Inhibition of Protein Synthesis by Didemnins: Cell Potency and SAR

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Synthetic and naturally occurring didemnins are potent and specific inhibitors of protein synthesis in vitro. Structure−activity analysis indicates a requirement for the intact macrocycle; however, the smaller ring size represented by the didemnin analogue, tamandarin A, is equipotent to didemnin B. Replacement of the *N*,*O*-dimethyltyrosine by a *N*-methylphenylalanine or *N*-methylleucine residue is also well-tolerated. The rank order for inhibition of protein synthesis in vitro appears to be retained in MCF-7 cells, albeit at much higher potency. This increase in potency is explained for the first time by data indicating that MCF-7 cells can accumulate didemnin B up to 2−3 orders of magnitude compared to the growth medium.

Introduction

Inhibitors of protein synthesis in prokaryotes have a well-established reputation as antimicrobial agents.[1] Antibiotics that function by inhibiting protein synthesis include tetracycline, streptomycin, and erythromycin. These agents can be used successfully in a therapeutic manner because they exhibit selectivity toward prokaryotic protein synthesis, either through selective uptake into microbial cells or because they are more potent toward prokaryotic protein synthesis than toward eukaryotic protein synthesis. With the emergence of drug-resistant antibiotic strains, translation has been revisited as a target for antimicrobial drugs resulting in the development of the new class of oxazolidinone antibiotics.[2] In contrast, compounds that inhibit protein synthesis in eukaryotes are generally toxic, and although many such agents have been examined as potential antitumor agents, none have been successfully developed for the clinical treatment of cancer. Large increases in the rate of protein synthesis are typically observed following signals for cell proliferation;[3] therefore, rapidly proliferating cells should be especially susceptible to inhibitors of protein synthesis. The relative success that has been achieved using cytotoxic agents that interfere with DNA synthesis[4] suggests that there may be a way to exploit protein synthesis inhibitors for treating some forms of cancer.

Didemnin B[5,6] (DB, **1**; Figure 1) displays growth inhibitory effects on a wide variety of human tumor cell lines at nanomolar concentrations.[7] At these concentrations, DB has a cytostatic effect upon cells, although this effect appears to be irreversible following extended exposure of cells to the drug.[8] At higher concentrations DB is cytotoxic and can induce programmed cell death through the activation of caspases.[9,10] DB inhibits protein synthesis in cells, and it has been proposed that this effect represents the primary mechanism of cytotoxicity for DB.[11,12] This mechanism of action has been disputed however, and we have shown recently that the inhibition of protein synthesis alone is insufficient to induce programmed cell death.[13]

The mechanism for the inhibition of protein synthesis by DB in vitro is mediated by EF-1α.[14,15] DB binds to ribosome·EF-1α complexes and prevents the binding of EF-2.[16] The requirement for EF-1α to be present, as well as the binding of EF-1α/GTP to didemnin affinity columns,[14,17] implicates this protein as the molecular target for DB in vitro and possibly also in cells. Indeed, dehydrodidemnin B (aplidine) has been shown to inhibit protein synthesis in Erlich ascites tumor cells specifically at the elongation stage, consistent with its mechanism of action in vitro.[18] Thus, while inhibition of protein synthesis itself may be only a contributor to the cytotoxicity of didemnins,[19−24] this effect provides a useful read-out for measuring the relative affinity of various didemnins for their EF-1α target. Here, we describe further evidence that the mechanism that we have described for the inhibition of protein synthesis by DB in vitro also operates in cells, and we summarize our initial structure−activity relationship (SAR) data for inhibition of protein synthesis by didemnins.[25]

Results

To establish a SAR for the inhibition of protein synthesis, natural and synthetic didemnins (Figures 1 and 2) were tested in a rabbit reticulocyte cell-free translation assay.[15] For comparison, a selection of other compounds also was examined, including some known protein synthesis inhibitors and several biologically active cyclic and acyclic peptides to examine nonspecific effects on the translation machinery. The results are shown in Tables 1 and 2. Fusidic acid, cycloheximide, and aurintricarboxylic acid, all known inhibitors of

