Mechanism of Protein Synthesis Inhibition by Didemnin B *in Vitro*†

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ABSTRACT: The cytotoxic and immunosuppressive marine depsipeptide didemnin B is a potent inhibitor of protein biosynthesis in intact cells. Here, didemnin B is shown to inhibit protein synthesis *in vitro* during the elongation cycle, by preventing eukaryotic elongation factor 2- (eEF-2-) dependent translocation. No inhibition of aminoacyl-tRNA delivery or of peptidyltransferase activity is observed. Didemnin B stimulates eEF-1α-dependent aminoacyl-tRNA binding to rabbit reticulocyte ribosomes, and eEF-1α is required for inhibition of the subsequent translocation of phenylalanyl-tRNA^{Phe} from the A- to the P-site. These observations suggest that didemnin B prevents translocation by stabilizing aminoacyl-tRNA bound to the ribosomal A-site, similar to the antibiotic kirromycin, and consistent with the known affinity of didemnins for elongation factor eEF-1α [Crews et al. (1994) *J. Biol. Chem. 269*, 15411]. Unlike kirromycin, didemnin B does not prevent peptide bond formation, so inhibition is observed only at the translocation step. Inhibition of translocation by didemnin B is attenuated by increasing concentrations of eEF-2.

Marine organisms provide a rich source of biologically active natural products, some of which show potential as novel chemotherapeutic agents (Flam, 1994). However, progress in identifying these compounds, and elucidation of their structures, has outpaced the determination of their various mechanisms of action. One class of marine natural products that has attracted considerable attention is the cyclic depsipeptides known as didemnins, originally isolated from the Caribbean tunicate *Tridemnun solidum* (Rinehart et al., 1981; Li & Joullié, 1992). Didemnin B (Figure 1) displays encouraging antineoplastic and antiviral activity *in vitro*; for example, it inhibits cell growth in human tumor stem-cell assays at concentrations from 1 to 100 nM (Jiang et al., 1983; Rinehart et al., 1983; Rinehart, 1985). However, results from phase II clinical trials indicate that this compound has little or no activity against cancer in humans, possibly due to its rapid conversion to an inactive metabolite (Malfetano et al., 1993; Jones et al., 1992; Dorr et al., 1988; Shin et al., 1991; Weiss et al., 1994; Sondak et al., 1994). Inhibition of T-cell proliferation by didemnin B has led to its evaluation as an immunosuppressive agent (Montgomery et al., 1985; Teunissen et al., 1992). Didemnin B is a more potent inhibitor of T-cell proliferation than cyclosporine and has been used successfully to prolong allograft survival in mice (Stevens et al., 1989; Yuh et al., 1989; Alfrey et al., 1992). However, no data are available pertaining to the effect of didemnins as immunosuppressive agents in humans.

The structure of didemnin B is known from X-ray crystallography (Hossain et al., 1988) and solution NMR studies (Searle et al., 1989; Kessler et al., 1990), and several syntheses have been completed (Rinehart et al., 1987; Schmidt et al., 1988; Hamada et al., 1989; Li et al., 1990; Jouin et al., 1989, 1991; Mayer et al., 1994). However, an understanding of this compound's mechanism of action and structure−function requirements has been slow to develop (Li et al., 1984; Crampton et al., 1984; Gschwendt et al., 1987, 1989; Legrue et al., 1988; Shen et al., 1992; Crews et al., 1994; Dominice et al., 1994). Didemnin B inhibits DNA and protein synthesis, and to a lesser extent RNA synthesis, in intact tumor cells, and it has been suggested that didemnin B cytotoxicity is primarily a consequence of its effect upon translation (Rinehart et al., 1983; Li et al., 1984). In accord with this hypothesis, didemnin B completely inhibits the *in vitro* translation of mouse liver RNA in rabbit reticulocyte lysates (Legrue et al., 1988). Shen and co-workers have provided evidence for a cytosolic receptor for didemnin B in the 100000*g* supernatant derived from Nb2 node lymphoma cells (Shen et al., 1992) and recently Crews and co-workers reported the GTP-dependent binding of didemnin A derivatives to elongation factor eEF-1α[1] (Crews et al., 1994). However, other workers have shown that the closely related analog nordidemnin prevents WRK$_1$ cell growth by

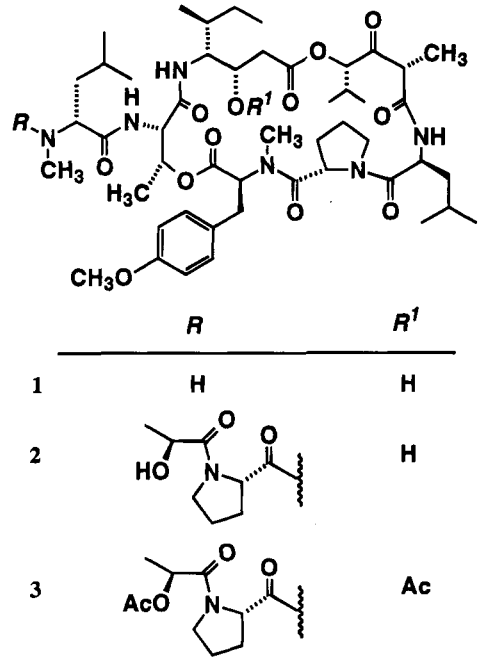

FIGURE 1: Structures of the didemnins. **1**, didemnin A; **2**, didemnin B; **3**, diacetyldidemnin B.

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interfering with *myo*-inositol uptake and reducing the intracellular inositol phosphate pool (Dominice et al., 1994).

During our studies of the molecular basis of didemnin cytotoxicity, we have examined the effect of didemnin B upon *in vitro* translation in eukaryotic cell lysates and we have investigated the mechanism of protein synthesis inhibition (SirDeshpande & Toogood, 1993). Here, we provide evidence that didemnin B exerts a direct effect upon the cell's translation machinery without a requirement for the intact cell, stabilizing aminoacyl-tRNA bound to the ribosomal A-site and inhibiting translocation. These results are discussed in relation to the biological effects of didemnin B upon intact cells and in the context of other known protein synthesis inhibitors.

