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Differential Chemosensitizing Effect of Two Glucosylceramide Synthase Inhibitors in Hepatoma Cells

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It has been proposed that ceramide mediates anthracyclin-induced apoptosis and that drug resistance may arise due to upregulated removal of this active lipid through glucosylation. We report that HepG2 hepatoma cells displayed only a modest apoptotic response to doxorubicin treatment, accompanied by a substantial elevation of ceramide levels only at toxic drug concentrations. D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and D,L-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP), used at concentrations causing a 90% inhibition of ceramide glucosylation, enhanced doxorubicin-elicited ceramide elevation, but only PDMP potentiated apoptosis. Exogenously administered ceramide had only a marginal apoptotic effect on HepG2 cells; moreover, even in this case, apoptosis was propagated by PDMP but not by PPPP. PDMP moderately inhibited *P*-glycoprotein activity only at the highest concentration tested, but its chemosensitizing effect was still outstanding at lower concentrations, at which P-gp inhibition was no longer observed. These results demonstrate that the chemosensitizing effect of PDMP is, at least partly, independent from its activity as a glucosylceramide synthase inhibitor. Moreover, *P*-glycoprotein inhibition is not central to the phenomenon. © 2001 Academic Press

Key Words: **HepG2 cells; apoptosis; ceramide; *P*-glycoprotein; doxorubicin; glucosylceramide synthase.**

It is generally recognized that multiple mechanisms may operate whereby tumor cells circumvent the toxic activity of anticancer drugs. These range from overexpression of membrane proteins of the ATP-binding cassette family driving drug extrusion from the cell [e.g., *P*-glycoprotein (P-gp)] (1), to failure in the activation of the death program, due to unbalance in the proper expression of pro-apoptotic and/or anti-apoptotic proteins (2–4). Intriguingly, a further key of explanation for the phenomenon has come from the evidence that the apoptotic effect induced by anticancer drugs may be mediated by ceramide (Cer) (5, 6). This circumstance, in fact, has raised the possibility that drug-resistance may also result from alterations of sphingolipid metabolism, namely from impairment of drug-induced Cer elevation, either by block of its production or by acceleration of its removal (7, 8). In the latter respect, it has been demonstrated that glucosylation provides a major pathway through which Cer produced by anticancer drugs is downregulated and its apoptotic effect quenched (9, 10), thus suggesting that glucosylceramide synthase (GCS) inhibitors may provide precious tools in cancer chemotherapy (11). In line with this notion, a number of studies have shown that inhibition of GCS activity may reverse tumor cell resistance to treatment with anticancer drugs and that the chemosensitizing effect associates with upregulation of drug-induced Cer elevation (12–15). Hepatomas provide types of tumor markedly resistant to chemotherapy; moreover, it has been reported that exogenously administered Cer has negligible effect on hepatocyte viability (16). Herein, we investigated whether sustained glucosylceramide (GlcCer) synthesis may account for hepatoma cell resistance to the cytotoxic action of anticancer drugs. To this aim, we studied the effect of two GCS inhibitors, D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) and the more potent D,L-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP) (17) on the apoptotic response of HepG2 human hepatoma cells to doxorubicin.

MATERIALS AND METHODS

Materials. Material for cell culture was from Gibco BRL (MD). Doxorubicin, verapamil, Cer type III and rhodamine 123 (Rh123)

Abbreviations used: Cer, ceramide; C_6-Cer, *N*-hexanoylsphingosine, C_6-NBD, 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]hexanoic acid; GlcCer, glucosylceramide; GCS, glucosylceramide synthase; PDMP, DL-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol; PPPP, DL-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol; Z-VAD.fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; P-gp, *P*-glycoprotein; Rh123, rhodamine 123; PBS, phosphate-buffered saline; HPTLC, high-performance thin layer chromatography.

