight reagent for 20 min at 37°C. After centrifugation, they were resuspended in incubation buffer and analyzed by fluorescence-activated cell sorter scanner. MitoLight emits a red fluorescence (MitoLight aggregates, high $\Delta\Psi_m$) when sequestered in the mitochondrial membrane of healthy cells detectable in the PI channel. In apoptotic cells with altered mitochondrial membrane potential, the dye in its monomeric form remains in the cytoplasm, emitting a green fluorescence (MitoLight monomers, low $\Delta\Psi_m$) detectable in the fluorescein isothiocyanate channel.

siRNA transfections

Prior to electroporation, NB4-As^R cells were washed twice with serum-free Opti-MEM (Gibco BRL Paisley, UK) and resuspended to a final concentration of 8×10^6 cells/ml in Opti-MEM (Gibco BRL). Subsequently, 0.5 ml of cell suspension was mixed either with 0.5 nmol of Smart pool double-stranded RNA oligonucleotides (siRNA) against MEK1 (M-003571) or nonspecific control siRNA (D-001206-13-05) obtained from Dharmacon Tech (Lafayette, Co, USA) and electroporated in a 0.4-cm cuvette using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories Inc., Herculex, CA, USA) and using a single-pulse protocol (voltage 260 V and capacitance 1050 μF). At 48 h after transfection, cells were treated with ATO 1 μM for another 48 h. The cells were harvested for $\Delta\Psi_m$, annexin V, sub-G1 DNA content detection and immunoblotting.

Molecular analysis

Cell lysis, immunoblotting, immunoprecipitation, and ERK immunoenzymatic assay were carried out as described.[17,28,29] For immunoprecipitation, the following antibodies were used: rabbit polyclonal anti-p44/p42 ERK, rabbit polyclonal anti-Bad and rabbit polyclonal anti-phospho–Bad, all provided by Cell Signaling Technology Beverly, MA, USA. For immunoblotting, the following antibodies were used: rabbit polyclonal anti-p44/p42 ERK, rabbit polyclonal anti-phospho-p44/42 ERK (Thr202/Tyr204), rabbit polyclonal anti-MEK1/2, rabbit polyclonal anti-phospho-Bad, rabbit polyclonal anti-Bad, mouse monoclonal anti-Bax and rabbit anti-Bcl-xL all provided by Cell Signaling Technology, mouse monoclonal anti-Bcl-2 clone 124 (Upstate Biotechnology, Lake Placid, NY, USA), mouse monoclonal PARP (F2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); goat polyclonal anti-human actin (Santa Cruz Biotechnology); goat anti-rabbit IgG (H + L)-HRP conjugated (Bio-Rad); donkey anti-goat IgG (H + L)-HRP conjugated (Santa Cruz Biotechnology).

For coimmunoprecipitation analysis, lysates were precleared with the appropriate control IgG (normal rabbit IgG, Santa Cruz Biotechnology) together with Agarose A/G beads (Santa Cruz). Subsequently, the lysates (500 μg) were incubated with 2–4 μg of primary antibody at 4°C for 2 h. Agarose A/G beads (Santa Cruz) were added and samples were incubated at 4°C overnight. Samples were washed four times and the protein/bead mixture was denatured at 95°C for 5 min and then loaded onto 15% SDS-polyacrylamide gels. Blots were subjected to Western blotting as described.

Statistical analysis

For multiple comparisons, a statistical analysis was performed using analysis of variance for repeated measurements followed by a Tukey–Kramer or Dunnet post-tests.

Synergism, additive effects and antagonism were assessed using the Chou–Talalay method[30] and Calcusyn software (Biosoft, Ferguson, MO, USA). Briefly, the dose–effect curve for each drug alone was determined based on the experimental observations using the median-effect principle; the combination index (CI) for each experimental combination was then calculated according to the following equation: CI = (Ac)/(As) + (Bc)/(Bs) + (Ac) (Bc)/(As) (Bs), where (Ac) and (Bc) correspond to the concentrations of drugs A and B that have x effect when used in combination and (As) and (Bs) correspond to the concentrations of drugs A and B able to produce, alone, the same magnitude of effect. When CI = 1, this equation represents the conservation isobologram and indicates additive effects. CI values less than 1.0 indicate a more than expected additive effect (synergism). CI values more than 1.0 indicates an antagonistic effect.

Results

MEK1 inhibition enhances ATO-mediated apoptosis both in NB4 and in NB4-As^R cells, and in fresh APL primary blasts

We first studied the effect on cell growth inhibition in NB4 and NB4-As^R cell lines treated with PD and/or ATO. After 48 h of treatment, the combination of PDs (PD98059 40 μM or PD184352 1 μM) and ATO 1 μM significantly ($P<0.001$) decreased cell growth compared to PDs or ATO alone in both cell lines (Figure 1a and b). At later times (72–96 h), the combination of the two agents did not reach statistical significance in parental NB4 because of cell death due to ATO (1 μM)-induced apoptosis (data not shown).

We therefore studied the effect of PDs, ATO or both agents in combination on apoptosis in NB4, NB4-As^R leukemic cells and fresh APL primary blasts from patients.

After 72 h of treatment, we observed that the combination of MEK1 inhibitors (PD98059 40 μM or PD184352 1 μM) with ATO (ATO 1 μM) strikingly increased the percentage of sub-G1 apoptotic cells induced by ATO alone in both parental and NB4-As^R cells (Figure 2a). These data were confirmed by the exposure of phosphatidylserine on the outer leaflet of the plasma membrane (data not shown). Similar results were obtained in both cell lines treated with lower doses of PD98059 (20 and 10 μM) (data not shown).

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) are a critical step in cells undergoing apoptosis, regardless of the death signal. Therefore, we compared $\Delta\Psi_m$ in ATO- or PD + ATO-treated cells by using a mitochondrion-specific dye, MitoLight, which displays a green to red spectral shift proportional to $\Delta\Psi_m$ (see Materials and methods). The combined treatment with PD184352 and ATO strikingly potentiated loss of $\Delta\Psi_m$ induced by ATO in both cell lines (Figure 2b), indicating that the dual treatment affects the mitochondria apoptotic pathway. Similar results were observed with PD98059 (data not shown).

Finally, to characterize the pharmacologic interactions between PD184352 and ATO more rigorously and over a range of drug concentrations, median dose effect analysis was used (see Materials and methods). When apoptosis by sub-G_1 DNA content or annexin V/PI staining was measured after 48 h of drugs exposure, CI values considerably less than 1.0 were obtained in arsenic-resistant cells and to a lesser extent in the parental NB4 cells, corresponding to highly synergistic interactions in both NB4-As^R cells and parental cells (Figure 2c). Thus, these agents appear to synergize for the induction of apoptosis primarily in arsenic-resistant and also in parental NB4 cells.

