Biochemical Pharmacology, Vol. 58, pp. 1067–1074, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00193-8

Inhibition of Protein Synthesis by Didemnin B Is Not Sufficient to Induce Apoptosis in Human Mammary Carcinoma (MCF7) Cells

David R. Beidler,† Deepika Ahuja,‡ Max S. Wicha* and Peter L. Toogood‡§*

*Department of Internal Medicine and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109-0946; and ‡Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, U.S.A.

ABSTRACT. Didemnin B (DB) is one member of a class of natural cyclic depsipeptides that display potent cytotoxicity *in vitro*. The detailed mechanism of action of DB is unknown, although it appears to involve the inhibition of protein biosynthesis. Additional activities of DB have established DB as a rapid and potent inducer of apoptosis in HL-60 cells. Our aim was to determine if the induction of apoptosis by DB is mediated through inhibition of protein synthesis in MCF-7 human breast carcinoma cells. Apoptosis was observed only at ≥ 100 nM DB, even though inhibition of protein synthesis occurred at much lower DB concentrations (IC_{50} = 12 nM). DB-induced apoptosis was mediated by caspase activation, since cleavage of the caspase substrate poly(ADP-ribose) polymerase was observed as early as 6 hr after DB exposure. Two additional protein synthesis inhibitors, cycloheximide (CHX) and emetine (ET), failed to induce apoptosis at concentrations that completely inhibited protein synthesis. Moreover, DB-induced apoptosis was enhanced only slightly by pre- and co-treatment with CHX and ET. Thus, inhibition of protein synthesis alone was not sufficient to induce apoptosis in these cells. As a measure of antiproliferative potential, DB (1–5 nM) inhibited the colony forming ability of MCF7 cells regardless of pretreatment with CHX. In conclusion, additional effects of DB, independent of protein synthesis inhibition, are proposed to account for its ability to induce apoptosis and prevent cell proliferation. BIOCHEM PHARMACOL **58**;6:1067–1074, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. didemnins; apoptosis; caspases; protein synthesis; cycloheximide; emetine

The didemnins are potent antineoplastic and immunosuppressive natural products isolated from the sea [1, 2]. One of the more potent didemnins, DB[‖], displays cytotoxic effects against tumor cells *in vitro* at concentrations between 1 and 50 nM [3–5]. Following promising results with DB in small animal model studies [2, 6], DB was evaluated clinically as an antitumor agent against several human cancers [7–22]. In phase II clinical trials, no significant therapeutic effect of didemnin was observed in patients with ovarian or colorectal cancer [8, 11, 14] or carcinoma of the breast [7] or cervix [10], although a weak response was reported against glioblastoma [15]. Side-effects associated with the use of DB are largely restricted to nausea and vomiting, although neuromuscular toxicity and mild hepatotoxicity were reported in a small number of cases. Despite these results, the recent discovery of didemnins that are more potent than DB [5, 23–25] has renewed interest in these compounds as potentially efficacious clinical agents, and dehydrodidemnin B entered phase I human clinical trials in 1998 [26, 27]. However, appropriate use of these compounds would benefit from a clearer understanding of their mechanism of action.

Studies over the past 15 years have investigated the mechanism of action of didemnins, but currently there is little known about the primary target or the mode of action of didemnins in cells. Didemnins inhibit protein biosynthesis in cells and in cell-free assays [4, 5, 28–31]. This effect was originally proposed to be the primary mechanism of action for these compounds because the concentration dependence for inhibition of protein synthesis in L1210 cells matches closely the concentration dependence for inhibition of cell growth [4, 6]. The identification of EF-1α as a didemnin binding protein [32] and the delineation of a mechanism for inhibition of protein synthesis *in vitro* [29] lend support to this hypothesis, as do comparisons of didemnin analogs as cytotoxic agents and as inhibitors of macromolecule synthesis (Ahuja D and Toogood PL, unpublished results).

In contradiction to this hypothesis, discrepancies in the concentration dependence for inhibition of protein synthesis by didemnins and for other activities of these com-

† Corresponding author: David Beidler, Ph.D., Department of Internal Medicine, University of Michigan Medical School, 7110 Cancer and Geriatrics Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0946. Tel. (734) 763-4743; FAX (734) 647-9480; E-mail: dbeidler@umich.edu

§ Current address: Parke-Davis Pharmaceutical Company, 2800 Plymouth Road, Ann Arbor, MI 48105.

‖ *Abbreviations*: DB, didemnin B; CHX, cycloheximide; EF-1α, elongation factor-1 alpha; ET, emetine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; and zVAD-fmk, Z-Val-Ala-Asp(OMe)-Ch_2F.

Received 30 November 1998; accepted 8 February 1999.

pounds argue that inhibition of protein biosynthesis may not be entirely responsible for the cytotoxic and antiproliferative effects of didemnins. For example, DB inhibits T-cell blastogenesis at picomolar concentrations, but must be present in nanomolar concentrations to inhibit general protein synthesis in the same cells [33]. Nordidemnin inhibits *myo*-inositol uptake into WRK_1 cells at concentrations well below those necessary to inhibit the production of vasopressin receptors [34]. Moreover, inhibition of protein synthesis *in vitro* requires concentrations of didemnins that are approximately three orders of magnitude higher than are required to inhibit cell growth or to inhibit protein synthesis in intact cells [29]. Consistent with this latter observation, *N*-acetyldidemnin A binds relatively weakly to the translation factor EF-1α (K_d = 15 μM), which is believed to mediate the effect of didemnins upon protein synthesis in cell-free assays [32]. The discovery of a second didemnin-binding protein has fueled further speculation that protein synthesis may not be the most important target of didemnins in cells [35].

A recent report that DB is a potent and rapid inducer of apoptosis in HL-60 cells suggests that DB may be more effective as an inducer of programmed cell death than as a general cytotoxic agent [36]. However, previous studies were performed with relatively high concentrations of the peptide, which also would be expected to inhibit protein synthesis completely. At these concentrations it is not possible to distinguish between inhibition of protein biosynthesis and other mechanisms of action. Therefore, to determine whether DB triggers apoptosis as a consequence of its inhibition of protein synthesis or via a different mechanism of action entirely, we examined the effect of this drug upon cells from the MCF7 human breast carcinoma cell line. This cell line is well characterized for studying apoptosis [37, 38] and is known to be sensitive to low concentrations of DB. We compared the potencies of DB for the induction of apoptosis and for inhibition of protein biosynthesis in these cells, and performed parallel studies with other inhibitors of protein synthesis. From these experiments it is clear that inhibition of protein synthesis by DB is not sufficient, and may not be necessary, for the initiation of programmed cell death in MCF7 cells.