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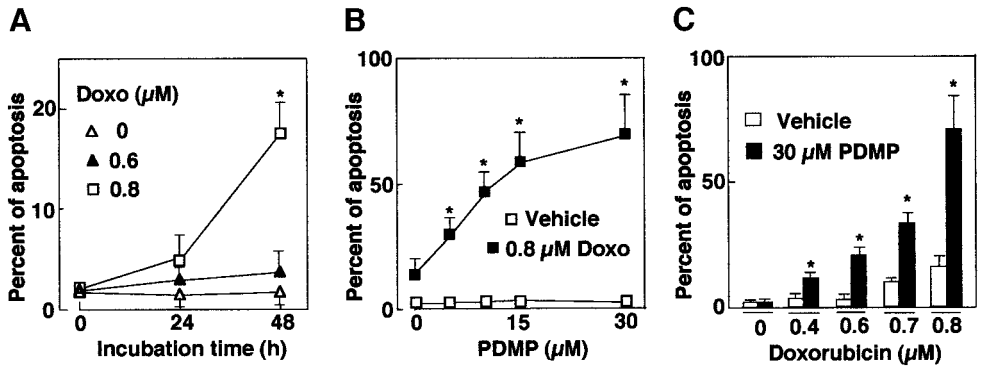

FIG. 1. PDMP potentiates doxorubicin-induced apoptosis in HepG2 cells. Apoptosis was monitored by flow cytometric analysis in cells treated with doxorubicin (Doxo) at the reported concentrations for 24 or 48 h (A); or with 0.8μM doxorubicin in the absence (vehicle) or presence of PDMP at the indicated concentrations for 48 h (B); or with doxorubicin at the reported concentrations in the absence (vehicle) or presence of 30 μM PDMP for 48 h (C). In all panels data are means ± SD of six different experiments. Statistical significance: $*P < 0.01$, as from Student's *t* test, in comparison with samples incubated in the absence of doxorubicin (A), or in the absence of PDMP (B and C).

were from Sigma (St. Louis, MO). PDMP, PPPP, *N*-hexanoyl-sphingosine (C_6-Cer), 6-[*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-amino]-hexanoic acid (C_6-NBD), diacylglycerol kinase from *Escherichia coli* and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD.fmk) were from Calbiochem (La Jolla, CA). [γ-^{32}P]ATP was from Amersham (Bucks, UK). Silica gel 60 high-performance thin layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany).

Cell culture and apoptosis evaluation by flow cytometric analysis. Cell were grown at 37°C in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% (v/v) CO_2. Cell treatments were performed in complete growth medium. Apoptosis was monitored by evaluation of the hypodiploid cell population by flow cytometric analysis, as previously reported (18). Briefly, cells were detached from the plates, washed with phosphate-buffered saline (PBS) (pH 7.4) and resuspended in 0.5 ml of a solution containing 50 mg/ml propidium iodide, 0.1% Triton X-100 and 0.1% sodium citrate. Cells were left in the dark at 4°C for at least 30 min prior to analysis by a FACScan Flow Cytometer (Becton–Dickinson, CA) (18).

Lipid extraction and separation and Cer measurement. Lipids were extracted according to the method of Bligh and Dyer (19), the chloroformic phase was dried under N_2 and subjected to mild alkaline hydrolysis with 0.1 M methanolic KOH for 1 h at 30°C. After re-extraction, the chloroformic phase was dried under N_2 and Cer content was estimated by the diacylglycerol kinase method, using Cer type III from Sigma for calibration, as previosuly described in detail (20). Briefly, Cer was resuspended in 100 μl of a mixture containing 150 μg of cardiolipin, 280 μM diethylenetriaminepentaacetic acid, 52 mM octyl-β-glucopyranoside, 50 mM NaCl, 51 mM imidazole, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 1mM ATP, 10μ Ci of [γ-^{32}P]ATP and 5 μg of diacylglycerol kinase from *E. coli*, at pH 6.5. The reaction was allowed to proceed for 30 min at 25°C, lipids were then extracted and Cer 1-phosphate was separated by HPTLC, using a solvent system of chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1, vol:vol). Radioactive spots were detected by autoradiography and quantitated by liquid scintillation counting. Cer mass was referred to cell protein content, measured according to (21).

