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13-Deoxytedanolide, a marine sponge-derived antitumor macrolide, binds to the 60S large ribosomal subunit

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Abstract—13-Deoxytedanolide is a potent antitumor macrolide isolated from the marine sponge *Mycale adhaerens*. In spite of its remarkable activity, the mode of action of 13-deoxytedanolide has not been elucidated. [11-^{3}H]-(11*S*)-13-Deoxydihydrotedanolide derived from the macrolide was used for identifying the target molecule from the yeast cell lysate. Fractionation of the binding protein revealed that the labeled 13-deoxytedanolide derivative strongly bound to the 80S ribosome as well as to the 60S large subunit, but not to the 40S small subunit. In agreement with this observation, 13-deoxytedanolide efficiently inhibited the polypeptide elongation. Interestingly, competition studies demonstrated that 13-deoxytedanolide shared the binding site on the 60S large subunit with pederin and its marine-derived analogues. These results indicate that 13-deoxytedanolide is a potent protein synthesis inhibitor and is the first macrolide to inhibit the eukaryotic ribosome.

1. Introduction

Marine organisms, for example, sponges and bryozoa, are important sources of biologically active natural products, some of which are clinically important.[1] Dolastatin 10 from *Dolabella auricularia* is an antitumor agent currently under clinical trials, which was shown to inhibit microtubule polymerization.[2] Didemnin B, isolated from a Caribbean tunicate, is a cyclic depsipeptide showing potent antitumor activity and the first marine natural product studied clinically.[3] Didemnin B has been shown to inhibit eukaryotic protein synthesis by specifically binding to the GTP-bound EF-1α, an essential factor for peptide elongation.[4]

Ribosomes are huge ribonucleoprotein complexes, which translate genetic messages into proteins. While genetic messages on mRNA are decoded by the small ribosomal subunit, the peptide bond formation is catalyzed by the peptidyltransferase center of the 50S and the 60S in prokaryotes and eukaryotes, respectively, the large ribosomal subunit. It is well known that many antibiotics bind to bacterial ribosomes, thereby inhibiting protein synthesis of bacteria. Particularly well studied are macrolide antibiotics of the erythromycin class,[5] which bind to a single site in the 50S large subunit located near the entrance of the nascent peptide tunnel as revealed by crystal structures of the complex of antibiotics and the subunit.[6] Some antitumor natural products have been reported to inhibit protein synthesis, suggesting that the process of eukaryotic protein synthesis contains potential targets for cancer therapy.

13-Deoxytedanolide (13-DT) is a potent antitumor macrolide isolated from the marine sponge *Mycale adhaerens*.[7] A parent compound, tedanolide, was isolated from the Caribbean sponge *Tedania ignis* (Fig. 1).[8] Both 13-DT and tedanolide showed remarkable cytotoxicity against P388 murine leukemia cells at pico- to nanomolar ranges. Moreover, 13-DT has been shown to have promising in vivo antitumor activity.[7] In spite of

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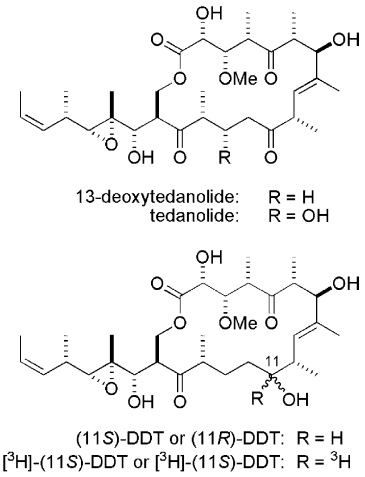

Figure 1. Chemical structures of 13-deoxytedanolide, tedanolide, (11*S*)-DDT, (11*R*)-DDT, and radiolabeled ligands.

