0026-895X/99/010118-08$3.00/0

MOLECULAR PHARMACOLOGY, **55:**118–125 (1999).

Daunorubicin- and Mitoxantrone-Triggered Phosphatidylcholine Hydrolysis: Implication in Drug-Induced Ceramide Generation and Apoptosis

ALI BETTAÏEB, ISABELLE PLO, VÉRONIQUE MANSAT-DE MAS, ANNE QUILLET-MARY, THIERRY LEVADE, GUY LAURENT, and JEAN-PIERRE JAFFRÉZOU

Contrat Jeune Formation Institut National de la Santé et de la Recherche Medicale 95-03, Institut Claudius Régaud, (A.B., I.P., V.M., A.Q., G.L., J.P.J.), Institut National de la Santé et de la Recherche Medicale U466, Centre Hospitalier Universitaire Rangueil, (T.L.), and the Service d'Hématologie, Centre Hospitalier Universitaire Purpan, (G.L.), Toulouse, France

Received July 27, 1998; accepted September 25, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

Several studies have suggested that diacylglycerol can affect the induction of apoptosis induced by toxicants and ceramide. The present study demonstrates that clinically relevant concentrations of the chemotherapeutic drugs daunorubicin and mitoxantrone (0.2–1 μM) transiently stimulated concurrently with sphingomyelin-derived ceramide generation and diacylglycerol and phosphorylcholine production within 4 to 10 min via phospholipase C hydrolysis of phosphatidylcholine. Pretreatment of cells with the xanthogenate compound D609, a potent inhibitor of phosphatidylcholine-phospholipase C, led to significant inhibition of drug triggered diacylglycerol and phosphorylcholine production and to a sustained increase in ceramide levels for a period up to 2 h. Moreover, D609 pretreatment induced both cell death and ceramide generation at daunorubicin and mitoxantrone concentrations previously shown to be ineffective (i.e., 0.1 μM). These results underline the importance of diacylglycerol in the regulation of programmed cell death and strongly argue for a balance between apoptotic (ceramide) and survival (diacylglycerol) signal transducers.

Daunorubicin (DNR) and mitoxantrone (MXT) are among the most active antitumor compounds used in clinical oncology, especially in the treatment of acute leukemias. Cytotoxicity mediated by these agents is generally thought to be the result of drug-induced damage to DNA. This damage is mediated by quinone-generated redox activity, intercalation-induced distortion of the double helix, or stabilization of the cleavable complex formed between DNA and topoisomerase II (Chabner and Myer, 1989). More recently, we demonstrated that at clinically relevant concentrations (0.5–1 μM), DNR and MXT can trigger apoptosis in certain myeloid leukemia cellular models (Quillet-Mary et al., 1996; Bailly et al., 1997). Present knowledge, however, does not allow us to determine whether apoptosis is a simple consequence of drug-induced DNA lesions or represents an independent cytotoxic mechanism triggered by a specific signaling pathway (Hannun, 1997).

We recently demonstrated that DNR activated the sphingomyelin (SM)-ceramide (CER) cycle leading to apoptosis. Indeed, DNR-stimulated neutral SMase activity responsible for SM hydrolysis and subsequent CER generation in U937 and HL-60 human leukemia cells (Jaffrézou et al., 1996). Such an apoptotic signaling pathway has also been described in vincristine, ionizing radiation (IR), anti-Fas, and tumor necrosis factor (TNF)α-induced apoptosis (Hannun, 1996). The fact that cell-permeant CER as well as natural CER (generated by exposure of the cells to bacterial SMase) induce apoptosis in these cells strongly suggests that CER was the mediator of DNR-induced apoptosis.

Several studies have shown that modulation of protein kinase C (PKC) activity can affect the induction of apoptosis induced by toxicants and CER (Haimovitz-Friedmann et al., 1994; Mansat et al., 1997b). Indeed, Jarvis et al. (1996) not only showed that PKC activators, including phorbol esters such as 12-*O*-tetradecanoyl phorbol-13-acetate and diacylglycerol (DAG), could inhibit the ability of cell-permeant CER to induce apoptosis, but also that PKC inhibitors enhanced

This work was supported by grants from the Actions Concertées Coordonnées-Sciences du Vivant ACCSV8:9508008 (to G.L.), La Fédération Nationale des Centres de Lutte Contre le Cancer (to J.P.J. and T.L.), the Conseil Régional Midi-Pyrénées (to J.P.J. and T.L.), l'Association pour la Recherche sur le Cancer Grants 6749 (to G.L.), 3002 (to T.L.), and 2069 (to J.P.J.), and by La Ligue Nationale Contre le Cancer (to G.L.), and la Société Française d'Hématologie, Paris (to A.B.).

ABBREVIATIONS: SM, sphingomyelin; CER, ceramide; DAG, diacylglycerol; PhoCho, phosphorylcholine; PC, phosphatidylcholine; PLC, phospholipase C; DNR, daunorubicin; MXT, mitoxantrone; IR, ionizing radiation; PKC, protein kinase C; DiC8, 1,2 dioctanoyl-*sn*-glycerol; TNF, tumor necrosis factor.

CER-induced apoptosis (Jarvis et al., 1994a). Furthermore, it has recently been reported that PKC inhibitors could increase neutral SMase activity (Chmura et al., 1996). These findings are consistent with those reported by Haimovitz-Friedmann et al. (1994), who observed that 12-*O*-tetradecanoyl phorbol-13-acetate inhibited CER generation and apoptosis in irradiated bovine endothelial cells.