MATERIALS AND METHODS

Cell Lines and Chemicals

The M1 subclone of MCF7 human breast carcinoma cells has been described previously [38]. Cells were cultured routinely in RPMI-1640 medium (GIBCO) supplemented with 10% heat-inactivated (50°, 15 min) fetal bovine serum (HyClone), L-glutamine (GIBCO), penicillin/streptomycin (GIBCO), and MEM non-essential amino acids (GIBCO). Cells were kept in a humidified 5% CO_2 incubator at 37°. DB was provided by the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute. CHX and ET (both obtained from Sigma) were prepared as 10 mg/mL stock solutions in ethanol and ddH_2O, respectively. zVAD-fmk (Enzyme Systems) was prepared as a 10 mM stock solution in DMSO. Aliquots of drug stocks were stored at -20°. PI was obtained from Sigma. [^{35}S]Methionine (~1000 Ci/mmol) was purchased from NEN.

Apoptosis Assay

Apoptosis was measured by the assessment of nuclear morphology of PI-stained cells as described previously [38]. Briefly, MCF7 cells were plated onto sterile glass coverslips (Fisher Scientific 22 x 22 mm^2 No. 1) in 6-well plates at a concentration of 2×10^5 cells/well. After drug exposure, the medium was aspirated, and wells were washed briefly with PBS. The coverslips were fixed in methanol at −20° for 10 min followed by two washes with PBS. Coverslips were incubated in PI (50 μg/mL, Sigma) in PBS for 5 min at room temperature. After three washes with PBS, the coverslips were mounted onto microscope slides using Vectashield mounting medium (Vector Laboratories). PI-stained nuclei were visualized by fluorescence microscopy using a FITC range barrier filter cube. Apoptotic and nonapoptotic nuclei were counted based on condensed nuclear morphology. For each sample a minimum of 200 cells was counted to determine the percentage of apoptotic cells present.

Protein Biosynthesis Assays

MCF7 cells in RPMI-1640 medium supplemented with 10% fetal bovine serum, l-glutamine, penicillin, and MEM non-essential amino acids were seeded in 6-well plates at an initial concentration of 4×10^4 cells/mL. After incubation in a CO_2 atmosphere for 24 hr at 37°, the cells were washed with PBS and resuspended in RPMI-1640 without methionine (4 mL) supplemented as described above. The appropriate inhibitor solution or carrier solvent as control also was added at this time. Following incubation of the cells for 1 hr, [^{35}S]methionine was added to each sample to a final concentration of 9 nM. Incubation was continued for 18 hr, and then the cells were washed with PBS and precipitated with cold 10% trichloroacetic acid. The precipitate was redissolved in 0.2 M NaOH at room temperature, and aliquots were removed for scintillation counting to determine the extent of methionine incorporation.

To examine the reversibility of protein synthesis inhibition, MCF7 cells were seeded in 6-well plates as described above. To plate A was added 4 mL/well of supplemented RPMI-1640 medium without methionine; to plates B and C was added 4 mL/well of supplemented RPMI-1640 medium with methionine. Inhibitor solutions (or EtOH for controls) were added to the culture medium in all three plates. Following incubation of plate A at 37° for 1 hr, 4 μL of [^{35}S]methionine (1175 Ci/mmol; final concentration 9 nM) was added to each well. All three plates then were incubated at 37° for 2 hr (or 18 hr). At the end of this time,

the cells in plate A were assayed for the incorporation of ^{35}S-label as described above. This plate was used to establish the extent of protein synthesis inhibition following a 2-hr (or 18-hr) incubation with drug. The drug-containing medium from plates B and C was aspirated, and the cells were washed with PBS (4 mL/well). Drug-free RPMI-1640 without methionine plus 4 μL of [^{35}S]methionine was added to each well in these plates. Plate B then was incubated at 37° for 1 hr, and the amount of ^{35}S-label incorporated into cells was determined as before. Similarly, plate C was incubated at 37° for 18 hr, and the amount of ^{35}S-label incorporated into cells was determined.

PARP Western Blot Analysis

For the analysis of PARP cleavage, 6×10^5 cells were plated onto 60-mm^2 dishes in drug-free medium. After drug exposure, cells were harvested by scraping, washed with ice-cold PBS, and lysed in 0.5 mL of a urea lysis buffer (6 M urea, 50 mM Tris pH 6.8, 6% 2-mercaptoethanol, 3% SDS, and 0.003% bromophenol blue). Whole cell lysates were sonicated for 40 sec on ice and heated to 95° for 8 min before 45-μL samples were loaded onto 7.5% SDS–PAGE gels. The proteins were transferred to nitrocellulose membranes, and immunoblotting was performed as described previously [37]. The anti-PARP monoclonal antibody (Enzyme Systems) was used at a dilution of 1:10,000, and the secondary antibody (anti-mouse Ig/horseradish peroxidase, Pierce) was used at a dilution of 1:4000. The peroxidase signal was visualized by chemiluminescence (Pierce).

Colony Forming Assays

To quantitate the effects of DB on colony forming ability, dilutions of 750, 250, and 100 cells per well were plated onto 6-well plates and further incubated for 18 hr. Test compounds were added to each of the wells, and incubation was continued for 18 hr. The medium was aspirated, and the wells were washed in drug-free medium for 1 hr followed by a 10-day incubation in drug-free medium to allow colony formation. At the end of this incubation, medium was aspirated, and the cells were fixed and stained by the addition of 0.5% methylene blue in 50% ethanol for 45 min at room temperature. The 6-well plates were washed gently with water and allowed to air-dry. Visible colonies were counted to determine the percent colony formation of plated cells for each drug treatment. Colony formation percentages for each drug treatment were compared with percentage values of untreated controls.

