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Promyelocytic leukemia retinoid signaling targets regulate apoptosis, tissue factor and thrombomodulin expression

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A B S T R A C T

Background and Objectives. Retinoids are involved in cell differentiation, morphogenesis, proliferation and antineoplasic processes. Thus, the retonoic acid receptor (RARα) agonist, AM80, regulates tissue factor (TF), thrombomodulin (TM) expression and granulocytic differentiation in promyelocytic cells, while the RARγ-selective retinoid, CD437, inhibits *in vitro* cell proliferation and induces apoptosis. The mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K) pathways constitute key integration points along the signal transduction cascades that link diverse extracellular stimuli to proliferation, differentiation and survival.

Design and Methods. PI3K and MEK/ERK kinase-dependent pathways were examined as potential targets of retinoid signaling. Likewise, by using specific inhibitors, the role of those kinases in retinoid-induced granulocytic differentiation, apoptosis, and TF and TM expression in NB4 cells were analyzed.

Results. AM80-treated NB4 cells had increased PI3K activity and phosphoinositide turnover. High steady-state pERK-1/-2 activity levels were not significantly changed by AM80. Yet, PI3K inhibitor LY294002 significantly reduced AM80-elicted ERK-1/-2 activity. Thus, the PI3K pathway might contribute to elevated ERK-1/-2 activity in NB4 cells. Inhibition of the PI3K and MEK/ERK pathways reversed AM80-induced granulocytic differentiation, TF down-regulation and TM induction. Besides, CD437 significantly reduced ERK-1/-2 activity of NB4 cells. Further ERK-1/-2 activity inhibition with the MEK inhibitor, PD98059, increased the retinoid pro-apoptotic effect with an additive effect.

Interpretation and Conclusions. Regardless the different regulation of PI3K and MAPK pathways promoted by AM80 and CD437, there is a varying degree of cross-talk between these pathways in the control of the overall response of promyelocytic cells to retinoids. Thus, disruption of targeted pathways, together with specific retinoids, might be an effective therapeutic treatment for acute promyelocytic leukemia.

Key words: promyelocytic leukemia, ERK/MAPK kinases, retinoids, procoagulant activities, apoptosis.

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Retinoids are involved in a wide range of biological processes including cell differentiation, morphogenesis, proliferation and antineoplasic activities in numerous tumor and cell systems, including myeloid cell lines.[1,2] Retinoids operate through their ability to bind to and activate a number of nuclear receptors (retinoic acid receptors or RARs) which function as transcription factors and in turn, regulate the expression of the retinoid target genes.[3] A new synthetic retinoid, 4-[(5,6,7,8-tetrahydro-5.5.8.8-tetramethyl-2-naphthyl)-carbamoyl] benzoic acid, AM80-specific RARα agonist-, is effective in treating acute promyelocytic leukemia which has relapsed from all-trans-retinoic acid-induced complete remission.[4]

In vitro, AM80 has been shown to exert anticoagulant effects by upregulating thrombomodulin (TM) and downregulating tissue factor (TF) expression, as well as inducing granulocytic differentiation in acute promyelocytic leukemia (APL) cells.[5] A RAR-γ selective retinoid, 6-[3-adamantyl-4-hydroxyphenyl]-2-naphthalene carboxylic acid (CD437) represents the prototype of a new class of synthetic retinoids with antiproliferative and apoptogenic properties in APL and other types of leukemia. This retinoid is likely to exert its apoptogenic effects through the activation of intracellular pathways that are distinctly stimulated by all-trans-retinoic acid (ATRA) and many of the available chemotherapeutic agents.[6,7]

The mitogen-activated protein kinase (MAPK) pathway is a key integration point along the signal transduction cascade that links diverse extracellular stimuli to proliferation, differentiation and survival.[8] Among MAPKs subfamilies, the ERK MAP kinase is known to mediate terminal differentiation

in several lineages of hematopoietic cells. In particular, activation of the ERK MAP kinase has been shown to be required for maturation of various myeloid cell lines.[9] While numerous studies have demonstrated an involvement of the MEK/ERK/MAP kinase pathway in hematopoietic cell differentiation, the detailed role of this pathway in retinoid-induced granulocytic differentiation (as well as TF and TM expression and apoptosis in promyelocytic cells) remains poorly understood.

There is evidence pointing to the existence of additional retinoid-induced signaling pathways. One such pathway involves the lipid kinase phosphatidylinositol-3-kinase (PI3K), which generates the putative signaling molecule inositol 3,4,5-triphosphate.[10] An increasing body of experimental evidence characterizes PI3K molecules as regulators of a variety of cellular functions including proliferation, differentiation and cell survival.[11] Several species of PI3K have been cloned and characterized. Among them, PI3Kγ was recently found to form a heterodimer with a protein named p101 and seems to be preferentially expressed in cells of the hematopoietic lineage.[12,13] It has been shown that a certain degree of cross-talk exists between these pathways, which may enhance or negate oncogenic extracellular signals.[14] Furthermore, there is now considerable evidence supporting a role of these evolutionary conserved protein kinases in cellular growth and differentiation. Yet, little is known about the function of these kinases in hematopoietic systems. Thus, a variety of potential signaling pathways have been implicated in the regulation of cell growth and differentiation by retinoids. In addition to these pathways, other as yet unspecified signaling routes may also be involved. In this study we have examined the PI3K and MAPK/ERK kinase-dependent pathways as potential targets of retinoid signaling, as well as the role of those kinases in retinoid-induced granulocytic differentiation, apoptosis and TF and TM expression in the promyelocytic NB4 cells. Our results demonstrate that regardless of the existence of a differential regulation of the PI3K and MEK/ERK pathways promoted by AM80 and CD437, there is a varying degree of cross-talk that occurs between these pathways in the control of the overall response of NB4 cells to the retinoids. Complex cross-talk between these signaling pathways could determine the eventual outcome of each retinoid-induced downstream response.

Design and Methods

Cell cultures

Two milliters of logarithmically-growing NB4 cells were plated in 12-well cell culture plates at a density of 5×10^5 cells/mL in medium alone, medium containing AM80 or medium containing CD437 for the indicated periods of time. To test the role of PI3Kγ or ERK/MAP kinase activation, cells were pre-incubated for 30 min with different doses of LY294002[23,24] or PD98059[25] (Calbiochem-Novabiochem, La Jolla, CA, USA), followed by retinoid treatment. In all experiments, cell viability was evaluated by triplicate counting of trypan blue dye-excluding cells under a light microscope.

