Inhibition of Class I Phosphoinositide 3-Kinase Activity Impairs Proliferation and Triggers Apoptosis in Acute Promyelocytic Leukemia without Affecting Atra-Induced Differentiation

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Abstract

We have investigated the role of phosphoinositide 3-kinases (PI3Ks) in the *in vitro* pathophysiology of acute promyelocytic leukemia (APL) and in the response to treatment with *all-trans*-retinoic-acid (ATRA), utilizing a range of novel inhibitors that target individual or all catalytic class I isoforms of PI3K (p110α, p110β, p110δ, and p110γ). ATRA-induced phosphorylation of the Akt kinase and ribosomal S6 protein in APL cells was sensitive to class I PI3K, and p110β or p110δ inhibitors, and to the mammalian target of rapamycin (mTOR) inhibitor rapamycin. In primary APL, inhibition of p110β or p110δ triggered apoptosis in the absence or presence of ATRA. Class I PI3K inhibition could also reverse ATRA-induced protection of these cells against doxorubicin and arsenic trioxide, correlating with impaired induction of the antiapoptotic MCL-1 protein. The differentiation-inducing effects of ATRA were not dependent on class I PI3K/mTOR. In summary, class I PI3K signaling, mediated by p110β and p110δ, plays an important role in basal and ATRA-induced cell survival mechanisms in APL. Addition of PI3K inhibitors to induction treatment regimens may provide therapeutic benefit. [Cancer Res 2009;69(3):1027–36]

Introduction

Acute promyelocytic leukemia (APL), the M3 (FAB) subtype of acute myeloid leukemia (AML), constitutes between 10% and 15% of adult AML (1). APL cells typically carry a specific reciprocal chromosome translocation, t(15;17), leading to the expression of the PML-RARα fusion protein, which plays a key role in pathogenesis. APL results from a deregulation in the process of myeloid cell self-renewal and differentiation, and is characterized by an accumulation of abnormal promyelocytes that fail to differentiate into neutrophil granulocytes and that are resistant to apoptosis (2). APL differentiation-based therapy using *all-trans* retinoic acid (ATRA) has proven to be efficient at inducing differentiation of leukemic cells and in combination with cytotoxic chemotherapy dramatically improves the long-term survival of APL patients (3). In addition to translocations involving RARα, APL cases may have deregulated signaling via the FLT3 or RAS pathways with a mutation rate of ~40% (4) and 10% (5), respectively.

Phosphoinositide 3-kinases (PI3Ks) catalyze the phosphorylation at the 3′ position of the inositol ring in phosphoinositide lipids and control a variety of functions including proliferation, survival, migration, and differentiation of many cell types. PI3K dysregulation is linked to the pathogenesis of several human diseases including inflammatory disorders and cancer (6). Mammals have eight distinct isoforms of PI3K, divided into three classes (7). The class I catalytic subunits (p110α, p110β, p110γ, and p110δ) produce the PIP_3 lipid and are acutely activated by extracellular stimuli, and signal downstream of Ras (7). Originally, a distinction was made between class IA (p110α, β, δ) and IB (p110γ) subsets based on the putative activation downstream of tyrosine kinases or G protein–coupled receptors (GPCR), respectively (7). However, this distinction between class IA and IB PI3Ks has recently been blurred, given the strong evidence for a key role of p110β downstream of GPCRs (8). p110s are found in heterodimeric complexes with a regulatory subunit, called p85 in the case of p110α, β, δ, and p87/p101 for p110γ. Whereas p110α and p110β are ubiquitously expressed, p110δ and p110γ are highly enriched in the hematopoietic system (9, 10). A combination of genetic (8, 11, 12) and inhibitor-based studies (13–15) have provided evidence for isoform-specific roles of the distinct p110s. Activating mutations in catalytic PI3K subunits have only been found for p110α (16), but overexpression of the nonmutated forms of the other class I PI3K isoforms can cause oncogenic transformation in avian cell model systems (17). PI3K pathways can also be activated by loss-of-function of the negative regulator PTEN, a lipid phosphatase that degrades PI3K lipids (18). Interestingly, the frequency of mutations in PTEN and p110α in hematologic malignancies is negligible (19, 20).[3] It is possible that the PI3K pathway is instead activated as a consequence of oncogenic activation of upstream pathways (such as FLT3 and RAS; refs. 4, 5), or by autocrine growth factor signaling (such as insulin-like growth factor (IGF-I); refs. 21, 22).

Class I PI3K signaling leads to activation of the Akt serine/threonine kinase. Akt is involved in the control of a number of signaling modules and one of its key targets is the mammalian target of rapamycin (mTOR) pathway, which regulates cell growth and metabolism. mTOR belongs to a family of PI3K-related Ser/Thr kinases and resides in two distinct signaling complexes, TORC1 and TORC2 (23–25). mTOR in the TORC1 complex is sensitive to the actions of the drug rapamycin (26).

Until recently, only broad-spectrum PI3K inhibitors (such as wortmannin and LY294002) were available, and use of these agents

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[3] http://www.sanger.ac.uk/genetics/CGP/cosmic

in vivo is associated with significant toxicities (27). LY294002 not only blocks PI3K but also inhibits mTOR and various other kinases. Novel agents that selectively target PI3K isoforms have now been generated (14). Using isoform-selective PI3K inhibitors for p110δ, we and others have reported that the constitutive activation of PI3K/Akt signaling in primary AML cells is predominantly attributable to p110δ (28, 29). Pharmacologic inhibition of p110δ can inhibit proliferation and potentiate the apoptotic effect of etoposide/VP16, without adverse effects on the biology of normal hematopoietic progenitors (28, 29).

Here, we investigate the roles of PI3K isoforms in APL. Several reports have provided evidence that the PI3K/Akt/mTOR pathway plays a role in the induction of retinoic acid responses in promyelocytic leukemic cells (30, 31). For example, Lal and colleagues (31) showed that ATRA activates Akt and mTOR pathways in the NB-4 APL cell line. Based on the observation that ATRA-induced differentiation could be blocked by LY294002, it was suggested that PI3K/mTOR inhibition could therefore compromise the efficacy of retinoid therapy. In the last few years, a number of reports have documented a broad range of inhibitory activities of LY294002 beyond PI3K, calling into question some of the conclusions reached in earlier studies utilizing this compound to evaluate the role of PI3K (14, 32). In the present study, we used novel selective class I PI3K inhibitors (Table 1), alone or in combination with ATRA, to evaluate the effects of blocking PI3K signaling on APL cell proliferation, survival, and differentiation, using the NB-4 cell line and primary APL samples. We report that class I PI3K inhibition blocked basal activation of PI3K/Akt/mTOR signaling and could reduce proliferation and induce apoptosis. Treatment of APL cells with ATRA led to the activation of PI3K and improved cell survival in the presence of cytotoxic agents such as doxorubicin. This could be reversed by class I PI3K inhibitors which, somewhat surprisingly, did not affect APL differentiation. These results suggest that addition of PI3K inhibitors to induction treatment regimens for APL might be of clinical value.