RESULTS

Induction of Apoptosis and Inhibition of Protein Synthesis by DB

To determine if DB induced apoptosis in MCF7 cells, a quantitative apoptosis assay was performed involving visual analysis of nuclear condensation in DB-treated and untreated cells. Significant levels of apoptosis were seen at concentrations of DB ≥ 100 nM after an 18-hr exposure (Fig. 1A). The concentration–response curve ranged between 100 and 500 nM DB with apoptotic percentages ranging from 10 to 80%, respectively. No increase in apoptosis was seen above 500 nM DB. Inhibition of protein biosynthesis in MCF7 cells was examined by measuring the incorporation of [^{35}S]methionine into cellular proteins. Following an 18-hr exposure of the cells to DB, protein synthesis was inhibited with IC_{50} = 12 ± 2.8 nM; 94% inhibition was achieved with 50 nM DB (Fig. 1B). It is clear from a comparison of panels A and B of Fig. 1 that inhibition of protein synthesis by DB was complete at concentrations below those at which any significant apoptotic effect was observed.

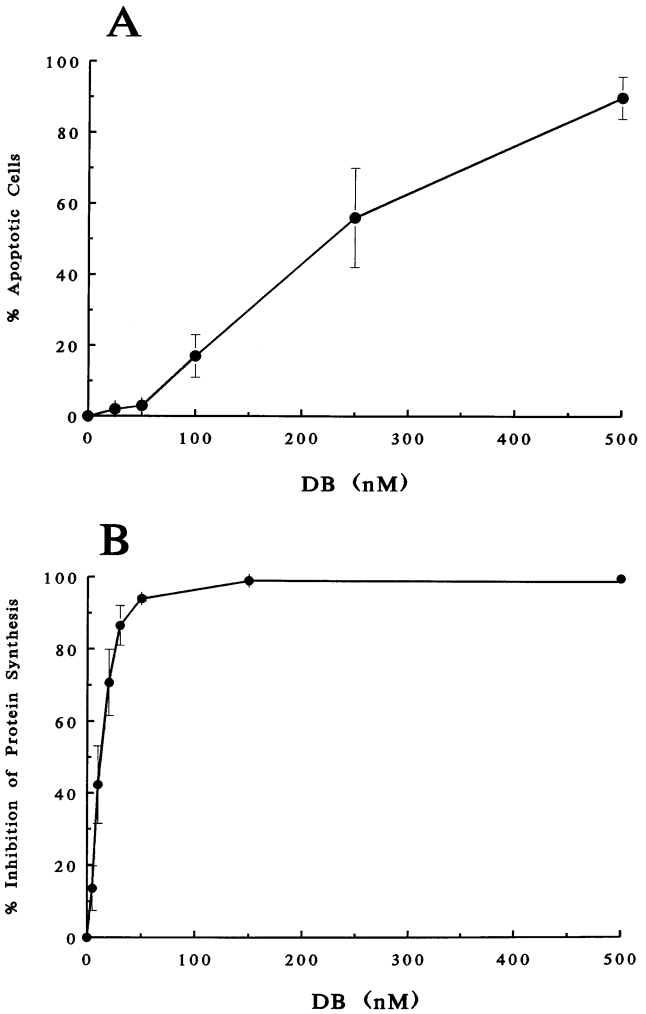

FIG. 1. Concentration–response profile of DB-induced apoptosis and protein synthesis inhibition in MCF7 cells. (A) Apoptosis. MCF7 cells were cultured in the presence of the indicated DB concentrations for 18 hr followed by quantitation of apoptosis as described in Materials and Methods. (B) Protein synthesis inhibition. MCF7 cells were cultured in the presence of the indicated concentrations of DB for 18 hr followed by quantitation of protein biosynthesis as described in Materials and Methods. Values represent means ± SD from at least three separate experiments.

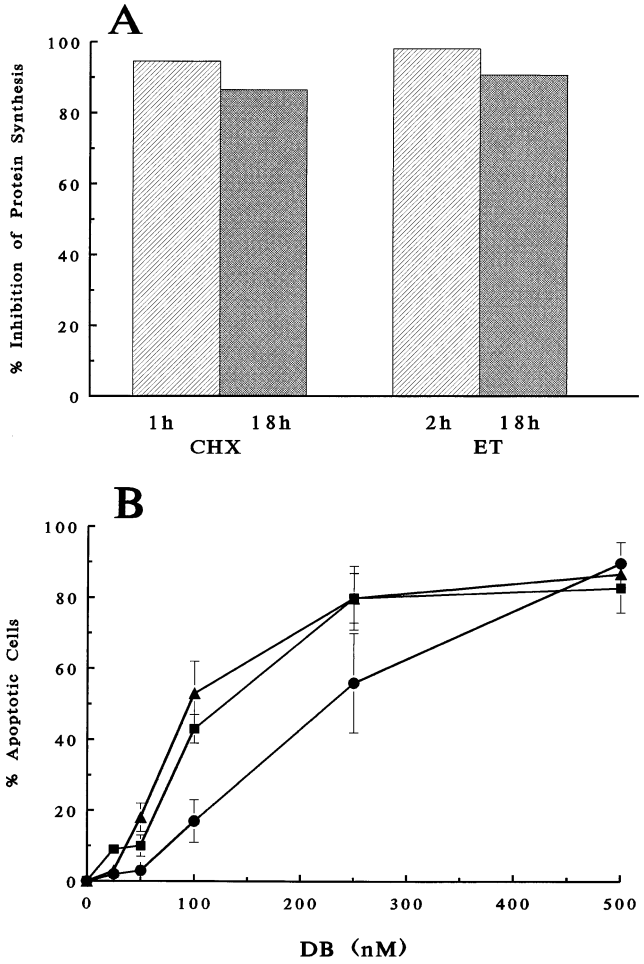

FIG. 2. Inhibition of protein synthesis and alteration of DB-induced apoptosis by CHX and ET. (A) Protein synthesis inhibition profiles. Protein synthesis was measured after a 1- and 18-hr exposure to CHX (10 μg/mL) and a 2- and 18-hr exposure to ET (1 μg/mL). Values are representative of an experiment that was run three times. (B) Apoptosis. CHX (10 μg/mL) was added 1 hr and ET (1 μg/mL) was added 2 hr before an 18-hr co-incubation with increasing concentrations of DB. Apoptosis was quantitated as described in Materials and Methods, and values represent the means ± SDfrom four separate experiments. Symbols: (●) DB alone; (▲) DB + CHX; and (■) DB + ET.