A variety of antitumor agents including anthracyclines, alkylating agents, and IR has been shown not only to trigger CER generation but also to increase cellular DAG levels and PKC activity. For example, doxorubicin and cisplatin led to both rapid DAG accumulation and PKC stimulation (Posada et al., 1989; Rubin et al., 1992). IR has also been described to induce a rapid PKC activation due to stimulation of phosphatidylinositol turnover (Uckun et al., 1993). Overall, the observation that toxicants can modulate both CER and DAG levels has led to the speculation for the existence of a balance between pro- and antiapoptotic mediators, opposing the cytotoxic and the cytoprotective roles for CER and DAG, respectively (Kolesnick and Fuks, 1995).

In light of the emerging concept of a cytoprotective function of DAG, and therefore PKC, in the regulation of leukemic cell survival, we attempted in this study to demonstrate the potential role of DAG in the response of leukemic cells to two chemotherapeutic agents used in the treatment of acute myelogenous leukemia, DNR and MXT. Here we show for the first time that DAG and phosphorylcholine (PhoCho) are produced in U937 cells by a DNR- and MXT-responsive phosphatidylcholine (PC)-specific phospholipase C (PLC). Furthermore, we present evidence that endogenous DAG can modulate both drug-triggered CER generation and apoptosis in the leukemic cell line U937.

Experimental Procedures

Drugs and Reagents. MXT was a gift from Lederle Laboratories (Rungis, France). DNR was obtained from the National Cancer Institute Drug Repository. [methyl-^{3}H]Thymidine (79 Ci/mmol) and [9,10(n)-^{3}H] palmitic acid (53 Ci/mmol) were purchased from Amersham (Les Ulis, France). Silica Gel 60 thin-layer chromatography plates were purchased from Merck (Darmstadt, Germany). D609 (xanthogenate tricyclodecan-9-yl) and DAG kinase inhibitor II (R 59 949) were purchased from Calbiochem (San Diego, CA). All other drugs and reagents were purchased from Sigma (St. Louis, MO), Carlo Erba Reactives (Rueil-Malmaison, France), or Prolabo (Paris, France).

Cell Culture. The human monocytic leukemia cell line U937, purchased from the American Type Culture Collection (Rockville, MD), was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Eurobio, Les Ulis, France). Cell stocks were screened routinely for *Mycoplasma* (Stratagene *Mycoplasma* PCR kit, La Jolla, CA).

Assay for PKC Activity. Exponentially growing cells (5×10^6/ml) were incubated for 1 h with drugs. Cells (5×10^6) were dissolved in 0.5 ml of ice-cold extraction buffer (25 mM Tris-HCl pH 7.4, 10 mM β-mercaptoethanol, 0.5 mM EGTA, 0.5 mM EDTA, 0.5 μM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. PKC activity was assayed by measuring the incorporation of ^{32}P from ATP into Neurogranin$_{28\ to\ 43}$ peptide (Chen et al., 1993) using the SignaTECT PKC Assay System (Promega, Madison, WI) as described by the supplier. Results are expressed as picomoles ^{32}P incorporated into neurogranin peptide per minute per milligram protein.

Determination of DNA Fragmentation. Exponentially growing cells (5×10^5/ml) were incubated for 1 h with drugs, washed twice, and cultured in drug-free medium for an additional 5 h. Viable cells were counted in the presence of trypan blue.

DNA fragmentation was quantified as previously described (Quillet-Mary et al., 1996). Exponentially growing cells (5×10^5/ml) were labeled for 24 h with 0.5 μCi/10^6 cells of [*methyl*-^{3}H]thymidine and washed 3 times with nonradioactive fresh medium. After drug treatment, cells were washed twice and cultured in drug-free medium for an additional 5 h. Cells were then harvested by centrifugation and the pellets were suspended in lysis buffer containing 15 mM Tris, 20 mM EDTA, and 0.5% Triton X-100, pH 8. After incubating on ice for 30 min, samples were centrifuged at 20,000g for 30 min. Pellets were resuspended in lysis buffer. The supernatant (detergent soluble low-molecular-weight DNA) and pellet radioactivity (intact chromatin DNA) were determined by liquid scintillation counting.

Analysis of Cellular Phospholipids, CER, DAG, and PhoCho. Quantitation of phospholipids, CER, and DAG was performed by labeling cells to isotopic equilibrium with [9,10(n)-^{3}H]palmitic acid (1 μCi/ml) (Levade et al., 1993; Andrieu et al., 1994). Cells were then washed and resuspended in serum-free medium for kinetic experiments. Aliquots were taken for protein determination (Smith et al., 1985). Lipids were extracted (Folch et al., 1957) and [9,10(n)-^{3}H]palmitic acid-labeled SM, PC, CER, and DAG were quantified as previously described (Andrieu et al., 1994; Augé et al., 1996; Jaffrézou et al., 1996). Alternatively, PC and SM were determined after labeling of cells with [*methyl*-^{3}H]choline. Similar results for CER and DAG quantitation were obtained using *Escherichia coli* DAG kinase (Amersham, kit RPN200) and [γ-^{33}P]ATP (3300 Ci/mmol; ICN, Orsay, France) according to previously published procedures (Van Veldhoven et al., 1992; data not shown). Presence and levels of PhoCho were determined in the aqueous phase of [^{3}H]choline-labeled cell extracts by thin-layer chromatography using the solvent system methanol/0.5% NaCl/ammonia (50:50:1) as described (Lacal et al., 1987). After autoradiography with EN^{3}HANCE (Dupont De Nemours, Les Ulis, France), the radioactive spots were scraped and the amount of radioactivity was determined by liquid-scintillation counting. Statistical analyses were performed using the Student's t test.

