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ORIGINAL ARTICLE

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Taxol-induced ceramide generation and apoptosis in human breast cancer cells

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Abstract *Purpose*: Taxol has emerged as a valuable antimitotic chemotherapeutic agent, particularly in advanced breast and ovarian cancers. Although much is known about cytotoxic mechanisms, the effectiveness of Taxol cannot be solely explained by microtubular interaction. This study was undertaken to determine whether ceramide generation plays a role in Taxol-induced apoptosis. *Methods*: Hormone-independent MDA-MB-468 and hormone-dependent MCF-7 breast cancer cell lines were employed, and ceramide metabolism was characterized using [^{3}H]palmitic acid as lipid precursor. *Results*: Exposure of cells to Taxol resulted in enhanced formation of [^{3}H]ceramide. Ceramide increased nearly 2-fold in MDA-MB-468 cells exposed to 50 n*M* Taxol, and more than 2.5-fold in MCF-7 cells exposed to 1.0 μ*M* Taxol. These concentrations mirrored the EC_{50} (amount of drug eliciting 50% cell kill) for Taxol in the two cell lines. Use of cell-permeable C_6-ceramide as a medium supplement revealed that MDA-MB-468 cells were 20-fold more sensitive to ceramide than MCF-7 cells ($P < 0.001$). Ceramide was generated as early as 6 h after exposure to Taxol in MDA-MB-468 cells, whereas the earliest signs of apoptosis were detected 12 h after treatment, and by 24 h the apoptotic index was six times that of untreated cells. Both fumonisin B_1, a ceramide synthase inhibitor, and L-cycloserine, a serine palmitoyltransferase inhibitor, blocked Taxol-induced ceramide generation, whilst sphingomyelin levels remained unchanged, indicating a de novo pathway of ceramide formation. L-Cycloserine reduced Taxol-induced apoptosis by 30% in MDA-MB-468 cells and totally blocked Taxol-induced apoptosis in MCF-7 cells. *Conclusions*: These results suggest that Taxol-induced apoptosis is, in part, attributable to ceramide and sphingoid bases. This is of relevance to drug mechanism studies, as ceramide is a known messenger of apoptosis. Clinical use of Taxol with ceramide-enhancing agents may maximize cytotoxic potential.

Key words Taxol · Ceramide · Apoptosis · Fumonisin B_1 · L-Cycloserine

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Introduction

Paclitaxel (Taxol) is an antineoplastic agent with broad antitumor activity. Since its introduction, it has become increasingly important in the treatment of a number of major solid tumors, particularly metastatic and drug-resistant breast and ovarian cancer [31]. Taxol is a compound extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) [34]. The cytotoxicity of Taxol is linked to both inhibition of cell proliferation and apoptosis. The antitumor activity of Taxol is unique. Unlike classical antimicrotubule agents that induce microtubular disassembly and/or paracrystal formation, Taxol acts by inducing tubulin polymerization and promoting the formation of unusually stable microtubules, thereby inhibiting the normal dynamic reorganization of the microtubular network required for mitosis and cell proliferation [25].

The cytotoxic effectiveness of Taxol cannot be explained solely by its action on microtubules. It is well recognized that Taxol can cause mitotic arrest in the G_2/M phase of the cell cycle and apoptosis in vivo and in

vitro [9]. However, it remains unclear whether Taxol-induced apoptosis is a secondary event of mitotic arrest or whether it represents an alternative mechanism of action against tumor cells.

Ceramide, a sphingolipid precursor and cellular second messenger, has been implicated in the initiation of apoptotic cell death in an assortment of physiologic settings [6, 10, 11, 12, 13, 37]. Ceramide can arise from either cleavage of the phosphodiester bond of sphingomyelin by neutral and acidic forms of sphingomyelinase, or via *N*-acylation of dihydrosphingosine by ceramide synthase, followed by desaturation [13, 20, 30]. An increase in intracellular availability of ceramide, through either pathway, is a hallmark of the initiation of apoptosis by cytotoxic stimuli [20]. Lethal effects of ceramide have been observed in the apoptotic response of both normal and malignant cells to a wide variety of cytotoxic stimuli including anthracyclines [3, 13], etoposide [24], vinca alkaloids [37], ionizing radiation [10], Fas/Apo 1 [6], and tumor necrosis factor-α [11, 15, 35].