EXPERIMENTAL PROCEDURES

Materials. L-[^{35}S]Methionine (1000 Ci mmol^{-1}) and [^{14}C]-phenylalanine (>450 mCi mmol^{-1}; ~1000 cpm pmol^{-1}) were obtained from New England Nuclear (Wilmington, DE). Rabbit reticulocyte lysates were obtained from Boehringer Mannheim (Indianapolis, IN) or from Green Hectares (Oregon, WI). Tobacco mosaic virus (TMV) mRNA, poly-(U), and 5′-guanylyl imidodiphosphate (GMPPNP) were purchased from Boehringer Mannheim. The GMPPNP contains less than 0.2% GTP according to the manufacturer's specifications. Liquid scintillation counting was performed using 5 mL of Scintiverse/sample (Fisher Scientific; Pittsburgh, PA) in a Packard 1600 TR liquid scintillation spectrometer. Didemnin B was a generous gift of Dr. Kenneth L. Rinehart Jr. (University of Illinois) and the Upjohn Chemical Co. Didemnin A was supplied by Dr. Madeleine Joullié (University of Pennsylvania). Sparsomycin was a gift from Mr. Don Harper of the Upjohn Chemical Co. Diacetyldidemnin B was prepared as described by Rinehart (1985). Rabbit elongation factors eEF-1α and eEF-2 were kindly donated by Dr. William C. Merrick (Case Western Reserve). Yeast eEF-1α was isolated following the procedure reported by Thiele et al. (1985). Aminoacyl-tRNA synthetases were prepared as described by Irvin and Hardesty (1972).

Aminoacylation and Acetylation of tRNA^{Phe}. Phe-tRNA^{Phe} was prepared using crude rabbit aminoacyl-tRNA synthetases as described by Odom et al. (1990) and further purified by passage through a Sephadex G-50 column eluting with 50 mM $Mg(OAc)_2$ and 10 mM NaOAc. It was stored as a 5 μM solution in 50 mM $Mg(OAc)_2$ and 10 mM NaOAc. The charging efficiency in these reactions is typically ≥900 pmol/A_{260} unit tRNA^{Phe}. Ac-Phe-tRNA^{Phe} was prepared according to Haenni and Chappeville (1966) and purified as described by Moazed and Noller (1989).

Preparation of Ribosomes. Salt-washed 80S ribosomes were isolated from rabbit reticulocyte lysates (Green Hectares, WI) as described by Merrick (1979) and sucrose-cushioned as described by Váquez et al. (1974). Ribosome activity was measured using the poly(U) assay (Merrick, 1979) and the synthesis of poly(phenylalanine) was demonstrated to be dependent upon the addition of elongation factors. From a typical preparation, 15 pmol of ribosomes was able to polymerize 6−7 pmol out of 10 pmol of added [^{14}C]Phe-tRNA^{Phe}, as determined by scintillation counting of the hot trichloroacetic acid- (TCA-) insoluble material. The amount of ribosomes used in individual assays was optimized for [^{14}C]Phe-tRNA^{Phe} binding, or the ability to translocate bound [^{14}C]Phe-tRNA^{Phe}, by examining the activities of different ratios of ribosomes to [^{14}C]Phe-tRNA^{Phe}.

Protein Synthesis Assays. In vitro translation was performed using commercially prepared cell-free translation systems as described in the manufacturer's instructions (Boehringer Mannheim), except that reaction mixtures (25 μL) contained 1 μL of inhibitor solution and 5 μL of TMV mRNA (40 μg mL^{-1} stock; the mRNA was determined not to be rate-limiting at this concentration and protein synthesis is linear with time for at least 1 h). Protein synthesis was initiated by the addition of TMV mRNA and the reaction mixtures were incubated at 37 °C for 60 min unless otherwise specified. The amount of protein synthesized was determined by precipitation onto a glass fiber filter (Whatman GF/C) using 10% TCA and scintillation counting of the dried filter.

Peptidyltransferase. Peptidyltransferase activity was assayed as described by Wurmbach and Nierhaus (1979). Sucrose-cushioned 80S ribosomes (28 pmol), and 6 μg of poly(U) were incubated in 50 μL of 50 mM HEPES-KOH, pH 7.5, containing 15 mM $MgCl_2$, 100 mM NH_4Cl, 5 mM β-mercaptoethanol, and inhibitor at 37 °C for 10 min, then 25 pmol of *N*-Ac-[^{14}C]Phe-tRNA^{Phe} was added, and incubation was continued for 20 min. A 10-μL aliquot of this mixture was removed, diluted with 1 mL of HEPES buffer, and filtered through a Millipore Type HA nitrocellulose filter to measure the amount of *N*-Ac-[^{14}C]Phe-tRNA^{Phe} bound to ribosomes. To the remaining mixture (40 μL) was added 5 μL of a 10 mM puromycin solution, and the sample was kept on ice for 1 h and then incubated at 37 °C for 5 min. NaOAc (1 M, 30 μL) was added and the *N*-Ac-Phe-puromycin was extracted (vortexed and then centrifuged) with ethyl acetate (1.4 mL). The amount of radioactivity in the organic extract was determined by scintillation counting of a 1-mL aliquot. In a typical experiment, approximately 20% of the *N*-Ac-Phe-tRNA^{Phe} binds to ribosomes, and 50% of the bound *N*-Ac-Phe-tRNA^{Phe} is reactive with puromycin, indicating that it is located in the ribosomal P-site.