Estimation of GCS inhibition by PDMP and PPPP in intact cells. Cells grown in 35-mm plates were preincubated in the absence or presence of PDMP or PPPP, at the reported concentrations, for 1 or 48 h. C_6-NBD (10 μM) was then added to the medium and the incubation allowed to proceed at 37°C for 2 h. Cells were then scraped in their medium and, after saving sample aliquots for cell protein determination, lipids were extracted and resolved by HPTLC in chloroform/methanol/water (65:25:4, vol:vol). Fluorescent C_6-NBD-GlcCer spots were detected under UV light, scraped off and extracted in 1.5 ml ethanol. Fluorescence was measured by a Perkin–Elmer LS-5 luminescence spectrometer, with λ_{exc} and λ_{em} set at 466 and at 535 nm, respectively. C_6-NBD-GlcCer quantitation was carried out referring to a C_6-NBD fluorescence calibration curve.

Estimation of P-gp activity. P-gp activity was monitored essentially as described in (22). Cell were harvested, washed and resuspended in PBS (2×10^6 cells/ml). After incubation at 37°C for 1 h with 10 μM Rh123 and 10 μM verapamil, cells were washed twice in ice-cold PBS, resuspended and split into portions to which PDMP or PPPP or verapamil or vehicle (dimethylsulfoxide) were added on ice. Samples were then incubated at 37°C for 20 min and centrifuged at 4°C for 3 min at 12,500*g*. Supernatants were collected and 1% Triton X-100 was added. The pellets were dissolved in PBS containing 1% Triton X-100 and Rh123 fluorescence monitored setting λ_{exc} and λ_{em} at 495 and 535 nm, respectively. Rh123 efflux was expressed as the fluorescence in the supernatant divided by the total fluorescence (pellet plus supernatant).

RESULTS

PDMP Sensitizes HepG2 Cells to Doxorubicin

Doxorubicin was administered at nanomolar concentrations—i.e., at doses regarded to as of clinical relevance (23)—to HepG2 cells maintained in complete growth medium and apoptosis monitored over a 48 h period. Figure 1A shows that apoptosis was not observed within 24 h of treatment up to a drug concentration of 0.8 μM; moreover, as the treatment was prolonged to 48 h, cell viability was still unaffected by 0.6 μM doxorubicin, whereas only a modest apoptotic response (averaging less than 20%) was observed at a 0.8 μM drug concentration. The GCS inhibitor PDMP, used at concentrations that were non-toxic per se, sensitized HepG2 cells to doxorubicin. Figure 1B shows that, in the presence of 0.8 μM doxorubicin, chemosensitization was already evident at 5 μM PDMP, turning outstanding at a GCS inhibitor concentration of 30 μM (about 70% of apoptosis). In addition, Fig. 1C shows

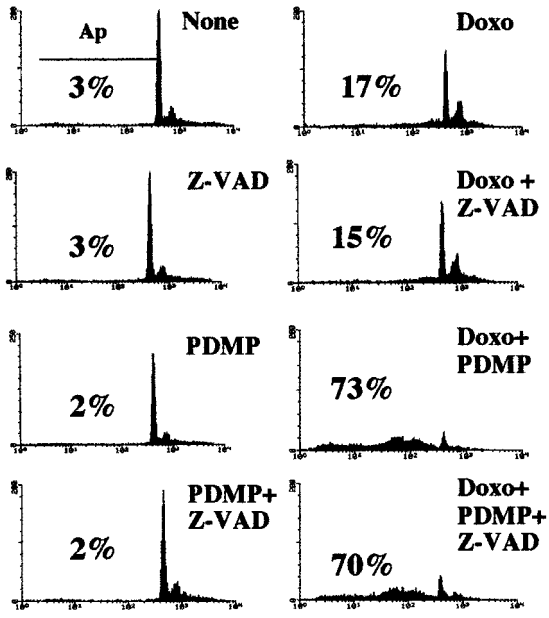

FIG. 2. Doxorubicin-induced and PDMP-potentiated apoptosis is not prevented by Z-VAD.fmk. Typical DNA histograms, after propidium iodide staining, of cells treated for 48 h with vehicle (left panels) or 0.8 μM doxorubicin (Doxo, right panels), in the absence of any other compound or in the presence of 100 μM Z-VAD.fmk, or 30 μM PDMP or 100 μM Z-VAD plus 30 μM PDMP. Horizontal axes represent fluorescence emission at 580 nm, vertical axes the number of events.The percent of the apoptotic (hypodiploid) cell population (Ap) is given in each panel.

that PDMP lowered the threshold toxic concentration of doxorubicin. Notably, the pan-caspase inhibitor Z-VAD.fmk (100 μM) neither prevented doxorubicin-induced apoptosis, nor had effects on apoptosis potentiation induced by PDMP (Fig. 2).