Of particular relevance to the present study, Taxol-induced apoptosis has been shown to be enhanced by ceramide in leukemia cells [23]. This observation prompted our study to characterize the interplay of ceramide with the cytotoxic response to Taxol. To expand what is known about the molecular basis of Taxol-induced cell death, we investigated the relationship between ceramide metabolism and the cytotoxicity of Taxol in hormone-independent MDA-MB-468 and hormone-dependent MCF-7 breast cancer cells. To this end, we demonstrated, for the first time, an association between the generation of ceramide and the cytotoxic impact of Taxol.

Materials and methods

Materials

Taxol was a gift from Bristol-Myers Squibb (Princeton, N.J.). RPMI-1640 medium was obtained from Life Technologies (Grand Island, N.Y.), and FBS was from HyClone (Logan, Utah). Fumonisin B_1 (FB_1) and L-cycloserine were purchased from Biomol (Plymouth Meeting, Pa.). C_6-ceramide and sphingomyelin (brain-derived) were from Avanti Polar Lipids (Alabaster, Ala.). [9,10-^{3}H]Palmitic acid (50 Ci/mmol) was from DUPONT/NEN (Boston, Mass.), and water-compatible liquid scintillation fluid (EcoLume) was from ICN Biomedicals (Costa Mesa, Calif.). TLC plates (Silica Gel G) were purchased from Analtech (Newark, Del.). Plastic tissue cultureware was from Costar (Cambridge, Mass.), and 96-well plates, 6-well plates, 6- and 10-cm dishes, and T-75 flasks were from Corning (Corning, N.Y.).

Cell culture

The MDA-MB-468 cells were purchased from the American Type Culture Collection (Rockville, Md.). Dr. Kenneth Cowan of the Eppley Cancer Center, Omaha, Neb., and Dr. Merrill Goldsmith, National Cancer Institute, Bethesda, Md., provided the MCF-7 cell line. Cells were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 584 mg/l L-glutamine. Cells were grown in a humidified tissue incubator at 37°C under an atmosphere containing 5% CO_2, and subcultured weekly using a 0.05%/0.53 m*M* trypsin/EDTA solution. For experiments, cells were subcultured in 6-well or 96-well plates, or 6-cm dishes, and the FBS content of the medium was reduced to 5%. Culture medium containing Taxol, FB_1, and L-cycloserine was prepared just prior to use. Ethanol vehicle was present in controls.

Cell radiolabeling and lipid analysis

[^{3}H]Palmitic acid (1.0 µCi/ml culture medium) was used as lipid precursor to trace the cellular metabolism of ceramide and sphingomyelin. After cell radiolabeling for specified times, 100-µl aliquots of medium were removed and analyzed by liquid scintillation counting (LSC) to determine cellular uptake of the [^{3}H]palmitic acid. After experiments, the radiolabeled medium was aspirated, and cell monolayers were rinsed twice with ice-cold phosphate-buffered saline. Ice-cold methanol containing 2% acetic acid was added, and cells were scraped free of the substratum (using a plastic scraper) for lipid extraction using chloroform and water with vortex mixing in 1-dram glass vials as described previously [1].

After centrifugation, the resulting organic lower phase of the biphasic extraction was withdrawn, transferred to a glass vial, and evaporated to dryness under a stream of nitrogen. Lipids were dispersed in 50–100-µl aliquots of chloroform/methanol (2:1) for analysis by thin-layer chromatography (TLC). [^{3}H]Ceramide was resolved from other radiolabeled cellular lipids by TLC in a solvent system containing chloroform/acetic acid (90:10, v/v), and [^{3}H]sphingomyelin was resolved by TLC in chloroform/methanol/acetic acid/water (60:30:7:3, v/v). Commercial lipid standards were cochromatographed. After drying, lipids were visualized by iodine vapor staining. Spots of interest were scraped onto 0.5 ml water, vortexed, and 4.5 ml EcoLume added for analysis of tritium by LSC.