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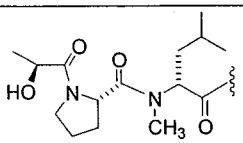
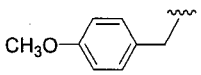
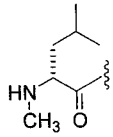
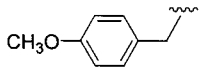
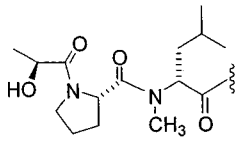
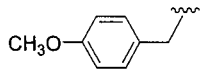
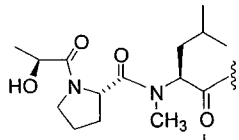
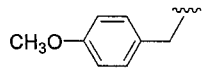
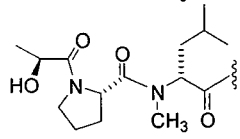
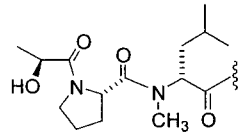
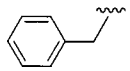
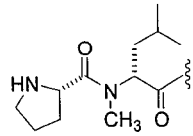
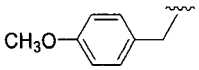
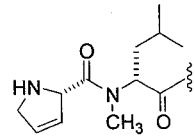
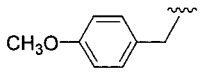
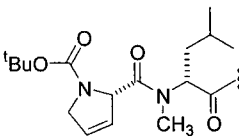
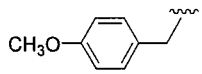
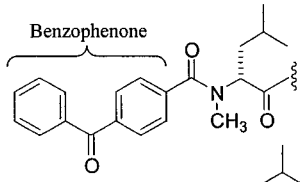
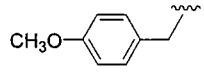
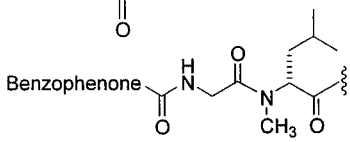
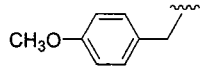
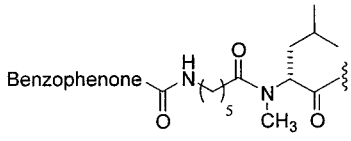
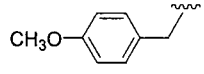
1		CH_3		5
2		CH_3		5
3		H		42
4		H		43
5		CH_3		44
6		CH_3		44
7		CH_3		55
8		CH_3		55
9		CH_3		55
10		CH_3		56
11		CH_3		56
12		CH_3		56
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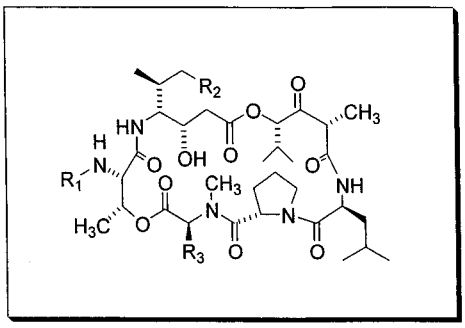

Figure 1. Didemnin derivatives tested for inhibition of protein synthesis in vitro.

eukaryotic protein synthesis, inhibited protein synthesis in our assay at concentrations that are consistent with the literature reports for these compounds.[26−28] Berninamycin,[29] a member of the thiostrepton class of antibiotics that inhibit prokaryotic protein synthesis, was weakly inhibitory with IC_{50} = 600 μM. Adipokinetic hormone has been reported to inhibit protein synthesis in *Locusta migratoria*,[30] and a fragment of adreno-