Nitroblue tetrazolium (NBT) reduction assay, inositol uptake and in vitro PI3K assays

The degree of differentiation was assessed by the ability of cells to produce superoxide (measured by reduction of NBT) as previously described by Collins *et al.*[15] Inositol uptake assays were performed as previously reported.[16] Total *in vitro* PI3K activity from whole cell lysates was assayed as described elsewhere.[17]

Western blot analysis

Aliquots of cells from all experiments were frozen at –80°C until required. Whole cell lysates were prepared by resuspending thawed cells in lysis buffer [20 mM HEPES, 150 mM NaCl, 3.0 mM $MgCl_2$, 2 mM EGTA, 0.2 mM Na_2–EDTA and 1% (v/v) Triton-X100 supplemented with aprotinin (2.8 mg/ml), leupeptin (20 μg/mL), PMSF (0.1 mM), pepstatin A (15 μg/mL), β-glycerophosphate (5 mM) and Na-orthovanadate (1 mM)], followed by incubation for 10 min on ice. Cell lysates were cleared by centrifugation for 15 min (20,000 g at 4°C), and supernatants were saved for protein analysis.

Fifty micrograms of protein were size-frationated by SDS-PAGE buffer on a 10% gel and transferred onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK). The following antibodies were used in this study: rabbit polyclonal antibody to PI3Kγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody to ERKs 1 and 2 (EMD Biosciencies) and monoclonal antibody specific for phosphorylated Thr202 and Tyr204 ERKs 1 and 2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Visualization of immune complexes was performed using secondary antibodies conjugated to horseradish peroxidase and the Luminol Reagent detection system (Santa Cruz Biotechnology,).

Flow cytometry analysis

Flow cytometry analyses were performed as previously described,[16] by using specific monoclonal antibodies to human TF [clone TF9-6B4, fluorescein isothiocyanate (FITC)-conjugated; American Diagnostica (Greenwich, CT, USA)], to human TM (unlabeled; American Diagnostica) or to human CD11b [R-phycoerythrin (PE)-conjugated]; Caltag Laboratories (San Francisco, CA, USA). Flow cytometry analysis was performed on a FACScan (BD Biosciences, San Jose, CA, USA).

Gene expression analysis

Cultured NB4 cells were processed for RNA isolation as previously described,[27] using either 50 pM each of forward and reverse TF-specific oligonucleotide primers (forward 5'-CTA CTG TTT CAG TGT TCA AGC AGT GA-3'; reverse 5'- CAG TGC AAT ATA GCA TTT GCA GTA GC-3') or 50 pM each of forward and reverse TM-specific oligonucleotide primers (forward 5'-ACG ACT GCT TCG CGC TCT AC-3'; reverse 5' –TCT CCC GTA ACC CAC TGG AA- 3') or 50 pM each of forward and reverse PI3Kγ-specific oligonucleotide primers (forward 5'-ACA GAT TCT ACG AAT CAT GG-3'; reverse 5'-GCA TTC CTG TCA TCA GCA TC-3'). The mRNA levels were quantified using the image analysis software *Intelligent Quantifier* version 2.1.1 (Bio Image, Ann Arbor, MI, USA). Results were calculated in terms of integrated optical density and expressed in arbitrary units.

Assessment of apoptosis: analysis of C3 activity and annexin V binding

Specific C3 activity in cell extracts was measured through a colorimetric assay, following the manufacturer's specifications of a commercial kit (Calbiochem-Novabiochem), and expressed as pmol of C3 per min and per µg of total protein. For annexin V binding studies, cells were washed twice with the binding buffer (10 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 4% BSA). Samples were incubated with a 1:500 solution of FITC-conjugated annexin V for 15 min at room temperature. Stained cells were analyzed by flow cytometry, while membrane integrity was simultaneously assessed by the propidium iodide exclusion procedure.

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were carried out with the SigmaStat software package (Systat, Point Richmond, CA, USA). Before comparing two data groups, a normality test and an equal variance test were performed. If data groups passed both tests, a comparison was made by a parametric test (paired Student's t-test). If the normality and/or equal variance test was violated, a comparison was made by a non-parametric test (Mann-Whitney rank sum test). Differences were considered statistically significant at $p < 0.05$.

Results

PI3Kγ and ERK/MAPK kinase signaling pathways induced by AM80 and CD437

Figure 1 show that PI3Kγ responded in a dose-dependent manner to AM80 treatment. Significant induction of mRNA and protein PI3Kγ expression, PI3K activity and [^{3}H]-myo-inositol incorporation were already observed at 1 nM AM80, and reached a maximum at 10 nM. The reverse transcription polymerase chain reaction (RT-PCR) analysis revealed a significant increase in gene expression, visible after 6 hours of incubation with 10 nM AM80, reaching a maximum after 48 h. In addition, both Western blot analysis and assay of PI3K activity (by measuring the levels of PtdIns^{3,4,5} P_3 synthesized *in vitro*) revealed a parallel and strong up-regulation of PI3Kγ protein synthesis and activity within 12 hours. After 48 hours, PI3K activity was about 25-fold higher than the initial activity (*not shown*). Treating the cells with AM80 10 nM of cells induced a three-fold increase in [^{3}H]-myo-inositol uptake with respect to that of untreated cells after 72 h of exposure, whereas CD437 did not markedly modify the amount of myo-inositol incorporated (*data not shown*).

Dose-response analysis of AM80 treatment after 48 hours (a time when maximal PI3K activity was observed) showed no change in the high steady-state levels of p-ERK-1/-2 activity seen in NB4 cells, even at very low doses (Figure 2A). On the other hand, a dose-response reduction in the levels of kinase activity was observed after 72 h of treatment with CD437 Figure (Figure 2B). Kinetic studies revealed a significant inhibition of ERK-1/-2 activity in 100 nM CD437-treated cells after 48 hours of exposure. Moreover, ERK-1/-2 activity remained significantly reduced even after 96 hours, and such changes were not explained by differences in the amounts of total ERK-1/-2 protein, which remained unchanged (Figure 2B).

On the other hand, combined treatments with both retinoids, did not significantly modify the specific response caused by each retinoid given separately (*data not shown*).