Results

Activation of PC Hydrolysis and DAG Generation by DNR and MXT. [^{3}H]palmitic acid-labeled U937 cells were treated with either 1 μM DNR or 1 μM MXT, and intracellular DAG and PC contents were determined at various time points. Both DNR (Fig. 1A) and MXT (Fig. 1B) transiently stimulated the rapid production of DAG, increasing within 4 to 10 min to a maximum of ~45% and ~35% above baseline, respectively. This burst in DAG occurred concurrently with a rapid PC cycle (hydrolysis and resynthesis) and reached a maximum of ~15% at 4 to 10 min (Fig. 1, insets). Dose-effect studies showed significant PC hydrolysis starting at 0.1 μM DNR and MXT (~6% and ~4%, respectively) (Table 1).

To demonstrate the possible origin of DAG generation, we used a potent inhibitor of PC-PLC, the xanthogenate tricyclodecan-9-yl D609 (Müller-Decker, 1989; Pörn-Ares et al., 1997). Preincubation of cells for 1 h with 10 μg/ml D609 induced a 20% reduction in basal DAG levels and inhibited both DNR- and MXT-triggered DAG production (Fig. 1). In addition, D609 was able to block PC hydrolysis induced by, for example, DNR (Fig 1A, inset), supporting the hypothesis that PC is the substrate of a putative drug-responsive phospholipase. To confirm the contribution of PC-PLC to DAG formation, we analyzed the presence and the levels of Pho-

Cho after drug stimulation. DNR treatment of [^{3}H]choline-labeled cells led to a rapid increase of the amount of intracellular PhoCho, which reached ~ 120% of unstimulated values at 4 to 10 min. However, the increase of the amount of intracellular PhoCho was inhibited by treatment of cells with D609 (Fig 2).

Effect of DNR and MXT on PKC Activation. We have demonstrated that DNR and MXT induced a transient increase of DAG production in U937 cells. Because DAG is a well known activator of PKC, we determined PKC activity in U937 cells treated with DNR or MXT by measuring the incorporation of ^{32}P from ATP into neurogranin peptide. Results summarized in Table 2 indicate that both 1 μM DNR and 1 μM MXT increased PKC activity. However, PKC activation was not dependent on PC hydrolysis, as pretreatment of U937 cells with D609 decreased basal PKC activity but did not prevent the drug-induced increase of PKC activity (Table 2).

TABLE 1

Effect of DNR and MXT on cellular PC in U937 cells

PC hydrolysis induced by DNR and MXT were estimated in U937 cells prelabeled with [^{3}H]palmitic acid or [^{3}H]choline for 48 h. Cells were then washed and resuspended in serum-free medium with DNR or MXT at the indicated concentrations. After incubation, aliquots were collected and lipids were extracted. Labeled PC was resolved and quantitated as described in *Experimental Procedures.* Results shown are those observed at peak PC hydrolysis (8 min) and are the mean of at least three independent experiments and are expressed as the percentage of untreated controls (mean ± S.D.). The baseline level of PC in untreated controls did not change significantly.

Drug Concentration	PC Hydrolysis (% of controls)
μM	
DNR	
0.1	6.6 ± 4.5
0.5	8.6 ± 3.8
1.0	15.0 ± 6.0
MXT	
0.1	4.1 ± 1.9
0.5	6.9 ± 3.4
1.0	17.0 ± 7.2

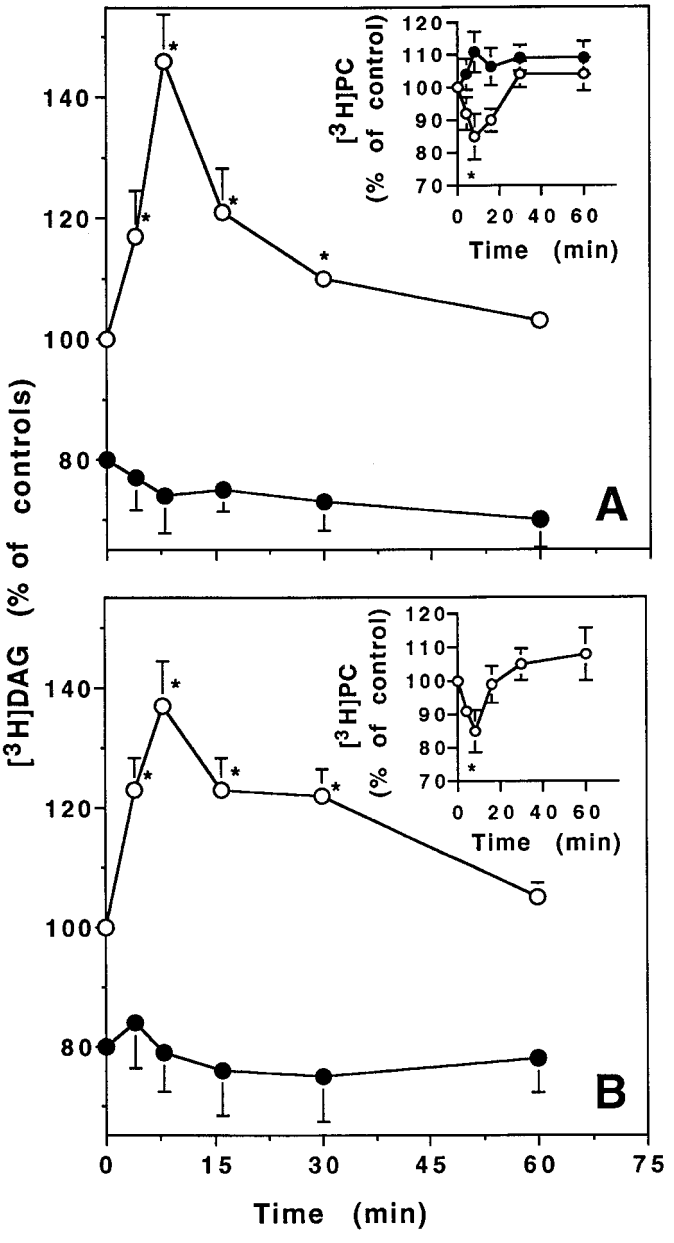
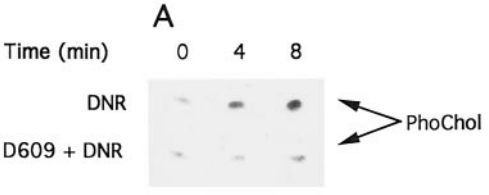