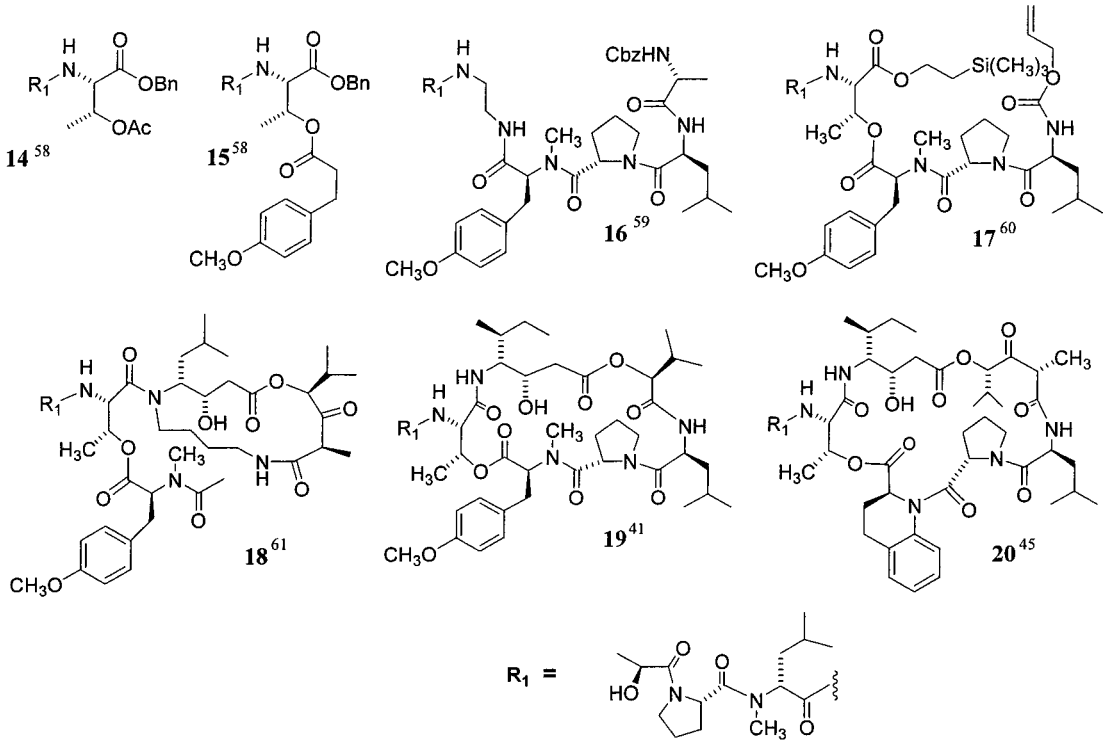

Figure 2. Didemnin analogues tested for inhibition of protein synthesis in vitro.

Table 1. Inhibition of Protein Synthesis in Vitro by a Selection of Small-Molecule Natural Products[a]

compound	IC_{50} (μM)
RA-VII	0.05 ± 0.02
bouvardin	0.1
adrenocorticotrophic hormone(4−10)	4 ± 0.5
cycloheximide	5 ± 5
aurintricarboxylic acid	10 ± 5
fusidic acid	400 ± 200
eledoisin	500
berninamycin	600
jasplakinolide	>500
adipokinetic hormone	>1000
destruxin B	>1000

[a] The amount of protein synthesis as a function of inhibitor concentration was determined by measuring the incorporation of [^{35}S]methionine into tobacco mosaic virus mRNA-encoded proteins in rabbit reticulocyte lysate.