Inhibition of the PI3Kγ and the ERK/MAP kinase signaling pathways by LY294002 and PD98059

Treatment with LY294002 (a specific inhibitor of the PI3-kinase family members)[19,20] abolished the PI3Kγ synthesis and PI3K activity induced by AM80 at both mRNA and protein levels in a dose-dependent way (Figures 3A, 3B and 3C). In parallel experiments, the addition of LY294002 significantly reduced the myo-inositol incorporation in AM80-treated cells, as expected (Figure 3D). On the other hand, as shown in Figure 4, there was a dose-dependent inhibition of ERK-1/-2 phosphorylation after treatment with AM80 plus PD98059 (a specific inhibitor of the upstream MAPK kinases, MEK1 and MEK2)[21] treatment. Similar results were seen in the case of combined treatments with CD437 and PD98059: a complete inhibition of ERK activation was seen in CD437 treated cells. By contrast, treatment with PD98059 did not significantly alter the levels of total ERK-1/-2 proteins (Figure 4A). As shown in Fig. 4B, LY294002 abolished the ERK-1/-2

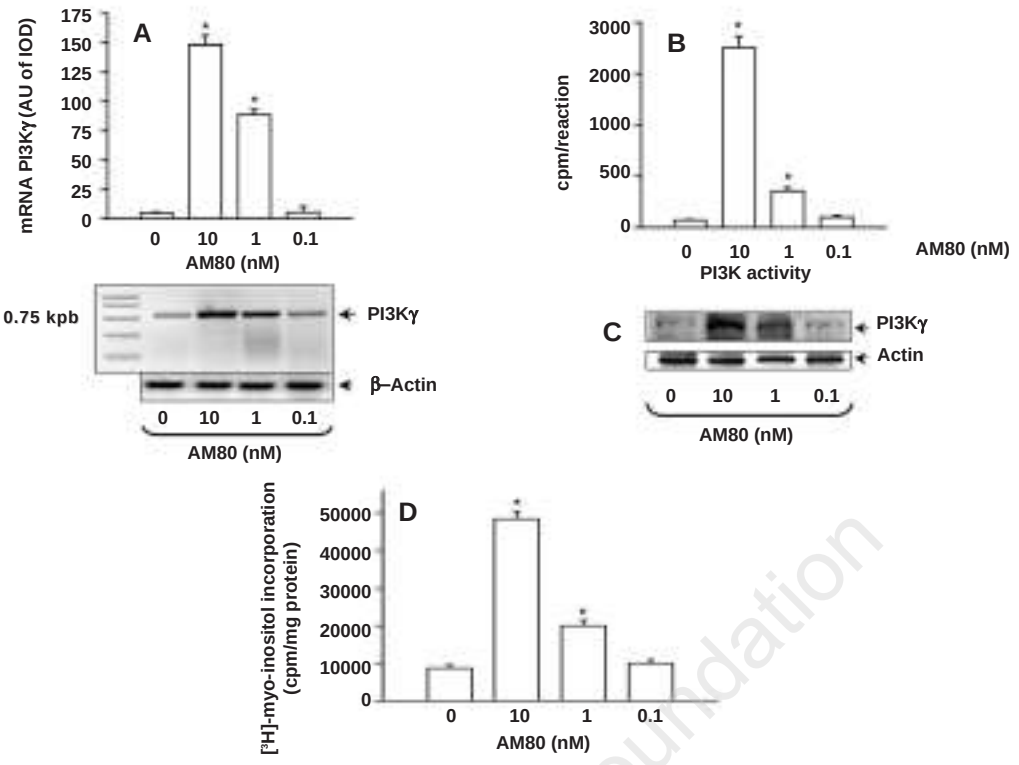

Figure 1. Dose-dependent AM80 induction of myo-inositol incorporation, PI3Kγ gene expression and PI3Kγ protein synthesis and activation. NB4 cells were treated with the indicated concentrations of AM80. After 24h, the cells were harvested, and aliquots were used for analysis of synthesis of RNA and protein. (A). The RT-PCR was performed on the total RNA, using primers corresponding to human PI3Kγ and β-actin cDNA sequences, as indicated. The values given in the graph (top a) represent the mean ± the standard error of the mean (SEM) of three independent experiments. (B). *In vitro* PI3K activity. Whole cell lysates were applied for *in vitro* lipid kinase assays. After autoradiography, the phosphorylated PI fractions were scraped off and quantified by liquid-scintillation counting. Data are the mean of three independent experiments ± SEM. (C) Western blot assay. After treatment, whole cell lysates were prepared and proteins (50 μg/lane) were electrophoresed on a 10% SDS/PAGE gel, and then transferred to nitrocellulose membranes. Blots were probed with a polyclonal antibody against PI3Kγ. (D). The myo-inositol incorporation was measured as described in Design and Methods. Results are the mean of three independent experiments ± SEM. Differences at $p < 0.05$: (*) vs. the cells incubated without the inducer (control).

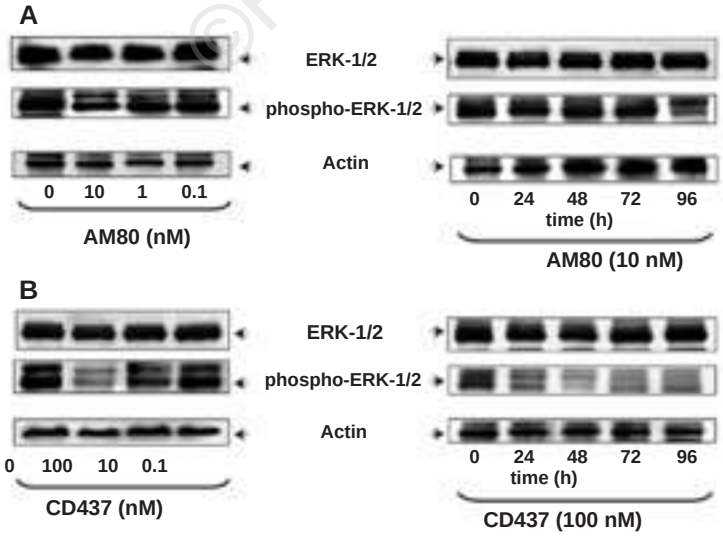

Figure 2. ERK-1/-2 activation in NB4 cells exposed to AM80 or CD437. Cells were treated with different doses of AM80 or CD437, and whole cell lysates were harvested at varying time-points. Protein aliquots (50 μg/lane) were electrophoresed on a 10% SDS/PAGE gel and transferred to nitrocellulose membranes. Filters were probed with anti-total ERK-1/-2 (upper) and anti-phospho-ERK-1/-2 (Thr202/Tyr204) (middle) antibodies. The anti-phospho-ERK-1/-2 filters were stripped and reprobed with anti-actin antibody (lower) to verify equal protein loading. Panels (A) and (B) are representative of one of three independent experiments performed by using, respectively, AM80 or CD437 as inducers.

