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Overexpression of Protein Kinase Cζ Confers Protection Against Antileukemic Drugs by Inhibiting the Redox-Dependent Sphingomyelinase Activation

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ABSTRACT

Induction of apoptosis by chemotherapeutic drugs involves the sphingomyelin-ceramide (SM-CER) pathway. This signaling is critically dependent on reactive oxygen species (ROS) generation and p53/p56 Lyn activation. In this study, we have investigated the influence of protein kinase C (PKC) ζ overexpression on the SM-CER pathway in U937 human leukemia cell line. We show that PKCζ overexpression resulted in delayed apoptosis and significant resistance to both 1-β-D-arabinofuranosylcytosine (ara-C) and daunorubicin (DNR), but there was no significant protection against cell-permeant C_6-CER. Moreover, PKCζ overexpression abrogated drug-induced neutral sphingomyelinase stimulation and CER generation by inhibiting ROS production. We further investigated p53/p56 Lyn activation in PKCζ-overexpressing U937 cells treated with ara-C or DNR. We demonstrate that PKCζ inhibited p53/p56 Lyn phosphorylation and stimulation in drug- or H_2O_2-treated cells, suggesting that p53/p56 Lyn redox regulation is altered in PKCζ-overexpressing cells. Finally, we show that PKCζ-overexpressing U937 cells displayed accelerated H_2O_2 detoxification. Altogether, our study provides evidence for the role of PKCζ in the negative regulation of drug-induced SM-CER pathway.

Daunorubicin (DNR) and 1-β-D-arabinofuranosylcytosine (ara-C) are the most active antileukemic agents; for this reason, they are often combined in front-line therapy of acute myeloblastic leukemia. DNR- and ara-C–induced cytotoxicity are thought to be the result of drug-induced damage to DNA. Among different mechanisms by which DNR and other anthracyclines exert their cytotoxicity, the most critical consists of the formation of stable drug/topoisomerase II/DNA ternary complexes. The ternary complex constitutes a latent DNA-damaging state, which is ultimately converted, when the cells enter S phase, to an irreversible double DNA strand break. ara-C, like other nucleoside analogs, once phosphorylated by intracellular enzymes, competes with deoxyribonucleotides for incorporation into DNA during replication, and analog incorporation inhibits DNA synthesis. DNR- or ara-C–induced DNA damage results in either apoptosis or mitotic catastrophe, depending on the dose and the cellular models. Recently, we demonstrated that high but clinically relevant doses of DNR (1 μM) or ara-C (40 μM) activate the sphingomyelin-ceramide (SM-CER) cycle, leading to apoptosis in U937 and HL-60 cells (Jaffrézou et al., 1996; Bezombes et al., 2001). Indeed, in these cells, DNR and ara-C stimulate neutral sphingomyelinase (N-SMase) activity responsible for SM hydrolysis and subsequent CER generation; CER, in turn, induces apoptosis by stimulating, through a redox-dependent mechanism, c-Jun N-terminal kinase module and AP-1 DNA binding affinity (Mansat-de Mas et al., 1999). Such an apoptotic pathway has also been described for vincristine, ionizing radiation, Fas agonist, and tumor necrosis factor α (Levade and Jaffrézou, 1999). More recently, we proposed that Lyn, a src family member, plays a critical role in drug-induced N-SMase stimulation. Indeed, in this recent article, we reported that ara-C activates Lyn through radical oxygen species (ROS) production and that activated Lyn interacts with N-SMase; moreover, Lyn depletion by antisense oligonucleotides results in abrogation of drug-induced N-SMase, CER production, and apoptosis, suggesting that Lyn is an important regulator of the SM-CER pathway (Bezombes et al., 2001).

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ABBREVIATIONS: DNR, daunorubicin; ara-C, 1-β-D-arabinofuranosylcytosine; SM, sphingomyelin; CER, ceramide; N-SMase, neutral sphingomyelinase; ROS, reactive oxygen species; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; DAPI, 4′,6′-diamidino-2-phenylindol; PBS, phosphate-buffered saline; AML, acute myeloid leukemia.

Previous studies have documented that the SM-CER apoptotic pathway is controlled by potent regulators that can operate either upstream or downstream CER production. Among them, protein kinase C (PKC) activity seems to play a critical role. Indeed, PKC stimulation induced by phorbol esters or diacylglycerol not only inhibits CER-induced apoptosis (Jarvis et al., 1994) but also limits DNR-induced N-SMase stimulation, CER generation, and apoptosis (Mansat et al., 1997). The role of PKC in regulating N-SMase is also supported by another study that showed that PKC inhibition by calphostin C resulted in N-SMase stimulation (Chmura et al., 1996).

However, very little is known about which PKC isoform mediates the inhibitory effect on drug-induced N-SMase stimulation. Indeed, 11 different isoforms of PKC have been characterized so far, and these have been grouped into three categories based on Ca^{2+} requirement for activation and phorbol ester binding activity. Conventional PKCs (α, βI, βII, and γ) are Ca^{2+}-dependent phorbol ester receptor kinases; novel PKCs (δ, ϵ, τ, η) are Ca^{2+}-independent phorbol ester receptor kinases; and atypical PKCs (ζ, ι/λ) are kinases independent of both Ca^{2+} and phorbol ester. Based on the inhibitory effect of phorbol esters on drug-induced N-SMase stimulation, it has been proposed that conventional or novel PKCs are involved in the regulation of the SM-CER apoptotic pathway. This hypothesis is also supported by the stimulatory effect of calphostin C on N-Smase, because this agent inhibits conventional or novel but not atypical PKC isozymes. However, the role of atypical PKCs in SM-CER pathway regulation has not been directly addressed.

Therefore, we investigated the influence of PKCζ overexpression on drug-activated SM-CER pathway. Among atypical PKC family members, PKCζ was selected rather than PKC ι/λ because this kinase plays a critical role in the propagation of signals activated by a large variety of growth factors and oncogenes, including p21Ras. PKCζ is also activated directly or indirectly by a variety of pivotal signaling molecules, including CER, phosphatidic acid, diacylglycerol generated from phosphatidylcholine hydrolysis, and phosphoinositide 3-kinase lipid products (Moscat and Diaz-Meco, 1996).

In this study, we have evaluated the functional consequences of PKCζ overexpression on each step of the signaling cascade resulting in N-SMase stimulation in U937 cells treated with either DNR or ara-C. We show here that PKCζ overexpression resulted in reduction of drug-induced ROS production, Lyn activation, N-SMase stimulation, CER production, as well as in apoptosis inhibition and drug resistance. These results suggest that PKCζ stimulation may confer significant protection against DNR and ara-C and could represent an adverse parameter in the treatment of leukemia.

Materials and Methods

Drugs and Reagents. ara-C was obtained from Upjohn (Paris, France). DNR was supplied by Laboratoire Roger Bellon (Neuilly-sur-Seine, France). Silica gel 60 thin-layer chromatography plates were from Merck (Darmstadt, Germany). All other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO), Carlo Erba (Rueil-Malmaison, France), or Prolabo (Paris, France). Sense and antisense oligonucleotides directed against PKCζ have been designed and manufactured by Biognostik (Göttingen, Germany).

Cell Culture. U937-ζJ and -ζB cells were obtained from separate cotransfections with a full-length rat PKCζ cDNA construct subcloned into the $pSV_2M(2)6$ vector and neomycin-resistant plasmid. U937-neo cells were obtained by transfection with the vector without the PKCζ insert. All these cell lines were kindly provided by Dr. D. K. Ways (Lilly Corporate Center, Indianapolis, IN) (Ways et al., 1994). All cell lines were cultured in RPMI 1640 medium at 37°C in 5% CO_2. Culture medium was supplemented with 10% heat-inactivated fetal calf serum, complemented with 2 mM L-glutamine, 200 units/ml penicillin, and 100 μg/ml streptomycin (all from Eurobio, Les Ulis, France).