PDMP Potentiates Doxorubicin-Evoked Cer Accumulation

As shown in Table 1, HepG2 cells actively glucosylate exogenously administered C_6-NBD; on the other hand, cell preincubation with 30 μM PDMP rapidly induced a dramatic inhibition C_6-NBD glucosylation (about 90% with respect to control) that was maintained for 48 h, at least. We studied whether doxorubicin treatment evoked Cer accumulation in HepG2 cells and whether the phenomenon was potentiated by PDMP. Figure 3A shows that cell treatment with doxorubicin alone elicited late accumulation of intracellular Cer in a fashion that fairly matched drug ability to induce apoptosis. In fact only a 20% Cer increase over basal was observed at 0.6 μM doxorubicin, approaching 100% at a 0.8 μM drug concentration. In the presence of 30 μM PDMP late Cer elevation was already observed at 0.4 μM doxorubicin and lipid accumulation elicited by a 0.8 μM drug concentration was about 350% of basal (Fig. 3B). Notably, PDMP did not increase basal Cer levels when administered alone, suggesting that, in the absence of doxorubicin, HepG2 cells can achieve disposal of the excess ceramide resulting from the block of glucosylation through alternative mechanisms.

TABLE 1

Effect of PDMP and PPPP on GlcCer Synthesis

Inhibitor	Preincubation time (h)	C_6-NBD-GlcCer (nmol/h/mg protein)
None		2.08 ± 0.28 (100%)
30 μM PDMP	1	0.29 ± 0.04* (13.9%)
30 μM PDMP	48	0.24 ± 0.03* (11.5%)
1 μM PPPP	1	0.22 ± 0.04* (10.6%)
1 μM PPPP	48	0.20 ± 0.02* (9.6%)

Note. Cells were preincubated either in the absence of any inhibitor or in the presence of PDMP or PPPP at the reported concentrations, for 1 or 48 h. C_6-NBD (10 μM) was then added to the medium and the incubation allowed to proceed at 37°C for 2 h. Synthesized C_6-NBD-GlcCer was estimated and referred to cell protein content as reported in the text. Data are means ± SD of four different experiments. Statistical significance: *$P < 0.01$, as compared to samples treated with carrier only (None), as from Student's t test. Percent of residual GCS activity is given in parentheses.

PPPP Inhibits GCS Activity and Enhances Doxorubicin-Induced Cer Elevation without Affecting Drug-Induced Apoptosis

We studied whether PPPP, a more potent GCS inhibitor than PDMP, also sensitized HepG2 cells to doxorubicin. Table 1 shows that, in cells exposed for 1 h to 1 μM PPPP, C_6-NBD glucosylation was inhibited by over a 90%; moreover, as observed for PDMP, the inhibitory effect of PPPP was fully sustained for 48 h at

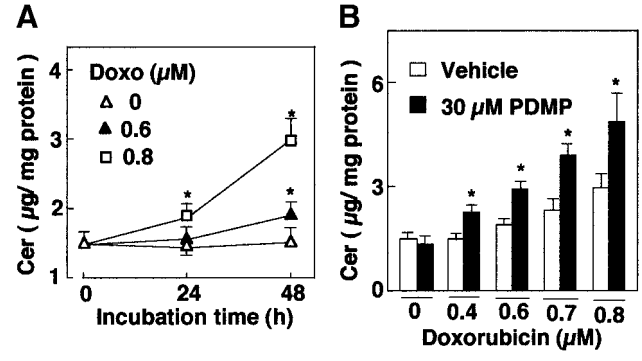

FIG. 3. PDMP potentiates doxorubicin-induced Cer accumulation. Cells were treated with doxorubicin (Doxo) at the reported concentrations for 24 or 48 h (A) or with doxorubicin at the reported concentrations in the absence (vehicle) or presence of 30 μM PDMP for 48 h (B). Cer was then quantitated and referred to cell protein content. In all panels data are means ± SD of four different experiments. Statistical significance: *$P < 0.01$, as from Student's t test, in comparison with samples incubated in the absence of doxorubicin (A), or in the absence of PDMP (B).

