The Antiproliferative Agent Didemnin B Uncompetitively Inhibits Palmitoyl Protein Thioesterase†

Lihao Meng, Ny Sin, and Craig M. Crews*

Department of Molecular, Cellular, and Developmental Biology, Yale University, 219 Prospect Street, New Haven, Connecticut 06520-8103

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ABSTRACT: Dynamic protein palmitoylation has been proposed to regulate GTP-binding proteins by controlling their membrane association and thus their access to key signaling proteins. While the palmitoyl protein thioesterase(s) responsible for depalmitoylation of plasma membrane-associated signaling proteins has (have) not been identified, the lysosomal palmitoyl protein thioesterase 1 (PPT1) has proven useful in in vitro studies of membrane localization requirements of GTP-binding proteins. We have previously reported the binding of the antiproliferative cyclic depsipeptide didemnin B to PPT1. To investigate the nature of this binding and its possible effects on PPT1 enzymatic activity, human PPT1 was expressed in an insect cell baculoviral system, and inhibition assays were performed using both [^{3}H]palmitoyl Ha-Ras and myristoyl-CoA as PPT1 substrates. Didemnin B was shown to inhibit recombinant human PPT1 with a K_i of 92 nM. Kinetic analysis of this inhibition revealed that didemnin B inhibits PPT1 uncompetitively. Providing biochemical support for an uncompetitive mode of inhibition, in vitro binding studies of PPT1 and didemnin indicate that the natural product binds preferentially to the enzyme−substrate complex PPT1−palmitoyl-CoA. As the first described inhibitor of PPT1, didemnin B may prove to be a useful tool in the investigation of protein palmitoylation regulation.

Didemnin B (DB)[1] (Figure 1), a cyclic antiproliferative depsipeptide isolated from the Caribbean tunicate *Tridemnum solidum*, was the first marine natural product to enter clinical trials as an antitumor agent (*1*). It has been investigated in Phase II clinical trials for the treatment of breast, ovarian, cervical, myeloma, glioblastoma/astrocytoma, and lung cancers (*2*−*7*). Moreover, didemnin B displays several in vitro biological activities, whose effective concentrations range over 5 orders of magnitude (*8, 9*), suggesting that the activities are mediated by different mechanisms. To investigate these modes of action, didemnin affinity matrixes have been used to purify didemnin-binding proteins. We have previously shown that a major intracellular DB-binding protein is an activated translation elongation factor, the GTP[1]-bound form of elongation factor 1 α (EF-1α) (*10*). These findings are consistent with the known protein synthesis inhibitory activity of didemnin B (*11*−*13*).

In addition to EF-1α, we have purified another DB-binding protein and identified it as palmitoyl protein thioesterase 1 (PPT1) (*14*), a 36 kDa glycosylated protein that was initially purified based on its ability to cleave palmitate from recombinant Ha-Ras and G_α proteins in vitro (*15, 16*). This enzyme is the first of a putative family of proteins capable of removing membrane-anchoring lipids from palmitoylated proteins (*17*). Despite its in vitro activity using Ha-Ras and GTP-binding proteins as substrates, PPT1 has a signal sequence (*14, 16*) that localizes it to lysosomal compartments (*18*−*20*). Molecular genetic studies have shown that inactivating point mutations in PPT1 result in infantile neuronal ceroid lipofuscinosis (INCL) (*21, 23*), one of a group of neuronal degenerative diseases that have an incidence of 1 in 12 500 births in the world. Children born with INCL have a flattened EEG by 3 years of age (*24*) and do not survive beyond an average age of 8−11 years (*25*).

Protein lipidation plays a key role in membrane association and protein function for a variety of different signal transducing molecules (*26*). Dynamic acylation of the membrane-anchoring lipid palmitate to certain GTP-binding proteins has been proposed to provide an additional level of regulation by controlling membrane association and subsequent access to membrane-localized downstream effector molecules (*26*−*28*). As the first reported protein palmitoyl thioesterase, PPT1 has proven useful in studies of the role of palmitoylation in GTP-binding protein localization and function (*29*).