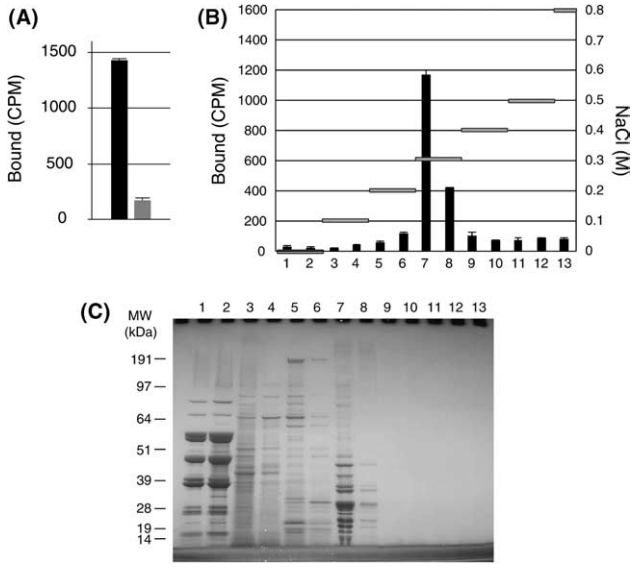

Figure 2. Selective binding of [^{3}H]-(11*S*)-DDT. (A) Binding of the radioligands to the yeast cell lysate. 10,000 DPM of either [^{3}H]-(11*S*)-DDT (black) or [^{3}H]-(11*R*)-DDT (gray) was incubated and filtered as described in Section 4. The experiment was carried out in duplicate, and the average is drawn (bars = SE). (B) Binding ability of [^{3}H]-(11*S*)-DDT to fractions obtained by DEAE Toyopearl column chromatography. NaCl concentrations are shown (gray bar). Yeast extract was separated on DEAE Toyopearl using stepwise elution. Aliquot (10 μL) of each fraction was examined for its binding ability as mentioned in Section 4. All data points were taken in duplicates, and the average is shown (bars = SE). (C) SDS-PAGE profile of each fraction obtained by DEAE Toyopearl column chromatography. Lane numbers correspond to those in (B).

their potent activity, their modes of action remained unknown. In this study, we searched for the target molecule of 13-DT by using radiolabeled analogues. From the budding yeast *Saccharomyces cerevisiae* cell lysate, we identified the 60S large ribosomal subunit as the 13-DT target, and showed that 13-DT strongly inhibited polypeptide synthesis. Furthermore, 13-DT was shown to share an unknown binding site on the 60S large ribosomal subunit with pederin,[9] a venom of blister beetles, and the related marine cytotoxins.[10] This is the first report that a macrolide binds to the eukaryotic ribosome.

2. Results and discussion

2.1. Synthesis of [^{3}H]-labeled analogues of 13-DT

In order to synthesize radiolabeled analogues retaining cytotoxicity, we attempted to reduce keto groups in 13-DT. Fortunately, a brief treatment of 13-DT with $NaBH_4$ resulted in the selective reduction of the C-11 keto group, furnishing two diastereomeric 13-deoxydihydrotedanolides (DDTs, Fig. 1). (11*S*)-DDT was as active as 13-DT (IC_{50} 14 pg/mL, P388 cells), while (11*R*)-DDT was significantly less active (IC_{50} 92 ng/mL). Therefore, we synthesized [11-^{3}H]-(11*S*)-DDT and its C-11 epimer by a treatment with [^{3}H]-$NaBH_4$ under the same conditions (Fig. 1).

2.2. Detection of 13-DT-binding molecule in the yeast cell lysate

13-DT and (11*S*)-DDT inhibited growth of *S. cerevisiae* while (11*R*)-DDT showed weaker growth inhibition (not shown). To identify the 13-DT binding protein, we incubated the cell lysate of *S. cerevisiae* with radio-labeled DDTs, and tested whether the radioactivity was detected in the fraction trapped on the glass filter. We found the potent radioactivity with [^{3}H]-(11*S*)-DDT more efficiently than with [^{3}H]-(11*R*)-DDT (Fig. 2A). Binding activity of these compounds correlated well with their antiyeast activities, suggesting the presence of a specific macromolecular target in yeast. Fractionation of the lysate on DEAE-Toyopearl concentrated the binding activity of [^{3}H]-(11*S*)-DDT in the 0.3 M NaCl fraction, only when performed in the presence of Mg^{2+} (Fig. 2B). SDS-PAGE of the active 0.3 M fraction displayed many bands smaller than 51 kDa (Fig. 2C). In the absence of Mg^{2+} in the eluting buffer, the binding activity of the radioligand was not observed for any fractions and totally different chromatographic profiles were obtained as observed by SDS-PAGE (not shown). Furthermore, we detected the 13-DT binding activity in the void volume fraction of the gel filtration column, which still contained a large number of proteins (not shown).