Table 2. Inhibition of Protein Synthesis in Vitro by Didemnins[a]

compound	IC_{50} (μM)	compound	IC_{50} (μM)
1 (DB sample b)	0.89 ± 0.1	**9**	5.3 ± 0.3
3	1 ± 0.5	**2** (DA)	5.4 ± 0.2
19 (tamandarin A)	1.3 ± 0.4	**5**	9 ± 2
1 (DB sample a)	2.0 ± 0.5	**13** (*O*,*O*-diacetyl-DB)	10 ± 2
6	2.5 ± 0.5	**12**	24 ± 1
20	2.7 ± 0.2	**17**	112 ± 12
4	3 ± 2	**16**	275 ± 25
10	4.0 ± 0.5	**14**	276 ± 40
7	4.3 ± 0.3	**15**	405 ± 40
8	4.3 ± 0.3	**18**	>1000
11	4.5 ± 0.5		

[a] The amount of protein synthesis as a function of inhibitor concentration was determined by measuring the incorporation of [^{35}S]methionine into tobacco mosaic virus mRNA-encoded proteins in rabbit reticulocyte lysate.

corticotrophic hormone (ACTH) comprising residues 4−10 has been reported to inhibit protein synthesis in a cell-free system from rat brain, albeit at relatively high concentrations (10^{-4} M).[31] Of these two hormones only $ACTH_{4-10}$ inhibited protein synthesis in rabbit reticulocyte lysate. Jasplakinolide[32−34] and destruxin B[35] are cytotoxic cyclic peptides whose mechanism of action was unknown at the time of testing. Neither compound displayed significant inhibition of protein synthesis. Jasplakinolide has subsequently been shown to cause disruption of the actin cytoskeleton.[36] In contrast, eledoisin[37] inhibited protein synthesis with an IC_{50} = 500 μM, essentially indistinguishable from that for fusidic acid. We concluded that an $IC_{50} \geq 500$ μM was very likely attributable to nonspecific effects on the translational machinary.

The most remarkable nondidemnin compounds that have been tested are RA-VII and bouvardin.[38] These RA peptides are derived from Rubiaciae plants, and over 15 such molecules have been characterized. All of the known RA peptides are cytotoxic, and RA-VII and bouvardin have been shown to inhibit protein synthesis in cells. RA-VII and bouvardin both inhibit protein synthesis at the elongation stage in vitro, similar to DB.[39] RA-VII is the most potent inhibitor that we have tested to date. It specifically inhibits the ribosomal peptidyltransferase. RA-VII is a very potent and rapid inducer of programmed cell death.[17]

Naturally occurring didemnins are generally active at low micromolar concentrations, i.e., at least 2 orders of magnitude lower than the inhibitory concentrations observed for the majority of the non-didemnin peptides examined. Moreover, synthetic didemnins and didemnin derivatives display a gradation of activity with changes in structure, typical of effects mediated through a discrete and structurally well-defined receptor or target molecule. Didemnin derivatives that display relatively conservative changes in the macrocycle or side chain (residues 7−9) retain their potency as inhibitors of protein synthesis. During the course of this study, two different samples of DB were employed, both obtained

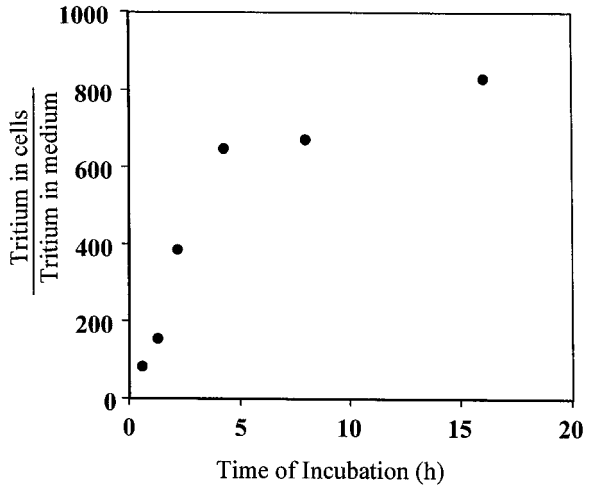

Figure 3. Accumulation of T_2-DB into MCF-7 cells.