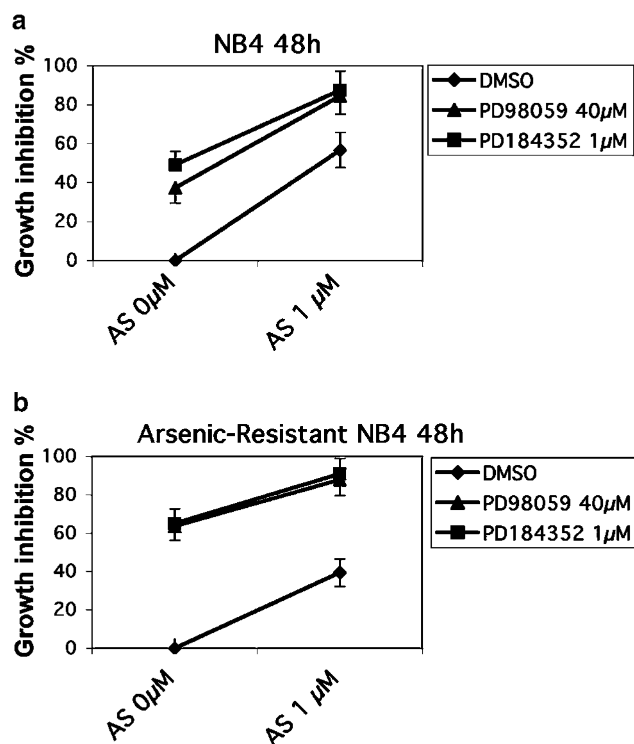

Figure 1 Combined effects of ATO and MEK1 inhibitors on cell growth in the NB4 and in NB4-As^R leukemic cell line. The cells NB4 (a) and NB4-As^R (b) seeded at 1×10^5 cells/ml were pretreated for 3 h with MEK1 inhibitors PD98059 40 μM or PD184352 1 μM, and then incubated with ATO. Each value represented the mean ± s.d. of at least three independent experiments. Cell count was determined using the trypan blue dye exclusion method. AS = ATO.

We next studied the apoptotic effect of the combined treatment in fresh APL primary blasts. Interestingly, the percentage of sub-G1 apoptotic cells in patients (cases # 1, # 2 and # 3) treated for 48 h with PD98059 40 μM and ATO at concentrations of 1 or 2 μM – concentrations that are usually achieved in the plasma of ATO-treated APL patients[25] – was greater (two- or three-fold more) than that found after treatment with ATO 1 or 2 μM alone (Supplementary Figure 2).

These data were confirmed by the exposure of phosphatidylserine on the outer leaflet of the plasma membrane (data not shown).

The treatment with PD98059 alone showed a low increase of cell death in patient # 2, while a stronger apoptosis induction was observed in patients # 1 and # 3 (relapse) (Supplementary Figure 2).

Moreover, no significant signs of cell differentiation were observed by microscopic examination of May–Grunwald-stained cells, NBT test and CD11b-positive cell count during this combined therapy (data not shown). Taken together, these findings showed that MEK1 inhibitors and ATO strongly cooperate in their activation of apoptosis effectors.

PD treatment inhibits ATO-mediated ERK1/2 activation and increases PARP fragmentation in NB4 and NB4-As^R cell lines as well as in fresh APL primary cells

In order to explain the molecular effectors involved in growth control and apoptosis, we first studied the kinetics of ERK1/2

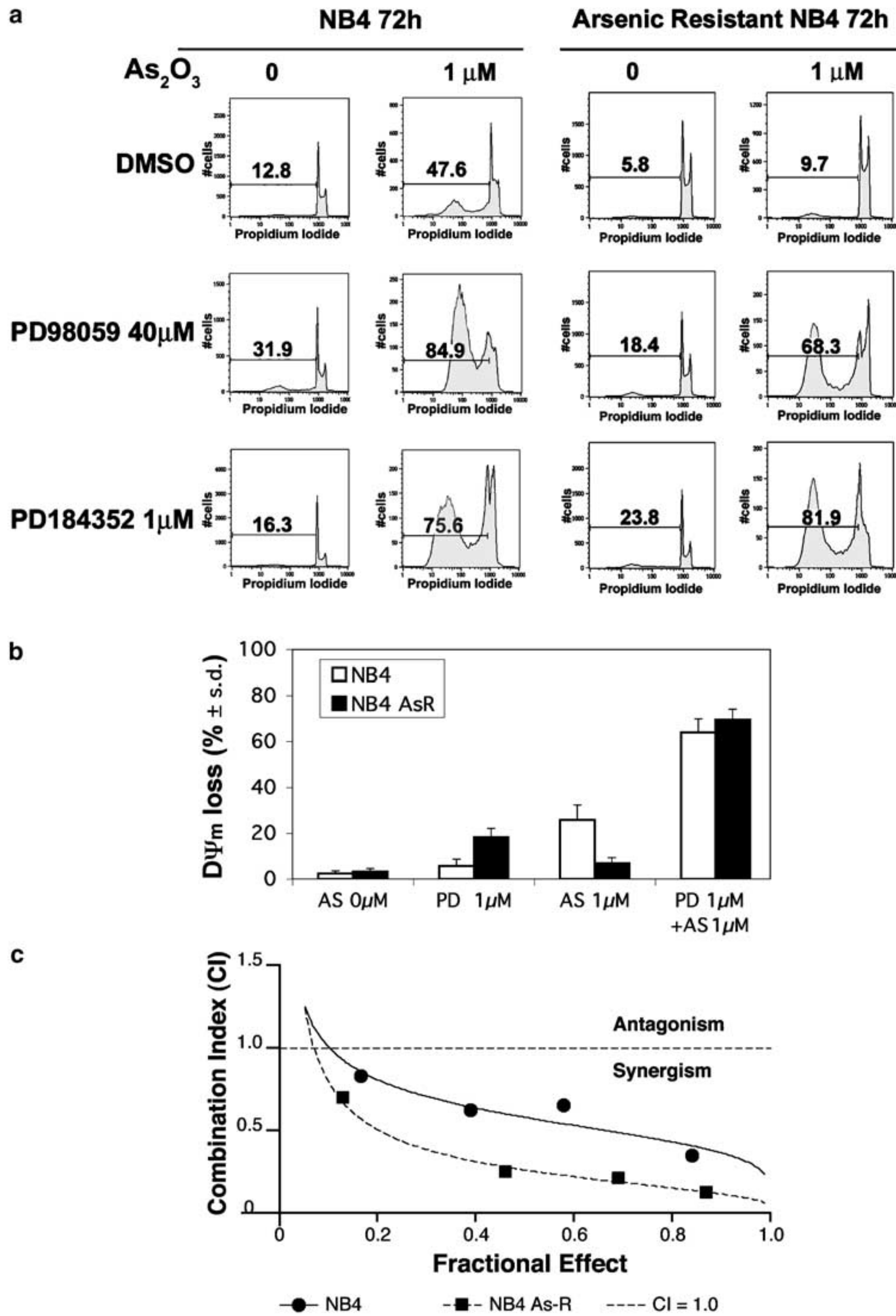

Figure 2 MEK1 inhibition sensitizes leukemic cells to spontaneous and ATO-induced apoptosis. (a) The NB4 and NB4As^R cell lines were seeded at 1×10^5 cells/ml in the presence of DMSO (vehicle) or MEK1 inhibitors for 3 h, and then incubated with ATO 1 μM for 72 h. Apoptosis was then measured as the percentage of cells with hypodiploid DNA content. Results are representative of one of three independent experiments. (b) NB4 and NB4-As^R were cultured in the presence of DMSO (vehicle) or PD 184352 1 μM for 3 h, and then incubated with ATO 1 μM. After 48 h, $\Delta\Psi_m$ was assessed by flow cytometry. Values represent the mean $\pm$ s.d. of three independent experiments and are expressed as the percentage of Parental NB4 (● solid line) and arsenic-resistant NB4 (■ dashed line) cell lines were cultured in the presence of escalating doses of PD184352 (0.1–20 μM), ATO (0.125–10 μM) or combinations of the two agents at a 1:1 ratio (0.25/0.25, 0.5/0.5, 1/1, 1.5/1.5). After 48 h, apoptosis was measured by sub-G1 DNA content. CI plots was then generated using the Calcusyn software (symbols represent actual data points for the combination). CI less than 1.0 indicates synergism; CI = 1.0 indicates additive effect; CI more than 1.0 indicates antagonistic effect. AS = ATO.

activity and poly(ADP-ribose) polymerase PARP fragmentation that reflects increased apoptosis[31] in parental and arsenic-resistant NB4 cells treated with PD and ATO.