Western Blot Analysis. Exponentially growing cells were then washed twice in serum-free medium, centrifuged, and lysed in radioimmunoprecipitation assay buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 0.1% SDS, 5 mM EDTA, 1 mM dithiothreitol, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.1 mM PMSF) for 20 min on ice, followed by centrifugation at 10,000*g* for 15 min. For each lysate, 40 μg of total protein was heated for 5 min at 95°C in the presence of 3% β-mercaptoethanol. Proteins were separated on 10% (w/v) SDS-polyacrylamide gel electrophoresis, and transferred electrophoretically onto nitrocellulose membranes (Hybond-C; Amersham Biosciences, Orsay, France). Nonspecific binding sites were blocked in 10 mM Tris-buffered saline containing 0.1% Tween-20 and 10% nonfat milk. Membranes were then incubated overnight at 4°C with anti-PKCζ (Santa Cruz Biotechnology, Santa Cruza, CA) or anti-actin (Chemicon, Hofheim, Germany) antibodies. Membranes were then washed three times at room temperature, and bound Ig was detected with anti-isotype monoclonal antibody coupled to horseradish peroxidase (Beckman-Coulter, Roissy, France). The signal was visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and autoradiography.

Cytochemical Staining. Changes in cellular nuclear chromatin was evaluated by fluorescence microscopy (Diaplan; Leica, Wetzlar, Germany) by 4′,6′-diamidino-2-phenylindol (DAPI) staining by the following method. Briefly, cells were fixed by 4% paraformaldehyde, pH 7.4, for 15 min. After washing in PBS, cells were dried and stained by 0.5 μg/ml DAPI.

Cell Cycle Analysis. One million cells were fixed by 70% ethanol at −20°C for 2 h. After two washes in PBS, pellets were resuspended in PBS containing 50 μg/ml propidium iodine and treated with 0.1 mg/ml RNase for 30 min. Cell cycle analysis was evaluated using flow cytometry on a FACScan (BD Biosciences, Pont-de Claix, France).

PKCζ Antisense Experiments. Blocking experiments were performed by preincubating cells for 48 h with antisense or sense phosphorothioate oligonucleotides (10 μM) directed against PKCζ. The decrease of PKCζ expression was evaluated by Western blot analysis as described above.

ROS Production. Production of ROS was detected with the C2938 fluorescent probe (Molecular Probes, Paris, France). Briefly, exponentially growing cells were labeled with 0.5 μM C2938 for 1 h and then incubated in the presence or the absence of 40 μM ara-C at 37°C. The cells were then washed in PBS, and cell fluorescence was determined using flow cytometry on a FACScan cytometer. Results correspond to the fluorescence difference (ΔFL1) between treated and untreated cells.

N-SMase Assay. N-SMase activity was assayed as described previously using choline-[methyl-^{14}C]SM (120,000 dpm/assay; PerkinElmer Life Sciences, Paris, France) as substrate (Jaffrézou et al., 1996).

Metabolic Cell Labeling and CER Quantitation. Total cellular CER quantitation was performed by labeling cells to isotopic equilibrium with 1 μCi/ml of [9,10-^{3}H]palmitic acid (53.0 Ci/mmol; Amersham Biosciences) for 48 h in complete medium as described previously (Jaffrézou et al., 1996). Cells were then washed and resuspended in complete medium for time-course experiments. Lip-

ids were extracted and resolved by thin-layer chromatography; CER was scraped and quantified by liquid scintillation spectrometry.

Analysis of p53/p56 Lyn Activity. p53/p56 Lyn kinase activity was measured in cells incubated in the absence or presence of 40 μM ara-C or 1 μM DNR for the times indicated in the legend to Fig. 7B. The reaction was stopped by the addition of ice-cold PBS containing 1 mM EDTA, 500 μM Na_3VO_4, and 10 mM NaF. Cells were immediately pelleted at 4°C and lysed with 100 μl of ice-cold tyrosine kinase extraction buffer containing 10 mM HEPES, pH 7.2, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% Triton X-100, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml leupeptin. After 30 min on ice and centrifugation, supernatants were collected. Supernatant (200 μg) was incubated with 0.5 μg/ml p53/p56 Lyn antibody (Lyn-44; Santa Cruz Biotechnology) for 1.5 h at 4°C and then with 25 μl of protein G-Sepharose for 1.5 h at 4°C. Immunoprecipitates were washed and resuspended in 20 μl of tyrosine kinase extraction buffer. Ice-cold tyrosine kinase reaction buffer (30 μl; 50 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$, 0.02% Triton X-100, 60 μM ATP, 1 mM Na_3VO_4, 2 μCi of ATP-γP^{33}) with or without 60 μg of poly(Glu-Tyr) as substrate was then added and incubated for 20 min at 37°C. The reaction was stopped by adding 25 μl of the mix on filters (Invitrogen, Pontoise, France). Filters were then washed twice for 30 min and once for 2 h in a cold washing buffer containing 10% trichloroacetic acid and 10 mM sodium pyrophosphate. Filters were washed once in 95% ethanol and dried; radioactivity was determined by scintillation counting.

p53/p56 Lyn Tyrosine Phosphorylation. p53/p56 Lyn tyrosine phosphorylation was measured in cells incubated in the absence or presence of 40 μM ara-C for the times indicated in Figs. 7A and 8A. After being washed twice in cold PBS, cells were lysed with 100 μl of lysis buffer containing 20 mM HEPES, pH 7.2, 2 mM EDTA, 125 mM NaCl, 0.1% Nonidet P40, 1 mM Na_3VO_4, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine. After 30 min on ice and centrifugation, supernatants were collected. Supernatants (200 μg) were incubated with 3 μg of phosphotyrosine antibody (PY-20; BD Biosciences) for 1.5 h at 4°C. Immune complexes were collected by incubation with 25 μl of protein G-Sepharose for 1.5 h at 4°C, and eluted by boiling for 5 min in denaturation solution. Proteins were resolved by electrophoresis in 10% SDS-polyacrylamide, transferred on nitrocellulose membrane (Hybond-C; Amersham Biosciences), and probed with p53/p56 Lyn antibody (Lyn-44; Santa Cruz, CA). The signal corresponding to the total p53/p56 Lyn tyrosine phosphorylation was visualized by enhanced chemiluminescence. The exposed film (Roche Diagnostics, Meylan, France) was scanned using the EasyImage Digital System (Herolab, Wiesloch, Germany) and tyrosine phosphorylated p53/p56 Lyn was quantified by densitometry analysis.

Statistics. Quantitative experiments were analyzed using Student's *t* test. All *p* values resulted from the use of two-sided tests.

Results

PKCζ Status in Transfected Cell Lines. Immunocharacterization of U937-neo, U937-ζJ, and U937-ζB cells has been described previously (Ways et al., 1994; Mansat-de Mas et al., 2002). The doubling time was approximately 19 and 30 h for U937-neo and U937-ζJ or U937-ζB, respectively. However, the cell cycle distribution was similar, if not identical, among the three cell lines (data not shown). As shown in Fig. 1, U937-ζJ and -ζB cells displayed a 4-fold increase of PKCζ expression compared with U937-neo cells. Furthermore, PKCζ activity was 3-fold higher in U937-ζJ or -ζB than in control cells (data not shown; Mansat-de Mas et al., 2002).