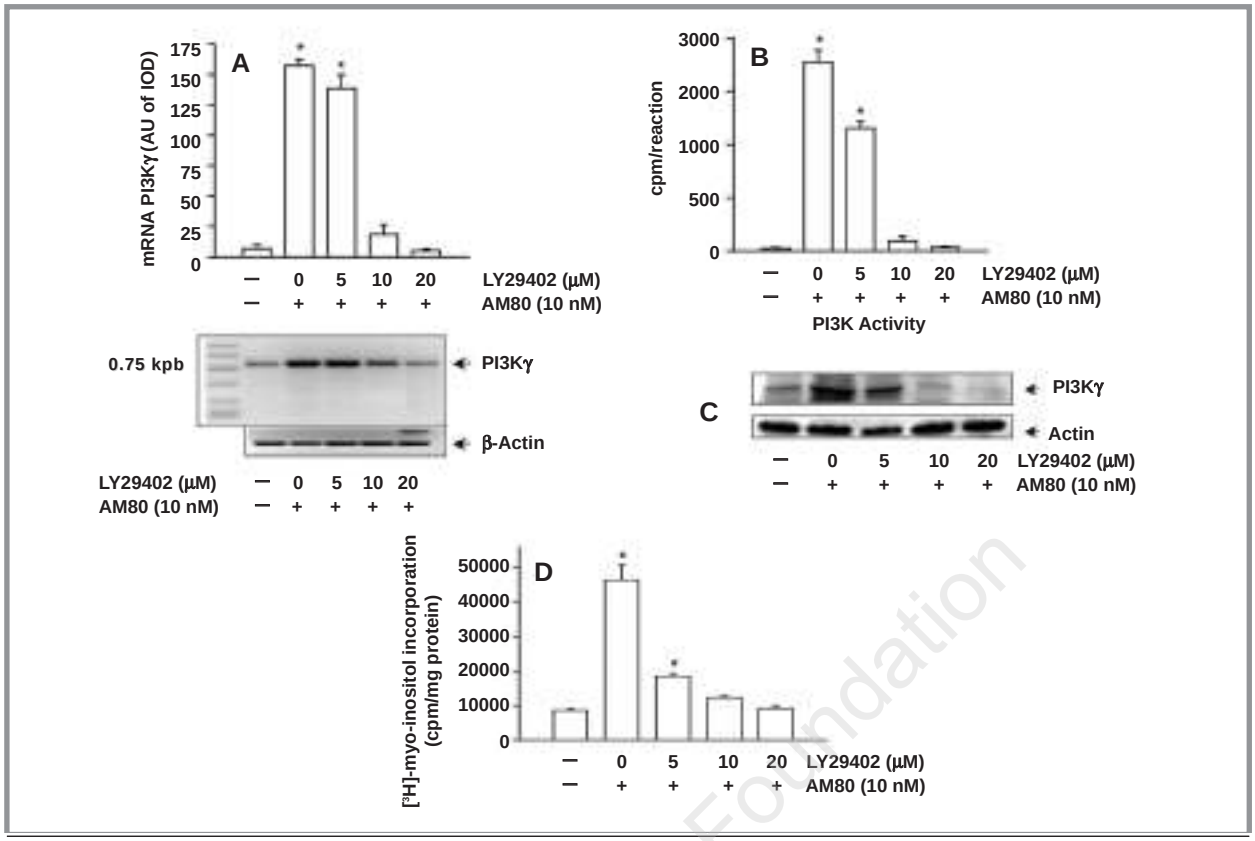

Figure 3. Inhibition of AM80-induced myo-inositol incorporation, PI3Kγ gene expression, PI3K activity and PI3Kγ protein synthesis by LY294002. NB4 cells were treated with varying doses of LY294002 during 30 min, followed by AM80 exposure for 48 h. Each experimental group was treated, as described in Figure 1, for analysis of PI3Kγ mRNA expression (A) and for PI3K activity (B) and for PI3Kγ protein synthesis (C). The values given in the graphs (A and B) represent the means ± SEM of three independent experiments. Panels (A and C) show one representative experiment. (D) The myo-inositol incorporation was measured as described in Design and Methods. Results are the mean of three independent experiments ± SEM. Differences at $p < 0.05$ (*) vs. untreated cells.