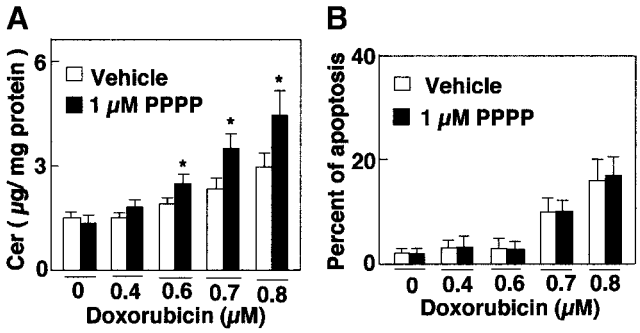

FIG. 4. PPPP enhances doxorubicin-induced Cer accumulation but not apoptosis. Cells were treated for 48 h with doxorubicin at the indicated concentrations either in the absence (vehicle) or presence of 1 μM PPPP. Thereafter, Cer was quantitated and referred to cell protein content (A) or apoptosis monitored by flow cytometric analysis (B). In both panels, results are means ± SD of four different experiments. Statistical significance: $*P < 0.01$, as from Student's *t* test, as referred to samples treated with the same doxorubicin concentration but without PPPP.

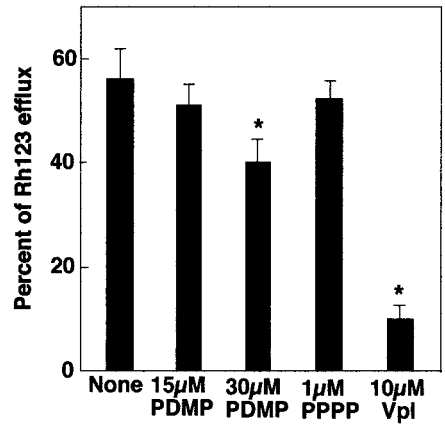

FIG. 6. Effects of PDMP on P-gp activity. Cells loaded with Rho123 were incubated with PDMP (15 μM or 30 μM), or 1 μM PPPP or 10 μM verapamil (Vpl) or vehicle (None) and fluorophore efflux quantitated as described in the text. Dare means ± SD of four different experiments. Statistical significance: $*P < 0.01$, as from Student's *t* test, in comparison with samples incubated with vehicle.

least. As shown in Fig. 4A, 1 μM PPPP did not increase basal Cer level when administered alone to HepG2 cells, but potentiated Cer elevation evoked by cell treatment with doxorubicin for 48 h; surprisingly, however, PPPP did not propagate the apoptotic response of HepG2 cells to doxorubicin (Fig. 4B).

PDMP but Not PPPP Propagates Apoptosis Induced by Short-Chain Cer

To further assess the role of Cer as a mediator of doxorubicin-induced apoptosis, we studied the effect of exogenously administered short-chain Cer on HepG2 cells. Figure 5 shows that cell treatment for 48 h with 30 μM C_6-Cer induced less than 10% of apoptosis. Most remarkably, this response was dramatically potentiated when short-chain Cer was used in combination with 30 μM PDMP (more than 50% of apoptosis); on the other hand, apoptosis potentiation was not observed when C_6-Cer was administered in combination with 1 μM PPPP.

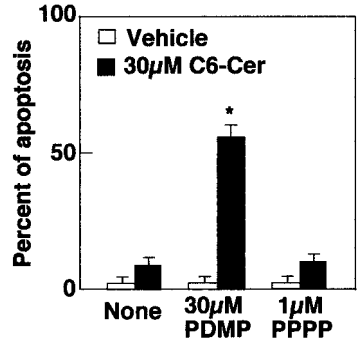

FIG. 5. PDMP but not PPPP potentiates short-chain Cer-induced apoptosis. Apoptosis was monitored in cells incubated for 48 h with 30 μM C_6-Cer or vehicle either in the presence of 30 μM PDMP, or 1 μM PPPP or in the absence of any GCS inhibitor (None). Data are means ± SD of four different experiments. Statistical significance: $*P < 0.01$, as from Student's *t* test, in comparison with samples incubated in the absence of any GCS inhibitor.