Aminoacyl-tRNA Binding. eEF-1α-Dependent aminoacyl-tRNA binding experiments were performed following the procedures outlined by Merrick (1979) using either GTP or GMPPNP. Reaction mixtures (50 μL) containing HEPES buffer (20 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 100 mM KCl, and 1.0 mM DTT), 0.2 μg of poly(U), 89 pmol of 80S ribosomes, 10 pmol of [^{14}C]Phe-tRNA^{Phe}, 1.5 μL of eEF-1α, (1.55 mg mL^{-1}), and 5 μL of 5′-guanylyl imidodiphosphate (1.5 mM, pH 7.0) were incubated at 37 °C for 30 min. To determine the amount of ribosome-bound [^{14}C]Phe-tRNA^{Phe}, an aliquot (5 μL) of the assay mixture was removed, diluted with 0.8 mL of HEPES buffer, and filtered through a Millipore Type HA nitrocellulose filter. The filter was rinsed with HEPES buffer (5 × 2 mL), dried at 60 °C for 10 min, and counted.

[1] Abbreviations: eEF-, eukaryotic elongation factor; NMR, nuclear magnetic resonance, Ac-Phe, *N*-acetylphenylalanine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); DTT, dithiothreitol; GTP, guanosine 5′-triphosphate.

Nonenzymic binding of [^{14}C]Phe-tRNA^{Phe} to ribosomes was performed as described in the literature (Ayuso et al., 1968; Carrasco et al., 1975).[2] Ribosomes (80S, 177 pmol) were incubated with 20 pmol of [^{14}C]Phe-tRNA^{Phe} and 40 μg of poly(U) in 100 μL of Tris-HCl buffer (50 mM Tris-HCl, pH 7.5, and 60 mM KCl) and 20 mM $MgCl_2$ at 37 °C for 30 min. To measure the amount of ribosome-bound [^{14}C]-Phe-tRNA^{Phe}, 10 μL of the assay mixture was removed and diluted into 0.8 mL of Tris-HCl buffer containing 20 mM $MgCl_2$, and filtered through a Type HA nitrocellulose filter. The filter was rinsed with buffer (2 × 5 mL), dried at 60 °C for 10 min, and counted.

Translocation. Inhibition of translocation was determined following the general approach described by Vázquez et al. (1974). Elongation factor-dependent [^{14}C]Phe-tRNA^{Phe} binding to 89 pmol ribosomes was performed as described above. The amount of P-site-bound [^{14}C]Phe-tRNA^{Phe} was determined for an identical sample using the puromycin reaction (Wurmbach & Nierhaus, 1979). Less than 5% of the bound [^{14}C]Phe-tRNA^{Phe} was puromycin-active, suggesting that all of the bound [^{14}C]Phe-tRNA^{Phe} is in the ribosomal A-site. Didemnin B (0.8 μL of a 3 mM solution in ethanol) or fusidic acid (1.5 μL of a 90 mM solution in H_2O) was added to the assay mixture, which then was incubated at 37 °C for 10 min. Next, eEF-2 (0.5 μL of a 0.79 mg mL^{-1} solution), 4.5 μL of 10× HEPES buffer, 10 μL of puromycin (10 mM in H_2O, pH 7.0), and 23 μL of H_2O were added, followed by addition of 6 μL of GTP (15 mM) to initiate translocation. Following incubation at 37 °C for a further 30 min, the amount of P-site-bound [^{14}C]Phe-tRNA^{Phe} was determined by extraction of the [^{14}C]Phe-puromycin into ethyl acetate and scintillation counting of the extract as described previously.

To assay translocation following nonenzymic binding of [^{14}C]Phe-tRNA^{Phe} to ribosomes, 9 μL of 10× Tris-HCl buffer (pH 7.5) was added to a 90-μL assay mixture, followed by 12 μL of GTP (15 mM in H_2O, pH 7.0), 5 μL of $MgCl_2$ solution (36 mM in H_2O), and inhibitor solution (or control) to a total volume of 180 μL ([Mg^{2+}] = 10 mM; [didemnin] = 50 μM or [fusidic acid] = 3 mM). Following incubation at 37 °C for 15 min, a 20-μL aliquot was diluted into 0.8 mL of Tris-HCl buffer containing 10 mM $MgCl_2$, and precipitated onto nitrocellulose as described to determine the amount of [^{14}C]Phe-tRNA^{Phe} bound to ribosomes immediately prior to translocation. Translocation was initiated by the addition of 2 μL of eEF-2 (0.79 mg mL^{-1} stock), 2 μL of 10× Tris-HCl buffer containing 100 mM $MgCl_2$, 10 μL of puromycin solution (10 mM in H_2O), and H_2O to increase the volume of the reaction assay to 200 μL. Following incubation at 37 °C for 30 min, the reaction was quenched with 400 μL of ammonium bicarbonate and extracted with 800 μL of ethyl acetate. The amount of [^{14}C]Phe-puromycin formed was measured by scintillation counting of a 600-μL aliquot of the organic layer.