Materials and Methods

Reagents. PIK4, PI-103, LY294002, and rapamycin were obtained from Calbiochem; TGX-155 and TGX-221 from Serono and Cayman, respectively; and IC87114 and AS604850 from Serono. All inhibitors (Table 1) and ATRA were dissolved in DMSO and stored at −20°C. ATRA, 12-0-tetradecanoylphorbol 13-acetate (TPA), doxorubicin, and arsenic trioxide were obtained from Sigma. Sources of other reagents are as follows: polyclonal anti–T308-pAkt, anti-S473-pAkt, anti–S235/236-pS6, anti-PTEN, anti–cleaved caspase-9, anti–pro-caspase-9 and monoclonal anti-Akt antibodies (Cell Signaling Technology), MCL-1 (sc-819; Santa Cruz), BCL-XL (sc-634; Santa Cruz), monoclonal anti–Bcl-2 and caspase-3 antibodies (BD Biosciences), polyclonal anti-BIM (R & D Systems), monoclonal antibodies against β-actin and α-tubulin (Sigma), monoclonal antibodies against glyceraldehyde-3-phosphate dehydrogenase (Abcam), phycoerythrin (PE)-conjugated polyclonal antibodies against CD11b (DakoCytomation). Polyclonal anti-p110α antibodies were a gift from Calle Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden), polyclonal anti-p110β (sc-602; Santa Cruz) antibodies were purchased from Santa Cruz, and polyclonal antibodies to p110δ have been described previously (9).

Cell culture. NB-4 cells, an established human APL cell line with t(15;17), were cultured in RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin. Patient APL samples from peripheral blood or bone marrow were obtained from the UK AML tumor cell bank or from patients presenting to University College Hospital, London. Written informed consent was obtained from all patients. Leukemic blasts were isolated by standard Ficoll-Hypaque density gradient centrifugation. All patient samples were used fresh within 24 h of collection. Cells were resuspended in RPMI 1640 and 10% FCS (RPMI/10% FCS). No exogenous cytokines were added. NB-4 and primary APL cells were cultured in RPMI/10% FCS in the presence of vehicle (DMSO), PIK4 (0.5 μmol/L), TGX-155/TGX-221 (0.5/1 μmol/L), IC87114 (5 μmol/L), AS604850 (1 μmol/L), PI-103 (0.5 μmol/L), Rapamycin (20 nmol/L; ref. 31), LY294002 (25 μmol/L), ATRA (1 μmol/L), doxorubicin (0.5 μmol/L), arsenic trioxide (1 mmol/L) in the indicated combination. The doses of drugs used throughout the study are as above, unless otherwise indicated, and the IC_{50} of each isoform-selective PI3K inhibitor are listed in Table 1.

Cell proliferation, apoptosis, and measurement of mitochondrial membrane potential assays. Using a cell counter (Casy Model TT; Scharfe System), NB-4 cell number from aliquots of time course experiments was estimated after 72 h of continuous treatment. Cell proliferation/survival assays using the CellTiter96 aqueous nonradioactive cell proliferation kit (Promega), which is based on the MTS (a tetrazolium compound) colorimetric dye reduction, were performed as in previous studies (29). The apoptosis assay based on Annexin V cell surface binding [flow cytometric analysis of FITC-conjugated Annexin V expression and propidium iodide (PI) exclusion] was carried out according to the Manufacturer's instructions (BD Biosciences) and was performed as previously described (29). For each assay, results were quantitated from 3 to 5 independent experiments to calculate the mean and SE, and values were normalized to those obtained for control cells. Loss of mitochondrial membrane potential was detected using the fluorescent dye chloromethyl-X-rosamine (CMXRos, Molecular Probes/Invitrogen) by incubating primary APL cells in 200 nmol/L CMXRos for 15 min at 37°C and analyzing for reduced fluorescence by flow cytometry.

Table 1. *In vitro* IC_{50} (μmol/L) of isoform-selective PI3K inhibitors

	p110α	p110β	p110δ	p110γ	mTORC1	mTORC2
PIK-4	0.002	0.016	NA	0.66		
TGX-155	>20	0.03	0.34	>20		
TGX-221	5	0.007	0.1	3.5		
IC87114	>100	1.82	0.07	1.24		
AS-604850	3.4	>20	>20	0.19		
PI-103	0.008	0.088	0.048	0.15	0.02	0.083
LY294002	0.7	0.306	1.33	7.26	8.9	

NOTE: Data were compiled from published work: PIK-4 (p110α-selective inhibitor; ref. 49), TGX-155 (p110β-selective inhibitor; ref. 13), TGX-221 (p110β-selective inhibitor; ref. 50), IC87114 (p110δ-selective inhibitor; ref. 13), AS-604850 (p110γ-selective inhibitor; ref. 13), PI-103 (class I PI3K and mTOR inhibitor; ref. 14), and LY294002 (pan-PI3K and mTOR inhibitor; ref. 13).