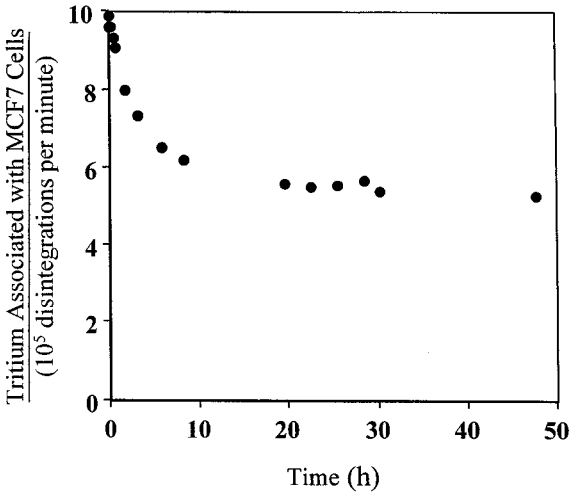

Figure 4. Release of T_2-DB from saturated MCF-6 cells.

from the National Cancer Institute. These two samples exhibited identical analytical properties (single peak by reverse-phase HPLC, ^{1}H NMR) but unexplainably the second sample was approximately 4-fold more potent as an inhibitor of protein synthesis in vitro. Both results are given in Table 2. Two other didemnins (**3**, **19**) were found to be as potent as the second DB sample.

Accumulation and Release of DB from MCF-7 Cells. When MCF-7 cells were treated with 50 nM T_2-DB[16] (7.4 Ci mmol^{-1}), the cells accumulated tritium as shown in Figure 3. Similar results were observed when the starting concentration of T_2-DB in the medium was 10 or 1 nM (data not shown). Replacement of the medium by fresh RPMI-1640 containing no inhibitor resulted in a slow release of tritium from the cells into the medium (Figure 4). The release of tritium reached a plateau after approximately 16 h, when the ratio of radioactivity between the cells and the medium was 3500:1. In a separate experiment, replacement of the medium again at 16 h led to a second release of tritium (data not shown). These observations appear to indicate an equilibration of T_2-DB between the cells and the medium with the cells sustaining an equilibrium concentration approximately 1000-fold higher than the medium under these experimental conditions.

Correlation of Cell-Free and Cell-Based Data. A comparison of potencies for inhibition of protein synthesis in rabbit reticulocyte lysate and in MCF-7 cells was made for five compounds. The results are shown in Figure 5. Four of the compounds appear to lie on a straight line indicating a correlation between the cell-free and cell-based data. The fifth compound, *O*,*O*-diacetyldidemnin B, is clearly an outlyer, possibly due to hydrolysis of the acetate esters resulting in the formation of DB inside the cells. In this case, the cellular potency expected would be nearer to that for DB, as observed.

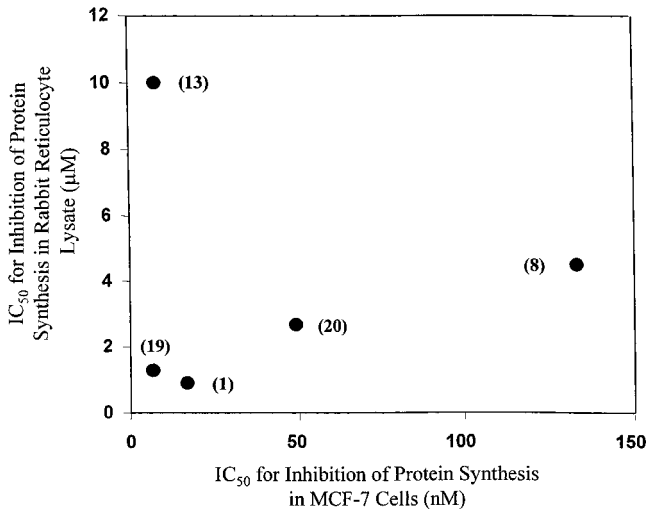

Figure 5. Comparison of protein synthesis inhibition potencies in cells vs cell-free assays.