In NB4 cells, the analysis of ERK1/2 activity demonstrated that ATO induced, in a dose-dependent manner, a sustained kinase activity that was clearly evident after 15 min, 1 h (data not

shown) and 24 h of treatment (Figure 3a). At a later time point (48 h), the ATO-mediated ERK1/2 activity was still detectable, although at very low levels (Figure 3a).

Even in the NB4-As^R, ATO 1 μM strongly increased ERK1/2 activity and the activation of these mitogen-activated protein kinases (MAPK) was clearly evident after 24 and 48 h of ATO treatment (Figure 3b).

The treatment with MEK1 inhibitors downmodulated the steady level of ERK activity and prevented the ATO-mediated ERK1/2 activation in both cell lines (Figure 3a and b).

Moreover, the combined treatment also led to an increased fragmentation of PARP that occurred earlier in arsenic-resistant cells (24 h) as compared to parental cells (48 h) (Figure 3a and b).

Since ATO is able to enhance the activity of ERK in both NB4-As^R and parental NB4 cell lines, we decided to compare its activity in these cell lines by means of an immunoenzymatic MAP kinase assay. Interestingly, ERK1/2 activity was greatly enhanced in the NB4-As^R as compared to parental cells (2.7- and 24-fold increase after 24 and 48 h of ATO treatment, respectively) as shown in Figure 3c. Moreover, in NB4-As^R treated with ATO, similar levels of ERK activity were observed at 24 and 48 h, whereas a significant decrement in activity occurred after 72 h. In parental NB4 cell lines, the decrement in this MAPK activity was clearly evident as from 48 h (Figure 3c).

Subsequently, fresh blasts from APL patients were treated with ATO 0.25, 1 and 2 μM for 24 h with or without PD98059 pretreatment. In APL primary blasts, ATO induced a strong

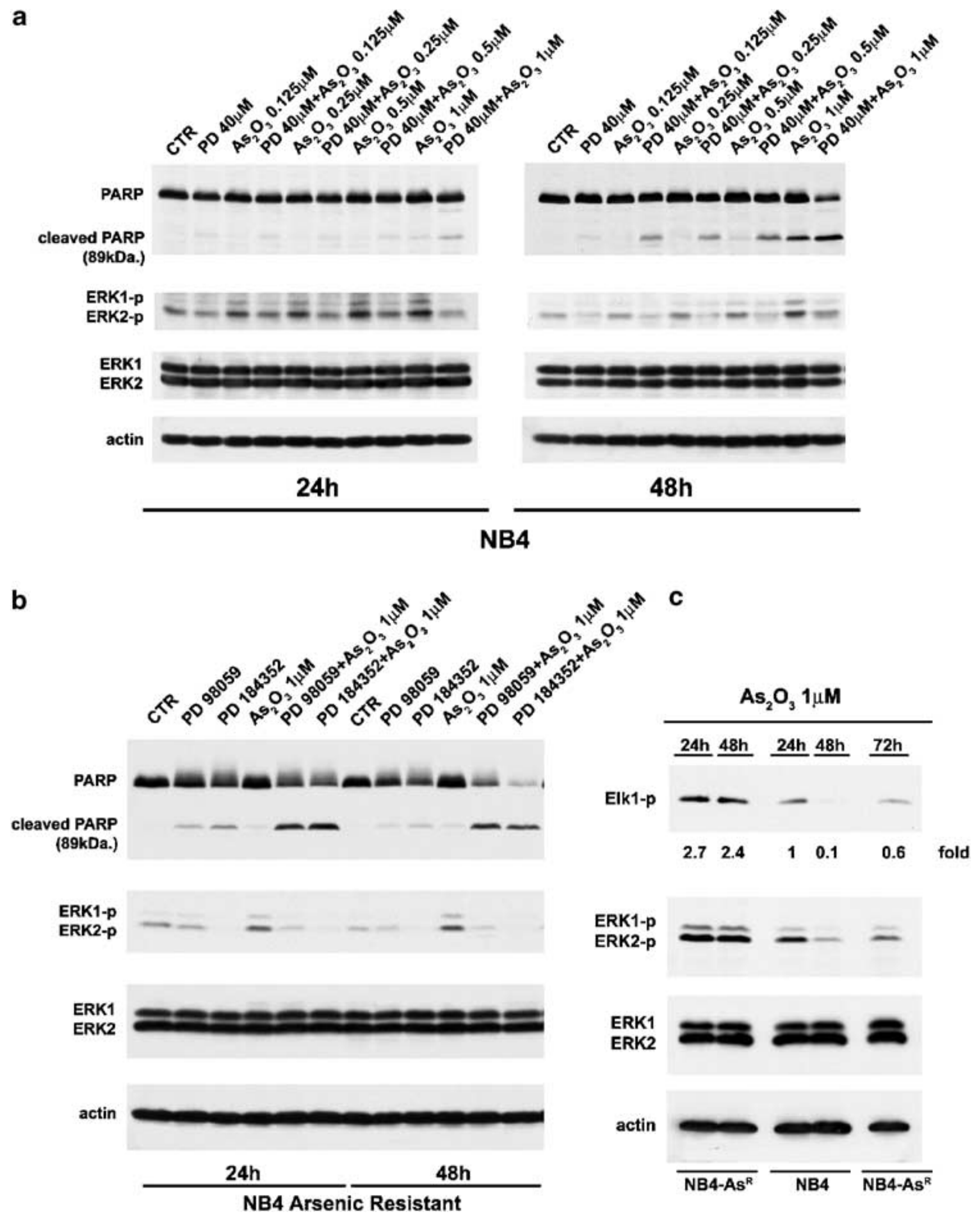

Figure 3 Downmodulation of ERK1/2 dual phosphorylation by MEK1 inhibitors increases ATO-induced PARP fragmentation. (a) The NB4 cell line and (b) NB4-As^R were stimulated with indicated doses of ATO for the times indicated, both with and without MEK1 inhibitors. Immunoblots show cleaved PARP fragments, ERK1 and ERK2 dual phosphorylation and expression after treatment. (c) Immunoenzymatic MAP kinase assay was performed in NB4 and in NB4-As^R cell lines treated with ATO for the indicated time. After cell lysis, ERK activity was assayed by immunoprecipitation with an anti-ERK antiserum in immunocomplex kinase assays with ELK1 as substrate or with dual phosphorylation ERK1/2-specific antisera (p-ERK1/2).

increase of ERK1/2 activity in a dose-dependent manner; PD98059 pre-treatment was able to inhibit the ATO-mediated ERK1/2 activation, leading to increased PARP fragmentation (Supplementary Figure 3).

Finally, to determine the contribution of MEK-ERK pathway activation in mediating ATO resistance, the MEK1 mRNA was selectively knocked down by means of specific double-stranded RNA oligonucleotides (siRNA). Recently, siRNAs have been shown to achieve a high degree of specificity with low toxicity also in mammalian cells acting through a degradative chain reaction catalyzed by the activation of a cellular RNA-dependent RNA polymerase.[32,33] Transfection of MEK1 siRNA, but not the nonspecific control siRNA, led to the decrease of MEK1 in NB4-As^R without affecting the levels of the unrelated protein actin (Figure 4a). When NB4-As^R were treated with ATO, an increased PARP fragmentation and an important shift in green fluorescence, indicating loss of $\Delta\Psi_m$, was observed in cells transfected with MEK1 siRNA relative to cells transfected with control siRNA (Figure 4a and b). The apoptotic nature of the cell death triggered by ATO after the disruption of MEK-ERK pathway was confirmed by the exposure of phosphatidylserine on the outer leaflet of the plasma membrane and by demonstration of a decrease in the DNA content to sub-G_1 levels (data not shown). These findings indicate that the MEK-ERK pathway is determinant in the development of ATO resistance.