As described above, the target molecule of 13-DT showed two characteristic features; the magnesium ion requirement revealed by the anion exchange column and the extremely large molecular mass consisting of dozens of proteins indicated by the gel filtration. These features hinted that the target molecule of 13-DT was the ribosome.[11–13]

2.3. Binding of 13-DT to ribosomes

As expected, the radioligand bound to the purified salt-washed 80S ribosome. Scatchard plots analyzed by the least square method[14] revealed the presence of a high-

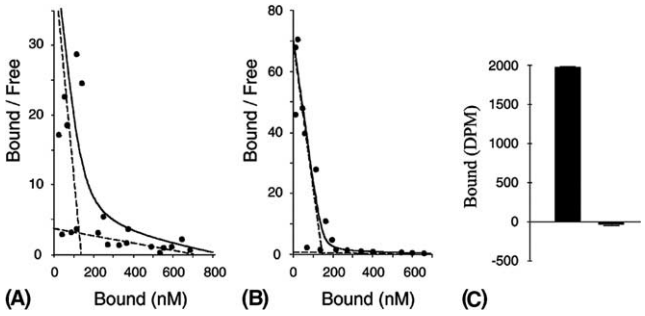

Figure 3. Binding of [^{3}H]-(11*S*)-DDT to ribosomes. Scatchard plots of data for binding of [^{3}H]-(11*S*)-DDT (A) to salt-washed 80S ribosome or (B) to 60S subunit. (A) Salt-washed 80S ribosome (0.22 μM) was incubated with the radioligand (30.1 nM–9.65 μM). (B) 60S ribosome (0.23–0.39 μM) was incubated with the radioligand (30.1 nM–9.65 μM). (C) Selective binding of the radioligand to the 60S large subunit. [^{3}H]-(11*S*)-DDT (38.6 nM) was incubated with 60S subunit (0.18 μM, left) or 40S subunit (0.18 μM, right). Binding experiments were carried out as described in Section 4. Results from two independent experiments were averaged and each experiment was carried out in duplicates (bars = SE).

affinity binding site (Fig. 3A, K_d 3.3 nM; n^s, 0.64) and a low-affinity binding site (K_d 0.19 μM; n^s, 3.3). Moreover, the radioligand bound to the 60S subunit with high affinity (Fig. 3B, K_d 2.1 nM; n^s, 0.65), but did not bind to the 40S subunit (Fig. 3C). The 60S subunit had only a high-affinity binding site, although 13-DT showed a high-affinity binding and a low-affinity binding to the 80S ribosomal complex. Although the molecular basis for this difference is currently unknown, it seems possible that it is due to the presence of the heterogeneity of the high-salt washed 80S complex as observed previously for [^{3}H]-anisomycin binding.[15]

The presence of the high-affinity binding (K_d 2.1 nM) suggested that a covalent bond formed between the ribosome and the macrolide, since the epoxide in the side chain of 13-DT plausibly seemed to participate in the bond formation. However, this was not the case. Neither ribosomal RNAs nor ribosomal proteins, extracted from the incubated mixture of the 60S subunit and the radioligand, showed radioactivities. This indicated that the radioligand was dissociable from the subunit (data not shown).

2.4. Inhibition of in vitro polypeptide synthesis by 13-DT

The strong binding of 13-DT to the 60S subunit of the ribosome suggests that 13-DT inhibits protein synthesis. To test this possibility, we examined the effects of 13-DT and DDTs on poly(U)-directed poly(Phe) synthesis in the yeast S30 fraction. 13-DT and (11*S*)-DDT inhibited this reaction at the IC_{50} value of 0.15 μM, whereas (11*R*)-DDT showed only moderate inhibition (IC_{50} = 0.80 μM) (Fig. 4). Under these conditions, the inhibitory activity of 13-DT was more potent than cycloheximide (IC_{50} = 0.30 μM). In contrast, 13-DT and DDTs did not inhibit the polypeptide synthesis in the cell lysate prepared from *Escherichia coli* even at a concentration of 10 μM (data not shown).