Fig. 1. Effect of the xanthogenate D609 on DNR- and MXT-triggered DAG production and PC hydrolysis. DAG and PC levels were estimated in U937 cells prelabeled to isotopic equilibrium with [9,10-^{3}H]palmitic acid for 48 h. Cells were then washed and preincubated for 1 h in serum-free medium with (●) or without (○) 10 μg/ml D609. Cells were then treated with 1 μM DNR (A) or 1 μM MXT (B). At different time points, aliquots were collected and lipids were extracted. Labeled DAG and PC (inserts) were quantitated as described in *Materials and Methods*. Results are the mean of three independent experiments and are expressed as a percentage of controls at time 0 (± S.E.M.). *, $p < .05$.



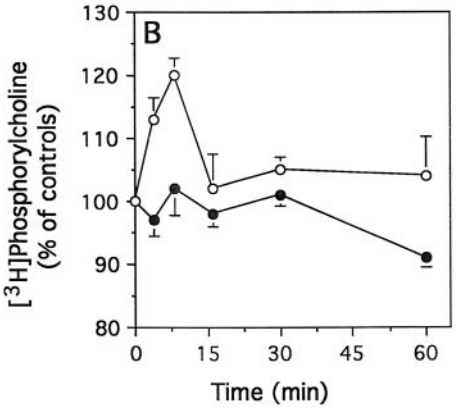


Fig. 2. Effect of DNR and D609 on PC-PLC activation. Activation of PC-PLC was attested by the generation of PhoCho derived from PC hydrolysis. U937 cells were labeled for 48 h with [^{3}H]choline, washed, and then treated with 1 μM DNR in the presence (●) or the absence (○) of 10 μg/ml D609. Water-soluble [^{3}H]choline metabolites were analyzed by TLC and the content of [^{3}H]PhoCho was determined as described in *Experimental Procedures*. Results are the mean of three independent experiments and are expressed as a percentage of controls at time 0 (± S.D.).

Effect of D609 on the SM-CER Pathway Triggered by DNR and MXT. Because DAG has been described to antagonize the SM-CER pathway (Kolesnick and Fuks, 1995), we evaluated the influence of D609 on DNR- and MXT-triggered SM hydrolysis and CER generation. [^{3}H]palmitic acid or [^{3}H]choline-labeled U937 cells were pretreated for 1 h with 10 μg/ml D609 before addition of either 1 μM DNR or 1 μM MXT. Both DNR and MXT triggered a rapid cycle of SM hydrolysis (Fig. 3, A and B) and CER generation (Fig. 3, C and D). Peak SM hydrolysis of ~27% and ~20% for DNR and MXT treated cells, respectively, was reached between within 4 to 10 min, concurrently with CER generation ~25% and ~20%, respectively. In U937 cells pretreated with D609, DNR and MXT induced a similar increase in CER but also led to a sustained increase in CER for a period up to 2 h (Fig. 3, C and D). This increase was observed concurrently with a long-term decrease in SM content (Fig. 3, A and B). This observation suggests that endogenous D609-sensitive DAG modulates the SM-CER cycle. D609 alone was unable to induce either CER generation or SM hydrolysis (data not shown).

TABLE 2

Effect of DNR and MTX on PKC activation in U937 cells

PKC activity was assayed by measuring the incorporation of ^{32}P from ATP into neurogranin$_{28-43}$ peptide using the SignaTECT PKC assay system (Promega) as described by the supplier. Results are expressed as picomoles of ^{32}P incorporated into neurogranin peptide per min per 10^6 cells. Results are the mean of three independent experiments and are expressed as a percentage of controls at time 0 (± S.E.M.). Values in parentheses are the difference of PKC activity compared to control.

	PKC activity (pmol/min/10^6 cells)	
	− D609	+ D609 (10 μg/ml)
Control	84.93 ± 7.50	48.70 ± 7.84
DNR (1 μM)	106.86 ± 12.10 (21)	71.38 ± 5.81 (23)
MXT (1 μM)	119.44 ± 20.60 (34)	78.15 ± 12 (30)

Effect of D609 on CER Generation Triggered by Low Doses of DNR and MXT. Because we observed that down-regulation of PC-PLC potentiated CER generation at optimal drug concentrations (i.e., 1 μM), supporting the concept that DAG antagonizes the SM-CER pathway, we decided to investigate the effects of D609 on low doses of DNR and MXT. Indeed, we previously demonstrated that below 0.2 μM, DNR and MXT could not induce apoptosis in myeloid leukemia cells (Quillet-Mary et al., 1996; Bailly et al., 1997) and that below 0.2 μM, DNR failed to trigger SM hydrolysis and CER generation (Jaffrézou et al., 1996). However, we now describe PC hydrolysis which started at 0.1 μM DNR and MXT (Table 1). Hence, we evaluated the biological consequences of D609-mediated inhibition of DAG generation. As shown in Fig. 4, in D609-pretreated U937 cells, 0.1 μM DNR and 0.1 μM MXT triggered significant CER production (~32% and ~22% increase, respectively).