ATO-mediated ERK1/2 activation induces phosphorylation at Ser112 of the endogenous proapoptotic Bad protein in APL cells

Recent studies have demonstrated the involvement of MEK-ERK kinases upstream of Bad phosphorylation at serine 112 in promoting cell survival.[34–37] The proapoptotic function of Bad is characterized by its ability to heterodimerize with Bcl-2 and Bcl-xL, blocking the antiapoptotic function of these proteins.[38,39] Phosphorylation at Ser112, Ser136 or Ser155 inhibits the function of Bad. We then investigated phosphorylation at Ser112 and the expression of the proapoptotic Bad protein after ATO treatment, both with and without MEK1 inhibitors in NB4, NB4-As^R and APL primary cells. Immunoblotting analysis of cellular extracts from the same experiments described above showed that, in NB4 and NB4-As^R cell lines as well as in APL primary cells, ATO-mediated ERK1/2 activation was related to an increased phosphorylation at Ser112 of Bad (Figure 5 and Supplementary Figure 5). Interestingly, after ATO treatment, the NB4-As^R cells showed a larger increment of Bad phosphorylation at Ser112 (2.4-fold more) than NB4 (Figure 5) that correlated well with the stronger ERK activity found in this cell line (Figure 3c). The downmodulation of ERK1/2 activity by MEK1 inhibitors resulted in decreased phosphorylation of Bad in NB4, NB4-As^R cell lines as well as in APL primary cells (Figure 5 and Supplementary Figure 5). Interestingly, the PD-mediated downmodulation of phospho-Bad completely restored the sensitivity to ATO treatment in NB4-As^R cells, leading to a strong increment of fragmented PARP (Figure 5). The expression of the Bcl-2 family proteins, Bcl-xL, Bcl-2 and Bax, involved in apoptotic pathways were also studied. The death antagonists Bcl-2 and Bcl-xL proteins were expressed at similar levels in untreated parental or NB4-As^R (Figure 5). After 24 h of treatment, we did not observe significant Bcl-2 degradation in NB4-As^R or NB4 cells treated with 1 μM of ATO (Figure 5); conversely, both cell lines nearly doubled the expression of Bcl-xL (Figure 5). Neither MEK1 inhibitors alone nor their combination with ATO 1 μM were able to induce significant degradation of the Bcl-2 protein (Figure 5); conversely, MEK1 inhibitors significantly inhibited the Bcl-xL increased protein expression mediated by ATO in both cell lines (Figure 5). The base-line level of the death agonist Bax protein was significantly greater in NB4 (1.7-fold more) than in NB4-As^R. After ATO or PD + ATO treatment, Bax expression nearly doubled in both cell lines. Together, our results showed that the more marked difference between these two lines was the status of ERK and Bad phosphorylation after ATO treatment.

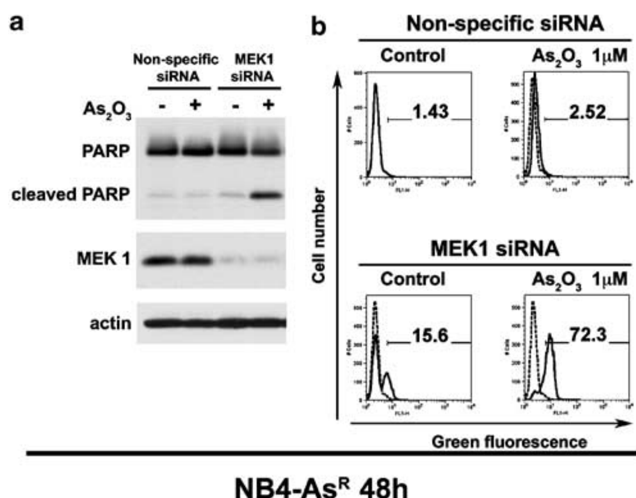

Figure 4 Abrogation of MEK1 expression sensitizes NB4-As^R cells to ATO treatment. NB4-As^R cells were transfected with the indicated siRNAs. At 48 h after transfection, cells were treated with ATO 1 μM for another 48 h. Subsequently, the cells were harvested for immunoblotting and $\Delta\Psi_m$ detection. (a) Immunoblots show cleaved PARP fragments, MEK1 and actin expression after treatment. (b) Disruption of mitochondrial membrane potential in transfected NB4-As^R cells treated or not with ATO. The percentage of cells with a low $\Delta\Psi_m$ is indicated.

ATO triggers dissociation of Bcl-xL and Bcl-2 from Bad in APL cells

We proceeded to investigate whether the phosphorylation status of Bad affected its capacity to associate with death antagonists Bcl-xL and Bcl-2 in NB4-As^R and parental NB4 cell lines.

To this end, we examined the constitutive and ATO-induced protein–protein interactions of Bad pathway components using co-immunoprecipitation. Preliminary studies confirmed that the Bad antibody effectively precipitated Bad (data not shown). In untreated NB4-As^R and NB4 cells, we observed a significant binding of Bcl-xL and Bcl-2 proteins to Bad immunoprecipitates, indicating the presence of endogenous Bad/Bcl-xL or Bad/Bcl-2 heterodimers (Figure 6). The levels of Bcl-xL and Bcl-2 that co-immunoprecipitated with Bad were comparable in both cell lines (Figure 6). After treatment with ATO, the binding of Bcl-xL and Bcl-2 to endogenous Bad sharply decreased in both cell lines (Figure 6). Interestingly, after ATO treatment, we observed a reduced amount of Bad/Bcl-xL and Bad/Bcl-2 heterodimers in NB4-As^R compared to NB4 (Figure 6); this behavior correlated well with the different phosphorylation status of Bad in these two lines treated with ATO (Figure 5).

In order to examine the amounts of free Bcl-xL and Bcl-2 that did not co-immunoprecipitate with Bad, the supernatants after Bad immunoprecipitation were Western blotted for Bcl-xL and