Cytotoxicity assay

MDA-MB-468 and MCF-7 cells were counted using a hemocytometer and seeded in 96-well plates (5000 cells/well) in 0.1 ml medium containing 5% FBS. We did not use the perimeter wells of the 96-well plates for cells; perimeter wells contained 0.2 ml water. Cells were cultured for 24 h before the addition of the drugs. Taxol was diluted into 5% FBS-containing medium and added to each well in a volume of 0.1 ml. Cells were incubated at 37 °C for 72 h. Cell viability was determined using a Promega Cell Titer 96 Aqueous cell proliferation kit (Promega, Madison, Wis.), as measured spectrophotometrically at λ 490 nm. Absorbance was recorded using an FL600 microplate reader (Biotek, Winooski, Vt.).

Apoptosis detection

Apoptosis was quantitatively measured using a Cell Death Detection ELISA^{PLUS} (Roche Diagnostics, Indianapolis, Ind.). This is a photometric enzyme immunoassay using a mouse monoclonal antibody directed against DNA and histone. The assays were conducted as recommended in the company instructions.

Results

Taxol cytotoxicity

MDA-MB-468 cells are extremely sensitive to Taxol [18]. Initial experiments were conducted to compare the response of MDA-MB-468 and MCF-7 cells to Taxol treatment. Viability dropped sharply in response to Taxol treatment in both cell lines (Fig. 1), but MDA-

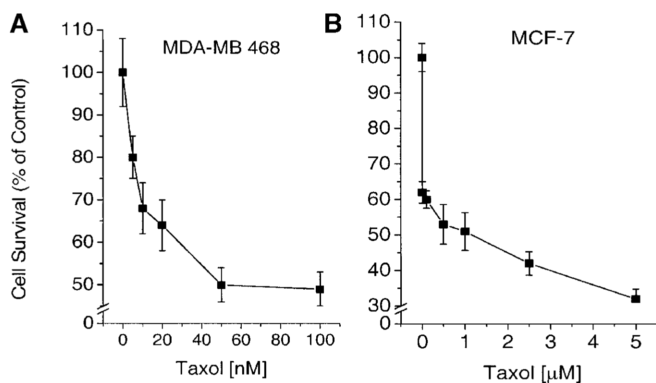

Fig. 1A, B Taxol cytotoxicity in MDA-MB-468 (**A**) and MCF-7 cells (**B**). Cells were seeded in 96-well plates and treated the following day with either vehicle (control) or various doses of Taxol. Cell viability was determined 3 days later using cell proliferation reagents as described in Methods. Each point represents the mean ± SD ($n = 6$)

MB-468 cells were sensitive in the nanomolar range (Fig. 1A), whereas MCF-7 cells required low micromolar dosing to reduce survival (Fig. 1B). The EC_{50} for Taxol in MDA-MB-468 and MCF-7 cells was 50 ± 5.3 nM and 1.0 ± 0.12 μM, respectively, with MDA-MB-468 cells displaying a 20-fold heightened sensitivity over MCF-7 cells.

Taxol induces ceramide generation in breast cancer cells

As shown in Fig. 2A, exposure of MDA-MB-468 cells to Taxol resulted in a concentration-dependent elevation of ceramide. Significant ceramide generation was noted at Taxol doses as low as 25 nM (141% above control). Similarly, albeit at higher concentrations, exposure of MCF-7 cells to Taxol resulted in a dose-dependent elevation of ceramide (Fig. 2B). At 1.0 μM Taxol, ceramide increased more than 250% above control values. Ceramide was generated in MCF-7 cells with concentrations of Taxol as low as 0.5 μM (210% over control). With higher concentration of Taxol, the ceramide response began to plateau in both cell lines.

Differential sensitivity of breast cancer cells to ceramide

The decline in cell viability (Fig. 1) and the concomitant increase in endogenous ceramide (Fig. 2) occurring with Taxol treatment suggested that ceramide may be part of the cytotoxic response mechanism. This prompted us to examine cellular response to the addition of exogenous ceramide, to determine whether ceramide is cytotoxic. When challenged with C_6-ceramide, MDA-MB-468 cell viability dropped precipitously (Fig. 3). The EC_{50} for C_6-ceramide was 0.36 ± 0.03 μM, but MCF-7 cells were much more refractory. The EC_{50} for C_6-ceramide in MCF-7 cells was 7.9 ± 0.34 μM, representing a 20-fold heightened resistance compared with MDA-MB-468 cells, much in line with the differential EC_{50} for Taxol (Fig. 1).