Peptide Bond Formation. Peptide bond formation between Ac-Phe-tRNA^{Phe} and Phe-tRNA^{Phe} was assayed in 100-μL reaction mixtures containing 10 μL of sucrose-cushioned ribosomes (83 pmol), 20 μg of poly(U), 10 μL of a 10 mM GTP solution (pH 7.0), 10 μg of yeast eEF-1α (1 mg mL^{-1}), 0.5 μL of eEF-2 (0.79 mg mL^{-1}), 10 pmol of Ac-[^{14}C]Phe-tRNA^{Phe}, and either didemnin B (final concentration 50 μM), sparsomycin (final concentration 150 μM), or carrier, in buffer (50 mM Tris-HCl, pH 7.5, 80 mM KCl, 10 mM $MgCl_2$, and 2.5 mM DTT). The mixtures were incubated at 37 °C for 10 min. A 10-μL aliquot of this mixture was used to measure the amount of Ac-[^{14}C]Phe-tRNA^{Phe} bound to ribosomes by dilution into 1 mL of buffer and filtration through a nitrocellulose filter, which was rinsed and counted as before. The amount of P-site-bound Ac-[^{14}C]Phe-tRNA^{Phe} was determined for an identical sample using the puromycin reaction. Mixtures were quenched with 400 μL of 0.1 M NaOAc, pH 5.5, and extracted with 800 μL of ethyl acetate by vortexing. The amount of Ac-[^{14}C]Phe-puromycin formed was measured by scintillation counting of 600 μL of the organic layer. To identical samples were added 10 pmol of [^{14}C]Phe-tRNA^{Phe}, 20 μL of yeast eEF-1α (1 mg mL^{-1}), 5 μL of buffer (10× previous concentrations), 10 μL of KCl (280 mM), and 10 μL of H_2O, increasing the volume of each assay to 140 μL. These samples were incubated at 37 °C for a further 20 min, and a 10-μL aliquot was precipitated onto nitrocellulose as described earlier to estimate the amount of ribosome-bound ^{14}C label. The remainder of the reaction mixture was diluted with 2 volumes of 0.3 M KOH and incubated at 50 °C for 15 min. The hydrolyzed samples were neutralized with acetic acid and supplemented with unlabeled Phe, *N*-Ac-Phe, and *N*-Ac-Phe-Phe (prepared from commercially available phenylalanine). HPLC analysis of these samples was performed on a Waters System 625 LC equipped with a 3.9- × 150-mm Delta Pak C-18 column and a Waters 994 photodiode array detector. Elution was performed using a convex gradient of 0−65% acetonitrile in 10 mM K_2HPO_4, pH 2.1, at 0 °C; elution times were Phe, 9.5 min; Ac-Phe, 13.2 min; and Ac-Phe-Phe, 14.8 min (Gast et al., 1985). Radioactivity was monitored by counting 200-μL aliquots from 2-mL fractions.

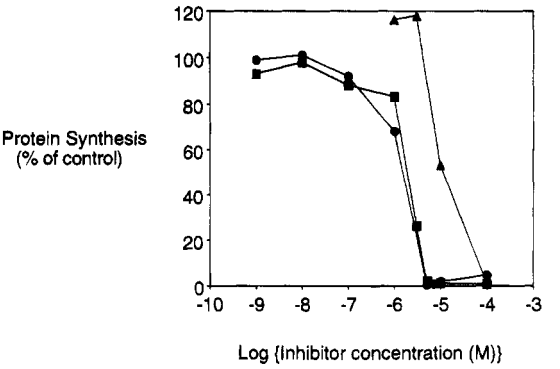

FIGURE 2: Inhibition of protein synthesis by didemnins. Protein synthesis assays were performed as described in the experimental section: (●) didemnin A; (■) didemnin B; (▲) diacetyldemnin B. The data are expressed as a percentage of the amount of protein obtained in the absence of the inhibitor (control). Five picomoles of protein was synthesized in the control. Each point represents the mean of at least three independent determinations and is reproducible to ±10%.

RESULTS

Inhibition of *in vitro* protein synthesis in rabbit reticulocyte lysates was observed with didemnin A (IC_{50} = 3 μM), didemnin B (IC_{50} = 3 μM), and diacetyldidemin B (IC_{50} = 50 μM; Figure 2). Similar results were obtained for didemnin B using β-globin or TMV mRNA, and in yeast or

[2] Nonenzymic binding of Phe-tRNA^{Phe} to ribosomes is performed at [Mg^{2+}] = 20 mM but the [Mg^{2+}] is readjusted to 10 mM to assay for translocation.

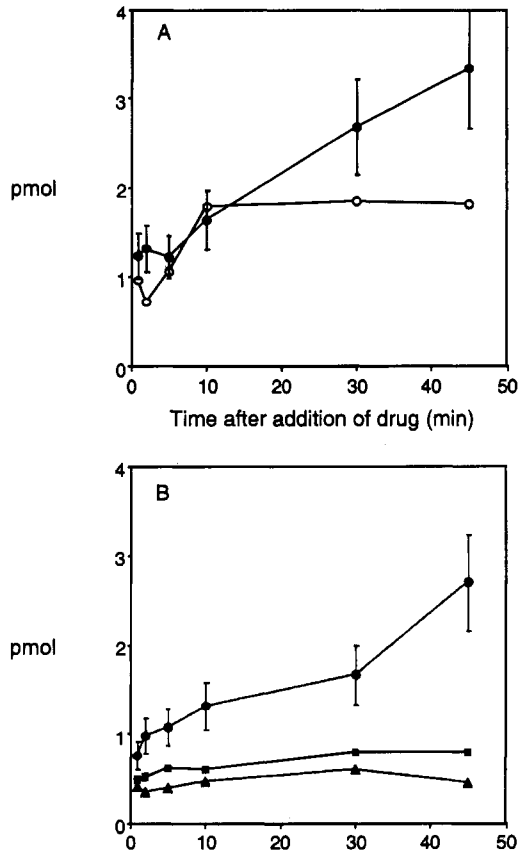

FIGURE 3: Time course for the inhibition of protein synthesis. (A) Inhibition of initiation; (B) inhibition of elongation. The effect upon *in vitro* protein synthesis of no drug (●), pyrocatechol violet (○), didemnin B (▲), and cycloheximide (■) was determined by the addition of each drug to an active rabbit reticulocyte cell-free translation mixture 15 min following the addition of TMV mRNA. Different samples were allowed to incubate for 1, 2, 5, 10, and 30 min following the addition of inhibitor. At each time point three separate 3-μL samples were withdrawn for precipitation and scintillation counting. Each point represents the mean of three determinations of the amount of ^{35}S-labeled protein synthesized in picomoles. For clarity, error bars are indicated for the controls only.

wheat germ cell-free translation systems. However, inhibition of cell-free translation in *Escherichia coli* S30 extracts was not observed with up to 50 μM didemnin B (data not shown). The time course for inhibition of protein synthesis by didemnin B in rabbit reticulocyte lysates was determined in comparison with the time course for inhibition by cycloheximide and pyrocatechol violet. Inhibition by pyrocatechol violet (2 mM) occurred approximately 8 min following its addition to the translation mixture (Figure 3A), as expected for an inhibitor of the initiation stage of protein synthesis (Lodish et al., 1971; Huang & Grollman, 1973). In contrast, the addition of cycloheximide (10 μM), a known inhibitor of polypeptide elongation (Baliga et al., 1970), resulted in immediate inhibition of translation. Didemnin B (10 μM) also causes immediate inhibition of protein synthesis (Figure 3B), and we conclude that didemnin B inhibits protein synthesis at the elongation stage.