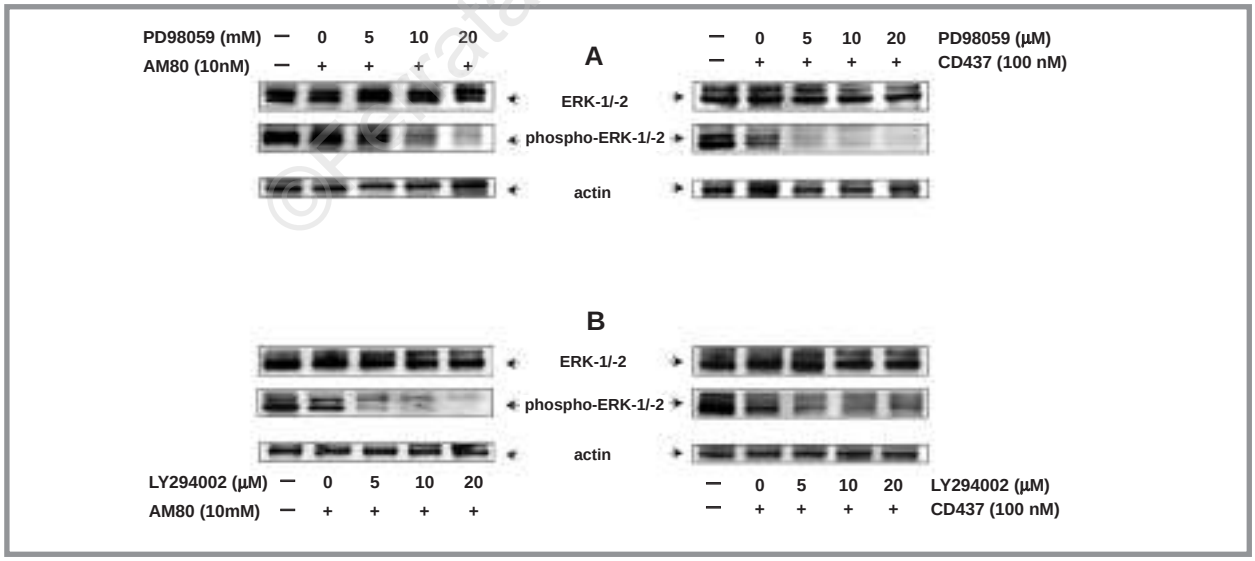

Figure 4. Inhibition of ERK-1/-2 activity by PD98059 and LY294002. The NB4 cells were pretreated with varying doses of either PD98059 or LY294002 during 30 min, followed by AM80 or CD437 exposure for 48 hours. Lysates were prepared and proteins (50 μg/lane) were electrophoresed on a 10% SDS/PAGE gel and then transferred to nitrocellulose membranes. The filters were probed with anti-total ERK-1/-2 or anti-phospho-ERK-1/-2 polyclonal antibodies. To demonstrate equal protein loading, the anti-phospho-ERK-1/-2 filter was stripped and reprobed with anti-actin antibody. (A) Western blots from combined treatments with AM80 or CD437 and PD98059. (B) Western blots from combined treatments with AM80 or CD437 and LY294002. Panels are representative for three independent experiments with similar results.

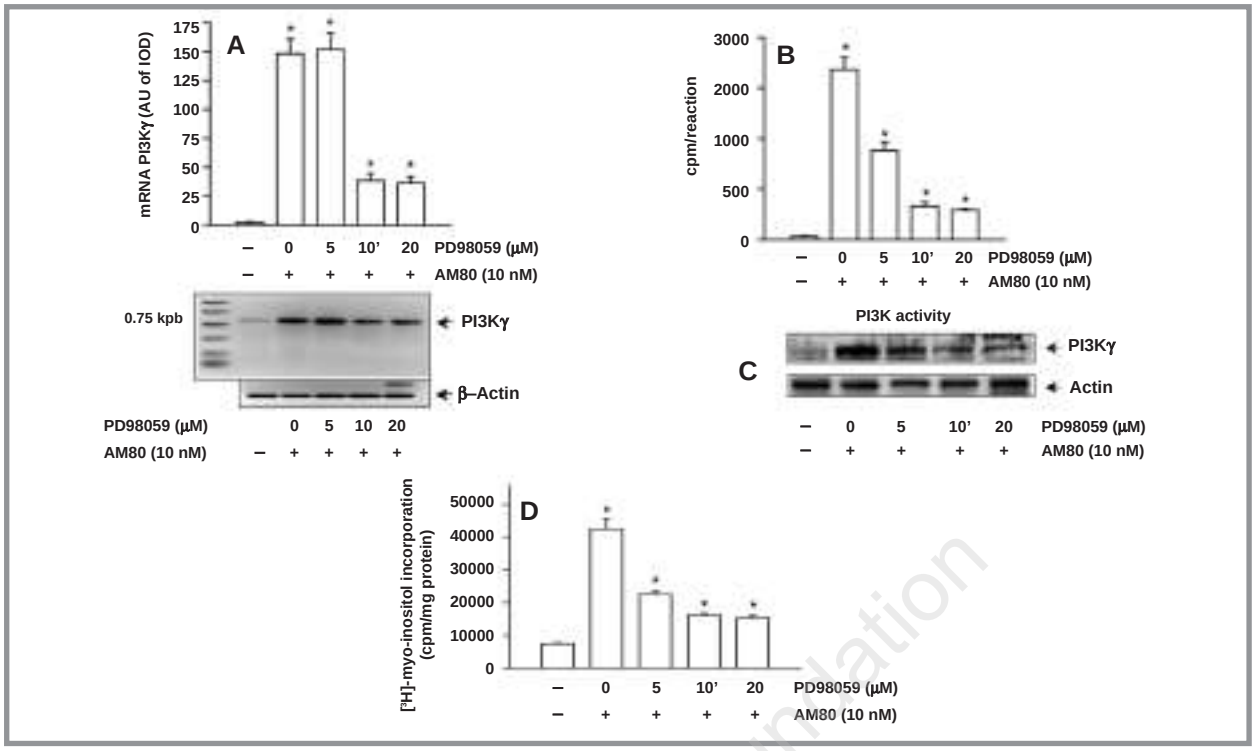

Figure 5. Inhibition of AM80-induced myo-inositol incorporation, PI3Kγ gene expression, PI3K activity and PI3Kγ protein synthesis by PD98059. NB4 cells were treated with varying doses of PD98059 during 30 min, followed by AM80 exposure for 48 h. Each experimental group was treated, as described in Figure 1, for analysis of PI3Kγ mRNA expression (A) for PI3K activity (B) and for PI3Kγ protein synthesis (C). The values given in the graphs (A and B) represent the means ± SEM of three independent experiments. Panels (A and C) show one representative experiment. (D) The myo-inositol incorporation was measured as described in Design and Methods. Results are the mean of three independent experiments ± SEM. Differences at $p < 0.05$ (*) vs. untreated cells.

activity seen in control and AM80-treated NB4 cells in a dose-response way. On the other hand, LY294002 treatment did not significantly modify the activity of ERK-1/-2 elicited by CD437. Additionally, the ERK inhibition caused by the different doses of PD98059 in combination with AM80 significantly reduced but did not abrogate PI3Kγ synthesis and activity, which were still significantly higher than in control cells (Figures 5A, 5B and 5C). Accordingly, the phosphoinositide turnover was significantly reduced but remained above the basal levels (Figure 5D). At a dose of 10 μM, LY294002 was not observed to have an effect over a 96 h period. However, at higher doses, LY294002 treatment resulted in a significant rapid loss of viability (*data not shown*). Similarly, doses higher than 20 μM PD98059 caused a sharp reduction in cell viability after 48 h of treatment. In view of these findings, LY294002 and PD98059 were used at doses of 10 μM and 20 μM respectively, in subsequent experiments.

PI3Kγ and MEK blockade inhibits the differentiation and reverses the effects induced by AM80 on TF and TM expression and synthesis

Granulocytic differentiation induced by AM80, assessed by the presence of CD11b on cell surfaces, was blocked by inhibition of the PI3Kγ and the MEK/ERK pathways (Figure6A). These results were also confirmed by analysis of the NBT-reducing ability of NB4 cells (Figure 6B). On the other hand, the down-regulation of TF expression and the up-regulation of TM induced by AM80 were blocked by the PI3Kγ and MEK inhibitors (Figure 7), LY294002 being more effective than PD98059. As described above for kinase regulation, combined treatments with AM80 and CD437 did not significantly modify the specific response caused by AM80 when given separately (data not shown).