Metabolic pathway of Taxol-induced cellular ceramide

Elevation of cellular ceramide has been shown to occur through hydrolysis of sphingomyelin by the action of sphingomyelinases and via ceramide synthase, the de novo pathway [30]. To determine the pathway of Taxol-induced cellular ceramide formation, we measured sphingomyelin levels following exposure of MDA-MB-468 cells to Taxol. Samples from the dose-response experiments shown in Fig. 2A were analyzed, and

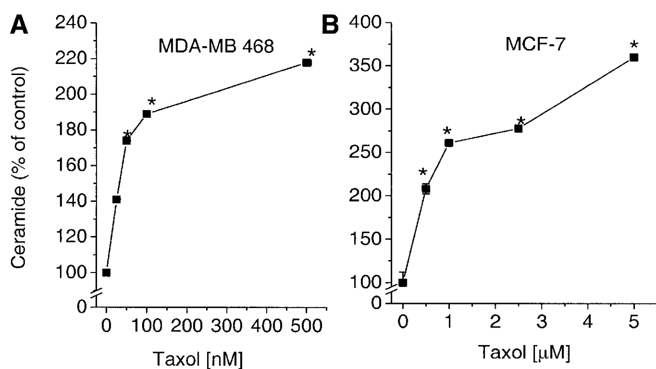

Fig. 2A, B Influence of Taxol on ceramide metabolism in MDA-MB-468 (**A**) and MCF-7 cells (**B**). Experiments were conducted in six-well plates, and Taxol was added when the cells were about 60% confluent. Cells were exposed to Taxol for 18 h in medium containing [^{3}H]palmitic acid (1.0 μCi/ml). Total cellular lipids were extracted, and [^{3}H]ceramide was analyzed as described in Methods. Each point represents the mean ± SD from three separate cultures. *$P < 0.01$, compared with control

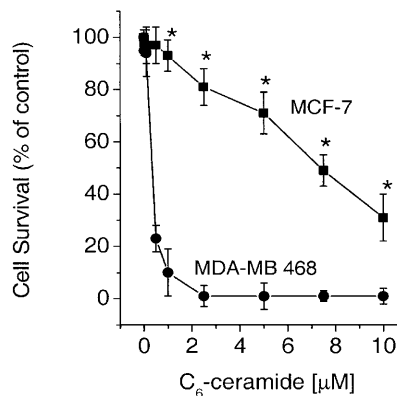

Fig. 3 Influence of C_6-ceramide on MDA-MB-468 and MCF-7 cell survival. Cells were seeded in 96-well plates and treated the following day with either vehicle (control) or various doses of C_6-ceramide. Cell viability was determined 3 days later using cell proliferation reagents as described in Methods. Each point represents the mean ± SD ($n = 6$). *$P < 0.0001$, compared with MDA-MB-468 cells

[^{3}H]sphingomyelin levels were found to remain constant throughout the concentration range of Taxol tested (data not shown). Similarly, increasing the exposure time to Taxol had little influence on cellular sphingomyelin decay (Fig. 4), whereas ceramide increased throughout the 24-h time course. MCF-7 cells displayed a similar time frame for Taxol-induced ceramide formation, with sphingomyelin remaining unchanged (data not shown).

Because cellular sphingomyelin pools are heavily radiolabeled, an increase in [^{3}H]ceramide at the expense of radiolabeled sphingomyelin may be difficult to assess. For this reason we employed FB_1, an inhibitor of ceramide synthase [19, 33], and L-cycloserine, a competitive inhibitor of serine palmitoyltransferase (SPT) [36], a key enzyme in upstream de novo synthesis of ceramide. As shown in Fig. 5A, in MDA-MB-468 cells treated with Taxol, [^{3}H]ceramide generation increased $245 \pm 12\%$ over control, whereas the addition of either FB_1 or L-cycloserine decreased Taxol-generated ceramide to $70 \pm 7\%$ and $55 \pm 6\%$, respectively. Similarly, in MCF-7 cells exposed to Taxol, ceramide increased to $250 \pm 8\%$ over control, whereas in the presence of FB_1 or L-cycloserine, Taxol-induced ceramide generation decreased to $40 \pm 13\%$ and $34 \pm 15\%$, respectively (Fig. 5B). Therefore, both FB_1 and L-cycloserine effectively abolished Taxol-induced ceramide generation and also impeded steady-state de novo formation of ceramide.