Effect of PKCζ Overexpression on Drug-Induced Cytotoxicity. The influence of PKCζ overexpression on drug-induced cytotoxicity was first evaluated when drugs were used at high doses. Therefore, U937-neo, U937-ζJ, and U937-ζB cells were treated with either 40 μM ara-C or 1 μM DNR. As shown in Fig. 2, A and B, under these conditions, both ara-C and DNR induced rapid cell loss in U937-neo cells, whereas drug-induced cytotoxicity was significantly delayed in U937-ζJ and in U937-ζB cells, as measured by trypan blue exclusion assay. DAPI staining revealed that, after 6 h of drug exposure, ara-C and DNR induced apoptosis in U937-neo cells, whereas no apoptosis could be detected in U937-ζJ or U937-ζB cells (Fig. 2C). Moreover, cytometry analysis after propidium iodide DNA staining revealed that, after 6-h incubation, both ara-C and DNR induced a dramatic increase in sub-G1 fraction (55.9 and 47.8%, respectively), with a concomitant decrease in S and G_2-M phase fraction (data not shown) in U937-neo cells, whereas in U937-ζJ cells, sub-G1 accumulation was significantly delayed: 40.2% for ara-C and 41.2% for DNR at 72 h. The same results were observed in U937-ζB cells treated with ara-C and DNR (data not shown). Taken together, these findings suggest that PKCζ overexpression resulted in delayed apoptosis and significant resistance to high doses of ara-C and DNR.

The influence of PKCζ overexpression on drug-induced cytotoxicity was also evaluated at low drug concentrations. Then, cells were treated with either 1 μM ara-C or 0.1 μM DNR for 24 h. As shown in Fig. 3A, PKCζ overexpression resulted in significant protection.

To reinforce the role of PKCζ in protection against drug-induced apoptosis, we have also investigated the influence of reduced PKCζ expression on drug-induced cytotoxicity in U937-ζJ cells. Repeated experiments showed that treatment with antisense oligonucleotides directed against PKCζ resulted in significant, although variable, reduction of PKCζ expression (Fig. 3B), which correlated with higher drug-induced cytotoxic effect, as shown for one representative experiment (Fig. 3C).

Effect of PKCζ Overexpression on Cell-Permeant CER-Induced Cytotoxicity. Because of the lack of apoptotic response in U937-ζJ or in U937-ζB, we hypothesized that PKCζ overexpression could interfere with drug-activated SM-CER pathway. We first examined the possibility that PKCζ overexpression confers significant protection against CER. Therefore, U937-neo, U937-ζJ, and U937-ζB cells were treated with cell-permeant C_6-CER and cell viability was measured by trypan blue dye exclusion assay. As shown in Fig. 4, U937-neo, U937-ζJ, and U937-ζB cells were sensitive to C_6-CER. Although the kinetics of CER-induced cytotoxicity seemed to be delayed in U937-ζB cells, no significant difference was found after 48 h exposure. Moreover, cell cycle analysis revealed no significant differences in sub-G1 frac-

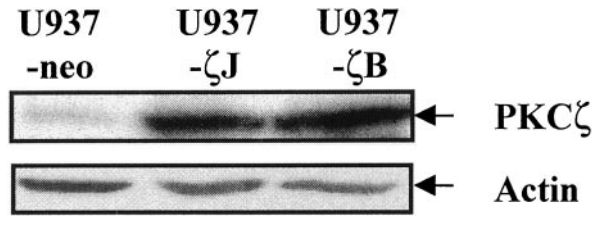

Fig. 1. PKCζ expression in PKCζ-overexpressing cells. Equal protein concentrations of whole-cell solubilized extracts were prepared from cells transfected with vector (U937-neo) or PKCζ (U937-ζJ and U937-ζB). PKCζ expression was measured by Western blot analysis as described under *Materials and Methods*. Results are representative of three independent experiments.

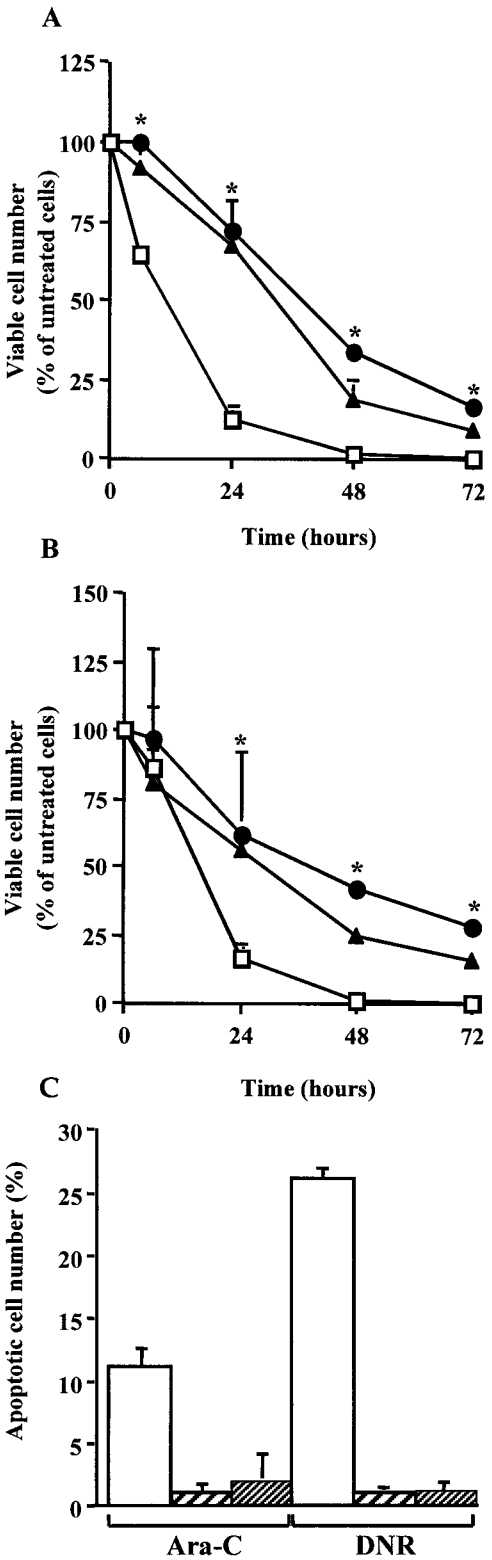

Fig. 2. Effect of PKCζ overexpression on drug-induced cytotoxicity. U937-neo (□), U937-ζJ (●), and U937-ζB (▲) cells were incubated or not in the presence of 40 μM ara-C (A) or 1 μM DNR (B), and cytotoxicity was evaluated after trypan blue exclusion. Results are mean ± S.E.M. of five independent experiments. *, $p < 0.05$ for U937-ζJ and U937-ζB cells compared with U937-neo treated cells. C, apoptotic cell number was evaluated at 6 h after DAPI staining in U937-neo (□), U937-ζJ (▨), and U937-ζB (▨) cells treated with 40 μM ara-C or 1 μM DNR. Results are mean ± S.E.M. of three independent experiments.

