Epolactaene binds human Hsp60 Cys⁴⁴² resulting in the inhibition of chaperone activity

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Epolactaene is a microbial metabolite isolated from *Penicillium* sp., from which we synthesized its derivative ETB (epolactaene tertiary butyl ester). In the present paper, we report on the identification of the binding proteins of epolactaene/ETB, and the results of our investigation into its inhibitory mechanism. Using biotin-labelled derivatives of epolactaene/ETB, human Hsp (heat-shock protein) 60 was identified as a binding protein of epolactaene/ETB *in vitro* as well as *in situ*. In addition, we found that Hsp60 pre-incubated with epolactaene/ETB lost its chaperone activity. The *in vitro* binding study showed that biotin-conjugated epolactaene/ETB covalently binds to Hsp60. In order to investigate the binding site, binding experiments with alanine mutants

of Hsp60 cysteine residues were conducted. As a result, it was suggested that Cys⁴⁴² is responsible for the covalent binding with biotin-conjugated epolactaene/ETB. Furthermore, the replacement of Hsp60 Cys⁴⁴² with an alanine residue renders the chaperone activity resistant to ETB inhibition, while the alanine replacement of other cysteine residues do not. These results indicate that this cysteine residue is alkylated by ETB, leading to Hsp60 inactivation.

Key words: chaperone activity, citrate synthase, covalent binding, epolactaene, heat-shock protein 60 (Hsp60), malate dehydrogenase.

INTRODUCTION

Epolactaene is a microbial metabolite isolated from the fungal strain *Penicillium* sp. BM 1689-P [1]. It was originally shown to be effective in promoting neurite outgrowth and arresting the cell cycle at the G₀/G₁ phase in a human neuroblastoma cell line, SH-SY5Y [2]. A study about epolactaene on DNA polymerase inhibition has been conducted [3,4]; however, there is no direct evidence that epolactaene interacts with those proteins in the cell. Therefore we began to study epolactaene-binding proteins in order to investigate how epolactaene affects cellular proteins and their functions.

The Hsp (heat-shock protein) 60 family is known to assist correct protein folding [5]; in addition, mammalian Hsp60 seems to have several functions in the cell, including apoptosis [6–9], an immunoregulatory function [10,11] and cell spreading [12]. Despite the importance of this molecule, little research has been conducted into mammalian Hsp60 compared with members of the prokaryotic Hsp60 family, such as GroEL. Although there is approx. 50 % sequence identity between mammalian Hsp60 and GroEL [13], they are not completely similar in structure. GroEL works only in the form of a 14-mer, double ring structure [14,15]. In contrast, human mitochondrial Hsp60 has been reported to be able to assist in protein folding in the form of a 7-mer, single ring structure [16,17]. It is also notable that mitochondrial Hsp60 is functionally active only with co-chaperone Hsp10, whereas GroEL can assist protein folding with either GroES or Hsp10 [14]. Further studies are needed to gain a detailed understanding of the molecular chaperone mechanism and functions of human Hsp60. In the present paper, we demonstrate that epolactaene and its derivative ETB (epolactaene tertiary butyl ester) bind to Hsp60 and inhibit its chaperone activity.

EXPERIMENTAL

Reagents

Monoclonal anti-Hsp60 antibody was purchased from BD Biosciences (San Jose, CA, U.S.A.). ETB was synthesized as reported in our synthetic study of epolactaene [18]. Biotin-conjugated ETB (bio-ETB) was synthesized by a coupling reaction using an activated biotin reagent [19]. The structures of ETB, its analogue and bio-ETB were determined by NMR analysis and MS.

Cell lines and culture

Human neuroblastoma SH-SY5Y cells and human cervix carcinoma HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, U.S.A.), supplemented with 10 % (v/v) heat-inactivated foetal calf serum (JRH Bioscience, Lenexa, KS, U.S.A.), 50 units/ml penicillin, 50 µg/ml streptomycin (Sigma) and 1 mM sodium pyruvate (Gibco, Invitrogen Corporation, Carlsbad, CA, U.S.A.) in 5 % CO₂ at 37 °C. T-lymphoma Jurkat cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10 % (v/v) heat-inactivated foetal calf serum, 50 units/ml penicillin and 50 µg/ml streptomycin.