ERK inhibition increases the proapoptotic effect of CD437, while PI3Kγ induction protects from apoptosis activation

Treatment of NB4 cells with CD437 10^{-7} M for 72 h induced a significant increase in apoptosis, which paralleled the inhibition of the ERK-1/-2 activity. Moreover, combined treatment of NB4 cells with CD437 and the MEK inhibitor PD98059 sensitized NB4 cells to the proapoptotic effects of CD437, promoting an additive effect on apoptosis induction, as assessed by caspase 3 activity and the presence of annexin V on the cell-surface (Figure 8). This pro-apoptotic effect also paralleled a further inhibition in ERK-1/-2 activity, which

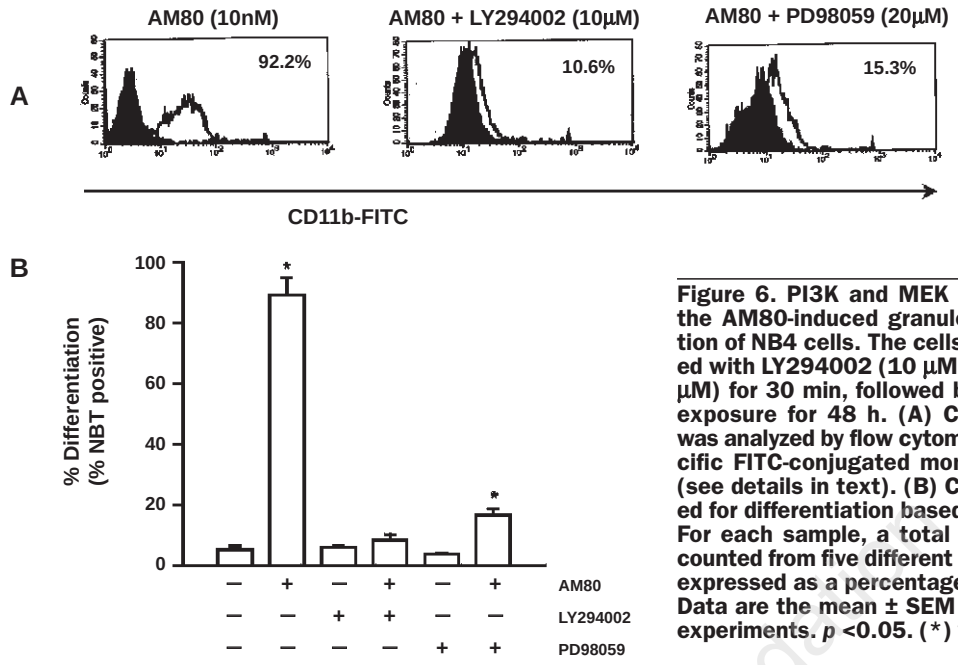

Figure 6. PI3K and MEK blockade inhibits the AM80-induced granulocytic differentiation of NB4 cells. The cells were preincubated with LY294002 (10 μM) or PD98059 (20 μM) for 30 min, followed by AM80 (10 nM) exposure for 48 h. (A) CD11b expression was analyzed by flow cytometry using an specific FITC-conjugated monoclonal antibody (see details in text). (B) Cells were evaluated for differentiation based on NBT staining. For each sample, a total of 200 cells was counted from five different fields. Results are expressed as a percentage of positive cells. Data are the mean ± SEM of three separate experiments. p <0.05. (*) vs untreated cells