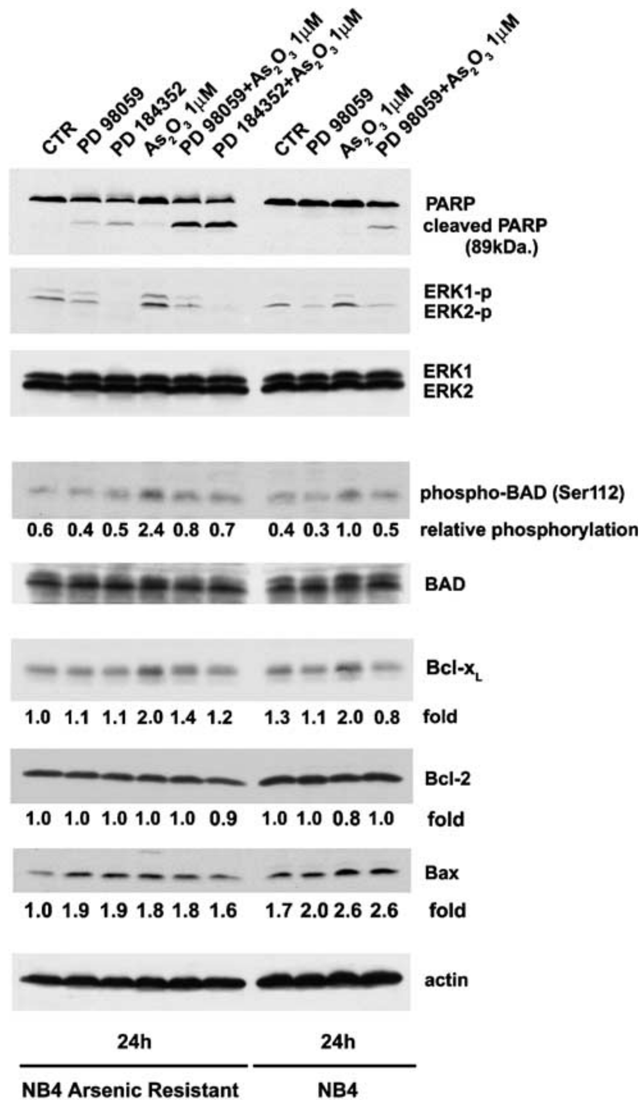

Figure 5 ATO induces the phosphorylation of Bad at serine 112. NB4-As^R and parental NB4 cell lines were pretreated with either DMSO (vehicle) or MEK1 inhibitors (PD98059 40 μM or PD184352 1 μM) for 3 h and then treated with 1 μM of ATO for 24 h. Immunoblots show cleaved PARP fragments, ERK1 and ERK2 dual phosphorylation and expression, Bad phosphorylation at Ser112 and Bad expression, Bcl-xL, Bcl-2 and Bax expression after treatment. For the analysis of Bad phosphorylation, lysates were subsequently immunoprecipitated with the anti-Bad antibody, and the immunoprecipitated samples were subjected to 15% SDS-PAGE, followed by Western blotting with the anti-phospho-Ser-112-Bad antibody. The filters were then stripped and reprobed with the anti-Bad antibody. To evaluate the relative levels of Bad phosphorylation, Bcl-xL, Bcl-2 and Bax expression, bands were subjected to densitometric scanning using the TINA 2 software.

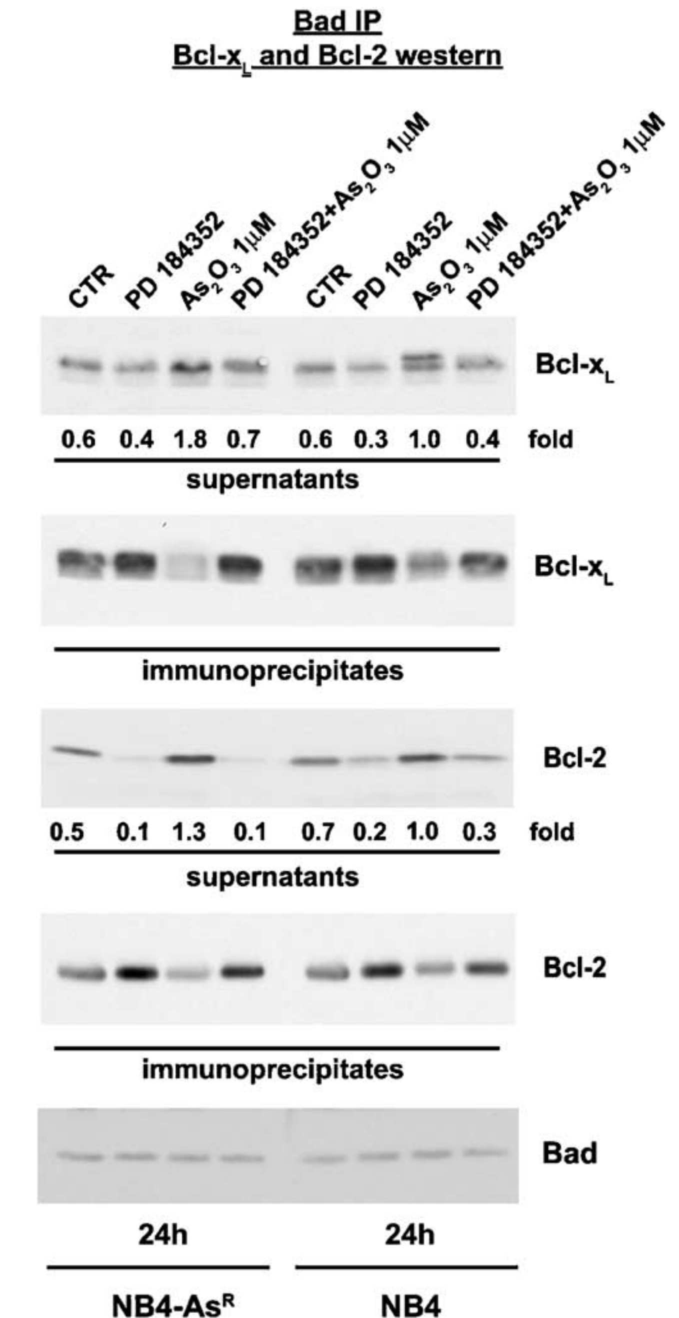

Figure 6 ATO triggers dissociation of Bcl-xL and Bcl-2 from Bad in NB4-As^R and in NB4 cell lines. NB4-As^R and parental NB4 cell lines were pretreated with either DMSO (vehicle) or PD184352 1 μM for 3 h and then treated with 1 μM of ATO for 24 h. Lysates were subsequently immunoprecipitated with the anti-Bad antibody, and the immunoprecipitates were analyzed by Western blotting with anti-Bcl-xL or anti-Bcl-2 antibodies to detect the amount of Bcl-xL and Bcl-2 proteins that associated with Bad. The supernatants after Bad immunoprecipitation were also analyzed to determine the amount of Bcl-xL and Bcl-2 that did not associate with Bad. Bands were subjected to densitometric scanning using the TINA 2 software. Bad levels in the immunoprecipitates are shown as control. Immunoblots are representative of at least two independent experiments.

Bcl-2. The amount of free Bcl-xL in untreated NB4-As^R or NB4 cells was comparable, but after ATO treatment, we observed a stronger increment of free Bcl-xL in NB4-As^R compared to parental NB4 (3- *vs* 1.7-fold increase compared to the control, respectively) (Figure 6). Interestingly, even if the total amount of Bcl-xL was comparable in both cell lines (Figure 5), free Bcl-xL was nearly doubled in NB4-As^R compared to NB4 in ATO-treated cells (Figure 6). In untreated cells, the amount of free Bcl-2 was more evident in NB4 than in NB4-As^R. As per free Bcl-xL, a stronger increment of free Bcl-2 was observed in NB4-As^R compared to NB4 (2.6 *vs* 1.4 compared to the control) after ATO treatment; the amount of free Bcl-2 was higher in NB4-As^R. The treatment with MEK1 inhibitors was able to downmodulate ERK1/2 and Bad phosphorylation (Figure 5), leading to a strong

increment of Bcl-xL and Bcl-2 protein that immunoprecipitated with Bad in both cell lines (Figure 6).

Discussion

In this report, an arsenic-resistant NB4 subline, NB4-As^R, was characterized and used to demonstrate that ATO and MEK1 inhibitors synergize to induce apoptosis. Resistance to the apoptotic effect of ATO was primarily linked to an altered activity of the Ras-MAPK pathway in response to this drug. In fact, also the parental NB4 showed, under ATO exposure, a significant increment in prosurvival ERK activity, although to a lesser extent as compared to NB4-As^R (Figure 3c). Interestingly, similar effects were observed in primary APL blasts from patients treated *in vitro* with ATO (Supplementary Figure 3).

The activation of the MEK/ERK pathway, which provides a survival signal against stress-induced apoptosis is not only reported as a result of ATO exposure[40,41] but also after stimulation by stress factors or inflammatory cytokines,[42] and subsequent to treatment with the chemotherapeutic drugs paclitaxel[43] and cisplatin,[44,45] or after ionizing and UV irradiation.[46–48]

Both in the parental and in the NB4-As^R cell lines as well as in primary APL blasts, the ATO-mediated ERK1/2 activation led to an increased phosphorylation at Ser112 of the proapoptotic Bad protein. Moreover, under ATO exposure, the higher ERK activity in NB4-As^R than in the parental NB4 cells correlated well with the stronger increment of Bad phosphorylation status at Ser112 observed in NB4-As^R (Figure 5).