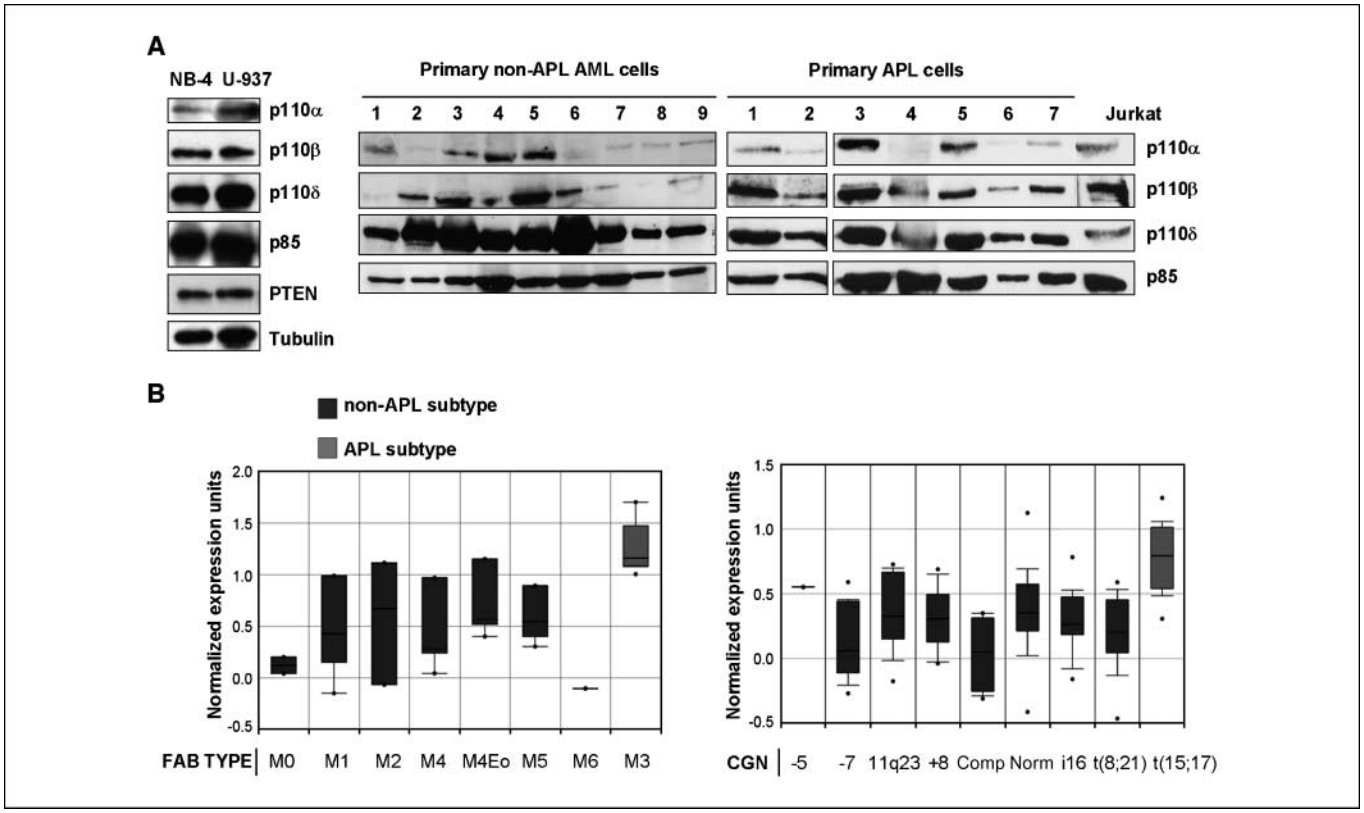

Figure 1. PI3K isoform expression in NB-4 and primary APL. *A,* class IA PI3K expression. Fifty micrograms of total cell lysates from NB-4 cells, 9 separate non-APL AML samples, and 7 separate APL samples were separated by SDS-PAGE and immunoblotted using the indicated antibodies. U937 (AML) and Jurkat (T-ALL) lysates were used as positive controls *B*. p110β mRNA levels in AML subtypes, as shown in the Oncomine cancer profiling database (http://www.oncomine.org/; accessed March 2008; ref. 34). *Left,* non-APL FAB TYPE (M0, M1, M2, M4, M4E0, M5, and M6) and APL FAB TYPE (M3). *Right,* different cytogenetic groups compared with the APL cases, t(15;17). *comp,* complex; *norm,* normal; *i16,* inversion 16.

Cell lysis and Western blot analysis. Cell lysis and Western blot analysis were performed as previously described (29).

Analysis of signaling pathway activation by flow cytometry. APL cells were incubated ± ATRA overnight with the indicated inhibitors or vehicle control. Cells were fixed and permeabilized using the Fix & Perm kit (Caltag/Invitrogen) according to the manufacturer's instructions. Primary antibodies used were anti–pS473-Akt (clone 193H12; unconjugated; Cell Signaling Technology) and anti–pS235/236-S6 (clone D57.2.2E; Alexa Fluor 488 Conjugated; Cell Signaling Technology). For pAkt staining, secondary anti–rabbit-APC antibody was used (Molecular Probes/Invitrogen). Samples were analyzed on a Cyan Analyzer (DAKO Cytomation) and results depicted as median fluorescent intensity of the whole population.

Morphologic assessment of leukemic cells. May-Grunwald Giemsa staining was used to analyze myeloid differentiation. Cytospins were prepared from cells cultured in suspension using the centrifugation (Shandon Cytospin 4; Thermo Electron Corporation) and washed once in PBS. Cytospins were fixed and stained in May-Grunwald solution (Sigma-Aldrich) for 5 min, rinsed in water for 5 s, followed by counter-staining of nuclei with 10% Giemsa solution (Merck kGaA) for 15 min. Photographs were taken at ×250 magnification using a Zeiss microscope coupled to a CCD camera (AxioCam; HPc; Zeiss).

Measurement of cell surface expression of CD11b. Neutrophil differentiation was assessed by analysis for the cell surface marker CD11b, which is associated with myeloid maturation. Cells were washed in PBS, resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide, and stained with PE-conjugated anti-CD11b polyclonal antibodies for 30 min at 4°C. After 2 washes in PBS containing 1% bovine serum albumin and 0.1% sodium azide, cells were fixed using CellFix 10× buffer (BD Biosciences). The proportion of cells expressing CD11b and the fluorescence intensity of CD11b were measured by flow cytometry using a FACS Scan (BD FACSCanto Flow cytometer, BD Biosciences or Cyan ADP Analyzer, DAKO), using an isotype-matched control antibody (used for subtraction of background signal). The results were analyzed using BD FACSDIVA (BD Biosciences) or SUMMIT (DAKO) software and were quantitated from three independent experiments to calculate mean and SE, and values were normalized to those obtained for the control cells.

Determination of reactive oxygen species generation. Acquisition of respiratory burst activity was assessed by the production of hydrogen peroxide using 2′7′dichlorofluorescein diacetate (DCFDA; Molecular Probes) as previously described (33). Briefly, APL cells were loaded with 10 μmol/L DCFDA for 15 min at 37°C and 5% of CO_2, followed by incubation in the absence or presence of 1 μg/mL TPA (Sigma) for 5 min at 37°C in 5% CO_2. The cells were pelleted by centrifugation, rinsed, and analyzed by flow cytometry for green fluorescence (excitation, 488 nm; emission, 515 nm) using a FACS Scan (Cyan ADP Analyzer; DAKO). The results were analyzed using SUMMIT (DAKO) software.

Results

Expression and activity of class IA PI3Ks in APL cells. We first evaluated the expression levels of class IA PI3K isoforms in the APL cell line NB-4 and in primary APL samples, by immunoblotting of total cell lysates (Fig. 1*A*). The p85 regulatory subunit of PI3K was found in all APL cells. Whereas NB-4 expressed all 3 class IA PI3K catalytic subunits, primary APL often lacked detectable expression of p110α, in contrast to p110β and p110δ, which were always detected. We and others have previously documented that