Taxol-induced apoptosis

As demonstrated in Fig. 4, ceramide generation in MDA-MB-468 cells in response to Taxol (50 n*M*), was measurable as early as 6 h, had increased further at 12 h, and thereafter further increased sharply. We next determined the time-frame for the onset of apoptosis in MDA-MB-468 cells in response to Taxol exposure. As shown by a shift in optical density (OD) in Fig. 6A, the earliest significant increase in apoptosis was observed at 12 h ($134 \pm 1\%$, $P < 0.005$), with further increases over control of 617% and 894% ($P < 0.005$) at 24 and 48 h,

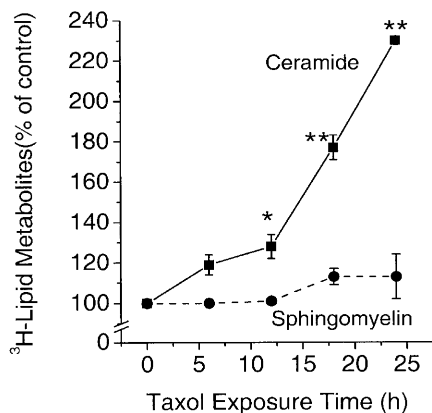

Fig. 4 The influence of Taxol exposure time on ceramide and sphingomyelin metabolism in MDA-MB-468 cells. Cultures at about 60% confluence were incubated in the absence or presence of Taxol (50 n*M*) in medium containing [^{3}H]palmitic acid for the times indicated. Total cellular lipids were extracted, and lipid metabolites were quantitated as described in Methods. Each value represents the mean ± SD from three separate cultures. *$P = 0.01$, **$P < 0.001$, compared with control

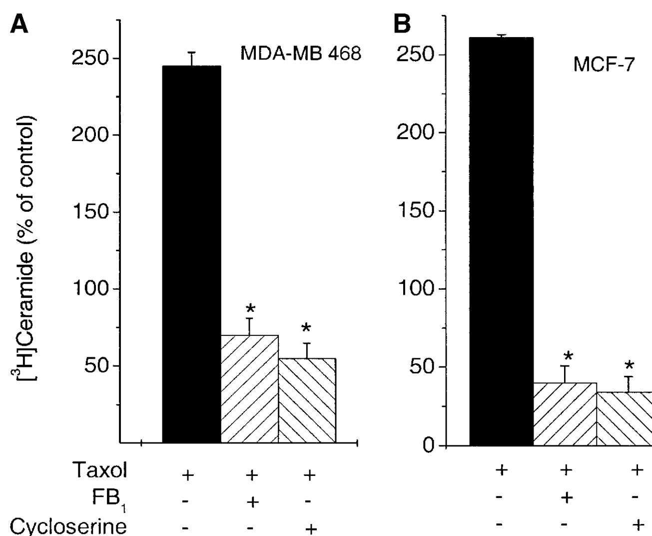

Fig. 5A, B Effect of FB_1 and L-cycloserine on Taxol-induced ceramide generation. MDA-MB-468 (**A**) and MCF-7 (**B**) cells in 6-cm tissue culture dishes were treated with Taxol (50 n*M* and 1.0 μ*M*, respectively) when about 60% confluent and either FB_1 (10 μM and 50 μM, respectively) or L-cycloserine (10 m*M*) as indicated for 24 h. FB_1 and L-cycloserine were dissolved in water. Cellular lipids were analyzed for [^{3}H]ceramide by TLC and LSC. Drug exposure had no influence on the uptake of [^{3}H]palmitic acid. Each value represents the mean ± SD from three separate cultures. *$P < 0.005$, compared with cells treated with Taxol only