To date, the only known function of the BH3-only protein Bad is to bind Bcl-2 family proteins Bcl-2 and Bcl-xL, thereby blocking the antiapoptotic action of these proteins by preventing them from binding Bax/Bak.[38,39,49] The apoptotic activity of Bad is clearly determined by its phosphorylation status and only nonphosphorylated Bad heterodimerizes with Bcl-xL or Bcl-2, neutralizing their protective effect and promoting cell death.[49]

To better understand Bad's role in this experimental model, we analyzed the total protein levels of Bcl-xL and Bcl-2 and their interaction with endogenous Bad. The death agonist Bax was also studied. Both in NB4 and in NB-As^R cell lines, the steady levels of Bcl-2 and Bcl-xL were comparable, whereas the Bax expression was higher in parental (1.7-fold more) than in NB4-As^R. At the earliest events, in response to ATO treatment, both cell lines increased the protein levels of Bcl-xL and Bax (a nearly two-fold increase compared to the control), whereas no significant modulation in the Bcl-2 protein was observed (Figure 5). This was consistent with previous reports, which showed that Bcl-2 degradation did not occur in NB4 cells treated with 1 or 2 μM of ATO.[50,51] Moreover, our findings that the ATO-induced upregulation of Bcl-xL was mediated by MEK/ERK pathway are in line with previous reports showing an MEK-dependent regulation of Bcl-2 family proteins.[52–55] Interestingly, the treatment with MEK1 inhibitors blunted the ATO-mediated Bcl-xL upregulation both in parental and in NB4-As^R cell lines (Figure 5).

Under ATO exposure, a more marked hyperphosphorylation of Bad took place in arsenic-resistant cells as opposed to parental cells, leading to an enhanced amount of free Bcl-xL and Bcl-2 capable of interacting with Bax/Bak proteins, thus favoring ATO resistance in the NB-As^R cell line. Interestingly, after ATO treatment, a more slowly migrating Bcl-xL band on immunoblots of supernatants was observed after Bad immunoprecipitation in parental but not in arsenic-resistant cells, suggesting that post-translational modifications of this protein occur only in the NB4 cell line (Figure 6). It has been demonstrated that the treatment with microtubule-targeted agents including paclitaxel, vincristine, vinblastine, colchicines and nocodazole or with DNA-damaging antineoplastic agents such as cisplatin and etoposide results in phosphorylation or in deamidation, respectively, of Bcl-xL protein;[56–60] both phosphorylation and deamidation alter Bcl-xL migration's during SDS-PAGE and have been shown to inhibit its antiapoptotic activities.[56,59] Further investigation will be necessary to determine which Bcl-xL post-translational modifications occurs in ATO-treated cells and their roles in drug resistance.

Pretreatment with MEK1 inhibitors strongly inhibited ERK and Bad phosphorylation in APL cells, enhancing apoptosis in ATO-treated NB4 and in primary APL cells, and completely restoring sensitivity to ATO in the NB-As^R cell line. Taken together, these results suggest that the Bad protein plays a pivotal role in determining the cell's susceptibility to drug-induced apoptosis and that compounds which affect its phosphorylation, such as MEK1 inhibitors PD98059 or PD184352, could be used in combination with existing therapies.[61,62] Accordingly, in BxPc3 tumors that had regressed in response to treatment with the MEK1 inhibitor PD184352, reduced phosphorylation of the serine 112 site on Bad was demonstrated *ex vivo* by Sebolt-Leopold.[63]

The compound PD184352 (now designated CI-1040) was formulated for use *in vivo per os*[24,63] and clinical trials are currently in progress for the purpose of studying its efficacy in advanced forms of cancer, lymphoma and leukemia refractory to treatment.[64] Interestingly, PD184352 even at 10 μM did not inhibit any protein kinase other than MEK1, as detected by an investigation of a large panel of kinases.[65]

Recently, we demonstrated that in leukemia cells, the combined treatment with MEK1 inhibitors and ATO promotes the up-regulation of p53AIP1 protein (p53-regulated apoptosis-Inducing protein 1) via p73 activation.[66] p53AIP1, a primary effector gene of wild-type p53 and TAp73-induced apoptosis,[67,68] interacts with Bcl-2 proteins at the mitochondrial membrane and the overexpression of Bcl-2 blocks the down-regulation of $\Delta\Psi_m$ and the proapoptotic activity of p53AIP1.[69]

Together, these findings support a model in which the p73-p53AIP1 apoptotic pathway is potentiated by Bad phosphorylation pathway through the increased capacity of Bad, after MEK 1 inhibition, to associate with Bcl-xL and Bcl-2, thereby blocking their antiapoptotic functions. Together with our results, this may encourage to design clinical trials aimed at investigating the efficacy of ATO, currently considered the treatment of choice for relapsed APL,[6] in combination with MEK1 phosphorylation-blocking agents.

Recently, it has been demonstrated that the combined inhibition of MAPK and phosphatidylinositol 3 kinase (PI3K) pathways, the two major protein kinase pathways involved in Bad phosphorylation, markedly increased the PI3K inhibition-mediated apoptosis in leukemia cells,[70] suggesting that multiple signal transduction pathways interact to regulate apoptosis in leukemic cells; both MAPK and PI3K pathways are also involved in the regulation of other downstream antiapoptotic members of the IAP family[71,72] and can synergize to induce cell survival and cellular transformation.[73] Moreover, the activation of PI3K contributes for MEK1-responsive growth and survival.[74] These findings together with our results provide a rationale background to further investigate the possible role of PI3K in ATO resistance and to determine whether the combined inhibition of MAPK and PI3K pathways is more effective than single targeting signal transduction pathway in increasing the antileukemic effect of ATO.

Recently, transient transfection of wild-type PML-RARα into nonhemopoietic CHO cells has been reported to cause little or no enhancement of cell death in response to ATO.[75] This finding is in agreement with the fact that the effects of ATO are cell type and dose specific.[76] Accordingly, the MEK1-ERK pathway may have opposite functions and may modulate cell life or death depending on the characteristics of the stimuli and the cell type involved, as well as on the doses utilized.[77] Indeed, PD184352 cannot inhibit any protein kinase other than MEK1,[65] and the antitumoral effects of MEK1 inhibition are well established and tightly consolidated by the reproducible results obtained by different groups.[17–22,63,78] In conclusion, our results provide an experimental basis for combined or sequential treatment with MEK1 inhibitors and ATO in APL.

Acknowledgements

This paper has been supported by grants from the 'Associazione Italiana per la Ricerca sul Cancro' (AIRC) from the 'Ministero dell'Istruzione dell'Università e della Ricerca Scientifica' (MIUR): FIN, FIL and 'Progetto Strategico Oncologia SP/4: Terapia preclinica molecolare in oncologia' and from 'Associazione Chiara Tassoni per la Lotta contro la Leucemia e il Cancro-Parma'.