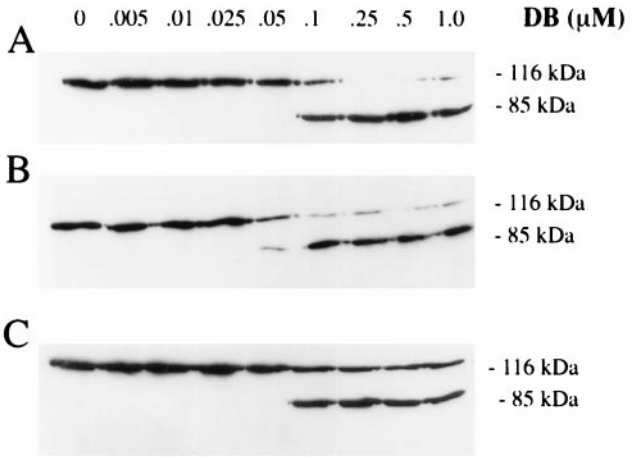

FIG. 3. Induction of PARP cleavage by DB and co-incubations of DB + CHX and DB + ET. (A) DB- induced PARP cleavage. After an 18-hr incubation with the indicated concentrations of DB, cells were prepared for PARP western blot analysis as described in Materials and Methods. The 116-kDa band represents the intact PARP protein, and the 85-kDa band represents a signature 85-kDa PARP cleavage product. Pre- and co-treatment with ET (B) and CHX (C) were as described in the legend of Fig. 2B.

Involvement of Protein Synthesis Inhibition in DB-Induced Apoptosis

Since DB inhibits protein synthesis at concentrations lower than those at which apoptosis is observed, we wanted to determine whether apoptosis is a consequence of the inhibition of protein biosynthesis. To address this question, we examined the effect on cells of two other protein synthesis inhibitors, CHX and ET. Protein synthesis in MCF7 cells was inhibited by these agents with IC_{50} values of 0.43 ± 0.19 and 0.07 ± 0.02 μg/mL for CHX and ET, respectively. Treatment of cells with 10 μg/mL CHX resulted in 95% inhibition of protein synthesis, and treatment with 1 μg/mL ET resulted in 98% inhibition of protein synthesis (Fig. 2A). The inhibition of protein synthesis by these two agents persisted for 18 hr since incorporation of the ^{35}S-label at 18 hr was only marginally greater than at 1 and 2 hr. Under these conditions, neither CHX nor ET induced apoptosis, indicating that the inhibition of protein synthesis alone was not sufficient to cause apoptosis.

To examine the effect of co-incubating CHX or ET with DB, MCF7 cells were treated with either 10 μg/mL CHX for 1 hr or 1 μg/mL ET for 2 hr to inhibit protein synthesis completely, followed by DB for 18 hr. As shown in Fig. 2B, pre- and co-treatment with CHX or ET slightly sensitized cells to DB-induced apoptosis at lower DB concentrations (50–250 nM). The levels of apoptosis observed at 100 and 250 nM DB were significantly ($P < 0.05$) higher when DB was combined with CHX or ET; however, by 500 nM DB the apoptotic levels were identical regardless of pretreatment with CHX or ET.

DB-Induced PARP Cleavage

To characterize the apoptosis induced by DB biochemically, the role of caspase activity was determined in DB-induced apoptosis. Caspases are a family of aspartic acid-specific cysteine proteases that have been shown to participate in a wide range of apoptotic responses [39]. Once activated, caspases in turn cleave a number of cellular substrates, one of the best known being PARP. Therefore, as an indicator of caspase activity, the cleavage of the 116-kDa intact PARP to a signature 85-kDa product was monitored by immunoblot analysis. The concentration response for PARP cleavage following incubation of cells with DB for 18 hr is shown in Fig. 3A. It closely resembled the concentration response of nuclear morphology shown

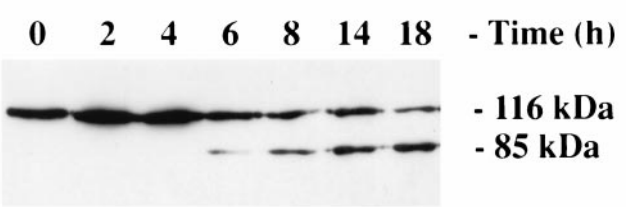

FIG. 4. Time–course analysis of DB-induced PARP cleavage in MCF7 cells. MCF7 cells were treated with 0.5 μM DB for the indicated times before PARP western blot analysis as described in Materials and Methods.

in Fig. 1A. PARP cleavage was observed at concentrations of DB ≥ 0.25 μM. A slight sensitization of cells to PARP cleavage was observed following pretreatment and co-incubation of cells with DB and ET (Fig. 3B), and the 85-kDa cleavage product was visible at 0.1 μM DB. This corresponds to the concentration response measured by examining nuclear morphology (see Fig. 2B). However, a similar increase in sensitivity was not observed following co-incubation of cells with DB and CHX (Fig. 3C). Importantly, incubations of up to 18 hr with ET or CHX alone did not produce any 85-kDa PARP cleavage product, in agreement with the inability of these compounds to induce apoptosis at these concentrations (Fig. 3, B and C; first lane).

To understand how quickly apoptosis was induced by DB in MCF7 cells, PARP cleavage was monitored at various time points after the addition of 0.5 μM DB. As shown in Fig. 4, the 85-kDa PARP cleavage product was visible as early as 6 hr following the addition of DB. Pretreatment and co-incubation with either CHX or ET did not alter the rate of PARP cleavage induced by DB (data not shown).

To directly assess the requirement for caspase activation in DB-induced apoptosis, MCF7 cells were pretreated with zVAD-fmk, a cell-permeable peptide inhibitor of caspase activity. Treatment of cells with zVAD-fmk itself did not induce apoptosis or PARP cleavage; however, zVAD-fmk (20 μM) completely abolished the apoptotic morphology and PARP cleavage induced by an 18-hr exposure to DB (data not shown).