Detection of epolactaene/ETB-binding proteins

The general procedure used to prepare the cell lysates for the detection of binding proteins has been described previously [20,21]. The lysates were incubated with or without ETB as a competitor, followed by the treatment with bio-ETB. After incubation for 1 h at 4 °C, proteins associated with bio-ETB were precipitated with streptavidin-agarose (Oncogene, San Diego, CA, U.S.A.). The bound proteins were eluted with SDS/PAGE loading buffer,

Abbreviations used: CS, citrate synthase; ETB, epolactaene tertiary butyl ester; bio-ETB, biotin-conjugated ETB; i-ETB, inactive ETB analogue; bio-i-ETB, biotin-conjugated i-ETB; HRP, horseradish peroxidase; Hsp, heat-shock protein; LC, liquid chromatography; MDH, malate dehydrogenase; NEM, N-ethylmaleimide; SAR, structure-activity relationship.

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separated by SDS/7.5 % PAGE, and visualized by silver staining. For immunological analysis, the separated proteins were subjected to Western blot analysis.

For the *in situ* binding assay, Jurkat cells were treated with several concentrations of biotin-conjugated derivatives for 2 h. The cells were collected in lysis buffer (10 mM Tris/HCl, pH 7.6, 1 % Nonidet P40, 0.1 % sodium deoxycholate, 0.1 % SDS and 0.15 M NaCl) containing Complete protease inhibitors (Roche, Mannheim, Germany), and the supernatants were then prepared by centrifugation at 800 g for 5 min, followed by centrifugation at 20 000 g for 15 min at 4 °C. Immobilized streptavidin magnetic beads (Promega, Madison, WI, U.S.A.) were added to the lysates, and the mixtures were incubated for 3 h at 4 °C. The bound proteins were washed and eluted by SDS/PAGE loading buffer with boiling. The separated proteins were transferred and analysed by immunoblotting with the anti-Hsp60 antibody.

Construction of human mitochondrial Hsp60-His₆ expression vector

A full-length cDNA encoding the precursor form of human mitochondrial Hsp60 [22] obtained from a Jurkat cDNA library (kindly provided by Dr S. Simizu of RIKEN) was modified so as to provide a translational start codon (AUG) at 5' to Ala²⁷, the first amino acid of the mature protein, as described previously [23]. To this was also added a C-terminal extension of eight amino acids (LEHHHHHH), the last six being a hexahistidine tag. Two PCR primers were employed: primer 1 (5'-GCCATATGGCCAAAGATGTAAATTTGGTG-3') introduced an NdeI site (underlined) at the initiator methionine, while primer 2 (5'-CGCCTCGAGGAA-CATGCCACCTCCCATAC-3') introduced an XhoI site (underlined) upstream of the stop codon. The PCR product was cut with NdeI and XhoI, and was ligated into the expression vector pET-21b(+) (Novagen, Darmstadt, Germany) digested with the same enzymes. The DNA sequence of this novel fusion protein was confirmed, and it will be referred to throughout the present paper as Hsp60-His₆.

Purification of recombinant mitochondrial Hsp60-His₆

Escherichia coli BL21(DE3) pLysS, transformed with the vector, was grown in LB (Luria-Bertani) medium with 100 µg/ml ampicillin at 28 °C. At a *D*₆₀₀ of 0.4, IPTG (isopropyl β-D-thiogalactoside) was added to 1 mM, and the cells were shaken for an additional 10 h. A soluble cell extract was prepared, and the mitochondrial Hsp60-His₆ was purified with Ni²⁺-nitrilotriacetate-agarose (Qiagen, Hilden, Germany) and desalted with PBS by dialysis.

Mutagenesis and mutant expression

The mutageneses of Cys²³⁷ to Ala (for Hsp60-1), Cys⁴⁴² to Ala (Hsp60-2) and Cys⁴⁴⁷ to Ala (Hsp60-3) were performed according to the method described previously [24], using a mutagenic primer for Hsp60-1 (5'-CAAAAGGTCAGAAAGCTGAGTTC-CAGGATG-3'), Hsp60-2 (5'-GTTTTGGGAGGGGGCGCCGC-CCTCCTTCG-3') or Hsp60-3 (5'-GTGCCCTCCTGCGGCC-ATTCCAGCCTTG-3'). The entire sequences of Hsp60-1, 2 and 3 were analysed to ensure that only the desired mutation had been introduced. The mutant Hsp60-His₆ proteins were prepared as described above.