Effect of D609 on DNR- and MXT-Induced Cell Death. To define the biological implications of the increase in CER levels induced by 0.1 μM DNR and 0.1 μM MXT in the presence of 10 μg/ml D609, studies were conducted to quantify cell death. In U937 cells pretreated with 10 μg/ml D609 for 1 h, we observed an increase in cytotoxicity as assessed by trypan blue exclusion (Fig. 5A); DNA fragmentation was assessed using the [^{3}H]thymidine release assay. Indeed, both

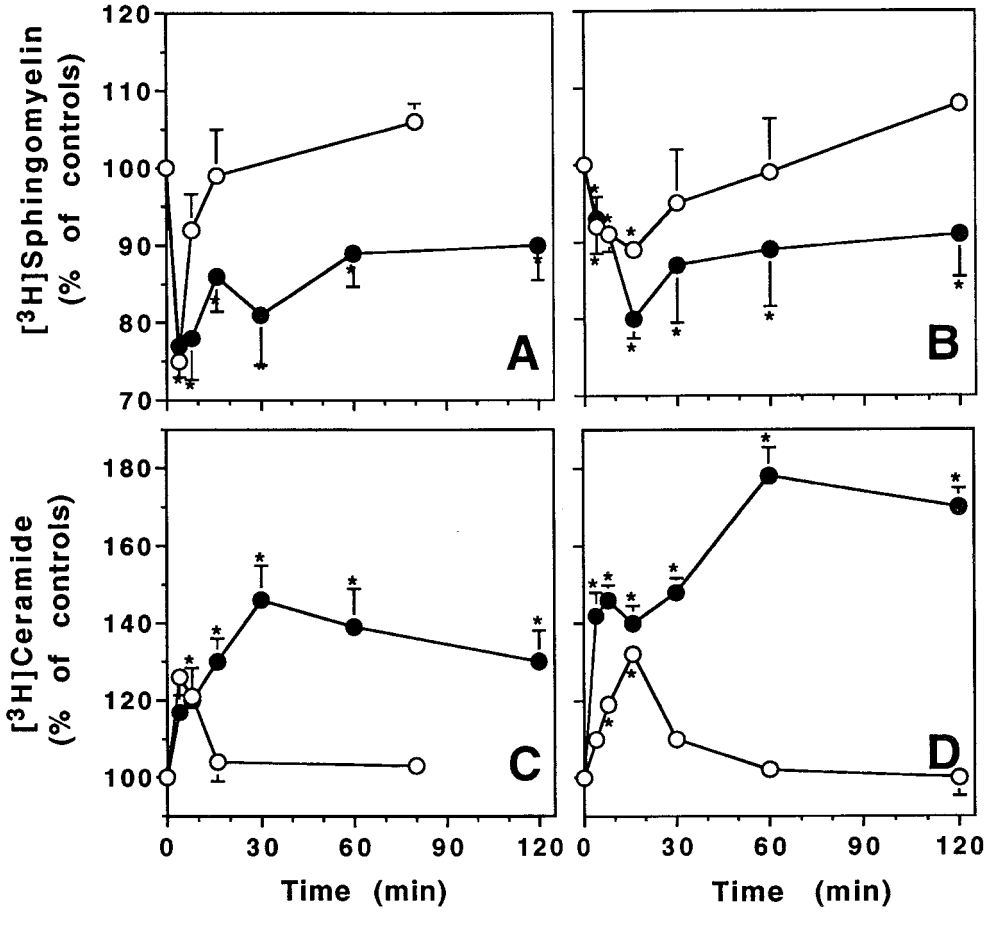


Fig. 3. Effect of D609 on DNR- and MXT-triggered CER production and SM hydrolysis. SM (A and B) and CER (C and D) levels were estimated in U937 cells prelabeled to isotopic equilibrium with [9,10-^{3}H]palmitic acid for 48 h. Cells were then washed and preincubated for 1 h in serum-free medium with (●) or without (○) 10 μg/ml D609 followed by 1 μM DNR (A and C) or 1 μM MXT (B and D) treatment. At different time points, aliquots were collected and lipids were extracted and quantitated as described in *Experimental Procedures*. Results are the mean of three independent experiments and are expressed as a percentage of controls at time 0 (± S.E.M.). *, $p < .05$.

DNR and MXT induced ~ 20% DNA fragmentation in U937 cells pretreated with D609 (Fig. 5B). Similar results were observed when the extent of apoptosis was evaluated by morphology (4′,6-diamidino-2-phenylindole staining) or poly-(ADP-ribose) polymerase cleavage (data not shown). As expected, no significant effect of 0.1 μM DNR and MXT alone was observed (Quillet-Mary et al., 1996; Bailly et al., 1997).

Effect of DAG and PhoCho on DNR- and MXT-Triggered DNA Fragmentation. To determine the contribution of PhoCho and DAG formation after drug treatment in the regulation of cell death, U937 cells were pretreated with either PhoCho or 1,2 dioctanoyl-*sn*-glycerol (DiC8) before the addition of drugs and DNA fragmentation was assessed using the [^{3}H]thymidine release assay. As shown in Fig. 6, 1-h pretreatment of cells with 25 μM PhoCho or DiC8 inhibited by 30% DNA fragmentation induced by 1 μM DNR and MXT.