Given our previously reported binding of PPT1 with didemnin, we investigated the possibility that didemnin binding results in PPT1 inhibition. In this study, baculovirally expressed human PPT1 was produced as a secreted epitope-tagged protein and was used in kinetic studies of didemnin-mediated inhibition employing Ha-Ras and myristoyl-CoA as substrates.

EXPERIMENTAL PROCEDURES

The reagents listed were obtained from the following sources: Myristoyl-CoA, palmitoyl-CoA, DTNB, and horse

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* Corresponding author. Telephone: (203) 432-9364. Fax: (203) 432-5713. Email: craig.crews@yale.edu.

[1] Abbreviations: GTP, guanosine triphosphate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); PPT1, palmitoyl protein thioesterase 1; DB, didemnin B.

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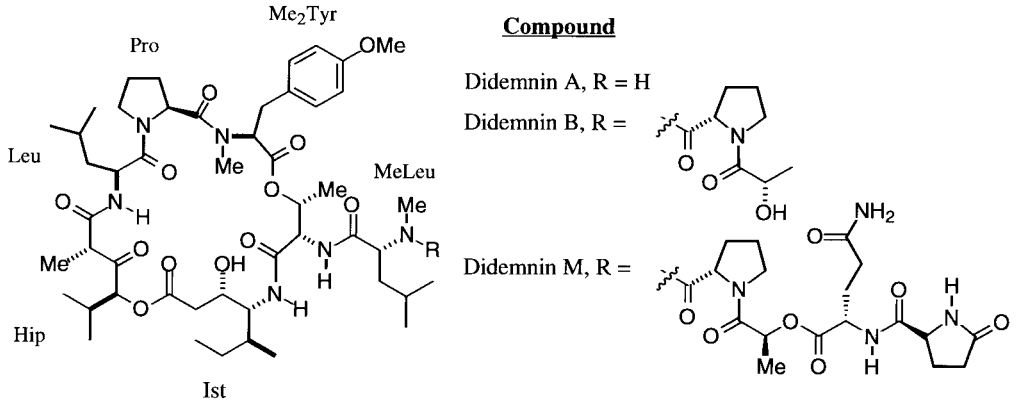

FIGURE 1: Structures of the didemnin family members tested in palmitoyl protein thioesterase 1 (PPT1) inhibition assays.

heart cytochrome *c* were purchased from Sigma Chemical Co. (St. Louis, MO). Serum-free medium ExCel-401 was obtained from JHR Bioscience, Inc. Pluronic F-68 and Tissue Culture grade antibiotic−antimycotic solution were purchased from Gibco-BRL. *N*-Octyl glucoside was obtained from Aldrich Chemical Co. The Enhanced Chemiluminescence kit was purchased from Amersham Corp. Affigel 10 and prestained protein markers were from BioRad. Didemnins A and B were kind gifts of Kenneth L. Rinehart (University of Illinois−Urbana), and [^{3}H]palmitate-labeled Ha-Ras was generously provided by Sandra Hofmann (UT−Southwestern Medical Center).

Semisynthesis of Didemnin M. As a starting point for didemnin M synthesis, the pseudotetrapeptide pGlu-Glnψ-[COO]Ala-Pro-OH was synthesized as previously described (*30*). To a stirring solution of this pseudotetrapeptide (4.1 mg, 9.53 μmol) in dimethylformamide (DMF) (1 mL) were added *O*-(7-azabenzotriazo-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (4.2 mg, 11.12 μmol), 1-hydroxy-7-azabenzotriazole (HOAt) (1.50 mg, 11.12 μmol), and diisopropylethylamine (DIEA) (6.6 μL, 38.12 μmol). After 10 min, didemnin A (3.0 mg, 3.18 μmol) was added to the reaction mixture. After stirring at room temperature for 24 h, the solvent was removed in vacuo, and the crude product was purified by flash column chromatography (97:3, CH_2Cl_2/MeOH) to give didemnin M (3.3 mg, 77%). The 500 MHz ^{1}H NMR and HRFABMS spectra were identical to the natural product (*31*).