Chaperone activity of Hsp60

The chaperone activity of human Hsp60 (Stressgen Biotechnologies Corp., Victoria, BC, Canada) was measured using porcine

heart CS (citrate synthase) (Sigma) [25] and porcine heart malate dehydrogenase (MDH) (Nacalai Tesque, Kyoto, Japan) [16], as described previously. Briefly, CS was denatured at a concentration of 15 µM in a buffer containing 6 M guanidinium chloride, 100 mM Tris/HCl, pH 8, and 20 mM dithioerythritol. Reactivation was initiated by diluting the denatured CS 100-fold into a buffer containing 100 mM Tris/HCl, pH 7.6, 10 mM MgCl₂, 10 mM KCl and 2 mM ATP at 20 or 35 °C with or without chaperones. Detailed information on the individual experiments are given in the legend to Figure 4. Human Hsp10 was also cloned and prepared as described previously [26]. To reconstitute the chaperone complex, Hsp60 and Hsp10 were mixed and incubated in reconstitution buffer (50 mM Tris/HCl, pH 7.6, 300 mM NaCl, 20 mM KCl, 20 mM magnesium acetate and 4 mM ATP) for 90 min at 30 °C.

For the MDH refolding assays, folding reactions were performed in 0.1 M Tris/HCl, pH 7.6, 7 mM KCl, 7 mM MgCl₂ and 10 mM dithiothreitol (buffer A). Porcine MDH (1 µM) was denatured in 10 mM HCl at room temperature (25 °C) for 2 h and diluted 10-fold into buffer A containing chaperones (2.1 µM Hsp60 and 4.2 µM Hsp10) pre-incubated in a reconstitution buffer. After incubation at 27 °C for 5 min, folding was initiated by the addition of 2 mM ATP. For the measurement of the ETB dose-response on the inhibition of Hsp60 mutant chaperone activities, wild-type and mutant Hsp60-His₆ proteins were used.

Hsp60 binding assay

For the *in vitro* binding assay, recombinant Hsp60-His₆ was pre-treated in the presence or the absence of 10 µM NEM (*N*-ethylmaleimide) as the competitor, and then treated with bio-ETB at 4 °C. The protein samples were separated by SDS/PAGE and detected by Western blotting using avidin-conjugated HRP (horse-radish peroxidase) (Pierce, Rockford, IL, U.S.A.).

Proteins were purchased from Stressgen for the binding experiment of bio-ETB with human Hsp60, Hsp70 and Hsp90. A mixture of 0.7 µM Hsp60, Hsp70 and Hsp90 was incubated with 0.35 µM bio-ETB for 1 h at 4 °C, and subjected to SDS/PAGE and Western blotting analysis.

RESULTS

Epolactaene derivatives for the screening of binding proteins

To investigate how epolactaene affects cellular proteins, we began to search for epolactaene-binding proteins in a cell lysate. During the SAR (structure-activity relationship) study for epolactaene [19], we synthesized a potent derivative, ETB, and an inactive derivative, i-ETB (Figure 1). We found that ETB is as effective as epolactaene in inhibiting the growth of several human cancer cell lines. Because of the ease of synthetic preparation, we used ETB instead of epolactaene in further studies.

On the basis of observations from the SAR studies, we also prepared bio-ETB and a biotin-conjugated inactive ETB (bio-i-ETB). ETB and i-ETB are conjugated with a biotin moiety at the ester position with an alkyl chain linker (Figure 1). Bio-ETB was found to retain the ability to inhibit growth on the SH-SY5Y and Jurkat cells, whereas bio-i-ETB was as inactive as i-ETB [19].

Identification of Hsp60 as an epolactaene/ETB-binding protein *in vitro* and *in situ*

With the biotin-conjugated probes in hand, we searched for the epolactaene/ETB-binding proteins. An SH-SY5Y cell extract was incubated with several concentrations of bio-ETB, and the bound

Figure 1 Chemical structures of epolactaene, ETB, i-ETB and biotin conjugates

Figure 2 Screening of ETB-binding proteins

A SH-SY5Y cell lysate was pre-incubated with 70 μ M ETB as a competitor, followed by treatment with 4 μ M biotinylated ETB. The bound proteins were precipitated with streptavidin beads. The proteins eluted were analysed by SDS/7.5% PAGE, followed by silver staining. Molecular masses are indicated in kDa to the left of the gel.