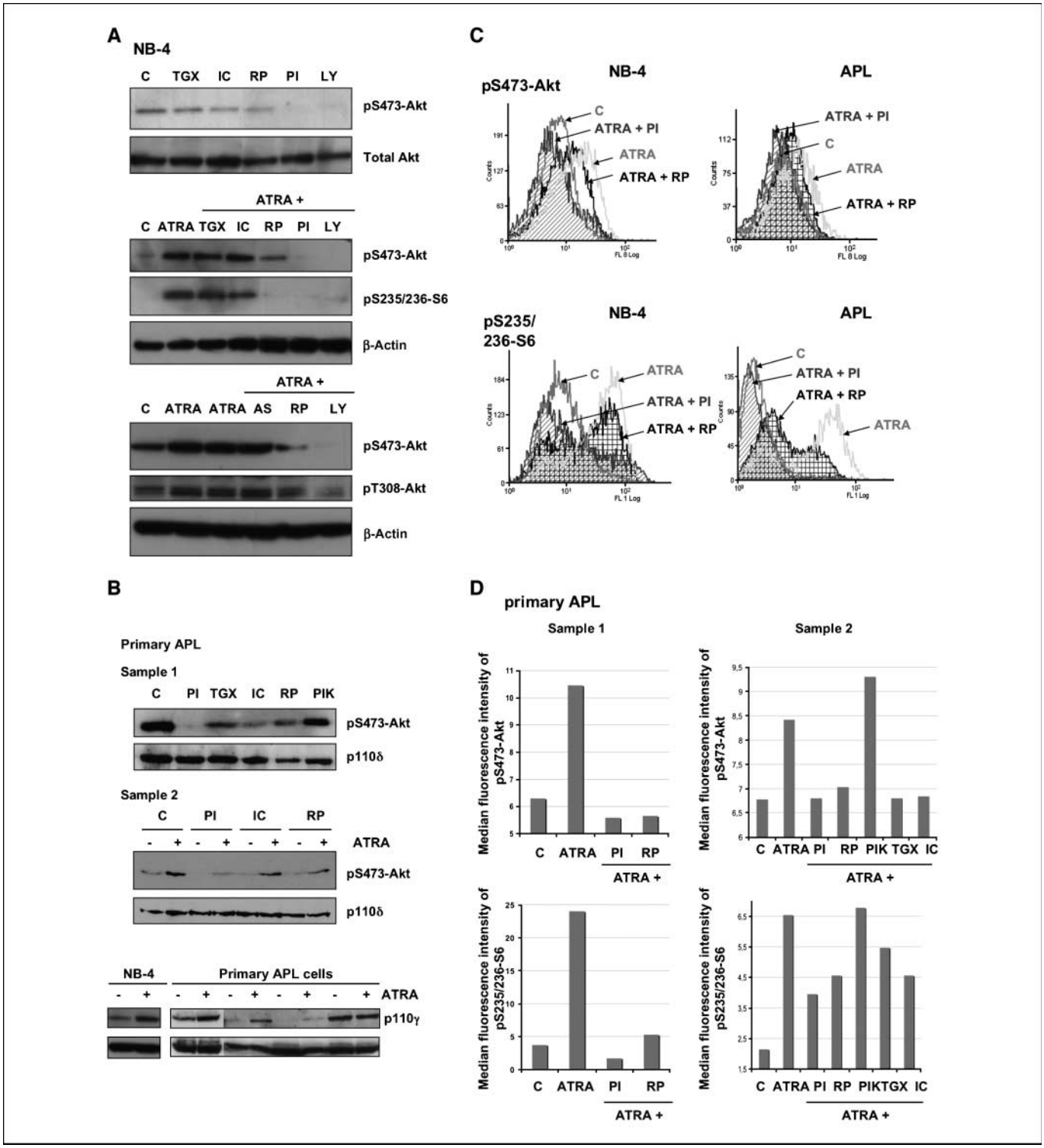

Figure 2. PI3K pathway activation and response to ATRA and PI3K inhibitors in NB-4 and primary APL. *A,* immunoblot analysis of pSer473-Akt in NB-4 cell extract (*top*). Cells were incubated in the presence of constant doses of the indicated PI3K and mTOR inhibitors. Total Akt was used as a loading control. *Middle* and *bottom,* immunoblot analysis of pS473-Akt, pT308-Akt, and pS235/236-S6 in cell extracts from control and NB-4 cells treated ± ATRA in the presence of constant doses of indicated PI3K and mTOR inhibitors. β-actin was used as a loading control. *B,* immunoblot analysis of pS473-Akt in two separate primary APL samples incubated ± ATRA in the presence of constant doses of the indicated PI3K and mTOR inhibitors (*top* and *middle*). p110δ expression was used as a loading control. *Bottom,* induction of expression of p110γ in response to ATRA in NB-4 and primary APL cells. NB-4 cells and four separate primary APL samples were incubated with ATRA overnight and immunoblotted for p110γ. β-actin and p110δ were used as a loading control for NB-4 and primary APL cells, respectively. *C,* overlay flow cytometry plots of pS473-Akt and pS235/236-S6 in NB-4 cells and primary APL treated ± ATRA and PI-103 or rapamycin. *D,* quantitative analysis by fluorescence-activated cell sorting (FACS; median cell fluorescence intensity) of pAkt (*top*) and pS6 (*bottom*) in two independent primary APL samples. *AS,* AS604850 (p110γ-selective inhibitor); *C,* control; *IC,* IC87114 (p110δ-selective inhibitor); *LY,* LY294002 (pan-PI3K and mTOR inhibitor); *PI,* PI-103 (class I PI3K and mTOR inhibitor); *PIK,* PIK4 (p110α-selective inhibitor); *RP,* rapamycin (mTOR inhibitor); *TGX,* TGX-155 (p110β-selective inhibitor) for NB-4 and TGX-221 (p110β-selective inhibitor) for primary APL cells.

p110δ was consistently present in primary non-APL subtypes of AML cells, with more variable expression of p110α and p110β (28, 29). Thus, whereas expression of p110α and p110δ in APL were analogous to primary non-APL subtypes of AML cells, APL seemed to have higher expression of p110β. The Oncomine gene expression profiling database[4] also revealed relatively higher p110β mRNA levels in morphologically or molecularly defined APL, compared with non-APL cells (Fig. 1*B*; ref. 34).

Basal PI3K pathway activation in APL cells is blocked by pan-class I inhibitors, but not by isoform-selective PI3K inhibitors or rapamycin. To determine PI3K pathway activation, we analyzed the phosphorylation of the PI3K target Akt by immunoblotting using antibodies specific for phosphorylated threonine 308 or serine 473 of this kinase (further called pT308-Akt and pS473-Akt). Basal Akt phosphorylation was detected in NB-4 cells (Fig. 2*A, top*) and in six of eight primary APL samples (Fig. 2*B, top* and *middle*; data not shown). This could be fully inhibited by PI-103 (a pan-class I PI3K/mTOR inhibitor) or LY294002, suggesting that Akt phosphorylation in these cells results from the constitutive activation of class I PI3Ks. Modest inhibition of basal Akt phosphorylation was induced by selective inhibition of the p110β or p110δ isoforms of PI3K (by TGX155 and IC87114, respectively; ref. 13) or of mTORC-1 [by rapamycin (31), both in NB-4 cells (Fig. 2*A, top*) and primary APL cells (Fig. 2*B, top* and *middle*)]. In contrast to the findings in primary APL cells, previously published data, including from our group, has suggested that in non-APL AML (28, 29), the p110δ isoform of PI3K is mainly responsible for the basal activation of PI3K/Akt.

ATRA-induced PI3K pathway activation is sensitive to isoform-selective PI3K inhibitors and rapamycin. ATRA has been shown to activate Akt and p70 S6 kinase in NB-4 cells, and it has been suggested, based on the use of LY294002, that the PI3K/Akt/mTOR pathway is essential for cellular differentiation in response to ATRA (31). Expression of the class IB PI3K p110γ was slightly increased upon ATRA treatment in NB-4 cells, in keeping with the results of Scholl and colleagues (30), and in three of four primary APL samples (Fig. 2*B, bottom*). However, pharmacologic inhibition of p110γ (using the selective inhibitor AS604850) did not affect Akt phosphorylation (Fig. 2*A, bottom*), suggesting that the ATRA-induced Akt activation is not a consequence of p110γ up-regulation.

Both in NB-4 cells (Fig. 2*A* and *C*) and in 6 of 8 primary APL samples (Fig. 2*B–D*; data not shown), ATRA treatment led to phosphorylation of Akt and S6, in a PI-103– or LY294002-dependent manner. In primary APL cells, but not in NB-4, this ATRA-induced phosphorylation of Akt and S6 was also inhibited by isoform-selective inhibitors of p110β or p110δ but not of p110α (Fig. 2*A–D*). Rapamycin partially inhibited ATRA-induced pS473-Akt in NB-4 cells (Fig. 2*A* and *C*), with an even more significant effect in three separate primary APL samples (Fig. 2*C* and *D*). Rapamycin also abrogated ATRA-induced S6 phosphorylation in NB-4 (Fig. 2*A* and *C*) and primary APL cells (Fig. 2*B–D*).