To determine how didemnin B inhibits polypeptide elongation, each step of this process was examined independently (Merrick, 1992). The effect of didemnin B upon elongation factor eEF-1α-dependent aminoacyl-tRNA delivery was investigated as follows: the amount of [^{14}C]Phe-tRNA^{Phe} bound to salt-washed ribosomes following incubation with eEF-1α and the nonhydrolyzable GTP analog 5′-guanylyl imidodiphosphate (GMPPNP) at 37 °C for 30 min was determined in the presence and absence of 50 μM didemnin B, by collection of the ribosomes on a nitrocellulose filter and scintillation counting to quantify the amount of bound radiolabel. No inhibition of [^{14}C]Phe-tRNA^{Phe} binding to ribosomes was observed; instead, didemnin B stimulates the binding of [^{14}C]Phe-tRNA^{Phe} to ribosomes by up to 90% (Table 1). Didemnin B also stimulates [^{14}C]Phe-tRNA^{Phe} binding to ribosomes in the presence of eEF-1α and GTP (data not shown). However, GMPPNP was routinely used in these and subsequent experiments to circumvent the possibility of translocation occurring due to small contaminating levels of eEF-2, although the absence of poly(U)-dependent poly(Phe) synthesis by purified ribosomes in the absence of added elongation factors suggests that the levels of eEF-1α and eEF-2 in these preparations are negligible.

Table 1: eEF-1α-Dependent [^{14}C]Phe-tRNA^{Phe} Binding[a]

[^{14}C]Phe-tRNA^{Phe} bound (pmol)	
no drug	+ 50 μM DB
4.9 ± 0.5	9.3 ± 0.5

[a] Elongation factor-dependent binding to rabbit reticulocyte ribosomes was measured using 10 pmol of [^{14}C]Phe-tRNA^{Phe} and 89 pmol of ribosomes as described in the experimental section. Results shown are the average of two independent determinations. However, stimulation of Phe-tRNA^{Phe} binding by didemnin B (DB) was observed consistently at all concentrations examined from 50 to 0.5 μM, in 10 independent experiments.

Table 2: Puromycin Assay for Peptidyltransferase Activity[a]

assay mixture	amount of Ac-Phe-puromycin (% of control)
control (no inhibitor)	100
+ 50 μM didemnin B	98 ± 17
+ 100 μM cycloheximide	125 ± 29
+ 20 μM sparsomycin	32 ± 9

[a] Peptidyltransferase activity was assayed as described in the experimental section with the specified inhibitor concentrations. Approximately 2.5 pmol of Ac-Phe-puromycin is formed in control reactions in the absence of inhibitors. The amount of Ac-Phe-puromycin formed is a measure of the level of peptidyltransferase activity.

The effect of didemnin B upon ribosome-catalyzed peptide bond formation was studied using the puromycin reaction (Wurmbach & Nierhaus, 1979). The formation of (*N*-acetylphenylalanyl)puromycin, following specific binding of Ac-Phe-tRNA^{Phe} to the P-site, was inhibited by sparsomycin (20 μM), a known peptidyltransferase inhibitor (van den Broek et al., 1987), but was not affected by the addition of cycloheximide (100 μM), an inhibitor of translocation (Vazquez, 1979), or by didemnin B (50 μM; Table 2). This result indicates that didemnin B does not inhibit the ribosome's intrinsic peptidyltransferase activity.

Ribosome-dependent synthesis of (Ac-phenylalanyl)phenylalanine (Ac-Phe-Phe) in the presence of eEF-1α was examined following eEF-1α-dependent binding of [^{14}C]Phe-tRNA^{Phe} to ribosomes already containing Ac-[^{14}C]Phe-tRNA^{Phe} in the P-site (puromycin-active: Haenni & Lucas-Lenard, 1968; Schilling-Bertetzko et al., 1992). The products were hydrolyzed and analyzed by high-performance liquid chromatography (Gast et al., 1985). Ac-Phe-Phe was formed

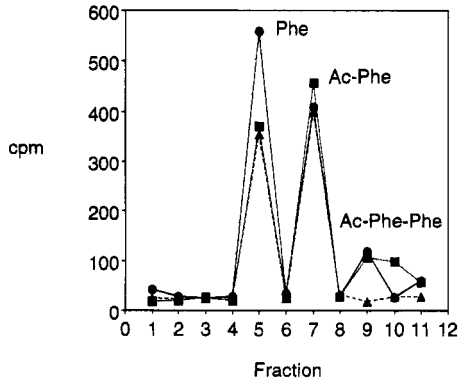

FIGURE 4: Assay for peptide bond formation. The graph shows radioactivity contained in 200-μL aliquots of 2-mL HPLC fractions following the preparation and hydrolysis of assay mixtures as described in the experimental section. The fractions containing Phe, Ac-Phe, and Ac-Phe-Phe were identified by UV absorbance monitoring of the eluant. Symbols represent (●) no drug, (■) + 50 μM didemnin B; (▲), no ribosomes. A sample containing 250 μM sparsomycin gave identical results to the no-ribosome control. The detection of ^{14}C-labeled Ac-Phe-Phe in samples containing didemnin B indicates that peptide bond formation is not inhibited.