Discussion

A comparison of didemnin derivatives and other peptides as inhibitors of protein synthesis in vitro confirms that didemnins are potent and specific inhibitors of translation. Despite the relatively small number of didemnins evaluated, and the narrow range of potencies observed, it is appropriate to make some comments regarding the relationship between didemnin structure and activity. For example, the potency of tamandarin[40] (**19**) indicates that an intact α-(α-hydroxyisovaleryl)-propionyl (HIP) unit is not required for inhibition of protein synthesis. Moreover, the removal of one methyl group from the isostatine residue in DB to produce nordidemnin[41,42] (**3**) also leads to no change, or a small increase in potency, possibly by relieving a weak steric conflict between the didemnin and its target. These results contrast with previous observations that stereochemical modification of the isostatine-HIP unit can significantly decrease cytotoxicity against human and murine cancer cell lines.[24]

Sakai et al.[24] reported that replacement of the *N*,*O*-dimethyltyrosine residue of DB by saturated analogues was well-tolerated producing didemnins with comparable bioactivities to the natural product. Similarly, we observed a small increase in potency upon replacement of the *N*,*O*-dimethyltyrosine residue by *N*-methylphenylalanine;[43] this change might be due to a requirement to desolvate the tyrosine methoxy group during formation of the protein−ligand complex. Comparison of compounds **1**, **6**, and **20** indicates that the aromatic methoxy group contributes negatively to the inhibition of protein synthesis and that the conformation of the aromatic side chain at this position in the bound state

closely matches the conformation that is imposed by the L-Tic residue.[44] Replacement of the aromatic residue by leucine, which presents slightly less hydrophobic surface area (92 Å^2 for the Leu side chain vs 106 Å^2 for the Phe side chain), confers a decrease in potency for inhibition of protein synthesis.[43] This observation suggests that residue 5 nestles into a substantial hydrophobic pocket in the target protein that must be filled in the protein−ligand complex for optimal binding.

As found previously for inhibition of protein synthesis in P388 cells,[24] changes to the characteristic didemnin side chain (residues 7−9) alter the ability of didemnins to inhibit protein synthesis (compare compounds **1**, **2**, **4**, **7**−**9**); however, the side chain itself (e.g., **14**) is insufficient for high potency. Other acyclic analogues of didemnins (**15**−**17**) and a conformationally constrained fragment of the natural product (**18**) similarly performed poorly in the protein synthesis assay indicating that the three-dimensional structure of the depsipeptide ring is important for activity. Also, the leucyl-prolyl-dimethyltyrosine tripeptide appears to be more important than the isostatine-HIP portion of the molecule, by comparison of compounds **16** and **17** with compound **18**. This conclusion is supported by the potency of tamandarin A (**19**).

Overall, the SAR that we have observed for the inhibition of protein synthesis in a cell-free assay is consistent with the SAR for cytotoxicity and for inhibition of protein synthesis in P388 cells reported by Rinehart's group.[24] With the exception of tamandarin (**19**), changes to the core macrocyclic ring structure are detrimental to biological activity although replacement of the side chains can be tolerated. The linear peptide portion is highly sensitive to changes, and modifications here can increase or decrease potency depending on the choice of replacement; small hydrophobic groups in place of the lactyl residue are preferred.[24] This consistency encourages us to believe that studies examining the effects of didemnins on cells and experiments using in vitro translation assays are addressing related phenomena.