Human Palmitoyl Protein Thioesterase Baculoviral Construction/Expression. Using PCR, the six amino acid Glu-Glu (EE) epitope (EYMPME) (*32*) was engineered into the amino terminus of the mature human PPT1 protein after the putative signal sequence cleavage site. This epitope-tagged EE-PPT1 gene was subcloned into the *Eco*RI/*Xba*I sites of the baculovirus expression transfer vector pFastBac (Gibco-BRL). The pFastBac-EE-PPT1 plasmid was then used to transfect Hi5 insect cells in 35 mm Petri dishes, and recombinant virus was obtained according to the manufacturer's protocol (Gibco Life Technologies).

Expression of Baculovirally Produced Palmitoyl Protein Thioesterase 1. Hi5 insect cells were cultured in suspension at 27 °C in serum-free medium ExCel-401 containing 2 mM L-glutamine, 0.1% Pluronic surfactant F68, and 50 μg/mL antibiotic/antimycotic (Gibco/Life Technologies) in a 100 mL flask. Grown to a density of 1×10^6 cells/mL, cells were pelleted at 4000*g* for 10 min. Ten milliliters of EE-PPT1 virus stock was incubated with cells for 30 min with occasional mixing. Infected cells were subsequently incubated for 4 days in 100 mL of medium containing one-third of Hi-5 conditioned media and two-thirds of fresh Ex-401 medium. The cell culture medium containing secreted EE-PPT1 was collected by cell centrifugation at 4000*g* for 10 min. EE-PPT1 protein concentration was determined by SDS−PAGE and Coomassie blue staining. EE-PPT1 protein stock solution retained enzymatic activity for several months after storage at 4 °C.

Palmitoyl Protein Thioesterase Assay. (A) [^{3}H]-Ha-Ras. Assays were performed as previously described (*15*). Briefly, [^{3}H]palmitate-labeled histidine-tagged Ha-Ras (1 μg, 20 000 cpm) was incubated with epitope-tagged human PPT1 and various concentrations of didemnin B or methanol control at 37 °C for 20 min. The proteins were precipitated by the addition of 500 μL of ethanol/ether (2:1, v/v) and centrifugation (4 °C, 13000*g*). Subsequently, 400 μL of each supernatant was subjected to liquid scintillation counting to determine the amount of [^{3}H]palmitate label released.

(B) Myristoyl-CoA Kinetic Assay. Assays were performed as previously described (*33*) with the following alterations: the reaction mixture contained 0.2 M Tris-HCl, pH 7.4, 1 mM EDTA, 0.2 mM DTNB, and varying concentrations of myristoyl-CoA and didemnin compounds. The enzyme activities were measured using the Spectronic Genesys 5 Spectrophotometer kinetic program in a disposable cuvette for 10 s at 410 nm, immediately after mixing 100 μL of PPT1 (10 μg/mL) with 800 μL of reaction solution.

Didemnin−Affigel Matrix Binding Assay. Didemnin A (DA)−Affigel and aminocaproic acid linker Affigel were synthesized as previously described (*14*). Binding assays were carried out as follows: 100 μL of EE-PPT1 (12 μg/mL) was incubated for 30 s with 10 μL of DA−Affigel in the absence or presence of 0.2 mM palmitoyl-S−CoA or 0.2 mM palmitate. The beads were washed twice with 1 mL of wash buffer (25 mM Tris, pH 7.5, and 150 mM NaCl) and boiled in 50 μL of SDS sample buffer. Proteins were electrophoresed on a 12% SDS−PAGE gel, transferred to a poly(vinylidene difluoride) membrane (Immobilon, Millipore), and immunoblotted using anti-Glu-Glu epitope monoclonal antibodies. EE-tagged proteins were detected by enhanced chemiluminescence (Amersham).