Effects of DB on Colony Forming Ability

A series of colony forming assays were performed in which MCF7 cells were exposed to DB for 18 hr, and then were incubated for 10 days in drug-free medium to allow the surviving cells to form colonies. As shown in Fig. 5, DB caused a loss of colony forming ability at concentrations between 1 and 5 nM, similar to the concentrations at which inhibition of protein synthesis was observed but well below the 100 nM threshold for DB-induced apoptosis. Pretreatment and co-incubation with 10 μg/mL of CHX did not alter the concentration–response profile for DB-induced reduction in colony formation (Fig. 5). CHX exposure alone reduced the colony forming ability less than 10% when compared with untreated controls.

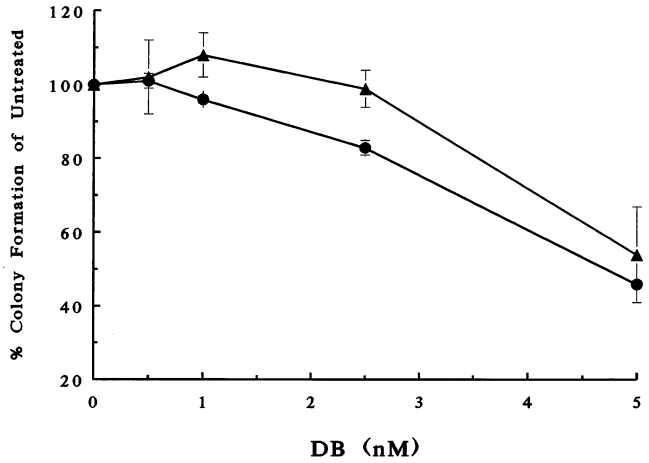

FIG. 5. DB and DB + CHX colony forming ability. MCF7 cells were exposed to the indicated concentrations of DB alone (●) or DB + 10 μg/mL of CHX (▲) for 18 hr. Colony formation was calculated as described in Materials and Methods. "Percent colony formation of untreated" represents the ratio of colony formation in drug-treated sample compared to untreated controls. Values represent the means ± SD from at least three experiments.

Reversibility of Protein Synthesis Inhibition

MCF7 cells were treated with DB or CHX for 2 or 18 hr, at the end of which time the cells were assayed for protein synthesis (Fig. 6). The drug was removed by washing, and protein synthesis was assayed again at 1 and 18 hr following removal of the drug. Cells treated with CHX for 2 hr displayed rapid and complete recovery of the ability to make proteins following removal of the inhibitor (Fig. 6A). Cells treated with CHX for 18 hr took longer to recover, but still recovered completely within 18 hr (Fig. 6B). Following treatment with 5 or 10 nM DB for 2 hr, cells recovered approximately 95% of their ability to synthesize proteins within 18 hr. Cells displayed 75% recovery following a 2-hr treatment with 30 nM DB (Fig. 6A). In contrast, cells treated for 18 hr with 5, 10, or 30 nM DB displayed diminished ability to make proteins 1 hr after the removal of the inhibitor, and did not recover beyond the level of inhibition observed following 18 hr of continuous exposure to the drug (Fig. 6B). The apparent increase in inhibition during the first hour following removal of the DB is currently unexplained and cannot be accounted for by a drop in cell number. These results establish that the effect of CHX upon protein biosynthesis in cells was rapidly and fully reversible, but inhibition of protein synthesis by DB following an 18-hr incubation was irreversible for at least 18 hr.

DISCUSSION

In this study, we have shown that the apoptotic response induced by DB in MCF7 cells involved the activation of a class of cysteine proteases called caspases. Caspases reside within the cell as larger, inactive precursors, which upon

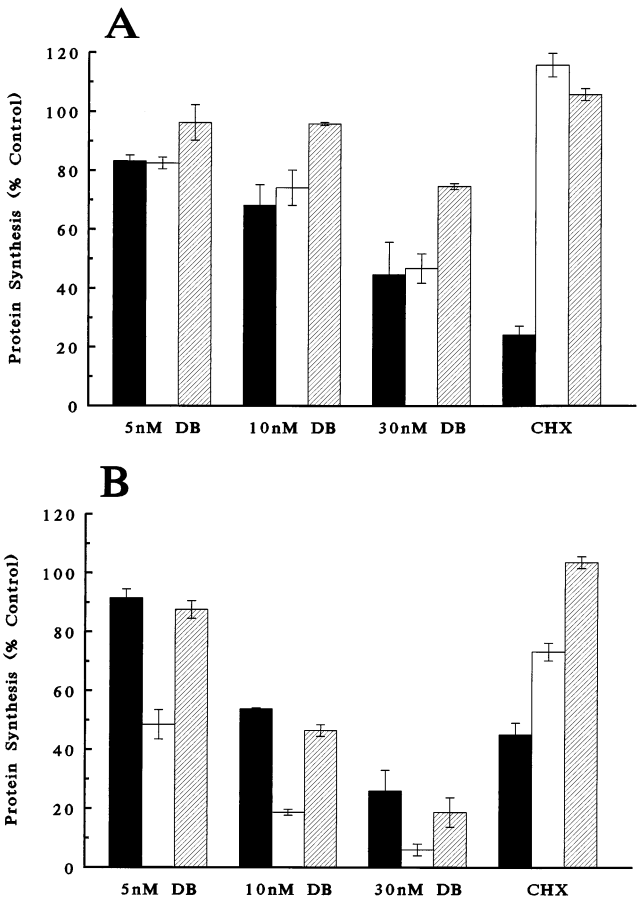

FIG. 6. Reversibility of protein synthesis inhibition by DB and CHX. Protein synthesis by MCF7 cells expressed as a function of controls containing no inhibitor (control counts were typically 1 × 10^4 cpm). Cells were treated with DB (5, 10, or 30 nM) or CHX (0.3 μg/mL) for (A) 2 hr or (B) 18 hr, and then were transferred to drug-free medium. Protein synthesis was assayed immediately after drug removal (black bars); 1 hr following removal of the drug (white bars); or 18 hr following removal of the drug (striped bars). Values represent the means ± SD from three experiments.