Although didemnins clearly inhibit protein synthesis in cell-free assays, there exists a significant difference in potency for inhibition of protein synthesis in cell lysate compared to inhibition of protein synthesis in intact cells. This discrepancy varies from 2−3 orders of magnitude depending on the cell line.[6,7,11,14] This large discrepancy in dose−response has led previous workers, and casual observers, to discount direct interactions between didemnins and the translational apparatus as an important mechanism of action with relevance to the cellular environment.[19,20] However, several possible explanations could be advanced to account for this discrepancy. For example, it may be necessary to inhibit protein synthesis completely (100%) in order to observe a cytotoxic effect. Alternatively, the intracellular concentration of didemnin may not reflect the concentration of inhibitor in the medium. For example, if cells were able to accumulate didemnin from the medium, then the concentration of inhibitor inside the cell would be significantly higher than the overall concentration added to the medium and could reach levels sufficient to inhibit ribosomal translocation via the mechanism that we have described.[16] Indirect support for this idea was provided by Orlowski and co-workers who suggested that DB could be accumulated by DC-3F Chinese hamster lung fibroblasts.[45] However, the data presented by these workers, while indicating that DB can passively diffuse across the cell membrane, do not provide any evidence for or against a higher concentration of DB inside the cell than in the growth medium. Having prepared tritiated DB (T_2-DB) of high specific activity,[16] we were in a position to be able to address this question directly.

The human mammary carcinoma cell line MCF-7 is sensitive to DB, which is antiproliferative at nanomolar concentrations and induces apoptosis in these cells with IC_{50} = 200 nM.[13] Protein synthesis in the MCF-7 cell line is inhibited by DB with IC_{50} = 12 nM. When MCF-7 cells were grown in medium containing 50 nM T_2-DB, the cells accumulated T_2-DB from the medium, reaching saturation after approximately 4 h. Assuming an average cell volume of 2 pL,[46] this accumulation corresponds to an intracellular concentration of tritiated DB or DB derivatives of 3.3 μM, or approximately 650× the concentration in the medium. Similar results were observed when the starting concentration of T_2-DB in the medium was 10 or 1 nM.

This remarkable ability of MCF-7 cells to accumulate DB from the medium easily could account for the 100−1000-fold discrepancy in potency for inhibition of protein synthesis in cells vs cell-free assays. However, a conclusion that the accumulated DB affects protein synthesis directly, rather than through some other pathway, requires that the temporal nature of protein synthesis inhibition match the profile of DB accumulation into the cell. In a single experiment, it was observed that incubation of MCF-7 cells with 50 nM DB resulted in 51% inhibition of protein synthesis after 2 h, 68% inhibition after 8 h, and maximal inhibition for this concentration of DB (76%)[13] at 18 h. Thus, the inhibition of protein synthesis in cells occurs with a similar time course to the accumulation of DB, consistent with a direct effect of this molecule on the translational machinery.

When cells bearing accumulated quantities of T_2-DB were placed into fresh medium that did not contain inhibitor, the efflux of DB into the medium could be determined by sampling the culture medium and measuring its level of radioactivity. Efflux was observed to occur rapidly at first and then to slow to a virtual halt (Figure 4). Further efflux could be prompted by once again replacing the medium from an inhibitor-free stock. Although the viability of T_2-DB-treated cells was not determined in this experiment, DB at the concentrations employed (≤50 nM) has been shown previously to irreversibly inhibit protein synthesis and to prevent cell proliferation as measured in a colony-forming assay.[13] Thus, an examination of whether protein synthesis recovers with a similar time course to DB efflux would not be meaningful.

A selection of didemnins was evaluated for inhibition of protein synthesis in MCF-7 cells to determine whether a correlation exists in relative potency for inhibition of protein synthesis in the intact cells compared to the cell-free system. With a single exception, a good correlation was observed between inhibition of protein synthesis in vitro and inhibition of protein synthesis in MCF-7 cells,

indicating that the same order of potency holds for didemnin derivatives in each system. The exception was *O,O*-diacetyldidemnin B (**13**), which is more potent in cells than anticipated based on data from cell lysate, possibly as a result of hydrolysis of the two acetate esters to produce DB in cells. While the possibility exists that different didemnins accumulate in cells to different extents, this scenario seems unlikely given the close structural similarity of the analogues examined. Moreover, differential accumulation would decrease the likelihood of a correlation between IC_{50} values rather than increase it. Consequently, overall these results lend credence to the cell-free assay as a representative measure of the relative potency of didemnin derivatives as protein synthesis inhibitors, and they suggest that conclusions reached regarding the SAR of didemnins based on the cell-free assay should substantially hold true in intact cells.