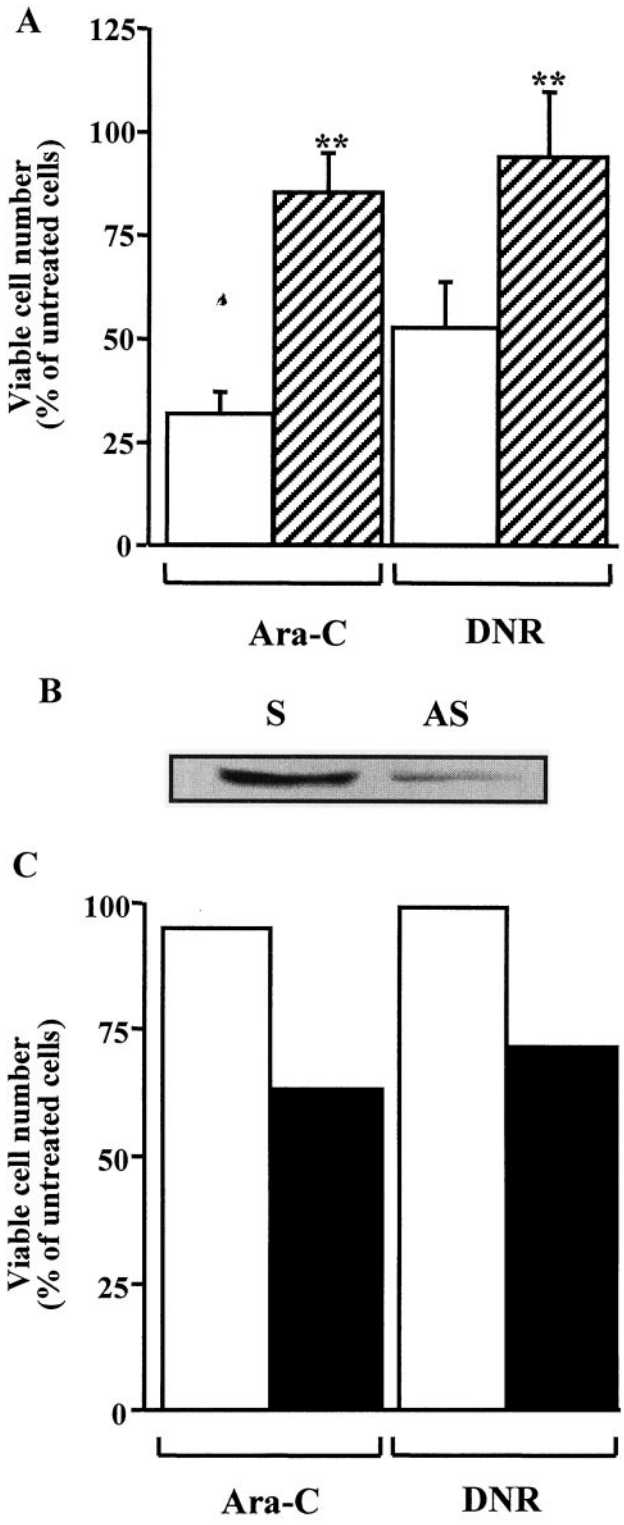

Fig. 3. Effect of antisense oligonucleotides directed against PKCζ. A, U937-neo (□) and U937-ζJ (▨) cells were incubated or not in the presence of 1 μM ara-C or 0.1 μM DNR for 24 h and cytotoxicity was evaluated after trypan blue exclusion. Results are mean ± S.E.M. of five independent experiments. **, $p < 0.01$ compared with U937-neo–treated cells. B, U937-ζJ cells were preincubated with 10 μM antisense (AS, ■) PKCζ or sense (S, □) oligonucleotides for 48 h and then treated with ara-C (1 μM) and DNR (0.1 μM) for 24 h. PKCζ expression was analyzed by Western blot. C, ara-C- and DNR-induced cell cytotoxicity was evaluated at 24 h on oligonucleotide-pretreated U937-ζJ cells by trypan blue exclusion assay. Results are representative of three independent experiments.

tion among U937-neo, U937-ζJ, and U937-ζB cells treated with CER (data not shown). Altogether, these results showed that U937-neo, U937-ζJ, and U937-ζB cells were equally sensitive to CER. These findings led us to speculate that PKCζ overexpression interfered with the SM-CER pathway by limiting CER production.

Effect of PKCζ Overexpression on CER Production and N-SMase Stimulation. Because U937-ζJ and U937-ζB cells give similar responses to cytotoxic agents, all the following experiments were done on the U937-ζJ clone. U937-neo and U937-ζJ cells were prelabeled with [9,10-^{3}H]palmitic acid to equilibrium for 48 h, then incubated with 40 μM ara-C or 1 μM DNR, and harvested at various times. As illustrated in Fig. 5A, ara-C and DNR induced time-dependent significant CER generation with a maximum at 15 min in U937-neo cells, as described previously (Jaffrézou et al., 1996; Bezombes et al., 2001), but not in U937-ζJ cells. This result suggested that PKCζ stimulation resulted in decreased N-SMase stimulation. At first, we observed that basal N-SMase activity was significantly decreased (40% reduction) in PKCζ-overexpressing cells compared with U937-neo cells (data not shown). Moreover, in U937-neo cells, as expected from previous studies (Jaffrézou et al., 1996; Bezombes et al., 2001), both ara-C and DNR induced weak but significant N-SMase stimulation that was detectable as early as 5 min and peaked at 10 to 12 min (Fig. 5B). However, neither ara-C nor DNR stimulated N-SMase activity in U937-ζJ. These results demonstrated that PKCζ overexpression abrogated drug-induced N-SMase stimulation and CER production. Previous studies showed that drug-induced ROS production played a critical role for N-SMase stimulation, so we investigated whether PKCζ overexpression might influence oxidative burst in drug-treated cells.

Effect of PKCζ Overexpression on ROS Generation. U937-neo cells and U937-ζJ cells were treated with 40 μM ara-C, and H_2O_2 production was evaluated by cytometry analysis of C2938 dye fluorescence. As described previously, ara-C induced a ROS burst (Bezombes et al., 2001) that peaked at 5 min in U937-neo cells but was dramatically reduced in U937-ζJ cells (Fig. 6). These results suggest that PKCζ-overexpressing cells displayed enhanced oxidative defenses that may account for the lack of drug-induced N-SMase stimulation. In a recent study, we showed that early ROS production plays an important role in the activation of p53/p56 Lyn, which in turn interacts with and activates N-SMase (Bezombes et al., 2001). Therefore, we hypothesized that the inhibitory effect of PKCζ on drug-induced ROS production could result in the abrogation of drug-induced p53/p56 Lyn activation.

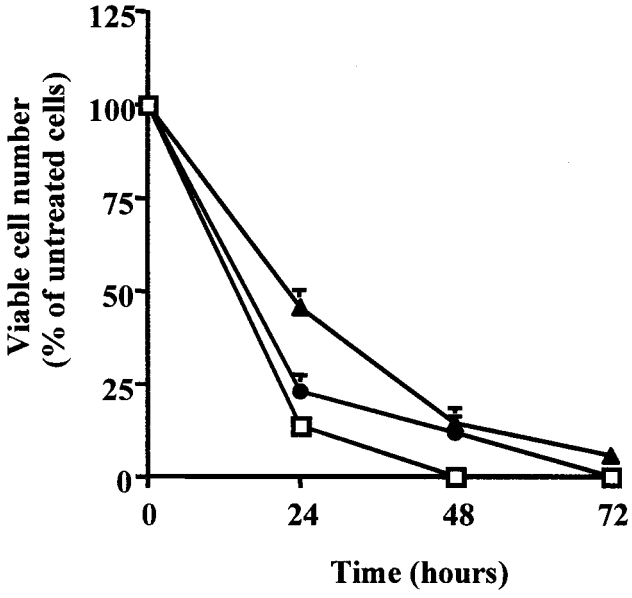

Fig. 4. Effect of PKCζ overexpression on cell-permeant CER-induced cytotoxicity. U937-neo (□), U937-ζJ (●), and U937-ζB (▲) cells were treated with 25 μM C_6-CER, and cell viability was evaluated after trypan blue exclusion. Results are mean ± S.E.M. of three independent experiments.