Effects of PDMP on P-gp Activity

PDMP has been reported to inhibit P-gp, namely a major determinant of drug-resistance in hepatoma cells (24). Therefore, we studied the effect of PDMP on P-gp activity in HepG2 cells, comparing it to that of PPPP and verapamil, a well-established and potent P-gp inhibitor (12). Figure 6 shows that 30 μM PDMP inhibited by less than 30% the efflux of the P-gp substrate Rh123; however, as PDMP concentration was decreased to 15 μM, significant inhibition of P-gp could no longer be detected. Figure 6 also shows that P-gp activity was not affected by 1 μM PPPP, whereas, as expected, it was dramatically inhibited by 10 μM verapamil (about 85% reduction of Rh123 efflux).

DISCUSSION

We have demonstrated that HepG2 cells displayed only a modest apoptotic response following doxorubicin treatment, accompanied by a substantial elevation of Cer levels only at toxic drug concentrations. Two well-established GCS inhibitors, PDMP and PPPP, used at equipotent concentrations, enhanced doxorubicin-induced Cer elevation: however, in spite of this common effect, only PDMP markedly enhanced the apoptotic effect of doxorubicin. These results clearly stand against the view that the chemosensitizing effect of PDMP simply impinges on potentiation of doxorubicin-induced Cer elevation and call for involvement of a mechanism that is dissociated from PDMP effects as a

GCS inhibitor. Indeed, failure to show a correlation between doxorubicin-evoked Cer elevation and the apoptotic response, as observed in the case of PPPP, also raises the question of whether Cer is indeed the key mediator of the apoptotic effect induced by the anticancer drug. In this respect, however, we found that also administration of short-chain Cer induced only a limited apoptotic response in HepG2 cells and, even in this case, apoptosis was propagated by PDMP but not by PPPP. Based on the bona fide assumption that short-chain Cer reproduces the effect of endogenously-generated lipid, it might be hypothesized that the apoptotic program of doxorubicin may be initiated by Cer generation but does not undergo completion because of a downstream restraint in the pathway that is overcome by PDMP through an "unspecific" mechanism.

It has been reported that PDMP inhibits P-gp activity (13), namely a major determinant of drug-resistance in HepG2 cells (24). With respect to this point, we found that, in our system, PDMP modestly inhibited P-gp activity only at the highest concentration tested, whereas the chemosensitizing effect of the compound strongly persisted at concentrations at which P-gp inhibition no longer occurred. As these results do not support a key role for P-gp inhibition in the chemosensitizing action of PDMP, it must be mentioned that a recent study performed on leukemia cells has shown that P-gp-mediated multidrug resistance may be overcome by agents that trigger caspase-independent apoptosis (25). On the basis of the results herein reported, it appears that this notion might also apply to HepG2 cells: in fact, we found that apoptosis induced by doxorubicin and propagated by PDMP is not prevented by the pan-caspase inhibitor Z-VAD.fmk, thus suggesting that the phenomenon is caspase-independent. To confirm this notion, we also found that the caspase substrate poly-(ADP)ribose polymerase was not cleaved in doxorubicin-induced and PDMP-potentiated apoptosis (not shown). It remains to be studied whether PDMP exerts its chemosensitizing effect by impinging on the function of those proteins, such as, for instance, the apoptosis-inducing factor (AIF) and Bax, involved in modulation of caspase-independent apoptosis (26, 27). Nevertheless, a further clue may be provided by the reported lysosomotropic properties of PDMP (28). Recent studies have shown that damage of the lysosomal membrane and the consequent release of their content into the cytosol may trigger apoptosis, either through caspase-dependent or -independent mechanisms (29, 30). On this basis, it could be hypothesized that PDMP, used at concentrations at which it is not toxic per se to the cell, is nonetheless able to produce sublethal damages at the lysosomal level that favour the action of an intervening apoptotic stimulus.

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