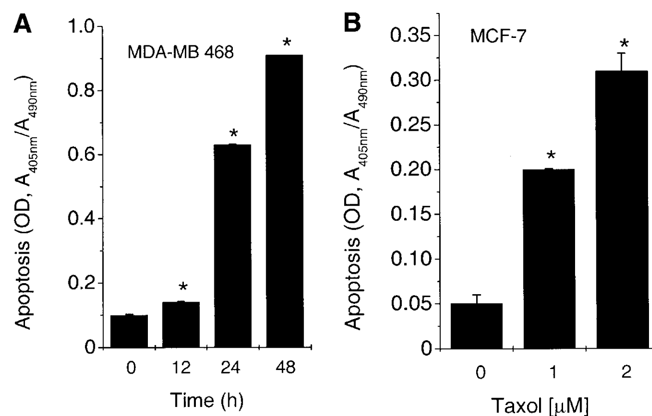

Fig. 6A, B Taxol-induced apoptosis in MDA-MB-468 and MCF-7 cells. **A** Time-course in MDA-MB-468 cells. Cells were cultured in the absence (vehicle only) or presence of Taxol (50 n*M*) and incubated for the times indicated. **B** Dose response in MCF-7 cells. Cells were cultured in the absence (vehicle only) or presence of Taxol (1 or 2 μ*M*) and incubated for 24 h. Following harvest, cells (4000/tube) were lysed and treated as described in the Cell Death Detection protocol, Roche Diagnostics. Apoptosis was quantitatively measured photometrically. Each value represents the mean ± SD ($n = 4$). *$P < 0.005$, compared with control

respectively. The apoptotic response of MCF-7 cells in response to Taxol was also assessed (Fig. 6B). After a 24-h exposure to Taxol, the ODs increased from 0.05 (control) to 0.2 and 0.3 with 1.0 and 2.0 μ*M* Taxol, respectively, representing a 400% and 600% increase over control in oligonucleosomal fragmentation ($P < 0.005$).

Inhibition of Taxol-induced ceramide generation checks cellular apoptosis

To more clearly define the relationship of sphingoid bases and ceramide to Taxol cytotoxicity, apoptosis was measured under conditions in which de novo Taxol-induced ceramide formation was blocked (see Fig. 5). MDA-MB-468 and MCF-7 cells were treated with Taxol in the absence or presence of L-cycloserine, and apoptosis was quantitated. As shown in Fig. 7A, the addition of L-cycloserine to the Taxol regimen suppressed apoptosis by 32% in MDA-MB-468 cells. Similarly, but to a greater extent in the MCF-7 cells, L-cycloserine checked Taxol-induced apoptosis nearly completely (Fig. 7B).

Discussion

The molecular consequences of Taxol exposure and the resultant induction of DNA fragmentation and apoptotic cell death have been documented in breast and ovarian cancers [18, 21]. However, the exact mechanism by which apoptotic cell death is accomplished has not been clearly defined. We have identified ceramide as a bioactive second messenger molecule in Taxol-induced cytotoxicity. It is interesting to note that glucocorticoids selectively inhibit Taxol-induced apoptosis without influencing either the microtubular effects of Taxol or cell cycle arrest at the G_2/M phase in some breast cancer cell lines [8]. It has also been shown that the antitumor effectiveness of Taxol correlates with Taxol-induced apoptosis but not with mitotic arrest in murine mammary tumors [22]. This suggests that both mitotic arrest and apoptotic cell death are two major but separate events in Taxol-treated cells [7]. Taxol can induce DNA fragmentation and typical morphological features of apoptosis via signaling pathways independent of its action on microtubules and subsequent mitotic arrest.

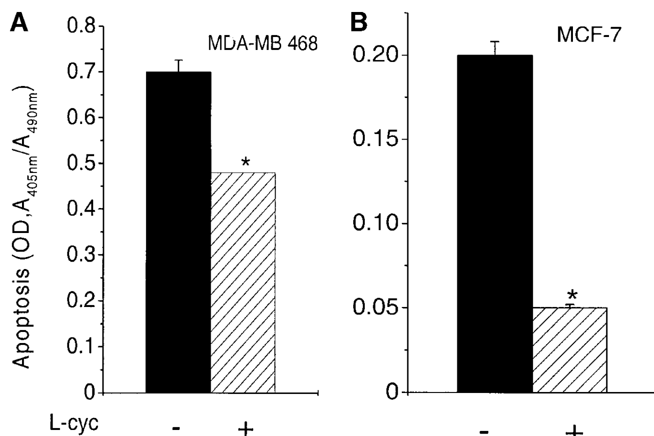

Fig. 7A, B Influence of L-cycloserine on Taxol-induced apoptosis in MDA-MB-468 (**A**) and MCF-7 (**B**) cells. Where indicated, cells were pretreated with L-cycloserine (10 m*M*) for 2 h, after which MDA-MB-468 and MCF-7 cells were treated with 50 n*M* and 1.0 μ*M* Taxol, respectively, for 24 h. Each value represents the mean ± SD ($n = 4$). *$P = 0.005$, compared with cells treated with Taxol only