RESULTS

Given the ability of palmitoyl protein thioesterase (PPT1) to hydrolyze palmitate from baculovirally produced Ha-Ras

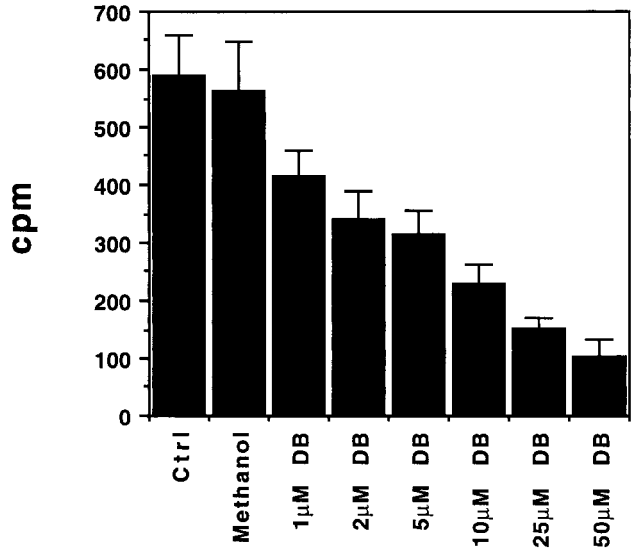

FIGURE 2: Didemnin B-mediated inhibition of PPT1 using Ha-Ras as substrate. [^{3}H]palmitate label released from baculovirally produced H-ras by PPT1 as measured in the presence of increasing concentrations of didemnin B.

(*15*) and our previous results showing didemnin B binding to PPT1 (*14*), we investigated the possibility that didemnin B inhibits PPT1 enzymatic activity in vitro. Using ^{3}H-palmitoylated Ha-Ras purified from metabolically labeled baculovirally infected insect cells as substrate, a range of didemnin B concentrations was tested for the ability to inhibit thioesterase activity. As shown in Figure 2, incubation of PPT1 with increasing concentrations of didemnin B resulted in decreased [^{3}H]palmitate released from acylated Ha-Ras. These results indicate that didemnin B not only binds PPT1, but also inhibits the in vitro human PPT1 thioesterase activity with an IC_{50} value of 5 μM.

To study the kinetics of human recombinant PPT1 inhibition by didemnins, hydrolysis of the thioesterase substrate myristoyl-CoA by PPT1 was measured because a sufficient quantity of palmitoylated Ha-Ras was not available. In addition, Hofmann and colleagues had shown that PPT1 has a higher specific activity for myristoyl-CoA as a substrate than palmitoyl-CoA (*16*). Under the enzymatic assay conditions we used, the apparent K_m for myristoyl-CoA was determined to be 91 μM and the V_{max} was calculated to be 30 μmol mg^{-1} min^{-1}. These values are higher than those previously reported using purified bovine PPT1 and palmitoyl-CoA (18 μM and 14 μmol mg^{-1} min^{-1}) (*15*) and may reflect the difference between the two substrates. To investigate the mechanism of didemnin-mediated PPT1 inhibition, double-reciprocal plots were generated from steady-state kinetic analyses (Figure 3). Employing myristoyl-CoA as the variable substrate, parallel Lineweaver−Burke plots were obtained for the three compounds tested, didemnins A, B, and M. Similar results were obtained using palmitoyl-CoA as a PPT1 substrate (data not shown). These kinetic results indicate an uncompetitive didemnin-mediated PPT1 inhibition with respect to myristoyl-CoA, implying that the didemnin family members tested bind preferentially to the enzyme−substrate (ES) complex. To biochemically confirm this binding model predicted by the kinetic studies, the affinity of recombinant PPT1 for an immobilized didemnin A matrix was analyzed in the presence and absence of the PPT1 substrate palmitoyl-CoA. As shown in Figure 4, approximately 100-fold more PPT1 was retained on the didemnin A affinity matrix in the presence of 0.2 mM palmitoyl-CoA. This increased binding was not due to