In conclusion, didemnins are moderately potent inhibitors of protein synthesis in vitro and the available SAR data indicate that the intact depsipeptide ring is required for this activity. DB is concentrated from the medium by intact MCF-7 cells, and this effect accounts for the discrepancy in potency observed for intact cells compared to cell-free assays. A correlation exists between inhibition of protein synthesis in cell lysate and inhibition of protein synthesis in cells, indicating that protein synthesis may be inhibited by the same mechanism in each case. Since DB acts to inhibit protein synthesis in vitro by binding to ribosome·EF-1α complexes,[16] these results suggest that EF-1α should be considered as a potential therapeutic target for antiproliferative agents. The essential role of this protein in translation and evidence that the increased expression of EF-1α correlates with higher levels of protein synthesis and tumorigenesis[47−53] argue that some cancers may be especially susceptible to EF-1α inhibitors. The cyclic depsipeptide didemnins provide a starting point for the identification of new EF-1α inhibitors as potentially therapeutic antiproliferative agents.

Experimental Section

Inhibition of Translation in Vitro. Inhibition of protein synthesis in vitro was assayed using rabbit reticulocyte lysate as described previously.[15]

Inhibition of Protein Synthesis in MCF-7 Cells. Protein biosynthesis was measured using a modification of the protocol described by Geier and co-workers.[54] MCF-7 cells in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan UT), l-glutamine (GIBCO), and penicillin/streptomycin (GIBCO) were seeded in 6-well plates at an initial concentration of 4×10^4 cells/mL and incubated for 24 h. The cells were washed with sterile phosphate-buffered saline (PBS) then resuspended in methionine-free, serum-supplemented RPMI-1640 (4 mL). Inhibitor solution or carrier was added at the required concentration, and the cells were incubated for 1 h at 37 °C. Protein labeling mix ([^{35}S]methionine/[^{35}S]cystein, specific activity 1175 Ci/mmol) was added to a final concentration of 9 nM, then the cells were incubated for an additional 18 h. The medium was removed and the cells were rinsed with PBS then precipitated by the addition of cold 10% trichloroacetic acid (1 mL) for 10 min. The precipitate was dissolved in 0.2 N NaOH (1 mL) for 5 min at room temperature, then aliquots were removed for scintillation counting.

Time Course for DB Binding to Cells. The incorporation of tritiated DB[16] (T_2-DB) into MCF-7 cells was quantified following the protocol described by Shen and co-workers.[20] Cells were seeded in three 25-mL flasks at 1.3×10^5 cells/mL using RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and incubated overnight. After removal of the medium the cells were briefly rinsed with PBS, then resuspended in serum-supplemented RPMI (7 mL). T_2-DB (specific activity 7.4 Ci/mmol, final concentrations of 1, 10 or 50 nM) or carrier solvent (EtOH) was added and incubation was continued. At predetermined time points the medium was aspirated from 1 flask and 3 aliquots (100 μL each) were removed for scintillation counting. The cells were washed in PBS buffer (1×7 mL), then lysed with 0.05% trypsin/EDTA (500 μL) for 5 min at room temperature. The lysed cells were resuspended in PBS (2 mL) then assayed for tritium content by scintillation counting.

The dissociation of T_2-DB from MCF-7 cells was examined by incubating cells (seeded as described above) with 50 nM T_2-DB for 4 h, by which time steady-state binding of the drug was achieved. The cells were briefly washed in PBS buffer, then resuspended in fresh serum-supplemented RPMI-1640 medium. Incubation was continued for at least 50 h. At predetermined time points during the incubation, 3 aliquots (100 μL each) were removed from the medium, taking care not to aspirate any of the cells, and their tritium content was determined by scintillation counting.

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