proteins were precipitated with streptavidin beads and detected by silver staining. To minimize false positives, we used ETB as a competitor. There was a band at approx. 60 kDa, which disappeared when ETB was added as a competitor (Figure 2). Next, a large amount of eluted proteins was analysed by Coomassie Brilliant Blue staining to apply liquid chromatography (LC)–MS analysis. The 60 kDa regions were excised and digested with lysyl endopeptidase, and then the resulting peptide mixture was

Figure 3 Identification of Hsp60 as the epolactaene/ETB-binding protein

(A) Immunoblot analysis of the 60 kDa protein bound to epolactaene/ETB. SH-SY5Y cell lysates were pre-incubated with 130 μ M of each competitor, and the Jurkat cell lysates were pre-treated with or without 70 μ M ETB, followed by 13 μ M bio-ETB. The bound proteins were precipitated with streptavidin beads. These proteins were analysed by immunoblotting with the anti-Hsp60 antibody. (B) Binding experiment of bio-ETB with Hsp60, Hsp70 and Hsp90 *in vitro*. An equimolar mixture of human Hsp60, Hsp70 and Hsp90 was incubated with bio-ETB for 1 h at 4 °C. The proteins were separated by SDS/PAGE and analysed by HRP-conjugated avidin blotting and Coomassie Brilliant Blue (CBB) staining. (C) Binding of bio-ETB to Hsp60 *in situ*. Jurkat cells in culture were treated with biotin-conjugated derivatives for 2 h. The cells were collected in a lysis buffer. Immobilized streptavidin was added to the lysates, and the mixtures were incubated for 3 h at 4 °C. The bound proteins (upper panel) and whole lysates (lower panel) were analysed by immunoblotting with the anti-Hsp60 antibody.

subjected to LC–MS and MS/MS analysis. The peptide fragment from the 60 kDa band was assigned to residues 370–387 of human Hsp60 [RIQEIIQLDVTTSSEYEK, (M + H)⁺ calculated, 2195.4; found, 2195.7]. To confirm Hsp60 as an epolactaene/ETB-binding protein, we analysed the binding protein using an anti-Hsp60 antibody (Figure 3). Western blotting experiments with the anti-Hsp60 antibody showed that Hsp60 bound to epolactaene/ETB specifically, because an excess of epolactaene/ETB blocked this association, but i-ETB did not. This association was also found in the Jurkat cell lysate, as shown in Figure 3(A). In addition, when we treated bio-ETB with an equimolar mixture of human Hsp60, Hsp70 and Hsp90 *in vitro*, Hsp60 was mainly labelled by bio-ETB (Figure 3B).

To examine whether the binding between epolactaene/ETB and Hsp60 can occur in the cells, we analysed this association *in situ* using bio-ETB derivatives. Jurkat cells were cultured with bio-ETB or bio-i-ETB for 2 h. The biotin-containing complexes were then isolated by streptavidin-immobilized beads, and the bound proteins were analysed by immunoblotting. The samples containing bio-ETB reacted with the anti-Hsp60 antibody in a concentration-dependent manner, whereas those containing bio-i-ETB showed no detectable band, demonstrating that Hsp60 bound specifically to bio-ETB (Figure 3C). These data indicate that epolactaene/ETB can bind to Hsp60 in living cells.

Inhibition by epolactaene/ETB of the chaperone activity of Hsp60

Hsp60 is a molecular chaperone which assists in protein folding. Mammalian Hsp60 was found to have two forms: cytoplasmic precursor Hsp60 and mitochondrial Hsp60. Although the two forms differ in that the former has a signal sequence in the N-terminus of the protein (1–26 amino acid residues), while the latter does not, both proteins exhibit molecular chaperone activity *in vitro* [27]. In the present study, we used recombinant human mitochondrial Hsp60 for the measurement of chaperone activity.

To determine whether epolactaene/ETB affects the chaperone activity of Hsp60, we assessed the effect of epolactaene/ETB binding on the activity of human recombinant Hsp60. CS refolding was analysed under permissive conditions (20 °C), where spontaneous reactivation could occur, or under non-permissive conditions (35 °C), where spontaneous reactivation could not occur. As shown in Figure 4(A), CS refolded to its native state in a chaperonin-independent manner under permissive conditions. ETB did not interfere with this reactivation, even at 7 μ M. In contrast, the spontaneous reactivation of CS was not observed at 35 °C (Figure 4B). When either of the proteins (Hsp10 or Hsp60) was added singly, only marginal reactivation was observed. In the presence of the complete chaperonin system consisting of Hsp60 and Hsp10, a remarkable recovery of active CS was observed. When we pre-treated Hsp60 with ETB (a final concentration of 2 μ M at the refolding step), the reactivation was almost completely blocked. In addition, when extra (the same amount as in the first addition) Hsp60 was added to the reaction mixture which included the ETB-pre-treated Hsp60, the reactivation was recovered, but such recovery did not occur when extra Hsp10 (the same amount as in the first addition) was added (results not shown).