proteolytic processing form active heterodimers that subsequently cleave a subset of endogenous substrates (including PARP, Lamin A, and actin) based on cleavage sequence recognition [39]. The activation of caspase activity is critical to numerous apoptotic responses, including apoptosis induced by tumor necrosis factor/Fas receptor activation, serum withdrawal, and DNA damage by UV, ionizing radiation or chemotherapeutic agents. DB-induced apoptotic changes, as shown by nuclear condensation, corresponded to cleavage of the caspase substrate PARP. Incubation with a known synthetic cell-permeable inhibitor of caspase activity (zVAD-fmk) not only blocked PARP cleavage but also blocked the morphological changes of DB-induced apoptosis. In previous studies, DB has been shown to induce a rapid apoptotic response in HL-60 cells (100% within 140 min) [36], and several protein tyrosine kinase inhibitors have been found to inhibit these apoptotic responses in HL-60 cells [40]. However, the molecular components involved in the apoptotic response were unknown. Here we show for the first time the necessity for caspase activation in DB-induced apoptotic response in MCF7 cells.

Previous work has established that DB inhibits protein synthesis in cell-free translation assays. This inhibition appears to arise through interaction between the inhibitor and EF-1α, ultimately leading to suppression of ribosomal translocation [29]. It has not been established whether the inhibition of protein synthesis in intact cells occurs via a similar mechanism, nor whether the inhibition of protein synthesis accounts for the cytotoxic effects of DB. However, observations that didemnins can inhibit T-cell blastogenesis and *myo*-inositol uptake in cultured cells at concentrations below those necessary to inhibit protein synthesis have led some workers to speculate that the inhibition of protein synthesis is not the primary mechanism of action of these compounds [32–34]. If protein synthesis inhibition were the sole mechanism for all of the DB-induced cytotoxic effects, then a close correlation between protein synthesis inhibition and apoptosis induction should be expected. For DB, the IC_{50} for protein synthesis inhibition was 12 nM, while apoptosis above background levels was only apparent at concentrations ≥ 100 nM, with IC_{50} = 200 nM (Fig. 1A). Thus, the concentration response for protein synthesis inhibition was at least 16-fold lower than the concentration response for apoptosis, and protein synthesis was inhibited completely at concentrations above 150 nM. Exposure of cells to DB for longer than 18 hr did not alter the apoptotic concentration–response curve (data not shown). This absence of a correlation between the concentration–response profiles for inhibition of protein synthesis and the induction of apoptosis by DB in MCF7 cells suggests that protein synthesis inhibition alone cannot account for the cytotoxic effects of didemnins. To further examine this point, the response of MCF7 cells to two additional protein synthesis inhibitors was examined. CHX and ET both inhibit protein synthesis *in vitro* at the stage of ribosomal translocation and in this regard appear to be mechanistically related to DB, although all three compounds possess different molecular targets [41]. In our assays, CHX or ET failed to induce apoptosis in MCF7 cells at concentrations at which they inhibited protein synthesis by 95–99% compared with controls. Thus, apoptosis is not a necessary consequence of the inhibition of protein synthesis, and inhibition of protein synthesis alone appears to be insufficient to trigger apoptosis.

From these data, we conclude either that the induction of apoptosis by DB is independent of the inhibition of protein synthesis, or that it requires the inhibition of protein synthesis plus one or more additional signals. If protein synthesis inhibition is not required for the induction of apoptosis by DB, then treatment of cells with agents that block protein synthesis, prior to the addition of DB, should not alter the concentration response for induction of apoptosis by DB. Conversely, if inhibition of protein synthesis is a necessary component of the apoptotic pathway, then prior treatment of cells with protein synthesis

inhibitors should display a synergistic effect, with less DB being required to obtain a given apoptotic response.

DB-induced apoptosis was enhanced only 2- to 3-fold at the lower apoptosis-inducing concentrations (50–100 nM) by pretreatment and co-incubation of cells with CHX or ET. While these results cannot exclude a role for the inhibition of protein synthesis in the apoptotic pathway, they do lend further support to the idea that inhibition of protein synthesis alone does not trigger apoptosis, because in this case the concentration–response curve for DB would be expected to shift sharply to much lower (sub-nanomolar) concentrations. Furthermore, co-treatment with CHX was found to slightly enhance apoptosis induced by camptothecin and doxorubicin in MCF7 cells (data not shown), suggesting that the sensitization to apoptosis by CHX was not unique to DB, but was a more general phenomenon.

In contrast to apoptosis, the concentrations of DB necessary to cause a loss of colony forming ability among MCF7 cells closely corresponded to the concentrations required for protein synthesis inhibition. Once again, however, inhibition of protein synthesis failed to account for the antiproliferative effects of DB since pre- and co-treatment with CHX did not alter significantly the colony forming ability of DB-treated MCF7 cells. Furthermore, CHX on its own did not inhibit colony forming ability. One explanation for these observations could be that CHX interacts with the protein synthetic machinery in a reversible manner, whereas DB generates an irreversible block to protein synthesis after an 18-hr exposure (Fig. 6). In the presence of CHX, DB is still able to generate an irreversible lesion that reduces the proliferative potential of the cell regardless of the effects of CHX. Curiously, after exposure of cells to DB for only 2 hr, inhibition of protein synthesis was still fully reversible. Apparently, a longer exposure of the cells to DB is required for the irreversible lesion to occur; however, this irreversibility is not a consequence of prolonged protein synthesis inhibition alone, since inhibition by CHX for 18 hr was reversible. Therefore, some additional event or interaction must occur between the 2-hr and the 18-hr DB exposure. This observation is consistent with a report by Lobo and coworkers [42] that incubation of human colon cancer cells with dehydrodidemnin B for only 3 hr is insufficient to inhibit cell proliferation permanently.