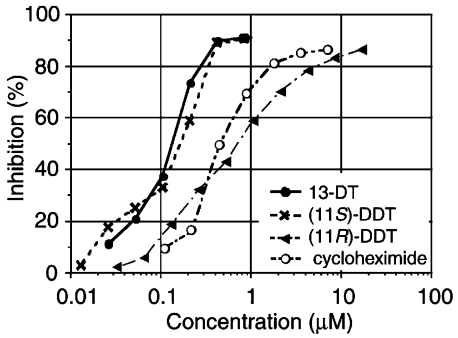

Figure 4. Inhibition of in vitro polypeptide synthesis by 13-DT, reduced analogues, and cycloheximide in yeast S30 extract. The lines are drawn to connect the data points. Data represent a mean of duplicate samples.

2.5. Competitive binding experiments

To investigate the binding site of 13-DT, competitive binding assays were carried out using ribosomal antibiotics, namely, peptidyltransferase inhibitors and translocation inhibitors. Typical peptidyltransferase inhibitors,[16] puromycin and anisomycin, which share a neighboring binding site around the peptidyltransferase center,[17,18] did not prevent the binding of the radioligand to the ribosome (data not shown).

The traditional translocation inhibitor, pederin (Fig. 5), was found to perturb the binding of the radioligand to the 60S large ribosomal subunit, while cycloheximide, another translocation inhibitor, did not inhibit it (Fig. 6). It is possible that cycloheximide has a binding site different from that of 13-DT, or binds to the 60S subunit much weaker than 13-DT.[19] Since Mandiyan et al. have suggested that the cycloheximide–ribosome interaction is a fast exchange reaction,[20] 13-DT may bind to the ribosome more efficiently than cycloheximide. However,

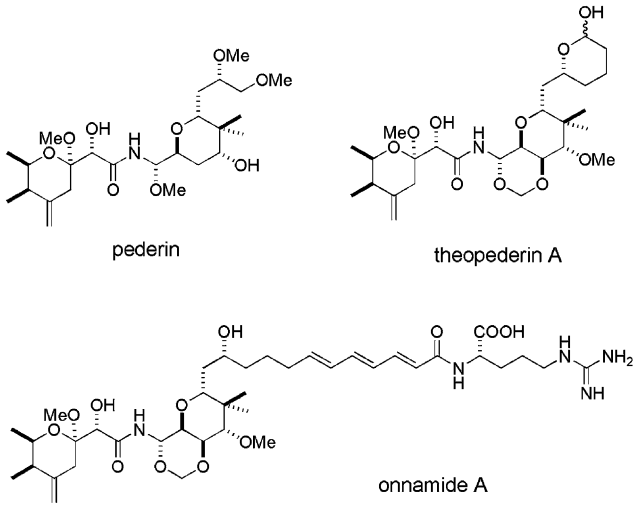

Figure 5. Chemical structures of pederin, theopederin A, and onnamide A.

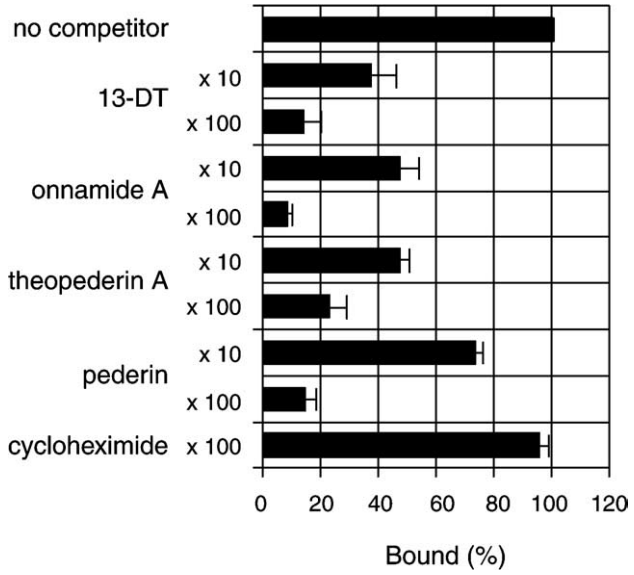

Figure 6. The effects of 13-DT, onnamide A, theopederin A, pederin, and cycloheximide on the binding of [^{3}H]-(11*S*)-DDT to 60S subunit. The radioligand (38.6 nM) was incubated with 60S subunits (0.18 μM), which had been pre-incubated with 10-fold excess amount (0.39 μM) or 100-fold excess amount (3.9 μM) of cold ligands. Binding experiments were carried out as described in Section 4. Results from two independent experiments are averaged. Each experiment was carried out in duplicates (bars = SE).