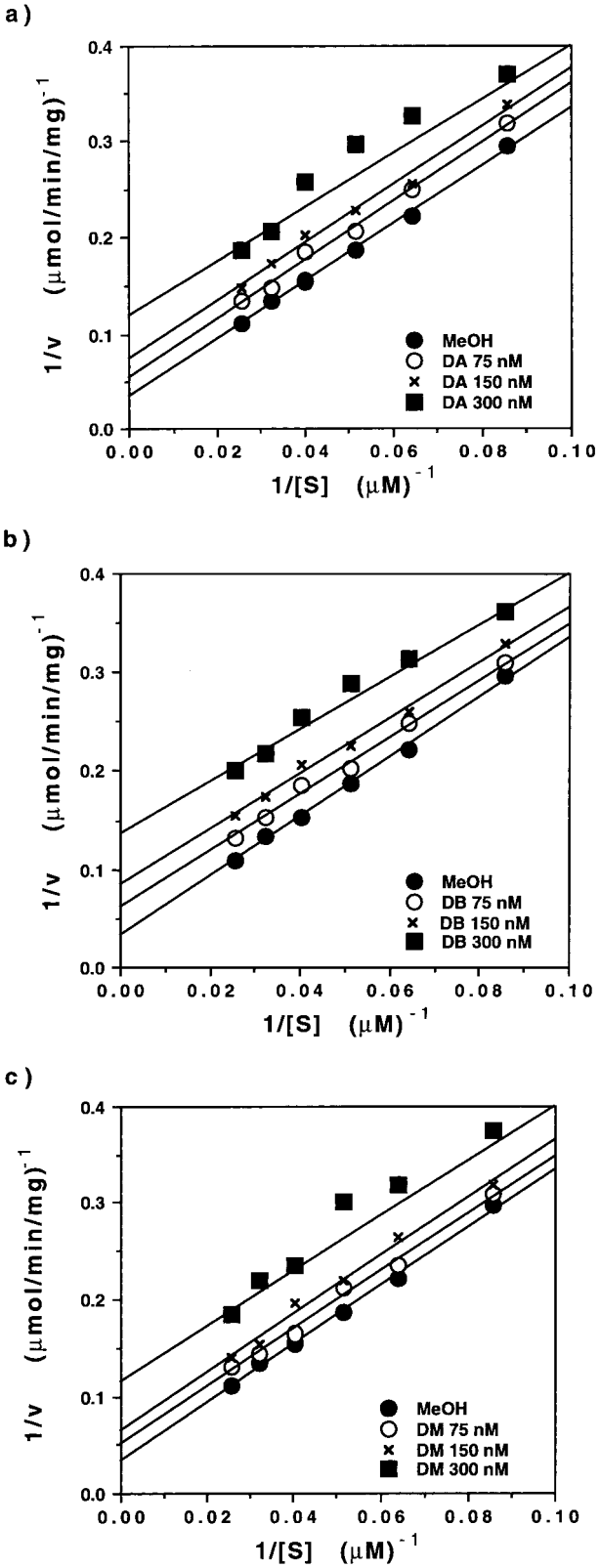

FIGURE 3: Kinetic analysis of didemnin-mediated PPT1 inhibition. Double-reciprocal plots of initial velocities versus myristoyl-CoA concentrations at fixed concentrations of PPT1 using varying concentrations of didemnin A (a), didemnin B (b), and didemnin M (c). The enzymatic rate (v) is expressed as micromoles of myristoyl-CoA cleaved per minute per milligram of PPT1.