Inhibition of class I PI3K + mTOR impairs NB-4 cell proliferation in the absence or presence of ATRA but does not lead to apoptosis. PI-103 and LY294002 strongly inhibited cell NB-4 cell proliferation over a 72-hour period, with a reduction in cell numbers (as assessed by CASY cell counting) of 60% and 72%, respectively (Fig. 3*A*). MTS assays yielded similar observations,

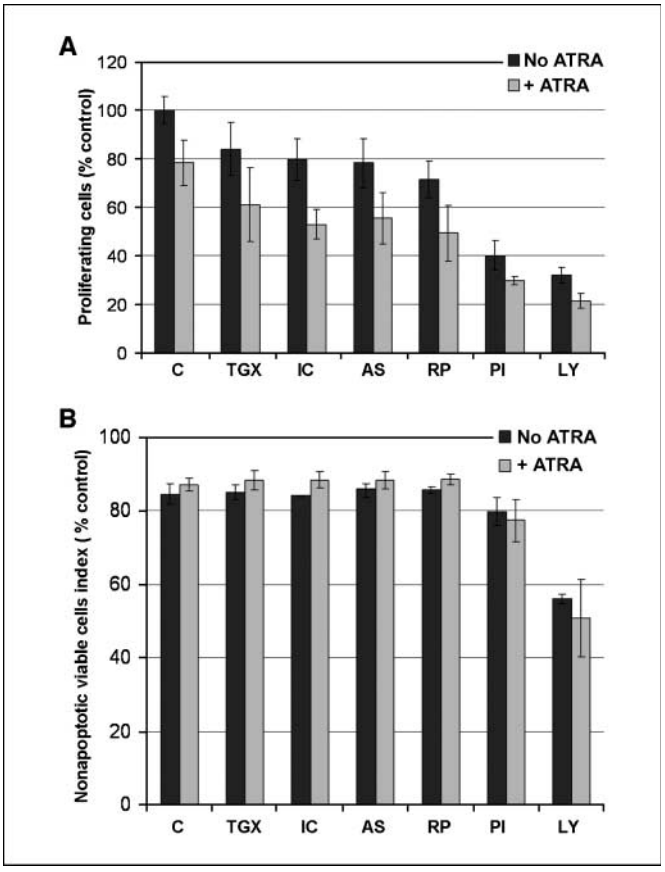

Figure 3. Effect of class I PI3K inhibitors on proliferation and apoptosis of NB-4. NB-4 cells were cultured in RPMI/10% FCS for 72 h ± ATRA ± constant doses of the indicated kinase inhibitors, followed by cell counting using the Casycounter (*A*) or by FACS analysis (*B*) to determine the % of nonapoptotic Annexin V^-/PI^- cells. Results are expressed as a % of control (*no ATRA,* no inhibitor). *AS*, AS604850 (p110γ-selective inhitor); *ATRA*, *all-trans* retinoic-acid; *C*, control; *IC*, IC87114 (p110δ-selective inhibitor); *LY*, LY294002 (pan-PI3K and mTOR inhibitor); *PI*, PI-103 (class I PI3K and mTOR inhibitor); *RP*, rapamycin (mTOR inhibitor); *TGX*, TGX-155 (p110β-selective inhibitor).

with a decrease of signal to 41% and 33% of control for PI-103 and LY294002, respectively (data not shown).

Whereas LY294002 induced a significant proapoptotic effect (Fig. 3*B*), PI-103 only induced a minor increase in apoptotic cells (Fig. 3*B*), despite reducing Akt phosphorylation to the same extent as LY294002 (Fig. 2*A*). Selective inhibitors of p110β, p110δ, p110γ, or mTOR all only induced a modest reduction in NB-4 cell proliferation compared with PI-103 and failed to trigger cell death (Fig. 3*A* and *B*).

Treatment of NB-4 cells with ATRA for a 72-hour period led to a 20% to 30% reduction of proliferation—a similar reduction was also detected in the presence of PI3K inhibitors or rapamycin (Fig. 3*A*).

Inhibition of class I PI3K + mTOR induces apoptosis of primary APL cells in the absence or presence of ATRA. Incubation of primary patient-derived leukemia cells with a dose-range of PI-103 for 5 days led to substantial inhibition in the MTS signal, with a maximal inhibition at 0.5 μmol/L (Fig. 4*A*). Interestingly, selective blockade of p110β, p110δ, or mTOR had a more significant effect on these primary cells than in NB-4 cells, inducing a reduction in MTS activity approaching the level observed with PI-103 (Fig. 4*B*). An inhibitor with selectivity for p110α (PIK) only had a minor effect on cell numbers (Fig. 4*B*). Flow

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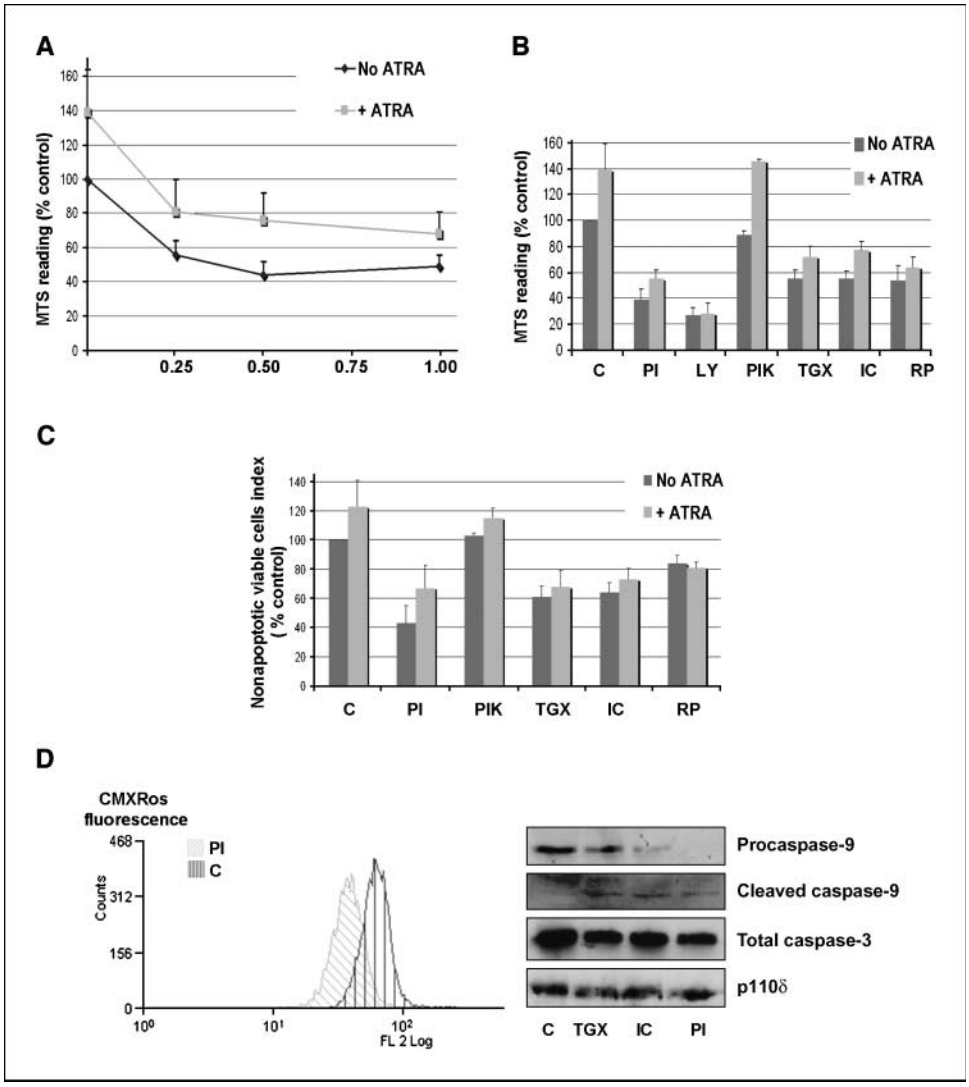