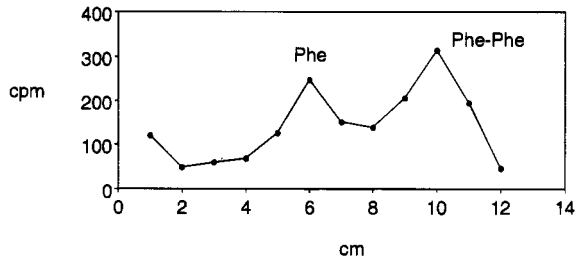

FIGURE 5: Peptide bond formation between Phe-tRNA^{Phe} (P-site) and Phe-tRNA^{Phe} (A-site). Didemnin B (50 μM) was added to posttranslocative ribosomes (89 pmol) containing P-site bound [^{14}C]-Phe-tRNA^{Phe} obtained as described in the experimental section following eEF-1α-dependent [^{14}C]Phe-tRNA^{Phe} binding and translocation, except that no puromycin was added. Samples were incubated at 37 °C for 15 min. Additional [^{14}C]Phe-tRNA^{Phe} (10 pmol) and eEF-1α (1.5 μL of a 1.55 mg mL^{-1} solution) were added, and incubation was continued at 37 °C for 30 min. Analysis of the products was performed by paper chromatography (Ravel, 1967) following filtration of the ribosomes onto nitrocellulose and hydrolysis overnight in 500 μL of 1 M NH_4OH containing 50 μg of phenylalanine and 50 μg of (phenylalanyl)phenylalanine. Radioactive products were detected by scintillation counting of 1-cm strips of the developed chromatogram and identified by chromatographic comparison with authentic samples of Phe and Phe-Phe detected by ninhydrin staining. Data are plotted as counts per min (cpm) for a 1-cm strip at the indicated distance from the origin. No [^{14}C]Phe-Phe was detectable in control samples not containing ribosomes. The presence of [^{14}C]Phe-Phe in samples containing didemnin B indicates that peptide bond formation is not inhibited.

even in the presence of 50 μM didemnin B (Figure 4), indicating that at a concentration 10-fold higher than is required to inhibit protein synthesis, didemnin B does not prevent peptide bond formation. Formation of Ac-Phe-Phe is inhibited by sparsomycin. In a similar experiment, 50 μM didemnin does not inhibit the formation of Phe-Phe following eEF-1α-mediating binding of [^{14}C]Phe-tRNA^{Phe} to posttranslocative ribosomes containing [^{14}C]Phe-tRNA^{Phe} in the P-site (Figure 5; Ravel, 1967).

The next step in the elongation cycle involves translocation of the A-site-bound peptidyl-tRNA to the ribosomal P-site. This process is catalyzed by the 100-kDa protein eEF-2 (Merrick, 1992). To study eEF-2-mediated translocation, salt-washed ribosomes were charged in their A-site (as indicated by lack of puromycin reactivity) with [^{14}C]Phe-tRNA^{Phe} by addition of eEF-1α, [^{14}C]Phe-tRNA^{Phe}, and GMPPNP; eEF-2 and GTP were added to initiate translocation. Following translocation, the [^{14}C]Phe-tRNA^{Phe} can react with puromycin to form [^{14}C]Phe-puromycin, which is extracted with ethyl acetate (Wurmbach & Nierhaus, 1979). Inhibition of translocation by didemnin B was observed whether the inhibitor was present during eEF-1α-mediated binding of [^{14}C]Phe-tRNA^{Phe} or whether it was added subsequent to binding but immediately prior to translocation (Table 3). Since both eEF-1α and eEF-2 are present in this experiment, it is possible that di- and tripeptides may form by delivery of more Phe-tRNA^{Phe} to the A-site following the translocation step. However, this event is unlikely in our experiments because the ribosomes are present in excess and approximately 90% of the available Phe-tRNA^{Phe} is ribosome-bound in the presence of didemnin B. Even if di- and tripeptides are formed in this assay, the observed inhibition most likely occurs at the translocation step, since inhibition of Phe-tRNA^{Phe} binding and peptide bond formation can be excluded on the basis of our independent determinations of the effect of didemnin B on these processes (*vide supra*). Inhibition of translocation was not observed when eEF-1α was omitted from the reaction mixture.

Inhibition of translocation by didemnin B requires eEF-1α-dependent delivery of the Phe-tRNA^{Phe}. eEF-2-Dependent translocation is not inhibited by didemnin B following nonenzymic binding of [^{14}C]Phe-tRNA^{Phe} to the ribosomal A-site (Ayuso et al., 1968; Carrasco et al., 1975).[2] Translocation following nonenzymic binding *is* inhibited by fusidic acid, which stabilies the eEF-2•GDP•ribosome ternary complex (0.05 pmol of [^{14}C]Phe-tRNA^{Phe} is translocated in the presence of 3 mM fusidic acid *versus* 1.5 pmol in the control). These data indicate that eEF-1α is essential for inhibition of translocation by didemnin B and that the mechanism of inhibition by didemnin B differs from the mechanism established for fusidic acid (Bodley et al., 1970a,b; Carrasco & Vázquez, 1973; Bermek & Mattaei, 1971).

Inhibition of translocation by didemnin B is diminished by increasing the concentration of eEF-2 (Table 4), and in experiments using stoichiometric quantities of eEF-2 with respect to ribosomes, only weak inhibition of translocation was observed in the presence of 50 μM didemnin B (data not shown).

DISCUSSION

The inhibition of protein synthesis by didemnins has been examined *in vitro* using rabbit reticulocyte lysates. Inhibition was observed for all three didemnins examined (Figure 2). The relative order of potency of didemnins A and B and diacetyldidemnin B in intact cells is diacetyldidemnin B > didemnin B > didemnin A (Rinehart et al., 1983; Rinehart, 1985; Shen et al., 1992). This order was not observed in cell-free translation assays, in which diacetyldidemnin B is the least effective inhibitor. This change in the relative potency of didemnins as inhibitors of protein synthesis might reflect a difference in mechanism, or could be due to the relative ability of these compounds to cross the cell membrane (Orlowski et al., 1988). Similar switches in the order of potency also have been observed by other workers studying derivatives of didemnin A (Shen et al., 1992).