13-DT inhibited the growth of *Schwanniomyces occidentalis* and *S. cerevisiae* Cy32, both of which acquire resistance to cycloheximide by expressing altered proteins of the 60S subunit.[21,22] Furthermore, pederin inhibited potassium-induced nonenzymatic translocation by polysomes from a cycloheximide-resistant yeast.[23] It is therefore most likely that 13-DT and cycloheximide do not share their binding sites on the 60S subunit.

Theopederin A[24] and onnamide A[25] (Fig. 5), which are closely related to pederin, also competed with 13-DT for binding, thus indicating that the potent cytotoxins of the pederin class share the binding site on the 60S large subunit common to 13-DT (Fig. 6).

3. Conclusion

In this report, we show that 13-DT, a potent antitumor macrolide isolated from the marine sponge *Mycale adhaerens*, inhibits polypeptide elongation by binding to the 60S large subunit of the *S. cerevisiae* ribosome. Although many protein synthesis inhibitory natural products are known to bind to the prokaryotic large subunit and inhibit the polypeptide elongation, 13-DT is the first macrolide that binds to the eukaryotic ribosome. Macrolide antibiotics, for example, erythromycin and carbomycin, inhibit protein synthesis by binding to the 50S large subunit of prokaryotic ribosomes, but do not bind to eukaryotic ribosomes. This difference may be due to the size of rings; 12- to 16-membered rings in prokaryotic antibiotics versus 18-membered rings in tedanoldies. It is noteworthy that 13-DT does not inhibit protein synthesis in in vitro translation assay using *E. coli* extract or its growth.

Interestingly, 13-DT shares the binding site on the 60S ribosomal subunit with pederin, a structurally-distinct venom from blister beetles, and its analogues of marine sponge origin. Since these compounds are highly antitumorous,[7,26] the insight into their modes of binding to the 60S subunit will apply to anticancer drug design.[27] It is also expected that 13-DT will be an important tool for elucidating structure and function of eukaryotic ribosomes.

4. Experimental

4.1. Materials

13-DT was isolated from a marine sponge *Mycale* sp., collected off the Kii Peninsula, western Japan, by the previously described procedures.[7] Onnamide A and theopederin A were isolated from the marine sponge *Theonella swinhoei* as described previously.[28,24] Pederin was purified from the EtOH extract of the female blister beetles, *Paederus fuscipes*, a kind gift from Jörn Piel. Puromycin, anisomycin, and cycloheximide were purchased from Wako Pure Chemicals (Osaka). [^{3}H]-$NaBH_4$ (15.0 Ci/mmol) was purchased from Perkin–Elmer, while [^{14}C(U)]-L-phenylalanine was from Moravek Biochemical. The yeast *S. cerevisiae* A364A was obtained from ATCC (ATCC No 22244).

4.2. Reduction of 13-deoxytedanolide

To a solution of 13-DT (12.0 mg) in MeOH (1.0 mL) was added $NaBH_4$ (20 equiv), and the mixture was stirred at rt for 10 min. The reaction mixture was diluted with 10 mL of water and desalted on an ODS column (5 mL). The product was separated by ODS HPLC with MeCN–H_2O (45:55) followed by ODS HPLC with MeOH–H_2O (65:35) to afford (11*S*)-DDT (2.4 mg) and (11*R*)-DDT (4.3 mg). Their structures were confirmed by interpretation of NMR and MS spectra. Absolute stereochemistry at C-11 was determined by NMR analysis and the modified Mosher's method.[29] Structural assignments are described in the following paper.[30]

4.3. Preparation of tritium-labeled analogues

13-DT (1.0 mg) was treated with [^{3}H]-$NaBH_4$ (15.0 Ci/mmol, 0.1 GBq) in 2-PrOH (25 μL) at rt overnight. The reaction mixture was processed as described above to afford [11-^{3}H]-(11*S*)-DDT (115 μg, 1.67 Ci/mmol) and [11-^{3}H]-(11*R*)-DDT (40 μg).