In order to confirm that ETB inhibits the chaperone function of Hsp60, the other substrate, mitochondrial MDH, was used. As shown in Figure 4(C), a substantial reactivation of the denatured enzyme was observed in the presence of Hsp60 and Hsp10, although neither protein could induce reactivation when added singly. Again, when we pre-treated Hsp60 with ETB, the reactivation was blocked. The reactivation was recovered by the addition of extra Hsp60 (the same amount as in the first addition), but not by the addition of extra Hsp10 (the same amount as in the first addition) (results not shown). These results indicate that the interaction between Hsp60 and epolactaene/ETB is sufficient to affect Hsp60 chaperone activity.

Cys⁴⁴² of Hsp60 is responsible for binding with epolactaene/ETB

To determine how epolactaene/ETB modifies the function of Hsp60, we first examined the binding site of Hsp60 with epolactaene/ETB. An *in vitro* binding study (Figure 5A) demonstrated that this binding could last in the SDS/PAGE condition, because it was stained by HRP-conjugated avidin. In addition, this binding was considerably inhibited in the presence of NEM. Because NEM is a Michael acceptor, it was suggested that the covalent binding occurs in a nucleophilic manner. The α,β -unsaturated ketone, epoxide and hemi-aminal carbon of epolactaene/ETB have an electrophilic character that is potentially reactive with biological nucleophiles, such as the thiols of cysteine residues.

Human Hsp60 has three cysteine residues in the molecule. To confirm that the modification of Hsp60 function occurs as described above, the alanine mutant proteins of each of three cysteine residues (C237A, C442A and C447A) in Hsp60 were prepared in *E. coli* with a C-terminal His₆-tag. They were then tested for the ability to bind with bio-ETB. As shown in Figure 5(B), the wild-type Hsp60–His₆, C237A and C447A mutants bound with

Figure 4 ETB inhibits the chaperone activity of Hsp60

The chaperone activity of Hsp60 was analysed under the following conditions. **(A)** Effects of ETB on CS reactivation at permissive conditions. Denatured CS was rapidly diluted to a concentration of 0.15 μ M into a buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 10 mM KCl and 15 % glycerol) with various concentrations of ETB at 20 °C. **(B)** Under non-permissive conditions, denatured CS was rapidly diluted to a concentration of 0.15 μ M into a buffer (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ and 10 mM KCl) containing additives (1.1 μ M Hsp60 or 3.2 μ M Hsp10, or both) at 35 °C. Hsp60 at 14 μ M was pre-incubated with or without 2 molar equivalents of ETB in PBS at 4 °C overnight. ATP (2 mM) was added to the mixture, and after 2 h of incubation, the reactivation of CS was measured. Bar 1, no additives; bar 2, Hsp10 alone; bar 3, Hsp60 alone; bar 4, Hsp60 and Hsp10; bar 5, ETB-pre-treated Hsp60 and Hsp10. **(C)** Effects of ETB using MDH as a substrate. Denatured MDH was rapidly diluted to a concentration of 0.1 μ M into a buffer (0.1 M Tris/HCl, pH 7.6, 7 mM KCl, 7 mM MgCl₂ and 10 mM dithiothreitol) containing additives (2.1 μ M Hsp60 or 4.2 μ M Hsp10, or both) at room temperature. Hsp60 was pre-treated with or without ETB, as described in **(B)**. ATP (2 mM) was added to the mixture, and, after 30 min, the reactivation of MDH was measured. Bar 1, no additive; bar 2, Hsp10 alone; bar 3, Hsp60 alone; bar 4, Hsp60 and Hsp10; bar 5, ETB-pre-treated Hsp60 and Hsp10.

bio-ETB, but C442A lost its binding. These results suggested that Cys⁴⁴² is responsible for covalent binding with bio-ETB.