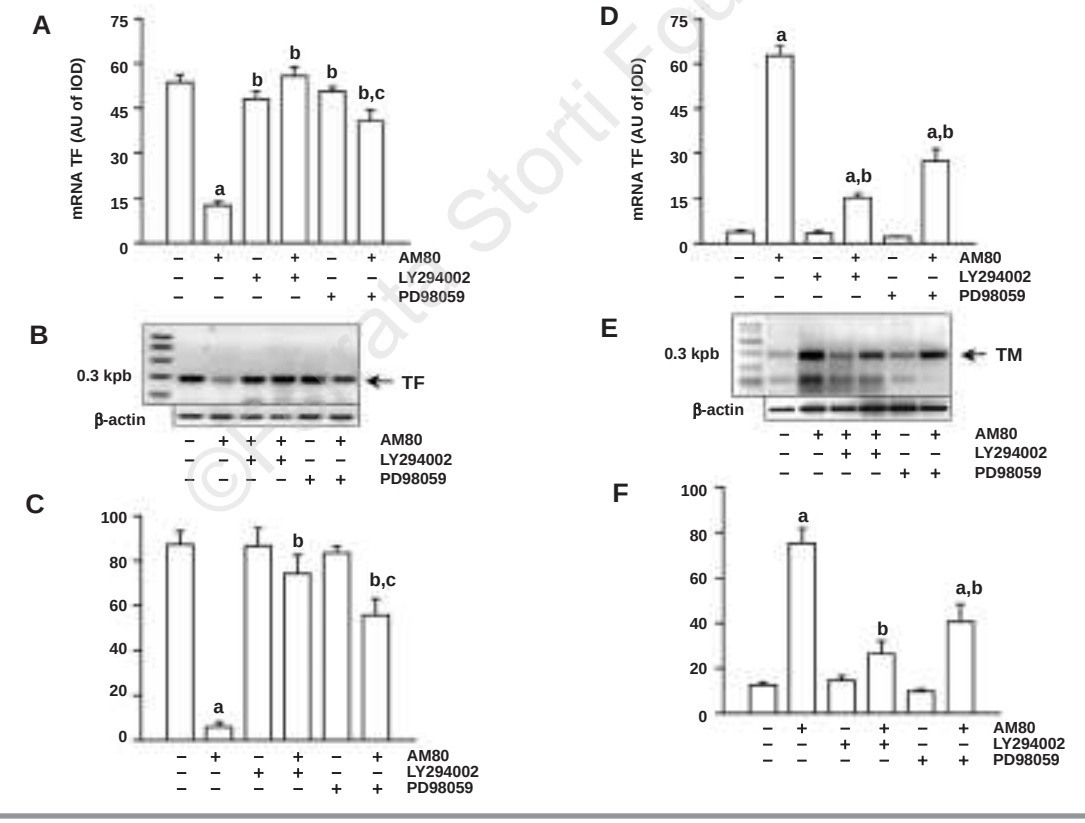

Figure 7. PI3K and MEK blockade reverses the effects induced by AM80 on TF and TM expression. The cells were preincubated with LY294002 (10 μM) or PD98059 (20 μM) for 30 min, followed by AM80 (10 nM) exposure for 48 h. The TF (A) and TM (D) mRNA were amplified by RT-PCR and results were calculated as described in Design and Methods and expressed as arbitrary units (AU) of integrated optical density (IOD). The panels (bottom, B and E) show one representative experiment for the TF and TM mRNA, respectively. The TF (C) and TM (F) presence on the cell surface was analyzed in intact cells by flow cytometry using specific monoclonal antibodies. Data are expressed as percentage of positive cells (se details in text). The results are the mean ± SEM of three separate experiments. p <0.05. a, vs cells incubated without the inducer (control); b, vs the cells incubated with the inducer alone; c, vs the cells incubated with AM80 plus LY294002.

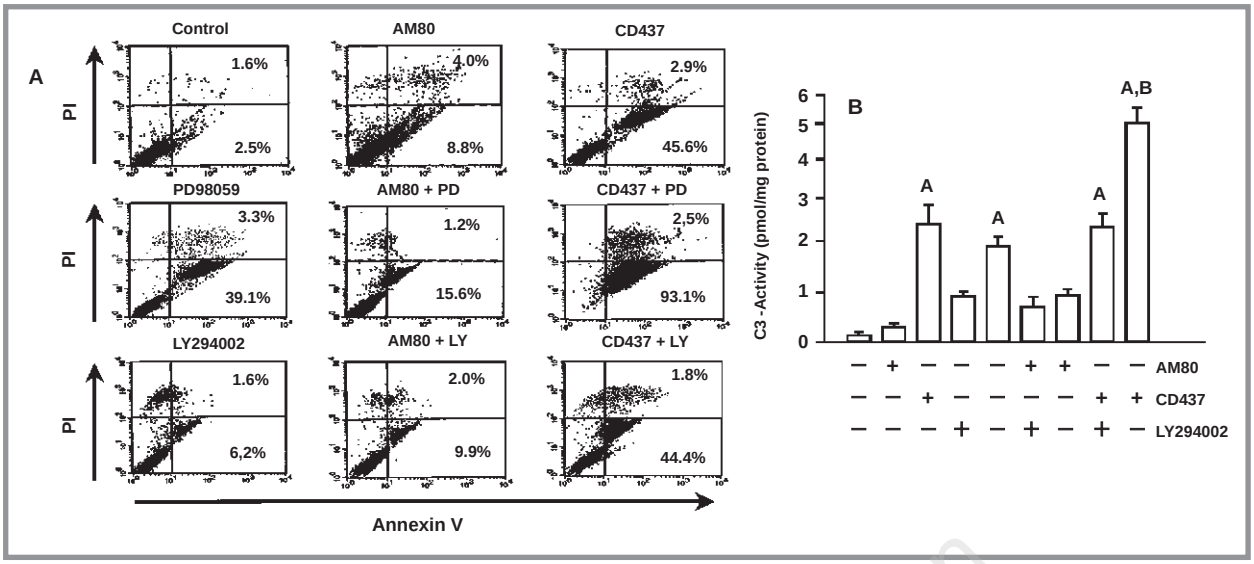

Figure 8. ERK-1/-2 inhibition increases the pro-apoptotic effect of CD437, while PI3Kγ induction protects from apoptosis activation. The NB4 cells were pre-incubated with PD98059 (20 μM) or LY294002 (10 μM) for 30 min, followed by AM80 or CD437 exposure for 72 h, and the apoptosis was evaluated by the presence of annexin V on the cell-surface and by the quantification of caspase 3 activity. (A) Cell-surface expression of phosphatidylserine. The results are representative of one of three independent experiments with similar outcomes. The lower right quadrants contain apoptotic cells (annexin-V$^+$/PI$^-$); the lower left quadrants contain viable cells (annexin-V$^-$/PI$^-$); the upper right quadrants contain necrotic cells (annexin-V$^+$/PI$^+$). (B) The state of caspase 3 activation was measured in NB4 cell extracts through a colorimetric assay, and expressed as pmol of C3 per min and per μg of total protein. Results are the mean ± SEM of three separate experiments. p <0.05. a, vs cells incubated without the inducer (control); b, vs the cells incubated with the inducer alone.

was abrogated after the combined treatment (Fig. 4A).

As described above, combined treatment of NB4 cells with AM80 and PD98059 failed to suppress PI3K activity (Fig. 5). Accordingly, no induction of apoptosis, but rather a slight protection from spontaneous cell death, was seen in NB4 cells after the combined treatment with AM80 and PD98059.

Discussion

The aim of the present study was to analyze the mechanism(s) of activation and function(s) of both pERK-1/-2 kinases and PI3K in promyelocytic leukemia cells. We also compared for the first time the effects of inhibiting these two signal transduction cascades on various aspects of promyelocytic cell functioning.

We found that treatment of NB4 cells with AM80 resulted in an early increased gene expression of PI3Kγ, indicating that the PI3Kγ gene may be a direct target for AM80. In addition, a sustained increase in the phosphoinositide turnover strictly paralleled the enhanced PI3K activity induced by AM80. Furthermore, the inhibition of both PI3K activity and phosphoinositide turnover after combined treatment with AM80 and LY294002 indicates the specificity of AM80 on PI3K activity. These data point to the PI3Kγ species as being the main source of elevated basal PI3K activity and suggest that phosphoinositides have a regulatory function in the response of NB4 cells to this retinoid derivative.

Various studies have demonstrated a high frequency of constitutive activation of MAPK (ERK-1/-2) in human primary acute leukemia cells.[22] Indeed, the promyelocytic NB4 cell line shows constitutive ERK-1/-2 activation, which did not change after AM80 treatment. Previous studies have shown that PI3Kγ could constitute an important signaling element, linking the G protein-coupled receptors to the MAPK pathways.[23,24] Our data showed that ERK-1/-2 activity was sensitive to the PI3K inhibitor LY294002, supporting the hypothesis that the PI3K pathway might participate, at least partially, in the maintenance of the elevated ERK1/-2 activity seen in NB4 cells after AM80 treatment.