Table 3: Inhibition of Translocation[a]

[didemnin B] (μM)	Phe-tRNA^{Phe} bound to ribosomes (pmol) (prior to addition of drug)	P-site-bound [^{14}C]Phe-tRNA^{Phe} (pmol) (puromycin-active)		
		before translocation	after translocation	
			−DB	+DB
		Following Enzymic Binding		
25	6.5	0.8	2.2	0.1
24	3.7	−0.1	2.5	0.3
5	6.9	−0.2	2.0	0.5
		Following Nonenzymic Binding		
25	6.9	na	1.5	1.3
13	4.6	0.3	1.0	1.0

[a] Translocation assays were performed as described in the experimental section either with or without eEF-1α (enzymic and nonenzymic binding, respectively). Didemnin B (DB) was added immediately prior to the addition of eEF-2. In assays incorporating eEF-1α-dependent Phe-tRNA^{Phe} binding, similar results were obtained when DB was present during the eEF-1α dependent binding step, except that the amount of Phe-tRNA^{Phe} bound to ribosomes was higher. The estimated error in each value is ±20%. na = data not available.

Table 4: Translocation Dependence on eEF-2[a]

amount of eEF-2 (μg)	translocation (% of control)
0.395	1.9
1.185	12
3.16	18

[a] Translocation was performed as described in the experimental section using 50 μM didemnin B, except that the amount of eEF-2 added was varied. In control experiments 2.2 pmol of [^{14}C]Phe-tRNA^{Phe} was translocated in the absence of didemnin B.

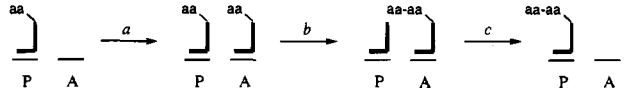

FIGURE 6: Steps in polypeptide elongation. (Step a) Binding of aminoacyl-tRNA; catalyzed by eEF-1α + GTP. (Step b) Peptide bond formation; catalyzed by ribosomal peptidyltransferase. (Step c) Translocation; catalyzed by eEF-2 + GTP. P and A refer to the peptidyl and aminoacyl sites in the ribosome active site; aa = aminoacyl group attached to a tRNA.

To determine which stage of protein synthesis is affected by didemnin B, the time course of inhibition was studied in comparison with inhibitors of known mechanism. Inhibitors of the initiation of protein synthesis are known to exhibit a delayed effect following their addition to an active translation system, as previously initiated polypeptides are completed (Huang & Grollman, 1973). In contrast, inhibitors of polypeptide elongation have an immediate effect upon translation by preventing the further growth of polypeptide chains (Lodish et al., 1971). Didemnin B has an immediate inhibitory effect upon translation, similar to the known inhibitor of elongation, cycloheximide, and in contrast to the known inhibitor of initiation, pyrocatechol violet (Figure 3). From these results, we conclude that didemnin B inhibits polypeptide elongation. This conclusion is supported by the inhibition of poly(U)-directed polyphenylalanine synthesis by didemnin B, which occurs at similar concentrations to those required to inhibit protein synthesis in cell lysates (data not shown).

Inhibition of polypeptide elongation may result from inhibition of aminoacyl-tRNA delivery, inhibition of peptidyltransferase, or inhibition of translocation (Figure 6). No inhibitory effect of didemnin B upon eEF-1α-dependent Phe-tRNA^{Phe} binding could be discerned; instead, didemnin B appears to stimulate or stabilize the eEF-1α-dependent A-site binding of Phe-tRNA^{Phe} (Table 1; a similar effect is not observed for nonenzymic binding in the absence of eEF-1α). Consistent with our observations, Crews and co-workers have found that a didemnin A derivative binds to eEF-1α with K_d = 15 μM and slightly stimulates the ability of this protein to hydrolyze GTP (Crews et al., 1994).

Peptide bond formation is catalyzed by the ribosomal peptidyltransferase. Known inhibitors of eukaryotic peptidyltransferse include the antitumor antibiotic sparsomycin (van den Broek et al., 1987). Peptidyltransferase activity can be assayed using the puromycin reaction, in which P-site-bound Ac-[^{14}C]Phe-tRNA^{Phe} is allowed to react with the polypeptide chain terminator puromycin. When compared to sparsomycin, didemnin B does not inhibit peptidyltransferase activity as measured by this method (Table 2).

Subsequent to peptide bond formation and release of the elongation factor eEF-1α as its GDP complex, a second elongation factor, eEF-2, catalyzes the translocation of peptidyl-tRNA from the A- to the P-site on the ribosome (Merrick, 1992). The effect of didemnin B upon eEF-2-mediated translocation was examined by prebinding [^{14}C]-Phe-tRNA^{Phe} to the ribosomal A-site, initiating translocation with eEF-2, and measuring the puromycin activity of the resulting complexes. The translocated [^{14}C]Phe-tRNA^{Phe} will occupy the ribosomal P-site and will be puromycin-active. Untranslocated [^{14}C]Phe-tRNA^{Phe} remains in the ribosomal A-site and is not reactive with puromycin. Spontaneous slippage of Phe-tRNA^{Phe} from the A- to the P-site, as has been observed with *E. coli* ribosomes at 37 °C, was not a problem in our experiments, possibly due to the presence of some uncharged tRNA^{Phe} in our [^{14}C]Phe-tRNA^{Phe} preparations (Schilling-Bertetzko et al., 1992; Ayuso et al., 1968).

Didemnin B (5 μM) completely inhibits eEF-2-catalyzed translocation following eEF-1α-dependent Phe-tRNA^{Phe} binding, but inhibition of translocation is not observed under similar conditions subsequent to nonenzymic binding of the Phe-tRNA^{Phe} (Table 3). In contrast, inhibition of translocation by fusidic acid is observed in both cases, using catalytic amounts of eEF-2. Fusidic acid is an antibiotic that inhibits protein synthesis by stabilizing the eEF-2•GDP complex, preventing its release from ribosomes (Bodley et al., 1970a,b; Carrasco & Vazquez, 1973; Bermek & Mattaei, 1971). It allows one round of translocation and GTP hydrolysis prior to the onset of inhibition. Didemnin B is a weak inhibitor of eEF-2 GTPase activity (results not shown) but appears not to inhibit protein synthesis by the same mechanism as fusidic acid.