Binding of ETB to Hsp60 via Cys⁴⁴² is also important for the inhibition of chaperone activity by ETB

Since the activities of the cysteine mutants of human Hsp60 are not known, we first examined the ability of each mutant to facilitate productive folding. We found that the ability of these altered forms of Hsp60–His₆ to productively fold MDH was unimpaired compared with that of the wild-type Hsp60–His₆ (Figure 6, open bars).

We next investigated the ETB inhibition effects on both the wild-type and mutant Hsp60–His₆ proteins. When each protein was pre-treated with ETB, the chaperone activities of the C237A

Figure 5 Cys⁴⁴² of Hsp60 is responsible for binding to epilactaene/ETB

(A) Competitive effect of NEM on the binding of Hsp60-His₆ to bio-ETB. Wild-type Hsp60-His₆ (1 μ M) was pre-incubated with or without 10 μ M NEM at 4 °C. After 4 h, 10 μ M bio-ETB was added to the mixture, which was then incubated for an additional 1 h. The proteins were resolved by SDS/7.5% PAGE and analysed by blotting with HRP-conjugated avidin (upper panel) and CBB staining (lower panel). (B) Binding experiments of the Hsp60 mutants with bio-ETB. The wild-type (wild) and the Hsp60-His₆ mutants (14 μ M) were each incubated with 2 molar equivalents of bio-ETB at 4 °C for 12 h. Blotting with HRP-conjugated avidin (upper panel) and Coomassie Brilliant Blue staining (lower panel) were performed as described in (A).

Figure 6 Inhibition by ETB of Hsp60 mutant chaperone activity on the chaperone-dependent MDH refolding

Denatured MDH was diluted 10-fold to 0.1 μ M at room temperature by suspending in the buffer containing 2.1 μ M each of Hsp60-His₆ mutants and 4.2 μ M Hsp10-His₆ or BSA at the same protein concentration. After incubation for 5 min, ATP (2 mM, final concentration) was added to the mixture. At 30 min after the addition of ATP, an aliquot of the mixture was withdrawn, and the recovered MDH activity was measured. The reactivation by each Hsp60-His₆ mutant pre-treated with ETB (grey bars; the mutant proteins at 14 μ M in PBS were incubated with 1.8 molar equivalents of ETB at 4 °C for 8 h) or without ETB (open bars) are shown. The reactivation by the wild-type (wild) Hsp60-His₆ pre-treated with 1.8 molar equivalents of NEM and 1.8 molar equivalents of mizoribine under the same conditions as ETB are shown by a hatched and a dotted bar respectively. The activity of the same amount of native MDH was taken as 100%, and then the reactivation (%) by BSA was subtracted as the baseline. The results are the means $\pm$ S.D. for three independent experiments.

and C447A mutants, and of the wild-type Hsp60-His₆, were considerably inhibited (Figure 6, grey bars). In contrast, the chaperone activity of the C442A mutant was not abolished by ETB pre-treatment. These results indicate that the inhibition of the

chaperone activity by ETB pre-treatment is derived mainly from the binding between the ETB and Cys⁴⁴² residue. In addition, these results are in agreement with the binding experiments described above (Figure 5B), which also suggested that ETB inhibits the chaperone activity by binding to Cys⁴⁴² of Hsp60.

Furthermore, we compared the inhibitory effect with NEM and mizoribine, which was reported to interfere with the chaperone activity of Hsp60 and Hsp10 at a higher concentration, 5 mM [28]. Under our experimental conditions, epilactaene/ETB inhibited Hsp60 chaperone activity at a concentration of 2–4 μ M in the refolding reaction mixture. Under the same conditions as epilactaene/ETB (3.8 μ M in the refolding reaction mixture), NEM and mizoribine did not show an inhibitory effect (Figure 6, hatched and dotted bars respectively).

DISCUSSION

The discovery of the binding proteins of bioactive natural compounds is one strategy for investigating their biological effects. There are many successful precedents, such as the studies on lactacystin [29], fumagillin [30], radicicol [20] and other compounds. With lactacystin, for example, the discovery that the binding target was the 20 S proteasome subunit, and that lactacystin inhibited proteasome activity [29], has promoted the exploration of not only the mode of action of lactacystin, but also the proteasome functions. In this way, the identification of binding proteins furthers biochemical research.