On the other hand, the ERK-1/-2 inhibition obtained with the MEK inhibitor PD98059 in combination with AM80 significantly reduced but did not abrogate the PI3K activity, which was still higher than in control cells. Accordingly, phosphoinositide turnover was significantly reduced but remained above the basal levels. Taken together, these results suggest an essential cooperation of PI3Kγ and enzymes of the MAPK cascade in the early response of NB4 cells to AM80 treatment, although the PI3Kγ pathway seems to be more specific and determinant in the response to the retinoid. We, therefore, investigated the functional consequences of the pharmacologic disruption of these pathways, using selective

inhibitors of MEK and PI3K activation. Granulocytic differentiation induced by AM80 was blocked by inhibition of the PI3K and the MEK/ERK pathways, suggesting that activities of both pathways are required for optimal cell differentiation in response to the retinoid derivative, as previously demonstrated in human myeloid cells after retinoic acid treatment.[24,25] Additionally, the down-regulation of TF expression and the up-regulation of TM expression induced by AM80 were blocked by PI3K and MEK inhibitors, further supporting the role of MEK/ERK and PI3K-dependent signals in the regulation of the procoagulant activity of NB4 cells in response to AM80. Moreover, TF suppression and TM induction in NB4 cells were found to be coherent with the induction of general myeloid differentiation markers. This might imply that the changes operated in the procoagulant activity of NB4 cells after AM80 treatment and the cell differentiation process are regulated concomitantly. This hypothesis is further supported by our previous reported results, showing a parallel modulation of the differentiation, TF and TM expression of NB4 cells after ATRA treatment.[16]

The main functional responses of NB4 cells to AM80 seemed to occur via activation of the PI3K pathway. This hypothesis was supported by two ways of evidence: 1) AM80 induced early induction of both PI3K expression and activity, which paralleled both granulocytic differentiation and suppression of procoagulant activities seen in basal NB4 cells; and 2) the retinoid did not induce *per se* any change in the elevated ERK-1/-2 activity seen in basal NB4 cells, and thus it could not explain the functional changes occurring in these cells. Furthermore, although the inhibition of both PI3K and MEK/ERK signaling pathways reversed the effects of AM80 on cell differentiation, TF and TM expression, the PI3K inhibition was always more effective in this reversal. Nevertheless, the present data highlight a certain degree of cross-talk between both pathways, demonstrated by the regulatory role of PI3K inhibition of the activity of ERK-1/-2, as also described in previous studies.[24,26] This may help to explain the concomitant participation of both pathways in the overall effects of AM80 on NB4 cells. The MEK blockade sets the stage for increased sensitivity to other apoptotic stimuli, such as retinoids and DNA-damaging agents.[8] Accordingly, CD437 concentrations as low as 100 nM resulted in ERK-1/-2 inactivation and apoptosis of NB4 cells. Moreover, further inhibition of ERK-1/-2 using the specific MEK inhibitor, PD98059, increased the pro-apoptotic effect of the retinoid, causing an additive effect. The *in vivo* antileukemic effect of CD437 is predominantly the result of a rapid and massive apoptosis-inducing action and is not the consequence of a cytodifferentiation phenomenon.[7] Our data indicate that CD437-induced apoptosis is preceded by the activation of the caspase-3-like enzymatic activity, which was further increased after MEK blockade.

Among the intracellular signals leading to caspase activation, Bcl-2 expression has been proposed to have a key function.[27,28] According with previous studies,[6,8] we found that neither the MEK blockade nor CD437 treatment affected the protein synthesis of Bcl-2 (data not shown), indicating that Bcl-2 regulation per se is not significant for CD437- and PD98059-induced apoptosis. On the other hand, we have shown in the present work that MEK inhibition also induced a significant activation of the caspase-3-like enzymatic activity, which might thus explain the pro-apoptotic synergism observed for the combination of the CD437 treatment and MEK inhibition. These results are in agreement with those of a recent study showing a direct correlation between inhibition of the MEK/ERK pathway and activation of caspase-9, an initiator protease that activates caspase-3 and down-stream caspases to initiate cellular destruction.[29] Additionally, it has recently been reported that both MEK and PI3-kinase inhibition sensitize the AML cells to ATRA-induced apoptosis.[8,30] A similar degree of sensitization has been observed in response to the pan-RA receptor agonist 9-*cis*-RA. However, this effect was not observed after a combined treatment with the RAR-α selective ligand (AM80) and PD98059 or with LY294002 at the doses used, thus suggesting that such a pro-apoptotic effect requires simultaneous activation of both the RAR and RXR pathways.

Increasing evidence has accumulated over the last two years suggesting various levels of cross-talk between the Raf-1-MEK-ERK and the PI3K-AKT pathways, which may also play a role in apoptosis regulation.[14] In this way, one feature of PI3K-dependent signaling is the recently described activation of an antiapoptotic pathway via PI3K-dependent kinase-1-mediated activation of AKT/PKB.[31] We have shown here that the combined treatment of NB4 cells with AM80 and PD98059 failed to suppress PI3K activity. Accordingly, no induction of apoptosis, but rather a slight protection from spontaneous cell death, was seen after treatment with AM80 plus PD98059. This suggests that just a slight PI3K activity is enough to protect against apoptosis, even if the MEK/ERK pathway is inhibited. Apoptosis frequently occurs at late stages after terminal differentiation of myeloid cells.[32] Therefore, it is possible that the transcriptionally induced endogenous PI3Kγ may provide a signal to activate a differentiation stage-dependent antiapoptotic program in NB4 cells, which might enable such cells to progress through the differentiation process initiated by AM80. Such an anti-apoptotic program, mediated at least in part via induced PI3Kγ, could allow the cells to execute their specialized functions before undergoing apoptosis.

Taken together, our results indicate that in NB4 cells: 1) AM80 mainly induces PI3K activity, while CD437 mainly inhibits ERK-1/-2 activation; 2) PI3K and

MEK/ERK blockade reverses the effects on cell differentiation, TF and TM expression induced by AM80; 3) ERK-1/-2 inhibition increases the pro-apoptotic effect of CD437, while PI3Kγ induction protects from apoptosis activation; and 4) regardless of the specific regulation of the PI3K and MEK/ERK pathways promoted by AM80 and CD437, there is a varying degree of cross-talk that occurs between these pathways in the control of the response of NB4 cells to retinoids.

Furthermore, our overall findings suggest that disruption of targeted pathways, in combination with specific retinoids, might represent an effective and relatively specific therapeutic strategy for APL patients.

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