Figure 4. Effect of class I PI3K inhibitors on cell number and apoptosis of primary APL. Primary APL cells were cultured in RPMI/10% FCS for 5 d ± ATRA, in the presence of (*A*) increasing concentrations of PI-103 (n = 9) or (*B*) constant doses of the indicated PI3K and mTOR inhibitors (n = 7), followed by MTS staining. Results are expressed as a % of control. *Columns,* mean; *bars,* SE. Primary APL cells (*C*) were processed as above and analyzed by FACS to determine the relative % of nonapoptotic Annexin V^-/PI^- cells. Results are expressed as a % of control. *Columns,* mean (n = 7); *bars,* SD. Primary APL cells (*D*) were incubated with the indicated inhibitors for 3 d and examined by flow cytometry for mitochondrial membrane potential using CMXRos (*left*) and by immunoblotting for the presence of proCaspase-9, cleaved Caspase-9, and Caspase-3 (*right*). *ATRA*, *all-trans* retinoic-acid; *C*, control; *IC*, IC87114 (p110δ-selective inhibitor); *LY*, LY294002 (pan-PI3K and mTOR inhibitor); *PI*, PI-103 (class I PI3K and mTOR inhibitor); *PIK*, PIK4 (p110α-selective inhibitor); *RP*, rapamycin (mTOR inhibitor); *TGX*, TGX-221 (p110β-selective inhibitor).

cytometric Annexin V/PI assays showed that inhibitors of p110β, p110δ or mTOR, as well as PI-103, potently induced cell death of primary APL cells (Fig. 4*C*), indicating that the reduction in the MTS signal is largely due to induction of apoptosis. Incubation of primary APL cells with PI3K inhibitors led to a loss of mitochondrial membrane potential as detected by CMXRos fluorescence, to a reduction in the level of full length procaspase-9 and the appearance of the cleaved, active, caspase-9 fragment (Fig. 4*D*). These results indicate that the effects of PI3K inhibitors are likely to be mediated by the mitochondrial pathway of cell death.

Treatment of primary APL cells with ATRA increased cell recovery at the end of the 5 day incubation period, with an increase in MTS activity (Fig. 4*A* and *B*) and a reduction in apoptotic cells (Fig. 4*C*), in keeping with previously published data (35, 36). The presence of ATRA did not significantly rescue the APL cells from the apoptotic effect of the PI3K and mTOR inhibitors (Fig. 4*C*).

ATRA-induced chemoresistance and up-regulation of MCL-1 are reduced by inhibition of class I PI3K. ATRA has previously been shown to attenuate anthracycline-induced cytotoxicity in APL cells (37). In NB-4 cells, ATRA preincubation significantly reduced apoptosis induced by the anthracycline doxorubicin but not by arsenic trioxide, another therapeutic agent used in APL (Fig. 5*A*). ATRA was unable to protect NB-4 cells from doxorubicin-induced apoptosis in the presence of PI-103. Arsenic trioxide induced apoptosis was enhanced in the presence of PI-103, with or without ATRA (Fig. 5*A*).

In primary APL, ATRA consistently reduced the apoptotic response to doxorubicin and, to a lesser extent, to arsenic trioxide. The addition of PI-103 and, less markedly, rapamycin, overcame this protective effect of ATRA, with some variation observed among patient samples (Fig. 5*B*).

To investigate the potential mechanism by which ATRA inhibits cell death, we measured the expression of Bcl-2 family members following overnight incubation with this compound. ATRA induced an increase in the level of the antiapoptotic MCL-1 protein, in a PI-103–dependent manner (Fig. 5*C*). ATRA did not affect the levels of BCL-XL (Fig. 5*C*), XIAP, or survivin (data not shown), and reduced the levels of Bcl-2 [as previously reported (37), and of the proapoptotic protein BIM in a PI-103–independent manner (Fig. 5*C*)].

Inhibition of class I PI3K or mTOR does not affect spontaneous or ATRA-induced APL cell differentiation. Treatment of NB-4 cells or primary APL cells with PI3K inhibitors

or rapamycin did not affect the basal levels of CD11b and p47/p67-phox (cytosolic components of the respiratory burst enzyme NADPH oxidase) or TPA-induced production of reactive oxygen species (data not shown).

Incubation of these cells with ATRA increased cell-surface CD11b expression (data not shown) and resulted in morphologic maturation (Fig. 6*A*) and up-regulation of TPA-induced respiratory burst activity (Fig. 6*B* and *C*). Addition of PI-103 or isoform-selective PI3K inhibitors or rapamycin did not significantly affect any aspect of ATRA-induced differentiation (Fig. 6*A–C*); a 25% reduction of ATRA-induced CD11b expression was seen with LY294002 in NB-4 cells (data not shown). These results indicate that although treatment of APL cells with ATRA increases PI3K/Akt/mTOR activity, this pathway does not play a significant role in cell differentiation.

Discussion

In malignant hematologic disease, PI3K signaling is most likely activated by oncogenic mutations of upstream regulators such as tyrosine kinases and Ras (38) and/or autocrine growth factor loops (21). Of the different PI3K isoforms, it has been shown that p110δ plays an important role in cell proliferation, survival, and chemoresistance in AML (28, 29). In this study, we have used a panel of novel, selective PI3K inhibitors to examine the role of class I PI3K signaling in APL pathophysiology. Previous work using the NB-4