Although a recent study has shown that Taxol toxicity can be enhanced by the inclusion of a ceramide glycosylation inhibitor [28], in a similar manner to the toxicity of other chemotherapy drugs [14, 16, 17], this is the first work demonstrating enhanced ceramide formation in response to Taxol exposure. Whether ceramide generation is independent of cell cycle arrest, or is consequent upon cell cycle arrest, remains to be seen. The increase in apoptosis, measured 12 h after Taxol addition, was preceded as early as 6 h by an increase in cellular ceramide. This upstream signal supports the idea that ceramide is the proapoptotic stimulus, similar to cell responses to daunorubicin [2], TNF-α [14] and the drug resistance modulator SDZ PSC 833 [4]. We identified the origin of Taxol-induced ceramide generation as being de novo, firstly by measuring [^{3}H]sphingomyelin levels, which remained unchanged despite increasing cellular ceramide generation, and by successful suppression of ceramide formation with FB_1 and L-cycloserine (Figs. 4 and 5). We did not measure the actual mass of ceramide formed in MDA-MB-468 and MCF-7 cells in response to Taxol. Although information of this type may be helpful in explaining Taxol sensitivity, multiple factors such as uptake and metabolism come into play. However, as the cells were radiolabeled to steady-state, radioactivity should have been representative of mass.

To further elucidate the dynamics of Taxol-induced apoptosis and ceramide generation, we examined the apoptosis pattern of Taxol alone or in combination with FB_1 or L-cycloserine, as prior studies have demonstrated that FB_1 is able to attenuate apoptosis induced by daunorubicin [2]. In the present study, suppressing ceramide generation by blocking ceramide formation with FB_1 at the level of ceramide synthase, did not alter the inevitability of apoptosis following Taxol treatment. On the other hand, our results clearly show that suppressing ceramide generation early in the de novo pathway by the use of L-cycloserine, significantly attenuated Taxol-induced apoptosis in both cell lines, particularly in MCF-7 cells. The differences in the cellular susceptibility to L-cycloserine inhibition of apoptosis (Fig. 7) may be associated with varying degrees of sensitivity to Taxol (Fig. 1). These findings also suggest that activation of SPT is the most critical event in de novo Taxol-induced ceramide synthesis, and inhibition of this enzyme protects against apoptosis. This is in accord with a recent study of etoposide induced-apoptosis and ceramide

generation, in which SPT activity was found to control de novo ceramide synthesis during apoptosis [24].

Both endogenous and exogenous sphingosine have been shown to induce apoptosis in a variety of cells including cancer cells of hematopoietic and carcinoma origin [5, 29, 32], and FB_1-induced apoptosis in colon cancer cells is mediated by accumulation of endogenous sphingoid bases [26]. Also of consequence, FB_1 can divert sphinganine, a substrate for de novo ceramide formation, toward degradation [29]. These findings may, in part, account for the inevitability of Taxol-induced apoptosis in the presence of FB_1, despite clear evidence of suppressed cellular ceramide generation. While not wanting to diminish the role of ceramide in the action of Taxol, apoptosis may also be a function of the sphingoid bases, as sphingosine and sphinganine are both cytotoxic.

One of the possible mechanisms of Taxol-induced apoptosis has been explored in this study. Ceramide is a recognized vital second messenger in the cellular signal transduction process leading to apoptosis. It has been shown that Taxol and ceramide in combination therapy interact in a superadditive manner to increase apoptosis in a leukemic cell line [23]. This further supports an essential distinction of ceramide in Taxol-induced apoptosis. There remains the possibility that ceramide generation represents a consequence rather than a cause of Taxol-induced apoptosis. However, the ability of L-cycloserine to abrogate Taxol-induced apoptosis, and the 20-fold difference in sensitivity in MCF-7 and MDA-MB-468 cells following independent treatment with Taxol and C_6-ceramide, corroborate the role of ceramide as crucial in Taxol-induced cellular apoptosis.

This study demonstrates a novel role of ceramide in the toxicity of Taxol at the molecular level. With pharmacological manipulation, we could clinically alter our approach to the use of Taxol. By using ceramide-enhancing drug combinations [16, 27], the availability of free ceramide could be increased, thereby inducing apoptosis more effectively and possibly overcoming cellular resistance to Taxol.

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