Effect of PKCζ Overexpression on Drug-Induced p53/p56 Lyn Stimulation. U937-neo and U937-ζJ cells were treated with 40 μM ara-C and 1 μM DNR, and p53/p56 Lyn activity was measured at various times using an immune kinase assay. In untreated cells, basal p53/p56 Lyn activity was 3-fold lower in U937-ζJ cells compared with U937-neo cells, whereas its expression level was similar (data not shown). As shown in Fig. 7A, in U937-neo cells, but not in U937-ζJ cells, ara-C induced early and sustained p53/p56 Lyn tyrosine phosphorylation with a maximum at 7 to 9 min as evaluated by immunoprecipitation using anti-phosphotyrosine antibody followed by immunoblotting with anti-p53/p56 Lyn antibody. This result suggested that PKCζ overexpression resulted in abrogation of drug-induced p53/p56 Lyn activation. To confirm the influence of PKCζ on drug-induced stimulation of Lyn activity, we used an immune kinase assay using poly(Glu-Tyr) as substrate. Immune kinase assay revealed that ara-C induced a time-dependent stimulation of Lyn activity (data not shown) that paralleled Lyn tyrosine phosphorylation kinetics in U937-neo cells but not in U937-ζJ cells. At the peak of stimulation (7 min), Lyn activity increased up to 275.3 and 230.9% in U937-neo cells treated with ara-C and DNR, respectively. However, Lyn activity remained unchanged in ara-C– and DNR-treated U937-ζJ cells (Fig. 7B). These results showed that PKCζ overexpression resulted in the inhibition of drug-induced p53/p56 Lyn phosphorylation and stimulation.

Effects of PKCζ Overexpression on Hydrogen Peroxide-Induced p53/p56 Lyn Phosphorylation and N-SMase Stimulation. Because PKCζ overexpression resulted in abrogation of drug-induced early ROS production on the one hand and it inhibited drug-induced p53/p56 Lyn activation and N-SMase stimulation on the other hand, we hypothesized that reduced ROS production was critical for the lack of N-SMase stimulation in U937-ζJ cells. To confirm this hypothesis, U937-neo cells and U937-ζJ cells were treated with H_2O_2, and p53/p56 Lyn tyrosine phosphorylation, as well as N-SMase activity, were examined from 0 to 10 min. As expected from our previous study (Bezombes et al., 2001), treatment with exogenous H_2O_2 (1 μM) resulted in p53/p56 Lyn tyrosine phosphorylation in U937-neo cells, whereas, p53/p56 Lyn tyrosine phosphorylation was significantly reduced in U937-ζJ cells (Fig. 8A). The lack of p53/p56 Lyn tyrosine phosphorylation was also observed for doses of H_2O_2 as high as 1 mM (data not shown). Moreover, whereas treatment with H_2O_2 resulted in N-SMase stimulation in U937-neo cells, this was not the case in U937-ζJ cells, even those treated at 1 mM (Fig. 8B). Taken together, these re-

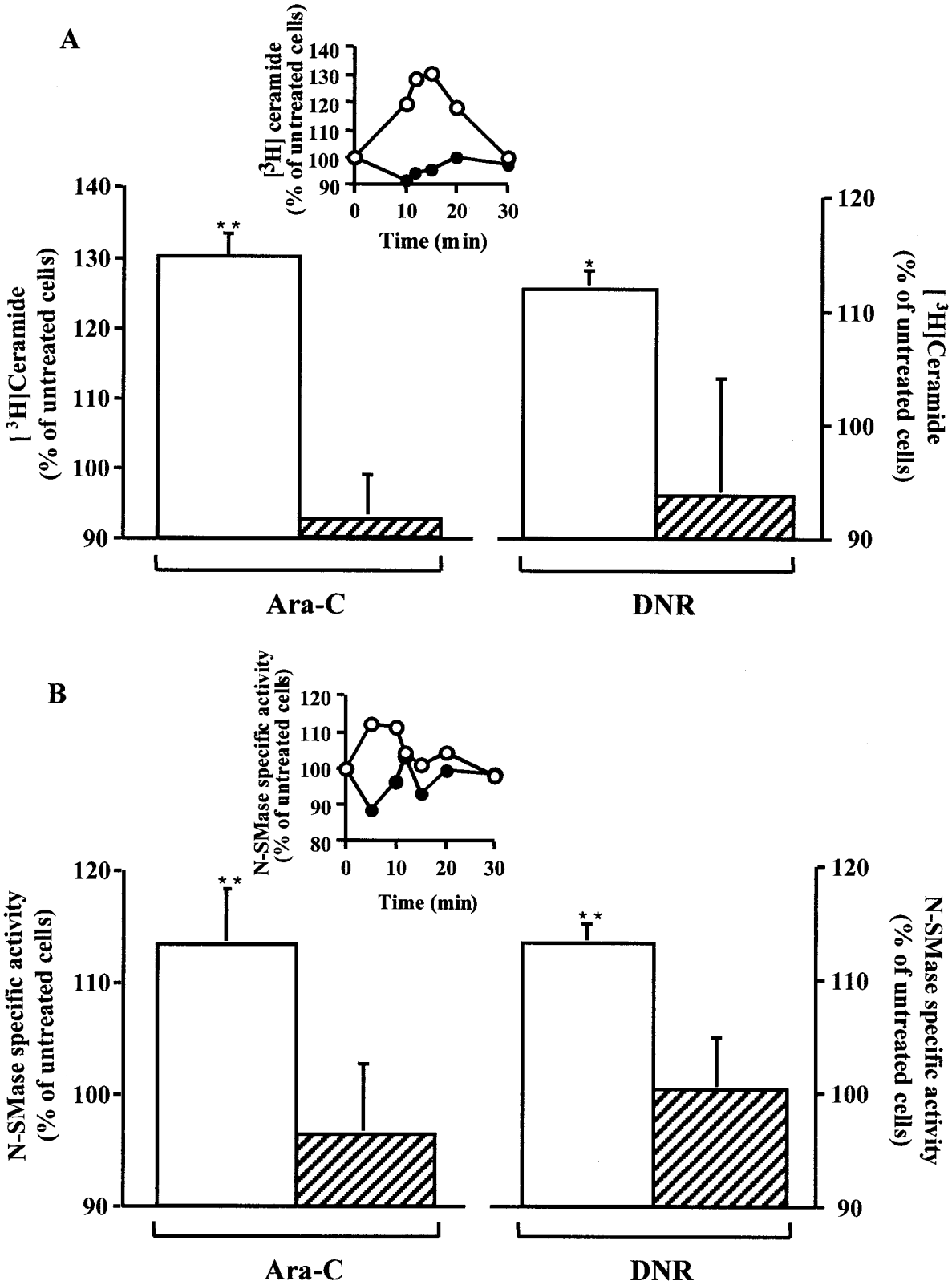

Fig. 5. Effect of PKCζ overexpression on CER production and N-SMase stimulation. U937-neo (□) and U937-ζJ (▨) cells were treated with either 40 μM ara-C or 1 μM DNR. A, CER levels were estimated in cells prelabeled with [9,10-^{3}H]palmitic acid for 48 h. Histograms illustrate intracellular CER accumulation at the peak of CER generation (12–15 min). Results are mean ± S.E.M. of three to four independent experiments. *, $p < 0.05$; **, $p < 0.01$, compared with untreated cells. Inset, kinetic of intracellular CER levels in U937-neo (○) or U937-ζJ (●) cells treated with 40 μM ara-C at the time intervals indicated. Graph is representative of three experiments. B, N-SMase was determined as described under *Materials and Methods*. Histograms illustrate N-SMase activity at the peak of N-SMase stimulation (10–12 min). Results are mean ± S.E.M. of three to five independent determinations. **, $p < 0.01$ compared with untreated cells. Inset, kinetic of N-SMase activity in U937-neo (○) or U937-ζJ (●) cells treated with 40 μM ara-C at the time intervals indicated. Graph is representative of three experiments.

sults suggested that PKCζ overexpression resulted in altered p53/p56 Lyn redox regulation.