ETB, which is a chemically synthesized derivative of epilactaene, is as effective as epilactaene in inhibiting the growth of several human cancer cell lines [19]. Although several studies on DNA polymerase inhibition have already been conducted [3,4], the IC₅₀ values of the DNA polymerase α and β inhibition by epilactaene were reported to be 25 and 100 μ M respectively *in vitro*, which are considerably higher than the effective dose on the cell growth inhibition. In the present study, we searched for the binding targets of epilactaene/ETB in order to obtain direct support for the probability that epilactaene interacts with cellular proteins and disturbs its function. There were several specific or non-specific binding proteins *in vitro*, and we successfully identified human Hsp60 using bio-ETB as a bioprobe (Figures 2 and 3). The binding between Hsp60 is specific for epilactaene/ETB because the binding was blocked by the addition of epilactaene/ETB, but not by the addition of inactive analogue i-ETB. Furthermore, the finding that bio-ETB can bind Hsp60 *in situ* indicated that this complex is also formed in living cells (Figure 3C).

To elucidate the details of the interaction between ETB and Hsp60, we prepared Hsp60 mutants. Our analysis of the binding between bio-ETB and the mutant proteins suggested that Cys⁴⁴² of Hsp60 is responsible for the binding (Figure 5B). To determine how this binding affects the Hsp60 chaperone activity, we first analysed the chaperone activity of each cysteine mutant, since the mutants of human Hsp60 have scarcely been explored [16]. We found that the chaperone activity of mutant Hsp60-His₆, using MDH as a substrate, was almost the same as that of the wild-type Hsp60-His₆ (Figure 6). ETB blocked the chaperone activities of the wild-type protein and of the C237A and C447A mutant proteins, but not that of the C442A mutant (Figure 6), indicating that epilactaene/ETB binding to Cys⁴⁴² disrupts the chaperone activity. Based on sequence alignment and a comparison with the reported GroEL crystal structures [31], Cys⁴⁴² is predicted to lie near the ATP binding site (see Supplemental Figure 1 at <http://www.BiochemJ.org/bj/387/bj3870835add.htm>), although none of the three cysteine residues in Hsp60 is conserved in GroEL. The ATP pocket is suggested to be important not only for ATPase activity, but also for oligomerization [14,32]. Therefore interaction with

ETB may disturb the nucleotide pocket and result in the loss of ATPase activity or the destabilization of oligomeric structures, or both, although it is difficult to determine which is responsible for the inhibition.

To date, there are scarcely any natural compounds known which bind and inhibit mammalian Hsp60 chaperone activity. The 60 kDa chaperones constitute a family of proteins that play an important role in the folding of nascent, translocated and stress-denatured proteins [5]. In addition, mammalian Hsp60 has been reported to have several functions in the cell [6–12]. Using mizoribine, which inhibits the chaperone activity of mammalian Hsp60/Hsp10 at 5 mM [28], the association of Hsp60 with integrin $\alpha_3\beta_1$ was investigated [12]. Hsp60-binding compounds would be very useful for studying mammalian Hsp60 functions. In the present study, we discovered that bio-ETB covalently binds to Cys⁴⁴² of Hsp60 and inhibits its chaperone activity. As far as we know, this is the first example of human Hsp60 covalently modified by a natural compound in a site-specific way.

In conclusion, we have demonstrated that epolactaene/ETB binds human Hsp60 *in vitro* and *in situ*, and that epolactaene/ETB inhibited the chaperone activity of human Hsp60. Furthermore, we have revealed that Cys⁴⁴² of Hsp60 is responsible for the covalent binding of epolactaene/ETB, and have shown the resistance of the C442A mutant to ETB inhibition, suggesting this binding resulted in the inhibition of the chaperone activity. These observations of how epolactaene/ETB inhibits the chaperone activity will be helpful for gaining an understanding of the human Hsp60 multifunctions and the mechanisms of molecular chaperones.