Our results indicate that the presence of eEF-1α is required for inhibition of *in vitro* protein synthesis by didemnin B. Since didemnin B stabilizes eEF-1α-dependent A-site bind-

ing of Phe-tRNA^{Phe}, the observed inhibition of translocation may simply reflect increased affinity of the aminoacyl-tRNA (presumably as a complex with eEF-1α and guanosine phosphate) for the ribosomal A-site. Increasing the concentration of eEF-2 reduces the level of inhibition by didemnin B (Table 4), possibly by competing with eEF-1α for binding to the ribosomes, since it is known that eEF-1α and eEF-2 occupy overlapping binding sites (Nygard & Nilsson, 1990).

Of the known protein synthesis inhibitors (Vázquez, 1979), didemnin B appears to be most similar in its mechanism of action to the well-studied antibiotic kirromycin (Parmeggiani & Swart, 1985). Kirromycin increases the affinity of EF-Tu·GDP for prokaryotic ribosomes, thereby inhibiting subsequent steps. Peptide bond formation is inhibited by the kirromycin·EF-Tu·GDP complex, which presumably prevents correct orientation of the aminoacyl-tRNA 3′-terminus. In contrast, didemnin B does not prevent the formation of Ac-Phe-Phe following eEF-1α-dependent binding of Phe-tRNA^{Phe} to ribosomes already containing P-site-bound Ac-Phe-tRNA^{Phe} (Figure 4). Thus, didemnin B inhibits translation *via* a previously unobserved mechanism and therefore may be useful for examining further details of the events and conformational changes mediated by eukaryotic elongation factors.

Despite indications that the inhibition of protein synthesis is an integral component of the effect of didemnin B upon intact cells, it is not obvious that this effect must arise due to direct interactions between didemnin B and the translational machinery. For example, didemnin B displays antiproliferative effects on intact cells at nanomolar concentrations (Li & Joullié, 1992; Rinehart et al., 1981). Moreover, it inhibits mitogen- and allogen-stimulated lymphocyte blastogenesis at doses several orders of magnitude below those necessary to inhibit protein synthesis in the same cells (Montgomery & Zukoski, 1985) and far below the concentrations that are required to inhibit protein synthesis *in vitro*. These discrepancies between the effective concentrations of didemnin B for inhibiting different cellular events suggest a pleiotropic effect upon intact cells. Indeed, recently published data indicate that didemnins may interfere with cell growth by inhibiting phosphoinositide turnover, independent of any effect upon protein synthesis (Dominice et al., 1994). Furthermore, Gschwendt and co-workers have observed that didemnin B inhibits phorbol ester-stimulated phosphorylation of elongation factor eEF-2, which might be expected to promote rather than to inhibit protein synthesis (Gschwendt et al., 1987, 1989).

Our results indicate that higher concentrations of didemnin B can exert a direct effect upon the translational machinery *in vitro*, by stabilizing aminoacyl-tRNA binding to the ribosomal A-site and preventing translocation. The effective concentration for inhibition of protein synthesis in intact cells *via* this mechanism may be lower than that suggested by our *in vitro* data for two reasons. (1) The highly organized state of the translational apparatus in cells can affect both the rate of protein synthesis and its sensitivity to inhibition. For example, permeabilized CHO cells synthesize proteins about 40-fold faster than a cell-free system derived from the same cells (Negrutskii et al., 1994). These permeabilized cells are approximately 10-fold more sensitive to didemnin B than rabbit reticulocyte cell lysates.[3] (2) The concentration of didemnin B required to inhibit translocation is dependent upon the concentration of eEF-2 (Table 4). While it is difficult to compare the amount of active eEF-2 in cell lysates with its concentration in intact cells, eEF-2 comprises only 0.05−0.1% of soluble cell protein and its concentration in intact cells may be much less than is present in our cell-free experiments. Moreover, the actual effective concentration of didemnin B in whole cell assays may be higher than reported due to rapid influx of this lipophilic compound, allowing its internalization inside cells and an increase in its concentration in the cytosol. Evidence in support of this hypothesis has been provided by Orlowski et al. (1988), who have examined the potency of didemnin B and other antitumor agents against electropermeabilized and nonpermeabilized DC-3F cells.

Since the discrepancy between the effective concentrations of didemnin B for intact cells and *in vitro* experiments remains to be explained, it is possible that inhibition of protein synthesis by the mechanism that we have described may represent a side effect of didemnin B, independent of its remarkable antineoplastic and antiproliferative effects. However, it is interesting that a comparison of didemnin B with other cytotoxic agents against the NIH collection of human tumor cell lines (mean graph analysis) indicates that it most closely resembles bouvardin (correlation coefficient = 0.870), deoxybouvardin (correlation coefficient = 0.862), bruceantin (correlation coefficient = 0.849), and sparsomycin (correlation coefficient = 0.848), all of which are inhibitors of polypeptide elongation [analogs of bouvardin are currently the subject of human clinical trials (Itokawa, 1991)].[4]

In summary, didemnin B is an effective inhibitor of *in vitro* protein synthesis and we have provided evidence that inhibition occurs by stabilization of aminoacyl-tRNA binding to the ribosomal A-site, preventing translocation but not peptide bond formation. These results are consistent with previous suggestions that eEF-1α is a low-affinity receptor for didemnins (Crews et al., 1994), and they support the hypothesis that inhibition of protein biosynthesis is one mechanism of didemnin B cytotoxicity. The recognition that didemnin B, similar to bouvardin and sparsomycin, inhibits protein synthesis at the elongation stage suggests that this mechanism should be explored further as a potential target for new antitumor agents.

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[3] Murray P. Deutscher, personal communication.

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