Effect of DAG Kinase Inhibition on DNR- and MXT-Triggered DNA Fragmentation. To further substantiate the role of DAG as an antagonist of CER-mediated apoptosis, we used a highly selective DAG kinase inhibitor R 59 949 (de Chaffoy de Courcelles et al., 1989). By blocking the phosphorylation of DAG, which results in phosphatidic acid, we hoped to increase the protective effect of DAG. Pretreatment of U937 cells with 1 μM R 59 949 for 30 min, indeed, protected U937 cells from apoptosis induced by 1 μM DNR and MXT, as illustrated by the significant decrease in DNA fragmentation (Fig. 7).

Discussion

Extensive studies of the biochemical and molecular pharmacology of DNR and MXT have revealed intimate drug-target interactions. Nevertheless, how and why such interactions (e.g., topoisomerase II cleavable complexes) should bring about cell death has been hitherto unclear (Hannun, 1997). Furthermore, while cytotoxicity of anticancer agents such as DNR and MXT is most often attributed to genotoxic effects, in many cases cellular damage actually caused by active doses of these agents is not sufficient to explain the observed toxicity (Chabner and Myer, 1989). In the case of DNR and MXT, the identification of apoptosis as a response of certain myeloid leukemia cells to clinically relevant doses of DNR and MXT suggests that the cytotoxic action of these drugs requires the active participation of the target cell (Quillet-Mary et al., 1996; Bailly et al., 1997). This implies that in response to DNR or MXT induced cellular damage, the tumor cell activates an apoptotic response.

Among the recognized bioactive molecules in signal transduction, CER has emerged as a potent mediator of apoptosis

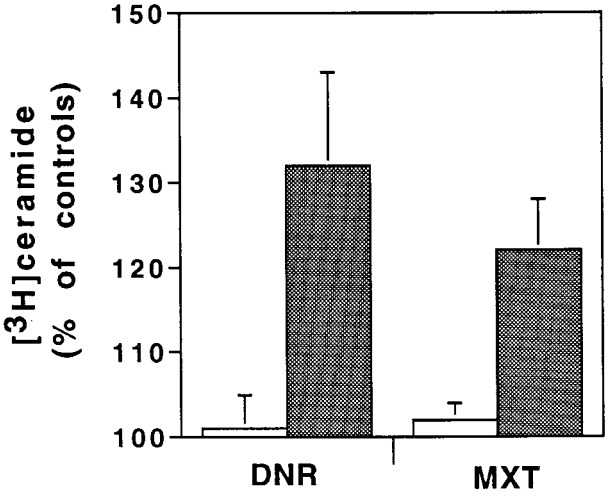


Fig. 4. Effect of D609 on CER production triggered by low doses of DNR and MXT. CER levels were estimated in U937 cells prelabeled to isotopic equilibrium with [9,10-^{3}H]palmitic acid for 48 h. Cells were then washed and preincubated for 1 h in serum-free medium with or without 10 μg/ml D609 followed by 0.1 μM DNR or 0.1 μM MXT treatment. At different time points, aliquots were collected and ceramide was extracted and quantitated as described in *Experimental Procedures*. The figure shows ceramide levels at 8 min (the peak) in DNR- and MXT-treated cells with (■) or without (□) D609. Results are the mean of three independent experiments and are expressed as a percentage of controls at time 0 (± S.E.M.).

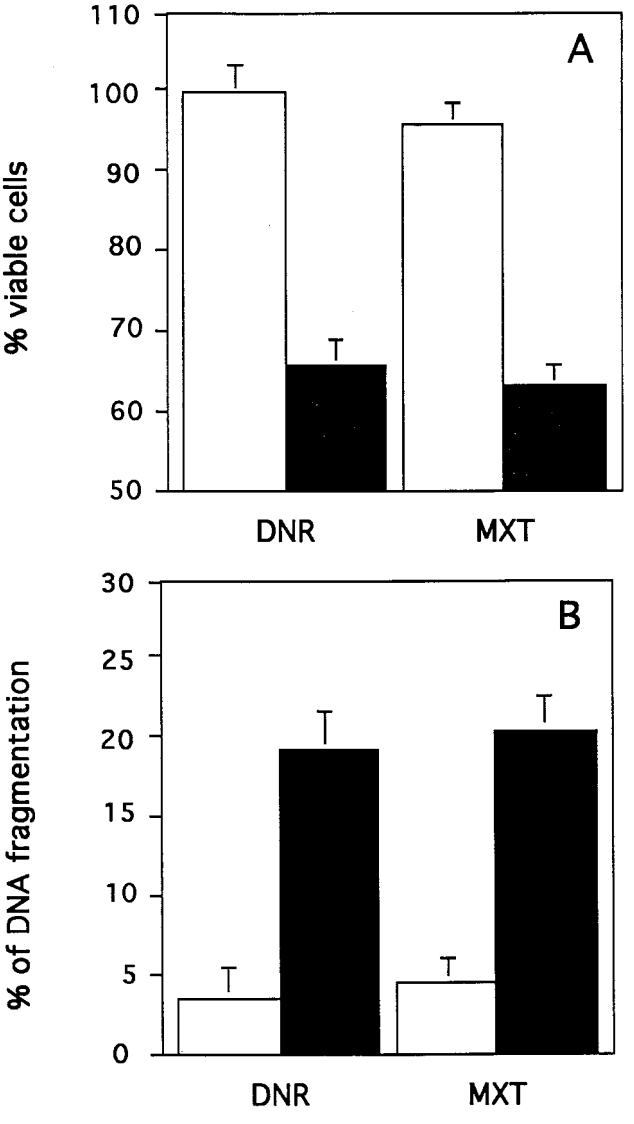


Fig. 5. Effect of D609 on low-dose DNR- and MXT-induced cell death. U937 cells were preincubated in the absence or in the presence of 10 μg/ml D609 for 1 h followed by a 1-h incubation with 0.1 μM DNR or 0.1 μM MXT. Cells were then washed and further incubated for 5 h in drug-free media. A, cell viability was assessed by trypan blue exclusion in DNR- or MXT- treated cells pretreated in the absence (□) or in the presence of D609 (■). B, DNA fragmentation was quantified using the [^{3}H]thymidine release assay, as described in *Experimental Procedures*, in U937 cells pretreated in the absence (□) or presence (■) of D609 followed by DNR or MXT. Results are the mean of three independent experiments and are expressed as a percentage of controls (± S.D.).