Supplementary Information

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

References

1 Soignet SL, Maslak P, Wang ZG, Wang ZG, Jhanwar S, Calleja E *et al.* Complete remission after treatment of acute promyelocytic leukemia with arsenic trioxide. *N Engl J Med* 1998; **339**: 1341–1348.
2 Soignet SL, Frankel SR, Douer D, Tallman MS, Kantarjian H, Calleja E *et al.* United States multicenter study of arsenic trioxide in relapsed acute promyelocytic leukemia. *J Clin Oncol* 2001; **19**: 3852–3860.
3 Sun HD, Ma L, Hu XC, Zhang TD. Treatment of acute promyelocytic leukemia by Ailing-1 therapy with use of syndrome differentiation of traditional Chinese medicine. *Chin J Comb Trad Chin Med West Med* 1992; **12**: 170–171.
4 Zhang P, Wang SY, Hu XH. Arsenic trioxide treated 72 cases of acute promyelocytic leukemia. *Chin J Hematol* 1996; **17**: 58–62.
5 Shen ZX, Chen GQ, Ni JH, Li XS, Xiong SM, Qiu QY *et al.* Use of arsenic trioxide (AS_2O_3) in the treatment of acute promyelocytic leukemia (APL): II. Clinical efficacy and pharmacokinetics in relapsed patients. *Blood* 1997; **89**: 3354–3360.
6 Tallman MS, Nabhan C, Feusner JH, Rowe JM. Acute promyelocytic leukemia: evolving therapeutic strategies. *Blood* 2002; **99**: 759–767.
7 Chen GO, Shi XG, Tang W, Xiong SM, Zhu J, Cai X *et al.* Use of arsenic trioxide (As_2O_3) in the treatment of acute promyelocytic leukemia (APL): I. As_2O_3 exerts dose-dependent dual effects on APL cells. *Blood* 1997; **89**: 3345–3353.
8 Cai X, Shen YL, Zhu Q, Cai X, Shen L, Zhu Q *et al.* Arsenic trioxide-induced apoptosis and differentiation are associated respectively with mitochondrial transmembrane potential collapse and retinoic acid signaling pathways in acute promyelocytic leukemia. *Leukemia* 2000; **14**: 262–270.
9 Waxman S, Anderson K. History of the development of arsenic derivatives in cancer therapy. *Oncologist* 2001; **6** (Suppl 2): 3–10.
10 Gianni M, Koken MHM, Chelbi-Alix MK, Benoit G, Lanotte M, Chen Z *et al.* Combined arsenic and retinoic acid treatment enhances differentiation and apoptosis in arsenic-resistant NB4 cells. *Blood* 1998; **91**: 4300–4310.
11 Jing Y, Wang L, Xia L, Chen GQ, Chen Z, Miller WH *et al.* Combined effect of all-*trans* retinoic acid and arsenic trioxide in acute promyelocytic leukemia cells *in vitro* and *in vivo*. *Blood* 2001; **97**: 264–269.
12 Zhu Q, Zhang JW, Zhu HQ, Shen YL, Flexor M, Jia PM *et al.* Synergistic effects of arsenic trioxide and c-AMP during acute promyelocytic leukemia cell maturation subtends a novel signaling cross-talk. *Blood* 2002; **99**: 1014–1022.
13 Lunghi P, Tabilio A, Pinelli S, Valmadre G, Ridolo E, Albertini R *et al.* Expression and activation of SHC/MAP kinase pathway in primary acute myeloid leukemia blasts. *Hematol J* 2001; **2**: 70–80.
14 Morgan MA, Dolp O, Reuter CWM. Cell-cycle-dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. *Blood* 2001; **97**: 1823–1834.
15 Kim SC, Hahn JS, Min YH, Yoo NC, Ko YW, Lee WJ. Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and down-regulation of a phosphatase, PAC1. *Blood* 1999; **93**: 3893–3899.
16 Towatari M, Lida H, Tanimoto M, Iwata H, Hamaguchi M, Saito H. Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia* 1997; **11**: 479–484.
17 Lunghi P, Tabilio A, Dall'Aglio PP, Ridolo E, Carlo-Stella C, Pelicci PG *et al.* Down-modulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. *Leukemia* 2003; **17**: 1783–1793.
18 Milella M, Kornblau SM, Estrov Z, Carter BZ, Lapillone H, Harris D *et al.* Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *J Clin Invest* 2001; **108**: 851–859.
19 Milella M, Estrov Z, Komblau SM, Carter BZ, Konopleva M, Tari A *et al.* Synergistic induction of apoptosis by simultaneous disruption of the Bcl-2 and MEK-MAPK pathways in acute myelogenous leukemia. *Blood* 2002; **99**: 3461–3464.
20 Baines P, Fisher J, Truran L, Davies E, Hallett M, Hoy T *et al.* The MEK inhibitor, PD98059, reduces survival but does not block acute myeloid leukemia blast maturation *in vitro*. *Eur J Haematol* 2000; **64**: 211–218.
21 Platanias LC. MAP kinase signaling pathways and hematological malignancies. *Blood* 2003; **101**: 4667–4679.
22 Lee JT, McCubrey JA. The Raf/MEK/ERK (MAPK) signal transduction cascade as a target for chemotherapeutic intervention in leukemia. *Leukemia* 2002; **16**: 486–507.
23 Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. PD098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J Biol Chem* 1995; **270**: 27489–27494.
24 Sebolt-Leopold JS, Dudley DT, Herrera R, Van Becelaere K, Wiland A, Gowan RC *et al.* Blockade of the MAP kinase pathway suppresses growth of colon tumors *in vivo*. *Nat Med* 1999; **5**: 810–816.
25 Shao W, Fanelli M, Ferrara FF, Riccioni R, Rosenauer A, Davison K *et al.* Arsenic trioxide as an inducer of apoptosis and loss of PML/RaRα protein in acute promyelocytic leukemia cells. *J Natl Cancer Inst* 1998; **90**: 124–133.
26 Mandelli F, Diverio D, Avvisati G, Luciano A, Barbui T, Bernasconi C *et al.* Molecular remission in PML/RARα-positive acute promyelocytic leukemia by combined all-*trans*-retinoic acid and idarubicin (AIDA) therapy. *Blood* 1997; **90**: 1014–1021.
27 Charrad RS, Li Y, Delpech B, Balitrand N, Clay D, Jasmin C *et al.* Ligation of the CD44 adhesion molecule reverses blockage of differentiation in human acute myeloid leukemia. *Nat Med* 1999; **5**: 669–676.
28 Bonati A, Carlo-Stella C, Lunghi P, Albertini R, Pinelli S, Migliaccio E *et al.* Selective expression and constitutive phosphorylation of Shc proteins in the CD34+ fraction of chronic myelogenous leukemias. *Cancer Res* 2000; **60**: 728–732.
29 Tan YI, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J* 1996; **15**: 4629–4642.
30 Chou TC, Talalay P. Quantitative analysis of dose–effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* 1984; **22**: 27–55.