While irreversible inhibition of protein synthesis may explain the loss of proliferative potential in colony forming assays, it is unknown if this time-dependent irreversible lesion also is necessary for the induction of apoptosis. The activation of an apoptotic response requires signals in addition to protein synthesis inhibition; a higher concentration of DB, not just a longer incubation time, is necessary to elicit an apoptotic response. These signals may or may not be mediated by EF-1α, the known target of DB for inhibition of protein synthesis *in vitro* [32]. However, the only other target of didemnins to have been identified to date is protein palmitoyl thioesterase [35], and this secreted protein appears not to mediate the cytotoxicity of didemnins based on comparisons of protein binding data *in vitro* and potency against intact cells [43]. We speculate that other, possibly more significant targets of didemnins remain to be identified.

In summary, we have demonstrated that DB induces apoptosis in the human breast carcinoma cell line MCF7 via the activation of caspases. Protein synthesis inhibition, which has been proposed previously to be the primary mechanism of action of didemnins, is not sufficient to explain the induction of apoptosis by DB. As yet, undefined additional events are necessary to elicit an apoptotic response, requiring significantly higher concentrations than those required for inhibition of protein synthesis. At lower concentrations, DB displays antiproliferative effects that may stem from the inhibition of protein synthesis but that require, in addition, a second irreversible step. The execution of this second step was found to be time-dependent, requiring exposure of the cells to DB for more than 2 hr. At this point, it is not known whether or not irreversible protein synthesis inhibition is also a prerequisite for apoptosis. In aggregate, these results suggest that besides EF-1α, at least one additional target exists for DB in cells. Since the clinical use of DB is limited by severe toxicity and immunosuppression, one possible approach to the discovery of improved didemnins may be to distinguish between these targets. For example, a series of DB analogs that more specifically target the apoptotic activation by DB but that minimally affect protein synthesis may prove to be better therapeutic tools than the parent natural product.

This work was supported by the National Cancer Institute through Grant R29 CA61978 (to P. L. T.), and a Cancer Center Support Grant, CA46592 (to D. R. B.).

References

1. Li W-R and Joullie MM, The didemnins: Biological properties, chemistry and total synthesis. *Stud Nat Prod Chem* **10:** 241–302, 1992.
2. Rinehart KL Jr, Gloer JB, Hughes RG Jr, Renis HE, McGovren JP, Swynenberg EB, Stringfellow DA, Kuentzel SL and Li LH, Didemnins: Antiviral and antitumor depsipeptides from a caribbean tunicate. *Science* **212:** 933–935, 1981.
3. Jiang TL, Liu RH and Salmon SE, Antitumor activity of didemnin B in the human tumor stem cell assay. *Cancer Chemother Pharmacol* **11:** 1–4, 1983.
4. Li LH, Timmins LG, Wallace TL, Krueger WC, Prairie MD and Im WB, Mechanism of action of didemnin B, a depsipeptide from the sea. *Cancer Lett* **23:** 279–288, 1984.
5. Sakai R, Rinehart KL, Kishore V, Kundu B, Faircloth G, Gloer JB, Carney JR, Namikoshi M, Sun F, Hughes RG Jr, Garcia Gravalos D, de Quesada TG, Wilson GR and Heid RM, Structure–activity relationships of the didemnins. *J Med Chem* **39:** 2819–2834, 1996.
6. Rinehart KL Jr, Gloer JB, Wilson GR, Hughes RG Jr, Li LH, Renis HE and McGovren JP, Antiviral and antitumor compounds from tunicates. *Fedn Proc* **42:** 87–90, 1983.
7. Benvenuto JA, Newman RA, Bignami GS, Raybould TJ, Raber MN, Esparza L and Walters RS, Phase II clinical and pharmacological study of didemnin B in patients with metastatic breast cancer. *Invest New Drugs* **10:** 113–117, 1992.