Effect of PKCζ Overexpression on H_2O_2 Detoxification. We hypothesized, therefore, that PKCζ overexpression resulted in stimulation of H_2O_2 detoxification. Consequently, U937-neo cells and U937-ζJ cells were treated with exogenous H_2O_2 (1 μM) and C2938 dye fluorescence was measured at 10 min. As shown in Fig. 9, H_2O_2-induced increase in C2938 dye fluorescence was significantly reduced in U937-ζJ cells compared with U937-neo cells, which suggested that PKCζ-overexpressing cells displayed enhanced antioxidant defenses that contributed to accelerate H_2O_2 detoxification. In fact, PKCζ-overexpressing cells were found to be significantly more resistant to exogenous H_2O_2 than control cells, with IC_{50} values of 100 and 20 μM, respectively, as evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium viability assay. Altogether these results suggest that PKCζ overexpression results in an increase in antioxidant defenses responsible of abnormal p53/p56 Lyn redox regulation, inhibition of drug-induced N-SMase stimulation, CER production, and cytotoxicity.

Discussion

In this study, we showed that PKCζ overexpression resulted in the inhibition of apoptosis and cytotoxicity induced by ara-C and DNR on U937 cells. This result suggests that PKCζ expression may represent an adverse factor for AML therapy. Although there is no information about PKCζ expression and/or activity levels in AML cells, there is some evidence that PKCζ could be activated in this clinical setting.

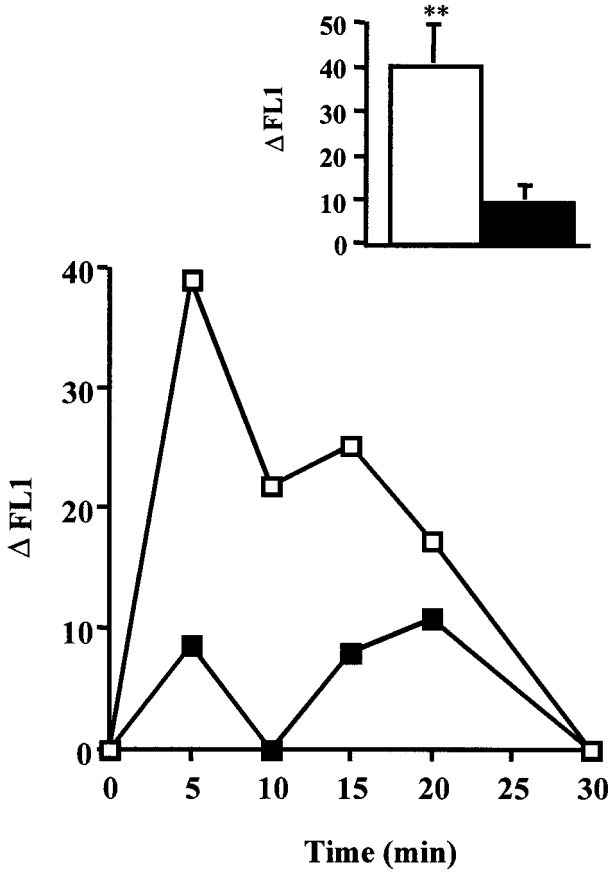

Fig. 6. Effect of PKCζ overexpression on ROS production. U937-neo (□) and U937-ζJ (■) cells were treated with 40 μM ara-C, and H_2O_2 production was determined using flow cytometry as described under *Materials and Methods*. Graph is representative of three experiments. Inset, results are represented at the peak of H_2O_2 production (5 min) and are the mean ± S.E.M. of five independent determinations. **, $p < 0.01$ compared with U937-ζJ–treated cells.

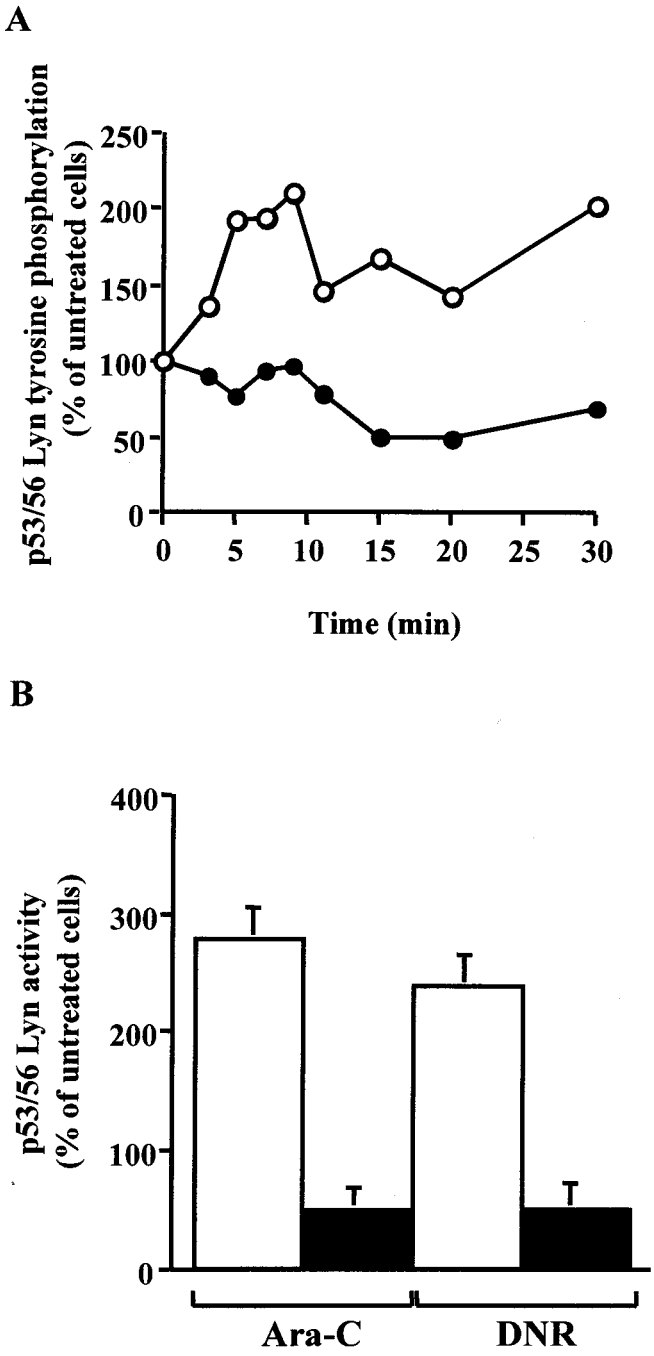

Fig. 7. Effect of PKCζ overexpression on drug-induced p53/p56 Lyn activity. A, U937-neo (○) and U937-ζJ (●) cells were treated with 40 μM ara-C, and kinetic of p53/p56 Lyn tyrosine phosphorylation was evaluated after phosphotyrosine immunoprecipitation as described under *Materials and Methods*. Graph is representative of three experiments. B, U937-neo (□) and U937-ζJ (■) cells were treated with either 40 μM ara-C or 1 μM DNR, and p53/p56 Lyn activity was evaluated by an immune kinase assay as described under *Materials and Methods*. Histograms illustrate p53/56 Lyn activity at the peak of p53/p56 Lyn kinase stimulation (7–9 min) and are mean ± S.E.M. of three independent determinations.