31 Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH, Smulson ME. Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose) polymerase in the early stages of apoptosis. *J Biol Chem* 1998; **273**: 13703–13712.
32 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–498.
33 Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci USA* 2001; **98**: 9742–9747.
34 Scheid MP, Duronio V. Dissociation of cytokine-induced phosphorylation of Bad and activation of PKB/akt: involvement of MEK upstream of Bad phosphorylation. *Proc Natl Acad Sci USA* 1998; **95**: 7439–7444.
35 Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 1999; **286**: 1358–1362.
36 Scheid MP, Schubert KM, Duronio V. Regulation of Bad phosphorylation and association with Bcl-xL by the MAPK/Erk kinase. *J Biol Chem* 1999; **274**: 31108–31113.
37 Fang X, Yu S, Eder A, Mao M, Bast Jr RC, Boyd D *et al*. Regulation of BAD phosphorylation at serine 112 by the Ras-mitogen-activated protein kinase pathway. *Oncogene* 1999; **18**: 6635–6640.
38 Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer S. Bad, a heterodimeric partner for Bcl-xL and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995; **80**: 285–291.
39 Ottilie S, Diaz JL, Horne W, Chang J, Wang Y, Wilson G *et al*. Dimerization properties of human BAD. *J Biol Chem* 1997; **272**: 30866–30872.
40 Ludwig S, Hoffmeyer A, Goebeler M, Kilian K, Hafner H, Neufeld B *et al*. The stress inducer arsenite activates mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 via a MAPK kinase 6/p38-dependent pathway. *J Biol Chem* 1998; **273**: 1917–1922.
41 Chen W, Martindale JL, Holbrook NJ, Liu Y. Tumor promoter arsenite activates extracellular signal-regulated kinase through a signaling pathway mediated by epidermal growth factor receptor and Shc. *Mol Cell Biol* 1998; **18**: 5178–5188.
42 Chang F, Steelman LS, Lee JT, Shelton JG, Shelton JG, Navolanic PM *et al*. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention. *Leukemia* 2003; **17**: 1263–1293.
43 MacKeigan JP, Collins TS, Ting JP. MEK inhibition enhances paclitaxel-induced tumor apoptosis. *J Biol Chem* 2000; **275**: 38953–38956.
44 Persons DL, Yazlovitskaya EM, Cui W, Pelling JC. Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin. *Clin Cancer Res* 1999; **5**: 1007–1014.
45 Wang X, Martindale JL, Holbrook NJ. Requirement for ERK activation in cisplatin-induced apoptosis. *J Biol Chem* 2000; **275**: 39435–39443.
46 Shonai T, Adachi M, Sakata K, Takekawa M, Endo T, Imai K *et al*. MEK/ERK pathway protects ionizing radiation-induced loss of mitochondrial membrane potential and cell death in lymphocytic leukemia cells. *Cell Death Differ* 2002; **9**: 963–971.
47 Kitagawa D, Tanemura S, Ohata S, Shimizu N, Seo J, Nishitai G *et al*. Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src-dependent epidermal growth factor receptor phosphorylation. *J Biol Chem* 2002; **277**: 366–371.
48 Mérienne K, Jacquot S, Zeniou M, Pannetier S, Sassone-Corsi P, Hanauer A. Activation of RSK by UV-light: phosphorylation dynamics and involvement of the MAPK pathway. *Oncogene* 2000; **19**: 4221–4229.
49 Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X_L. *Cell* 1996; **15**: 619–628.
50 Jing Y, Dai J, Cahlmers-Redman RME, Tatton W, Waxman S. Arsenic trioxide selectively induces acute promyelocytic leukemia apoptosis via a hydrogen peroxide dependent pathway. *Blood* 1999; **94**: 2102–2111.
51 Ma DC, Sun YH, Chang KZ, Ma XF, Huang SL, Bai YH *et al*. Selective induction of apoptosis of NB4 cells from G2+M phase by sodium arsenite at lower doses. *Eur J Haematol* 1998; **61**: 27–35.
52 Boucher MJ, Morisset J, Vachon PH, Reed JC, Lainé J, Rivard N. MEK/ERK signaling pathway regulates the expression of Bcl-2, Bcl-X(L), and Mcl-1and promotes survival of human pancreatic cancer cells. *J Cell Biochem* 2000; **79**: 355–369.
53 Jost M, Huggett TM, Kari C, Boise LH, Rodeck U. Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. *J Biol Chem* 2001; **276**: 6320–6326.
54 Pardo OE, Arcaro A, Salerno G, Raguz S, Downward J, Seckl MJ. Fibroblast Growth Factor-2 induces translational regulation of Bcl-XL and Bcl-2 via a MEK-dependent pathway. Correlation with resistance to etoposide-induced apoptosis. *J Biol Chem* 2002; **14**: 12040–12046.
55 Loza Coll M, Rosen K, Ladeda V, Filmus J. Increased Bcl-xL expression mediates v-Src-induced resistance to anoikis in intestinal epithelial cells. *Oncogene* 2002; **21**: 2908–2913.
56 Poruchynsky MS, Wang EE, Rudin CM, Blagosklonny MV, Fojo T. Bcl-xL is phosphorylated in malignant cells following microtubule disruption. *Cancer Res* 1998; **58**: 3331–3338.
57 Fang G, Chang BS, Kim CN, Perkins C, Thompson CB, Bhalla KN. 'Loop' domain is necessary for taxol-induced mobility shift and phosphorylation of Bcl-2 as well as for inhibiting taxol-induced cytosolic accumulation of cytochrome *c* and apoptosis. *Cancer Res* 1998; **58**: 3202–3208.
58 Basu A, Haldar S. Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. *FEBS Lett* 2003; **538**: 41–47.
59 Deverman BE, Cook BL, Manson SR, Niederhoff RA, Langer EM, Rosova I *et al*. Bcl-xL deamidation is a critical switch in the regulation of the response to DNA damage. *Cell* 2002; **111**: 51–62.
60 Takehara T, Takahashi H. Suppression of Bcl-xL deamidation in human hepatocellular carcinomas. *Cancer Res* 2003; **63**: 3054–3057.
61 Schimmer AD, Hedley DW, Penn LZ, Minden MD. Receptor- and mitochondrial-mediated apoptosis in acute leukemia: a translational view. *Blood* 2001; **98**: 3541–3553.
62 Tudor G, Aguilera A, Halverson DO, Laing ND, Sausville EA. Susceptibility to drug-induced apoptosis correlates with differential modulation of Bad, Bcl-2 and Bcl-xL protein levels. *Cell Death Differ* 2000; **7**: 574–586.
63 Sebolt-Leopold JS. Development of anticancer drugs targeting the MAP kinase pathway. *Oncogene* 2000; **19**: 6594–6599.
64 English JM, Cobb MH. Pharmacological inhibitors of MAPK pathways. *Trends Pharmacol Sci* 2002; **23**: 40–45.
65 Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000; **351**: 95–105.
66 Lunghi P, Costanzo A, Levrero M, Bonati A. Treatment with arsenic trioxide (ATO) and MEK1 inhibitor activates the p73-p53AIP1 apoptotic pathway in leukemia cells. *Blood* 2004; **104**: 519–525.
67 Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T *et al*. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000; **102**: 849–862.
68 Costanzo A, Merlo P, Pediconi N, Fulco M, Sartorelli V, Cole PA *et al*. DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. *Mol Cell* 2002; **9**: 175–186.
69 Matsuda K, Yoshida K, Taya Y, Nakamura K, Nakamura Y, Arakawa H. p53AIP1 regulates the mitochondrial apoptotic pathway. *Cancer Res* 2002; **62**: 2883–2889.
70 Zhao S, Konopleva M, Cabreira-Hansen M, Xie Z, Hu W, Milella M *et al*. Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias. *Leukemia* 2004; **18**: 267–275.
71 Carter BZ, Milella M, Altieri DC, Andreeff M. Cytokine-regulated expression of survivin in myeloid leukemia. *Blood* 2001; **97**: 2784–2790.

72 Carter BZ, Milella M, Tsao T, McQueen T, Schober WD, Hu W *et al*. Regulation and targeting of antiapoptotic XIAP in acute myeloid leukemia. *Leukemia* 2003; **17**: 2081–2089.
73 McCubrey JA, Lee JT, Steelman LS, Blalock WL, Moye PW, Chang F *et al*. Interactions between the PI3K and Raf signaling pathways can result in the transformation of hematopoietic cells. *Cancer Detect Prev* 2001; **25**: 375–393.
74 Blalock WL, Navolanic PM, Steelman LS, Shelton JG, Moye PW, Lee JT *et al*. Requirement for the PI3K/Akt pathway in MEK1-mediated growth and prevention of apoptosis: identification of an Achilles heel in leukemia. *Leukemia* 2003; **17**: 1058–1067.
75 Hayakawa F, Privalsky ML. Phosphorylation of PML by mitogen-activated protein kinases plays a key role in arsenic trioxide-mediated apoptosis. *Cancer Cell* 2004; **5**: 389–401.
76 Mann KK, Miller WH. Death by arsenic: implications of PML sumoylation. *Cancer Cell* 2004; **5**: 5307–5309.
77 Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 1995; **80**: 179–185.
78 Duesbery NS, Webb CP, Vande Woude GF. MEK wars, a new front in the battle against cancer. *Nat Med* 1999; **5**: 736–737.