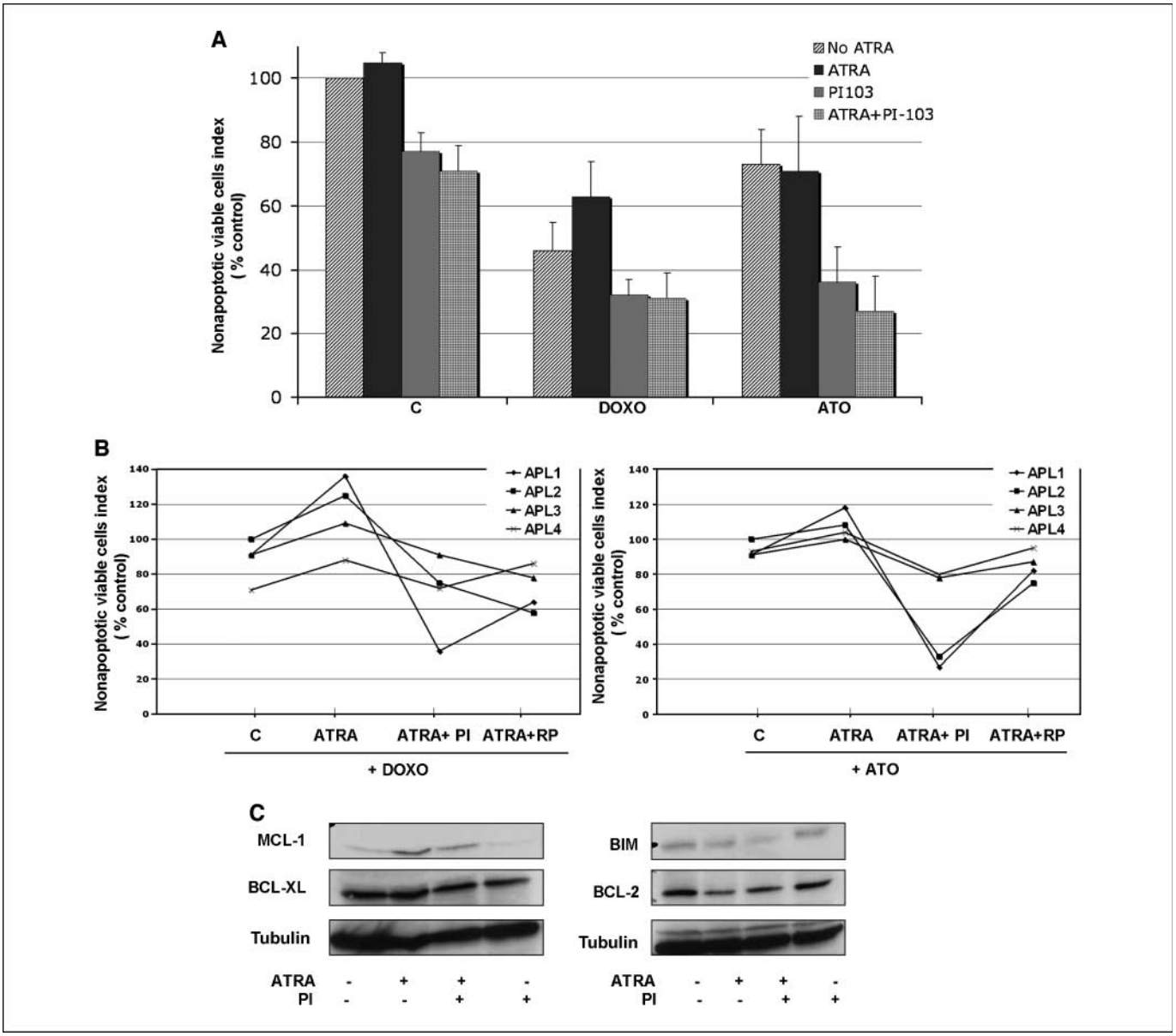

Figure 5. Effect of PI3K inhibition on ATRA-mediated protection from the apoptotic response to doxorubicin (*DOXO*) and arsenic trioxide (*ATO*). NB-4 (*A*) or primary APL cells (*B*) were treated overnight with vehicle, ATRA, PI-103, or Rapamycin. Doxorubicin or arsenic trioxide were added for a further 24 h and samples were analyzed by FACS to determine the relative percentage of nonapoptotic Annexin V^-/PI^- cells. Results are expressed as a % of control. *Columns,* mean ($n = 8$) for NB-4; *bars,* SE; *points,* mean ($n = 4$) for primary APL cells; *bars,* SE. *C,* NB-4 cells were treated overnight with vehicle, ATRA, or PI-103 in the indicated combinations, followed by immunoblotting of total cell lysates using the indicated antibodies. *ATRA, all-trans* retinoic-acid; *C*, control; *PI*, PI-103 (class I PI3K and mTOR inhibitor).

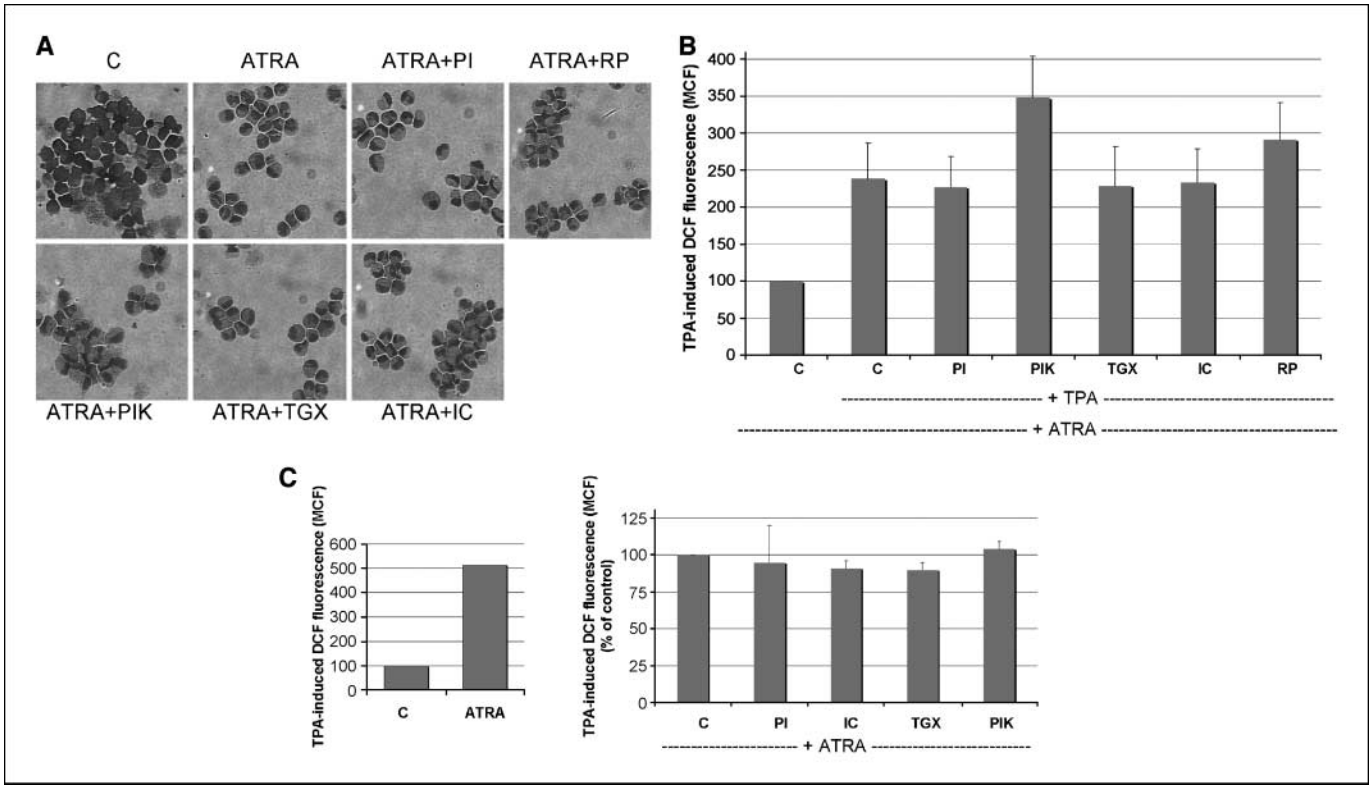

Figure 6. Effect of class IA PI3K and mTOR inhibition on APL cell differentiation. Primary APL cells were cultured for 5 d, in the presence of compounds as indicated, followed by cytospin and May-Grunwald-Giemsa staining (*A*). NB-4 (*B*) or primary APL cells (*C*) were cultured for 5 d in the presence of ATRA and inhibitors as indicated, followed by DCF-DA loading and TPA stimulation and measurement of respiratory burst by FACS. Results are expressed as a % of control (no ATRA, no inhibitor). *Columns,* mean ($n = 5$); *bars,* SE. *ATRA*, *all-trans* retinoic-acid; *C*, control; *IC*, IC87114 (p110δ-selective inhibitor); *PI*, PI-103 (class I PI3K and mTOR inhibitor); *PIK*, PIK4 (p110α-selective inhibitor); *RP*, rapamycin (mTOR inhibitor); *TGX*, TGX-221 (p110β-selective inhibitor).