4.4. Binding assays

Saccharomyces cerevisiae A364A was cultured in YPD media until A_{600} reached 2–3. Cells were harvested by centrifugation at 3000 rpm for 20 min. To the yeast pellet was added 1 mL of extraction buffer (10 mM Tris·HCl (pH 7.2)/20 mM $Mg(OAc)_2$/30 mM NH_4Cl/10 μg/mL of

leupeptin/10 μg/mL of pepstatin/10 μg/mL of AEBSF) and 2 g of acid-washed glass beads per 1 g of yeast, and the mixture was vortexed for ten 30 s bursts and 30 s chillings on ice between bursts. The result lysate was centrifuged at 5000*g* for 20 min, and the supernatant was adsorbed onto DEAE-Toyopearl, and eluted stepwisely with buffer (10 mM Tris·HCl (pH 7.2)/20 mM $Mg(OAc)_2$/30 mM NH_4Cl/0–0.8 M NaCl). The 0.3 M NaCl fraction was subjected to further separation. The binding activity was examined as follows: aliquots of each fraction (10–100 μL) was incubated with 10,000 DPM of radioligands in a total volume of 200 μL reaction mixtures (20 mM Tris·HCl (pH 7.2)/20 mM $Mg(OAc)_2$/30 mM NH_4Cl) on ice for 1.5 h. The samples were applied onto GF/C glass fiber filters (Whatman), which were washed three times with 200 μL of ice-cold incubation buffer. Filters were dried and the radio activity was measured by a liquid scintillation counter.

4.5. Preparation of the salt-washed 80S ribosome and ribosomal subunits

The salt-washed 80S ribosome was prepared according to Algire et al.[31] The 40S and the 60S ribosomal subunits were prepared by the method of van der Zeijst et al.[32] The 80S ribosome, the 60S large subunit, and the 40S subunit were suspended in a ribosome buffer (20 mM Tris·HCl (pH 7.5)/100 mM KCl/2.5 mM $Mg(OAc)_2$/2 mM β-ME), aliquoted, flash frozen in liquid N_2, and stored at −80 °C until use. The concentration of ribosomes were determined by A_{260} using coefficients of 5×10^7, 3×10^7, and $2\times10^7\,cm^{-1}M^{-1}$ for the 80S ribosome, the 60S subunit, and the 40S subunit, respectively.[33]

4.6. Binding of the radiolabeled 13-DT analogue to ribosomes

Binding of the radioligand to ribosomes was measured by the membrane ultrafiltration method[34] with some modifications. Either the salt-washed 80S ribosome (0.22 μM), the 60S ribosomal subunit (0.23–0.39 μM), or the 40S subunit (0.36 μM) was incubated with the radioligand (20 nM–9.7 μM) in 100 μL of ribosome buffer at 30 °C for 30 min. The incubated mixture was placed on ice and quickly removed to a centrifugation filter device, Microcon YM-100 (Millipore), followed by centrifugation at 500*g* for 10 min at 4 °C. A 50 μL portion of the filtrate was removed for scintilation counting. In the competitive binding experiments, a cold competitor was incubated for 30 min at 30 °C with ribosomes before addition of the radioligand. Nonspecific binding in the presence of 100-fold excess amount of cold (11*S*)-DDT was subtracted in all experiments.

4.7. Poly(U)-directed poly(Phe) synthesis

Reactions were carried out as described by Sutton et al.[35] and cell-free extract was prepared by the methods of Iizuka and Sarnow.[36] Poly(U)-directed poly(Phe) synthesis reactions were carried out in 50 μL volumes containing 25 μL of the prepared S30 fractions at 30 °C for 30 min.

4.8. Cytotoxicity assay

Cytotoxicity against P388 cells was evaluated by using MTT assay as described previously.[37]

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