For example, it has been documented that active Ras interacts with PKCζ and stimulates its kinase activity (Diaz-Meco et al., 1994), whereas Ras can be deregulated in AML cells or myelodysplastic syndrome not only by mutation but also by constitutive activation of proto-oncogenes, including c-kit and Flt-3 (Reuter et al., 2000). Therefore, although Ras mutation has been correlated with increased sensitivity to ara-C and to anthracyclines in solid tumors (Koo et al., 1996), it is conceivable that, in AML cells, Ras deregulation may result in PKCζ stimulation and subsequent drug resistance.

The mechanism by which PKCζ inhibited drug-induced apoptosis was also investigated. Based on different cell growth kinetics, it was conceivable that the slower cell growth of PKCζ-overexpressing cells may contribute to the protective effect. However, the fact that cell cycle distribution was similar, if not identical, between U937-neo and PKCζ-overexpressing U937 cells argued against this hypothesis. Moreover, although we could not rule out the possibility that PKCζ interfered with drug-induced DNA damage repair, the fact that ara-C and DNR induced different lesions strongly suggested that PKCζ acted after the damage occurred (for example, by inhibiting the drug-activated apoptotic pathway). This hypothesis was supported by previous studies, which have established that these two drugs share common apoptotic signaling pathways. Indeed, both ara-C and DNR activate the SM cycle, and there is now compelling evidence that the SM cycle plays an important role in drug-induced cytotoxicity (Laurent and Jaffrézou, 2001).

Therefore, we hypothesized that PKCζ interfered with either CER production or CER-induced apoptosis. Our study shows that whereas PKCζ overexpression had no effect on exogenous CER-induced apoptosis, it abrogated drug-induced N-SMase stimulation and CER production, suggesting that PKCζ acted by inhibiting one step of the signaling cascade leading to N-SMase stimulation. We have previously reported that, in U937 cells, treatment with ara-C (40 μM) results in a redox-dependent activation of Lyn and that activated Lyn interacted with N-SMase (Bezombes et al., 2001). Moreover, oxidative burst inhibition by antioxidants resulted in the abrogation of Lyn activation and N-SMase stimulation (Bezombes et al., 2001). The present study shows that whereas not only ara-C but also DNR induced ROS production and Lyn activation in control cells, this was not the case

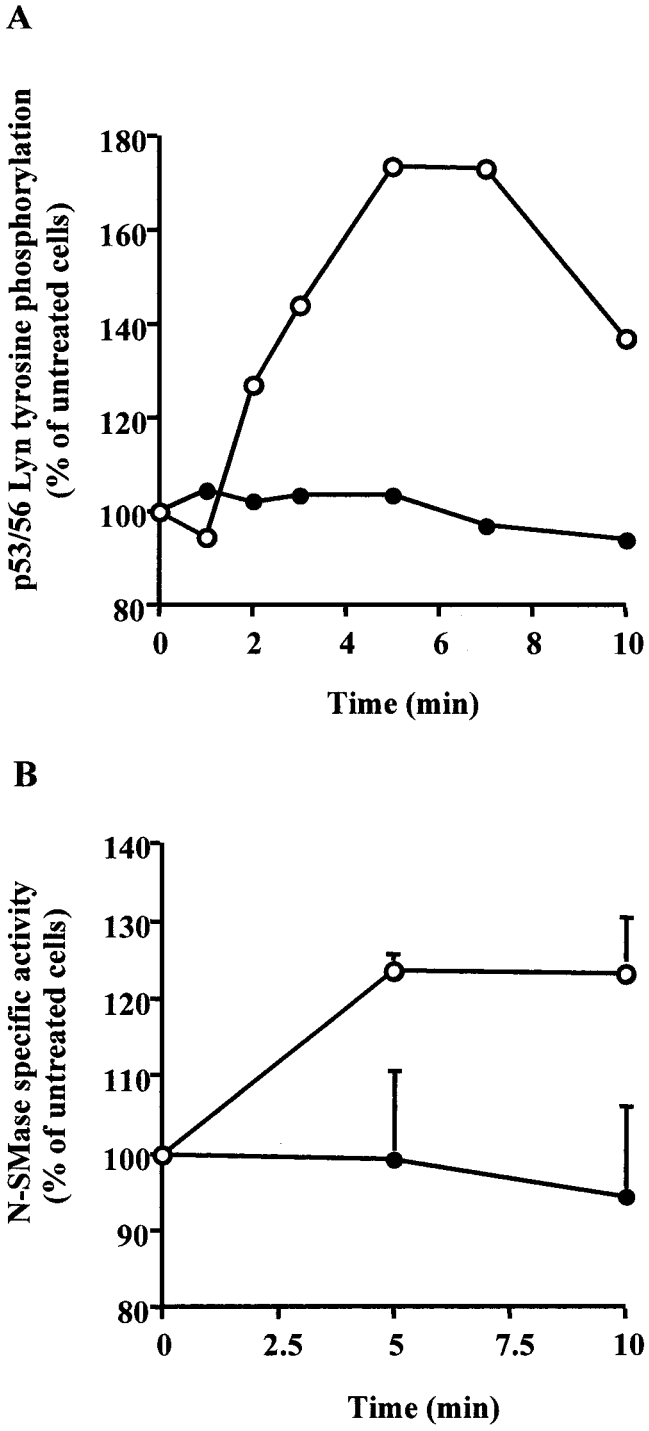

Fig. 8. Effect of PKCζ overexpression on hydrogen peroxide-induced p53/p56 Lyn phosphorylation and N-SMase stimulation. A, U937-neo (○) and U937-ζJ (●) cells were treated with 1 μM H_2O_2, and p53/p56 Lyn tyrosine phosphorylation was evaluated as described under *Materials and Methods* at the time intervals indicated. Graph is representative of three experiments. B, U937-neo (○) and U937-ζJ (●) cells were treated with 1 mM H_2O_2 and N-SMase stimulation was evaluated. Results are mean ± S.E.M. of three independent determinations.