induced by a diverse number of cytotoxic effectors including chemotherapeutic agents (Hannun, 1996). We first proposed the involvement of a SM-CER pathway in DNR-triggered apoptosis of leukemic cells (Jaffrézou et al., 1996). We reported that the CER was generated by the activation of a neutral SMase appearing within 4 to 10 min. We now describe, for the first time, similar findings using the anthracenedione MXT. There is growing interest in this field of research because the SM-CER pathway appears to be eminently regulated both upstream and downstream of CER generation. Upstream, the generation of CER appears to be regulated at once by the availability of substrate (Bettaïeb et al., 1996; Bezombes et al., 1998), by proteases (Mansat et al., 1997a; Dbaibo et al., 1997), and by PKC (Haimovitz-Friedmann et al., 1994; Mansat et al., 1997b). Moreover, stimulation of SMase activity by DNR is counter-regulated by PKC and is serine protease-dependent (Mansat et al., 1997a). Downstream, the apoptotic effect of CER is blocked by several factors such as Bcl-2 overexpression (Smyth et al., 1996; Allouche et al., 1997), caspase inhibitors (Kumar, 1995), antioxidants (Quillet-Mary et al., 1997; Garcia-Ruiz et al., 1997), and DAG (Jarvis et al., 1994b).

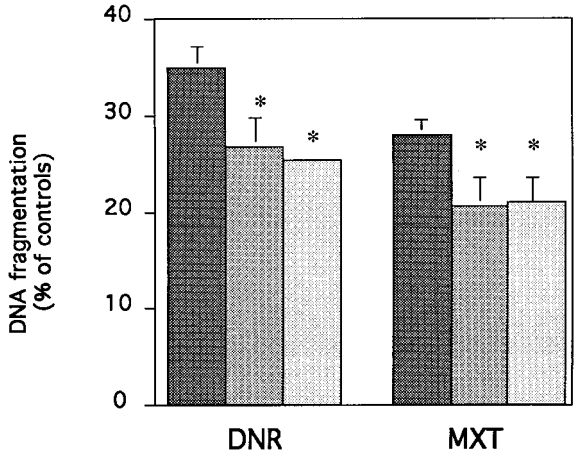


Fig. 6. Effect of PhoCho and DiC8 on DNR- and MXT-induced DNA fragmentation. U937 cells were preincubated in the absence or in the presence of 25 μM of either PhoCho or DiC8 for 1 h followed by a 1-h incubation with 1 μM either DNR or MXT. Cells were then washed and further incubated for 5 h in drug-free media. DNA fragmentation was analyzed in cells treated with 1 μM DNR and MXT before (■) or after pretreatment of cells with 25 μM PhoCho (▤) or 25 μM DiC8 (□). Results are the mean of three independent experiments (± S.D.). *, $p < .05$.

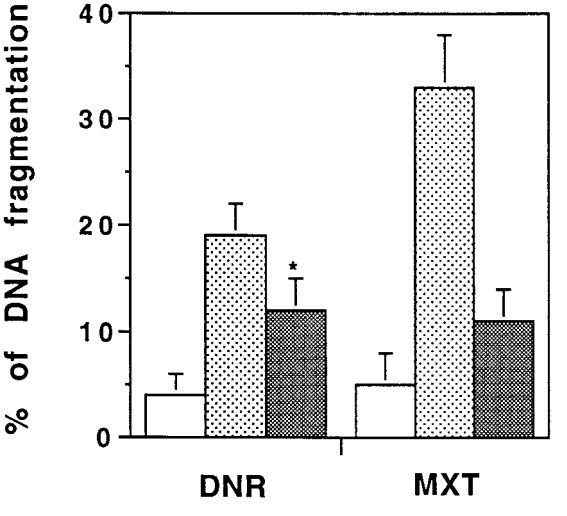


Fig. 7. Effect of DAG kinase inhibitor R59 949 on DNR- and MXT-induced DNA fragmentation. U937 cells were preincubated in the absence or in the presence of 1 μM R59 949 for 1 h followed by a 1-h incubation with 1 μM of either DNR or MXT. Cells were then washed and further incubated for 5 h in drug-free media. DNA fragmentation in control (□) and DNR- and MXT-treated cells pretreated in the absence (▤) or presence (▨) of 1 μM R59 949. Results are the mean of three independent experiments (± S.D.). *, $p < .05$.

The characterization of the apoptotic pathway activated by antitumor agents is made still more complex by the demonstrated or presumed existence of negative regulatory pathways. Although not clearly described, this field is, however, essential to the understanding at the molecular level of some phenotypes of resistance. It stands out that DAG plays a central role in the negative control of toxicant-induced apoptosis mediated by CER by potentially intervening upstream of its generation (by blockage of SMase activity) and downstream (by blocking the apoptotic effect of CER) (Hannun and Obeid, 1995). In this respect, it is advisable to recall that most antitumor agents able to trigger the SM-CER pathway are equally capable of simultaneously activating the production of DAG (Posada et al., 1989; Rubin et al., 1992; Nishio et al., 1992; Avila et al., 1993; Strum et al., 1994). For example, both doxorubicin and IR have been shown to induce in a dose-dependent manner the production of DAG (Posada et al., 1989; Avila et al., 1993).