APL cell line has shown that ATRA activates PI3K/Akt and mTOR signaling (30, 31) and studies using the broad-spectrum PI3K inhibitor LY294002 (which also inhibits mTORC1) have suggested a role for PI3K/Akt signaling in mediating ATRA-induced APL cell differentiation (30, 31, 39). The putative involvement of PI3K signaling in retinoid-induced differentiation of APL cells is of importance in understanding the mechanisms underlying this process and may also have implications for the use of PI3K inhibitors in the treatment of APL, in that agents that interfere with ATRA-induced differentiation could compromise the therapeutic benefit of ATRA.

In primary APL, we found that in addition to p110δ, p110β was expressed in all cases, whereas p110α expression was more variable. Analysis of the Oncomine database, a compendium of cancer transcriptome profiles from published case series, also shows consistently higher levels of p110β mRNA in APL cases compared with other FAB subtypes of AML or other cytogenetic groups (34). As judged by phosphorylation of Akt, the PI3K/Akt signaling axis is constitutively activated in APL and is further enhanced upon treatment with ATRA. Both in NB-4 and primary APL, this enhancement can be blocked by the pan-class I PI3K inhibitor PI-103 (which also inhibits mTORC1). The mechanism of ATRA-induced Akt phosphorylation is unclear. It has previously been suggested that this may be due to up-regulation of the p110γ isoform (30), but this is unlikely to be the main mechanism, given that we found that a selective p110γ inhibitor did not affect Akt phosphorylation induced by ATRA in NB-4 cells.

The mTOR pathway has a complex interrelationship with PI3K/Akt signaling. Indeed, Akt can activate mTOR (in the rapamycin-sensitive mTORC1 complex) via its effects on TSC2/Rheb and this can cause feedback inhibition of PI3K signaling, predominantly via p70 S6-kinase–mediated phosphorylation of the IRS-1 adaptor that is associated with insulin/IGF-I receptors (23). In addition, mTOR (in the rapamycin-insensitive mTORC2 complex) has been identified as a kinase that phosphorylates Akt at S473 thereby resulting in its full activation (24, 25, 40). To add further complexity to the regulation of Akt activation, prolonged treatment of some cell types with rapamycin can result in inhibition of mTORC2 assembly and reduction in pS473-Akt (41). The data presented in this article indicate that ATRA-treated APL cells can be included in the category of cells where Akt S473 phosphorylation is rapamycin-sensitive. Treatment of non-APL AML cells with rapamycin analogues has been reported to either activate Akt signaling via derepression of the IGF-I pathway (22) or to reduce mTORC2 signaling and inhibit Akt phosphorylation (42).

LY294002 has been widely used as a tool compound to evaluate the role of PI3K signaling in a variety of cellular functions. LY294002 inhibits all classes of PI3Ks and is also known now to have off-target effects on related kinases such as mTOR and DNA-PK and unrelated kinases including PIM and CK2 (14). Using LY294002, a number of investigators have implicated PI3K/Akt signaling in ATRA-induced differentiation of NB-4 APL cells (30, 31, 43). Using the more selective class I PI3K inhibitor PI-103 (which also inhibits mTORC1), we show here that ATRA-induced

phosphorylation of Akt and S6 can be blocked by this compound in NB-4 cells and primary APL, without any effect on cell differentiation. We also showed that direct inhibition of mTORC1 using rapamycin did not affect cell differentiation. Taken together, these results indicate that the PI3K/Akt/mTOR signaling pathway does not play a major role in differentiation signaling downstream of ATRA in APL cells.

The PI3K/Akt pathway has been shown to control apoptosis by various mechanisms, including via the regulation of Bcl-2 family members such as Bad, Bim, and Mcl-1 (44). We found that, although blockade of PI3K/Akt signaling in NB-4 cells inhibits proliferation, it did not trigger apoptosis. In contrast, class I PI3K inhibition promotes cell death of primary APL cells in a dose-dependent manner. The most potent proapoptotic effect was seen with combined blockade of all class I PI3Ks + mTOR with the compound PI-103. Inhibition of individual PI3K isoforms showed that p110α is not involved in survival signaling in primary APL cells, likely due to low/absent expression levels, but that p110β and p110δ play a significant part in preventing spontaneous apoptosis. In primary APL cells, a proapoptotic effect is also seen upon rapamycin treatment, possibly due to its combined effects on inhibition of mTORC1 signaling and inhibition of Akt phosphorylation, presumably by destabilizing mTORC2.

Consistent with findings from other investigators, we find that incubation of primary APL cells with ATRA improves viability and prolongs survival *ex vivo* (4, 35, 36). The clinical use of ATRA in APL, in particular as a single agent, can result in increased numbers of WBC in the initial treatment period before the onset of apoptosis associated with terminal differentiation (45). This is linked to clinical complications and the presence of a high leukocyte count predisposes to the development of the ATRA-syndrome and to premature death (46). In this study, we show that blockade of PI3K signaling reduces cell number and viability in response to ATRA in primary APL cells, which may have significant implications for the clinical use of agents targeting this pathway.

In addition to its effects on basal cell survival, treatment with ATRA has been shown to impair the cell death response of APL cells *in vitro* to clinically relevant therapeutic agents including anthracyclines (such as doxorubicin) and to arsenic trioxide (37). The prosurvival signaling effects of ATRA may be partially explained by ATRA-induced activation of NF-κB signaling (47, 48). We show that blockade of class I PI3K signaling negates the prosurvival effects of ATRA and promotes APL cell death when combined with doxorubicin or arsenic trioxide. ATRA addition to APL cells leads to an increase in the levels of the antiapoptotic protein Mcl-1 in a PI3K-dependent manner and the reversal of this increase with PI-103 may contribute to the proapoptotic effect of this compound.

In summary, we have shown that the class I PI3K/Akt/mTOR pathway plays a major part in controlling basal and ATRA-regulated cell proliferation, survival, and chemoresistance pathways in primary APL cells. Despite its potent activation by ATRA, this signaling module is not involved in APL cell differentiation processes. Our results suggest that addition of class I PI3K inhibitors to differentiation induction treatment regimens may be clinically useful in APL.

Disclosure of Potential Conflicts of Interest

B. Vanhaesebroeck: honoraria from speakers bureau, Piramed and Intellikine. A. Khwaja: honoraria from speakers bureau, Piramed. The other authors disclosed no potential conflicts of interest.

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