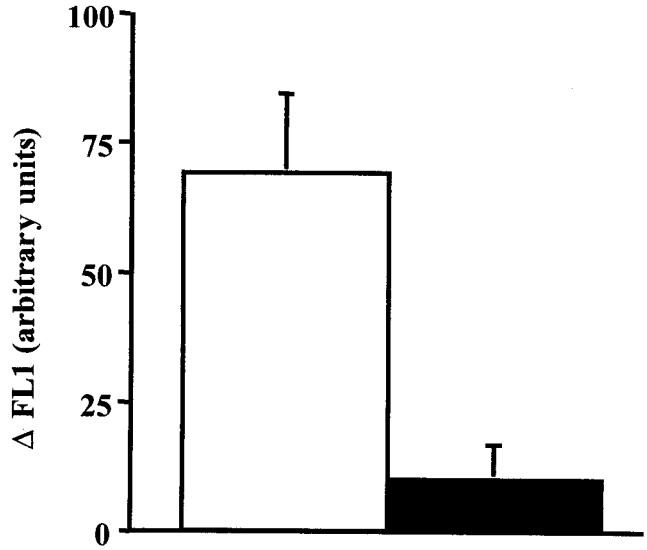

Fig. 9. Effect of PKCζ overexpression on H_2O_2 detoxification. U937-neo (□) and U937-ζJ (■) cells were treated with 1 μM H_2O_2 and detoxification was analyzed after 10 min by measuring intracellular H_2O_2 as described under *Materials and Methods*. Results are mean ± S. E. M. of three independent determinations.

in PKCζ-overexpressing cells. This result suggested that, in these cells, the lack of N-SMase stimulation was caused by the lack of ROS-induced Lyn stimulation. Therefore, we speculated that PKCζ-overexpressing cells displayed increased anti-oxidant defenses. The fact that PKCζ overexpression resulted in increased H_2O_2 detoxification and a high level of resistance to exogenous H_2O_2 supports this hypothesis. This could explain why even high doses of exogenous H_2O_2 could not restore p53/p56 Lyn activation and N-SMase stimulation. Collectively, these results suggest that, in PKCζ-overexpressing cells, increased H_2O_2 detoxification at least contributes to the inhibition of drug-activated SM cycle and apoptosis. H_2O_2 interacts with metal ions such as iron or copper as catalyst by the Haber-Weiss reaction to generate hydroxyl radicals (OH·), whereas antioxidant defenses may prevent the propagation of radical reactions. Indeed, glutathione may directly scavenge hydroxyl radicals, whereas H_2O_2 may be detoxified by a variety of enzymes, including catalase, glutathione peroxidase, or thioredoxin peroxidase (Briehl et al., 1997). Previous studies have established that phorbol ester-induced PKC stimulation may increase thioredoxin expression (Kumar and Holmgren, 1999). However, the role of the phorbol ester-insensitive PKCζ on this antioxidant system has not been yet documented. In an attempt to identify the mechanism by which PKCζ exerts its antioxidant properties, we first hypothesized that, in PKCζ-overexpressing cells, the inhibition of drug-activated SM cycle could be caused by an increase in glutathione levels. Indeed, previous studies have shown that glutathione is a critical regulator of N-SMase (Liu et al., 1998; Gouaze et al., 2001). However, we found that whereas U937-ζJ cells displayed higher intracellular glutathione content compared with U937-neo cells, buthionine-sulfoximine-induced glutathione depletion did not restore ara-C–induced p53/p56 Lyn tyrosine phosphorylation and N-SMase stimulation (data not shown). This result suggests that glutathione may not play an important role in PKCζ-induced abnormal p53/p56 Lyn redox regulation. Therefore, it is possible that PKCζ activity may influence the activities of catalase or thioredoxin system enzymes, including thioredoxin reductase and/or thioredoxin peroxidase. From this perspective, it is interesting to note that thioredoxin peroxidase has been previously identified as an important regulator of apoptosis through its antioxidant property (Zhang et al., 1997; Kang et al., 1998).

Although reduction of drug-induced oxidative burst seems to play an important role in the lack of Lyn activation in PKCζ-overexpressing cells treated with ara-C or with DNR, we cannot rule out the possibility that, in these cells, Lyn function is intrinsically altered. The fact that basal Lyn activity was decreased in PKCζ-overexpressing cells, compared with their parental counterparts, argues for this hypothesis. The role of PKCζ on Lyn activity has not been investigated before. Previous studies have established that SHP-1, a tyrosine phosphatase largely expressed in myeloid cells, including U937 cells, is a major negative regulator of Lyn (Somani et al., 2001). Therefore, it could be possible that PKCζ overexpression may influence SHP-1 expression and/or activity. Moreover, based on previous studies that have shown that SHP-1 activity could be directly influenced by ROS (Cunnick et al., 2000), it is conceivable that, in PKCζ-overexpressing cells, the abnormal redox balance may have resulted in SHP-1 stimulation and subsequent Lyn inactivation. These hypotheses are currently being tested in our laboratory.

Our study suggests that, in PKCζ-overexpressing cells, the inhibition of drug-induced apoptosis and cytotoxicity inhibition is caused by the blockage of the SM-ceramide pathway. However, we cannot rule out that PKCζ interferes with other apoptosis regulators. Indeed, it has been reported that PKCζ may activate protein IκB kinase-β, an upstream activator of nuclear factor-κB (Lallena et al., 1999), which, in turn, has been documented to confer significant protection against genotoxic agents, including DNR and ara-C (Wang et al., 1999; Romano et al., 2000). Moreover, other studies have shown that PKCζ may activate ERK proteins by stimulating either MEK (Berra et al., 1995) or Raf-1 (van Dijk et al., 1997) and that ERK activation may enhance cellular survival after exposure to ara-C (Anderson and Tolkovsky, 1999) or DNR (von Gise et al., 2001). Therefore, it is conceivable that PKCζ interferes at different levels of drug-induced apoptosis. The fact that this enzyme is inactivated by caspase-dependent proteolysis during apoptosis induced by UV radiation (Frutos et al., 1999) or by cisplatin (Basu and Akkaraju, 1999), supports this hypothesis and argues for a more general role for atypical PKC isozymes in the inhibition of apoptosis induced by genotoxic agents (Diaz-Meco et al., 1996; Murray and Fields, 1997).

To conclude, this study shows that PKCζ overexpression results in the inhibition of ara-C– and DNR-induced apoptosis and cytotoxicity, which is in turn caused, at least in part, by the blockage of the signaling cascade leading to CER production (Fig. 10). Previous studies have shown that this kinase can be activated by a large variety of internal and external stimuli, including growth factors and oncogenic

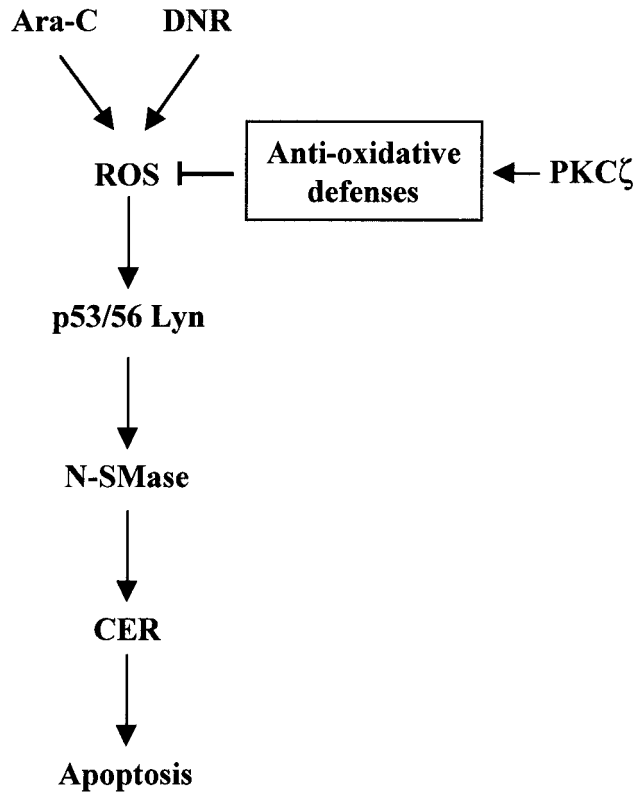

Fig. 10. Hypothetic model for the role of PKCζ in cellular protection against ara-C and DNR.

products; therefore, these results may have important implications in drug resistance and designate PKCζ as a putative pharmacological target for improving antileukemic effect of the DNR/ara-C combination.

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