In this study, we demonstrate that both DNR and MXT are capable of rapidly triggering significant DAG generation. We also show that this increase in DAG occurs concurrently with SM hydrolysis and CER generation. Concomitant increase of DAG and PhoCho induced by, for example, DNR, and inhibition of the DAG cycle by the potent PC-PLC inhibitor D609 strongly suggests that this enzyme is the one responsible for PC hydrolysis (Müller-Decker, 1989; Schütze et al., 1992). It is noteworthy that PC hydrolysis and DAG generation could be catalyzed by phospholipase D specific of PC. Because DNR-stimulated production of [^{3}H]choline was undetectable (data not shown), the implication of PC-PLD in DNR-triggered DAG production cannot be excluded. We observed that by blocking DNR- and MXT-triggered DAG and PhoCho production using D609, CER production was enhanced. In fact, the increased CER levels were maintained for at least 2 h compared to the rather rapid 15-min cycle in D609 untreated cells. The consequence of this increase in CER production was increased cytotoxicity. Indeed, this is most evident at the low dose (suboptimal drug concentrations). At 0.1 μM neither DNR nor MXT induced apoptosis, whereas these drug concentrations did trigger significant PC hydrolysis. By inhibiting the PC-PLC activation using D609, we were able to detect not only a boost in CER production but equally drug-induced apoptosis. Moreover, by addition of exogenous cell-permeant DAG or PhoCho, we were able to observe a significant decrease in DNR- and MXT-mediated apoptosis. One possibility is that PhoCho acts as a second messenger, but the more likely candidate appears to be DAG. However, it has been reported that the mitogenic effect of PhoCho occurs through extracellular target such as ATP and/or sphingosine 1-phosphate (Chung et al., 1997). We described that inhibition of DAG kinase activity that allows for a longer-lived DAG signal (Bishop et al., 1986) also led to a decrease in DNR- and MXT- mediated apoptosis. This result strongly suggests that DAG kinase activity counter-regulates the survival signal

mediated by DAG. Finally, we reported that DNR and MXT were able to activate PKC. This activation was not blocked by treatment of cells by D609. These observations suggest that drug-triggered DAG production through PC-PLC and PKC activation represents two distinct pathways. The origin of PKC activation is currently being investigated in our laboratory.

The mechanisms by which DAG modulates CER generation are unknown. Several possibilities exist and are under study in our laboratory. The most obvious is that DAG inhibits a SMase. However, we could not detect any effect of D609 or cell-permeable DiC8 on neutral SMase activity (data not shown). Nevertheless, DAG could affect another SMase. Finally, it is possible that DAG influences CER and SM metabolism, perhaps by preventing SM resynthesis. More recently, Pörn-Ares et al. (1997) have reported that D609-stimulated SMase activity potentiated TNF- and Fas-mediated apoptosis, and induced apoptosis on its own in U937 cells. The discrepancy is likely due to the high concentrations of D609 used by the authors [50 to 100 μg/ml versus 10 μg/ml (subtoxic concentration)]. Indeed, in our experiments, high concentration of D609 (50 μg/ml) also induced U937 cell death (data not shown).

Regardless of the mechanism by which DAG regulates SMase stimulation, our findings may have important implications in anthracycline pharmacology. Indeed, a number of intrinsic and environmental factors strongly influence DAG production and, therefore, may contribute to resistance to these drugs. For example, cytokines and growth factors such as TNFα, interleukin-3, or granuloctye macrophage-colony-stimulating factor induce DAG formation through hydrolysis of PC (Hannun and Bell, 1989; Schütze et al., 1991; Rao and Mufson, 1994, 1995). It is conceivable that paracrine or autocrine production of cytokines may lead to constitutive PLC activation, and may limit SMase stimulation, CER generation and apoptosis in anthracycline-treated cells. One could speculate that such mechanisms could account for the lack of apoptotic response to DNR and MXT observed in certain leukemia cells (Bailly et al., 1997).

In conclusion, we provide evidence for a balance between apoptotic (CER) and survival (DAG) mediators. These results imply that apoptosis triggered by chemotherapeutic drugs such as DNR and MXT may be linked to the overriding of a survival pathway by a cell death signal. The nature of the signaling pathway(s) which "referee" drug-triggered apoptosis is, of course, of fundamental importance in determining the chemosensitivity of the tumor cell. The implication of these signaling pathways in drug resistance opens intriguing avenues of research. Perhaps by pharmacologically manipulating key steps in the apoptosis/survival signaling cascade, one could increase chemosensitivity of neoplastic cells.

Acknowledgments

We thank Dr. M. Record, Dr. F. Tercé, G. Ribbes (Institut National de la Santé et de la Recherche Medicale U 326, Toulouse, France), and Dr. D. Lautier (CJF Institut National de la Santé et de la Recherche Medicale 95–03) for their support and critical reading of the manuscript and comments, and C. Bordier (CJF Institut National de la Santé et de la Recherche Medicale 95-03) for expert technical assistance.

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Send reprint requests to: Dr. Ali Bettaïeb, CJF Institut National de la Santé et de la Recherche Medicale 95–03, Institut Claudius Régaud, 20 rue du Pont St. Pierre, 31052 Toulouse, France. E-mail: inserm@icr.fnclcc.fr