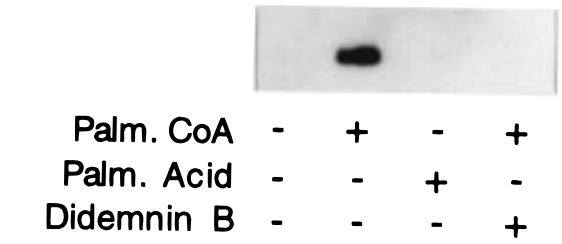

FIGURE 4: Palmitoyl-S−CoA induces PPT1 binding to didemnin−affigel. Baculovirally produced epitope-tagged human PPT1 was incubated briefly (30 s) with didemnin A-immobilized resin in the absence and presence of palmitoyl-CoA, palmitic acid, and didemnin B. After washing, retention of PPT1 on the resin was detected using SDS−PAGE and immunoblotting.

nonspecific action by the fatty acid since palmitate alone had little effect on didemnin A−PPT affinity (Figure 4, lane 3). However, excess didemnin B could compete for PPT−palmitoyl CoA-immobilized didemnin A binding, indicating a specific interaction between the enzyme−substrate complex and the natural product inhibitor.

DISCUSSION

The didemnin B−PPT1 binding results presented here are similar to the known binding mechanism of the other major didemnin B-binding protein, elongation factor 1 α (EF-1α). Our previous findings demonstrated that didemnin B selectively binds to the GTP-bound form of EF-1α (*10*), likely mediating the known protein inhibitory activity of didemnin B. Given the hydrophobic character of didemnin B and its limited solubility in aqueous solution, it is possible that the didemnin B-binding sites on both proteins are hydrophobic pockets, which are not normally solvent (or didemnin B) accessible. However, upon substrate (i.e., palmitoyl-CoA or GTP) binding to a didemnin-binding protein (PPT1 or EF-1α), a conformational change is induced unveiling a cryptic didemnin B-binding site. This model is consistent with the uncompetitive inhibition kinetic results reported here as well as the GTP-dependent binding of didemnin B previously described (*10*).

Molecular genetic studies have revealed a connection between PPT1 and the neuronal degenerative disease infantile neuronal ceroid lipofuscinosis (INCL) (*21*). Children with INCL possess two mutant nonfunctional PPT1 genes and accumulate large quantities of lipofuscin, a waxy autofluorescent lipopigment in lysosomal compartments (*23*). As a result, extensive neuronal death is evident by age 3, and few patients rarely live beyond a few years (*25*). Although it is possible that inhibition of in vivo PPT1 activity may mediate the biological activities of didemnin B, the relevance of PPT1 as a physiological target of the cytostatic activity of didemnin B is in question. For example, didemnin B-treated mice lack the characteristic lysosomal accumulation of lipofuscin associated with INCL, despite the lethal dosage administered (C. M. Crews and G. Faircloth, unpublished data).

The lack of a correlation between PPT1 inhibitory K_i values and IC_{50} values for reported in vivo biological activities among different didemnins further fails to support a role for PPT1 in mediating the reported biological activities of the didemnin family members. Whereas these biological activities (e.g., immunosuppressive) differ between didemnin A, didemnin B, and didemnin M by 3 orders of magnitude (0.98 nM, 0.42 nM, and 0.76 pM, respectively) (*34*), each didemnin family member tested inhibits PPT with comparable K_i values (111 nM, 92 nM, and 130 nM, respectively). This fact suggests that PPT1 binds to structural motifs shared among didemnins. As seen in Figure 1, didemnins share a macrocyclic ring but differ in the composition of the moiety attached to the *N*-methylleucine branching from the cyclic depsipeptide backbone. These relatively minor structural differences in didemnin family members contribute to the large differences observed in the cytostatic and immunosuppressive inhibitory concentrations of didemnins A, B, and M.

In conclusion, we have shown that different didemnin macrolides inhibit the in vitro thioesterase activity of palmitoyl protein thioesterase 1 (PPT1). Moreover, this inhibition is uncompetitively mediated in which PPT1 binding of didemnin family members requires the presence of substrate. Although the in vivo role of didemnin-mediated PPT1 inhibition is in question, the potent in vitro PPT1 inhibition by didemnin family members indicates that these natural products may serve as useful in vitro tools in exploring the role of protein palmitoylation.

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