8. Cain JM, Liu PY, Alberts DE, Gallion HH, Laufman L, O'Sullivan J, Weiss G and Bickers JN, Phase II trial of didemnin-B in advanced epithelial ovarian cancer. A Southwest Oncology Group study. *Invest New Drugs* **10:** 23–24, 1992.
9. Dorr FA, Kuhn JG, Phillips J and Von Hoff DD, Phase I clinical and pharmacokinetic investigation of didemnin B, a cyclic depsipeptide. *Eur J Cancer Clin Oncol* **24:** 1699–1706, 1988.
10. Jacobs AJ, Blessing JA and Munoz A, A phase II trial of didemnin B (NSC No. 325319) in advanced and recurrent cervical carcinoma: A Gynecologic Oncology Group study. *Gynecol Oncol* **44:** 268–270, 1992.
11. Jones DV Jr, Ajani JA, Blackburn R, Daugherty K, Levin B, Patt YZ and Abbruzzese JL, Phase II study of didemnin B in advanced colorectal cancer. *Invest New Drugs* **10:** 211–213, 1992.
12. Lad T, Mullane M, Swedler R, Kilton L, Johnson P, Albain K, Blough R and Weidner L, Phase II trial of didemnin B in non-small cell lung cancer. An Illinois Cancer Council study. *Proc Annu Meet Am Soc Clin Oncol* **10:** A891, 1991.
13. Malfetano JH, Blessing JA, Homesley HD, Look KY and McGehee R, A phase II trial of didemnin B (NSC #335319) in patients with advanced squamous cell carcinoma of the cervix. A Gynecologic Oncology Group study. *Am J Clin Oncol* **19:** 184–186, 1996.
14. Malfetano JH, Blessing JA and Jacobs AJ, A phase II trial of Didemnin B (NSC #335319) in patients with previously treated epithelial ovarian cancer. A Gynecologic Oncology Group study. *Am J Clin Oncol* **16:** 47–49, 1993.
15. Razis E, Mittelman A, Coombe N, Puccio C, Chun H, Ahmed T, Helson L, Cook P and Arlin Z, Phase II clinical trial of didemnin B (Dnb) in patients (Pts) with glioblastoma multiforme (Gm). *Proc Annu Meet Am Soc Clin Oncol* **11:** A432, 1992.
16. Shin DM, Holoye PY, Murphy WK, Forman A, Papasozomenos SC, Hong WK and Raber M, Phase I/II clinical trial of didemnin B in non-small-cell lung cancer: Neuromuscular toxicity is dose-limiting. *Cancer Chemother Pharmacol* **29:** 145–149, 1991.
17. Shin DM, Holoye PY, Murphy WK, Forman A, Papasozomenus S, Jones E, Raber M and Hong WK, Phase I clinical trial of didemnin-B (Nsc 325319:Dn-B) in non-small cell lung cancer; neuromuscular toxicity (Nmt) is dose-limiting. *Proc Annu Meet Am Soc Clin Oncol* **10:** A249, 1991.
18. Shin DM, Holoye PY, Forman A, Winn R, Perez-Soler R, Dakhil S, Rosenthal J, Raber MN and Hong WK, Phase II clinical trial of didemnin B in previously treated small cell lung cancer. *Invest New Drugs* **12:** 243–249, 1994.
19. Stewart JA, Low JB, Roberts JD and Blow A, A phase I clinical trial of didemnin B. *Cancer* **68:** 2550–2554, 1991.
20. Taylor SA, Goodman P, Crawford ED, Stuckey WJ, Stephens RL and Gaynor ER, Phase II evaluation of didemnin B in advanced adenocarcinoma of the kidney. A Southwest Oncology Group study. *Invest New Drugs* **10:** 55–56, 1992.
21. Weiss RB, Peterson BL, Allen SL, Browning SM, Duggan DB and Schiffer CA, A phase II trial of didemnin B in myeloma. A Cancer and Leukemia Group B (CALGB) study. *Invest New Drugs* **12:** 41–43, 1994.
22. Williamson SK, Wolf MK, Eisenberger MA, O'Rourke M, Brannon W and Crawford ED, Phase II evaluation of didemnin B in hormonally refractory metastatic prostate cancer. A Southwest Oncology Group study. *Invest New Drugs* **13:** 167–170, 1995.
23. Jouin P, Poncet J, Dufour M-N, Aumelas A and Pantaloni A, Antineoplastic activity of didemnin congeners: Nordidemnin and modified chain analogues. *J Med Chem* **34:** 487–491, 1991.
24. Mayer SC, Ramanjulu J, Vera MD, Pfezenmayer AJ and Joullié MM, Synthesis of new didemnin B analogues for investigations of structure/biological activity relationships (SAR). *J Org Chem* **59:** 5192–5205, 1994.
25. Sakai R, Stroh JG, Sullins DW and Rinehart KL, Seven new didemnins from the marine tunicate *Tridemnum solidum*. *J Am Chem Soc* **117:** 3734–3748, 1995.
26. Faircloth G, Grant W, Nam S, Jimeno J, Manzanares I and Rinehart KL, Scheduled dependency of aplidine, a marine depsipeptide with antitumor activity. *Proc Am Assoc Cancer Res* **40:** 394, 1999.
27. Urdiales JL, Morata P, De Castro IN and Sanchez-Jimenez F, Antiproliferative effect of dehydrodidemnin B (DDB), a depsipeptide isolated from Mediterranean tunicates. *Cancer Lett* **102:** 31–37, 1996.
28. Legrue SJ, Sheu T-L, Carson DD, Laidlaw JL and Sanduja SK, Inhibition of T-lymphocyte proliferation by the cyclic polypeptide didemnin B: No inhibition of lymphokine stimulation. *Lymphokine Res* **7:** 21–29, 1988.
29. SirDeshpande BV and Toogood PL, Mechanism of protein synthesis inhibition by didemnin B *in vitro*. *Biochemistry* **34:** 9177–9184, 1995.
30. Shen GK, Zukoski CF and Montgomery DW, A specific binding site in Nb2 node lymphoma cells mediates the effects of didemnin B, an immunosuppressive cyclic peptide. *Int J Immunopharmacol* **14:** 63–73, 1992.
31. Crampton SL, Adams EG, Kuentzel SL, Li LH, Badiner G and Bhuyan BK, Biochemical and cellular effects of didemnins A and B. *Cancer Res* **44:** 1796–1801, 1984.
32. Crews CM, Collins JL, Lane WS, Snapper ML and Schreiber SL, GTP-dependent binding of the antiproliferative agent didemnin to elongation factor 1α. *J Biol Chem* **269:** 15411–15414, 1994.
33. Montgomery DW and Zukoski CF, Didemnin B: A new immunosuppressive cyclic peptide with potent activity *in vitro* and *in vivo*. *Transplantation* **40:** 49–56, 1985.
34. Dominice C, Dufour M-N, Patino N, Manzoni P, Jouin P and Guillon G, Dual effects of nordidemnin on WRK_1 cells: Inhibition of phosphoinositide metabolism and cell proliferation. *J Pharmacol Exp Ther* **271:** 107–117, 1994.
35. Crews CM, Lane WS and Schreiber SL, Didemnin binds to the protein palmitoyl thioesterase responsible for infantile neuronal ceroid lipofuscinosis. *Proc Natl Acad Sci USA* **93:** 4316–4319, 1996.
36. Grubb DR, Wolvetang EJ and Lawen A, Didemnin B induces cell death by apoptosis: The fastest induction of apoptosis ever described. *Biochem Biophys Res Commun* **215:** 1130–1136, 1995.
37. Beidler DR, Tewari MT, Friesen PD, Poirier G and Dixit VM, The baculovirus p35 protein inhibits Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem***270:** 16526–16528, 1995.
38. Tewari M and Dixit VM, Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus *crmA* gene product. *J Biol Chem* **270:** 3255–3260, 1995.
39. Salvesen GS and Dixit VM, Caspases: Intracellular signaling by proteolysis. *Cell* **91:** 443–446, 1997.
40. Johnson KL, Vaillant F and Lawen A, Protein tyrosine kinase inhibitors prevent didemnin B-induced apoptosis in HL-60 cells. *FEBS Lett* **383:** 1–5, 1996.
41. Vazquez D, Inhibitors of protein synthesis. *FEBS Lett* **40:** S63–S84, 1974.
42. Lobo C, Garcia-Pozo SG, Nunez de Castro I and Alonso FJ, Effect of dehydrodidemnin B on human colon carcinoma cell lines. *Anticancer Res* **17:** 333–336, 1997.
43. Meng L, Sin N and Crews CM, The antiproliferative agent didemnin B uncompetitively inhibits palmitoyl protein thioesterase. *Biochemistry* **37:** 10488–10492, 1998.