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REFERENCES

- 1 Kakeya, H., Takahashi, I., Okada, G., Isono, K. and Osada, H. (1995) Epilactaene, a novel neurotogenic compound in human neuroblastoma cells, produced by a marine fungus. *J. Antibiot.* **48**, 733–735
- 2 Kakeya, H., Onozawa, C., Sato, M., Arai, K. and Osada, H. (1997) Neurotogenic effect of epilactaene derivatives on human neuroblastoma cells which lack high-affinity nerve growth factor receptors. *J. Med. Chem.* **40**, 391–394
- 3 Nakai, J., Kawada, K., Nagata, S., Kuramochi, K., Uchiro, H., Kobayashi, S. and Ikeita, M. (2002) A novel lipid compound, epilactaene, induces apoptosis: its action is modulated by its side chain structure. *Biochim. Biophys. Acta* **1581**, 1–10
- 4 Mizushima, Y., Kobayashi, S., Kuramochi, K., Nagata, S., Sugawara, F. and Sakaguchi, K. (2000) Epilactaene, a novel neurotogenic compound in human neuroblastoma cells, selectively inhibits the activities of mammalian DNA polymerases and human DNA topoisomerase II. *Biochem. Biophys. Res. Commun.* **273**, 784–788
- 5 Thirumalai, D. and Lorimer, G. H. (2001) Chaperonin-mediated protein folding. *Annu. Rev. Biophys. Biomol. Struct.* **30**, 245–269
- 6 Kirchhoff, S. R., Gupta, S. and Knowlton, A. A. (2002) Cytosolic heat shock protein 60, apoptosis, and myocardial injury. *Circulation* **105**, 2899–2904
- 7 Shan, Y. X., Liu, T. J., Su, H. F., Samsamshariat, A., Mestrlil, R. and Wang, P. H. (2003) Hsp10 and Hsp60 modulate Bcl-2 family and mitochondria apoptosis signaling induced by doxorubicin in cardiac muscle cells. *J. Mol. Cell. Cardiol.* **35**, 1135–1143
- 8 Samali, A., Cai, J., Zhivotovsky, B., Jones, D. P. and Orrenius, S. (1999) Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells. *EMBO J.* **18**, 2040–2048
- 9 Xanthoudakis, S., Roy, S., Rasper, D., Hennessey, T., Aubin, Y., Cassidy, R., Tawa, P., Ruel, R., Rosen, A. and Nicholson, D. W. (1999) Hsp60 accelerates the maturation of procaspase-3 by upstream activator proteases during apoptosis. *EMBO J.* **18**, 2049–2056
- 10 Bethke, K., Staib, F., Distler, M., Schmitt, U., Jonuleit, H., Enk, A. H., Galle, P. R. and Heike, M. (2002) Different efficiency of heat shock proteins (HSP) to activate human monocytes and dendritic cells: superiority of HSP60. *J. Immunol.* **169**, 6141–6148
- 11 Kol, A., Lichtman, A. H., Finberg, R. W., Libby, P. and Kurt-Jones, E. A. (2000) Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J. Immunol.* **164**, 13–17
- 12 Barazi, H. O., Zhou, L., Templeton, N. S., Kruttsch, H. C. and Roberts, D. D. (2002) Identification of heat shock protein 60 as a molecular mediator of $\alpha_3\beta_1$ integrin activation. *Cancer Res.* **62**, 1541–1548
- 13 Venner, T. J., Singh, B. and Gupta, R. S. (1990) Nucleotide sequences and novel structural features of human and Chinese hamster hsp60 (chaperonin) gene families. *DNA Cell Biol.* **9**, 545–552
- 14 Viitanen, P. V., Lorimer, G., Bergmeier, W., Weiss, C., Kessel, M. and Goloubinoff, P. (1998) Purification of mammalian mitochondrial chaperonin 60 through *in vitro* reconstitution of active oligomers. *Methods Enzymol.* **290**, 203–217
- 15 Weissman, J. S., Rye, H. S., Fenton, W. A., Beechem, J. M. and Horwich, A. L. (1996) Characterization of the active intermediate of a GroEL–GroES-mediated protein folding reaction. *Cell* **84**, 481–490
- 16 Nielsen, K. L. and Cowan, N. J. (1998) A single ring is sufficient for productive chaperonin-mediated folding *in vivo*. *Mol. Cell* **2**, 93–99
- 17 Levy-Rimler, G., Viitanen, P., Weiss, C., Sharkia, R., Greenberg, A., Niv, A., Lustig, A., Delarea, Y. and Azem, A. (2001) The effect of nucleotides and mitochondrial chaperonin 10 on the structure and chaperone activity of mitochondrial chaperonin 60. *Eur. J. Biochem.* **268**, 3465–3472
- 18 Hayashi, Y., Kanayama, J., Yamaguchi, J. and Shoji, M. (2002) Diastereoselective total synthesis of both enantiomers of epilactaene. *J. Org. Chem.* **67**, 9443–9448
- 19 Nagumo, Y., Kakeya, H., Yamaguchi, J., Uno, T., Shoji, M., Hayashi, Y. and Osada, H. (2004) Structure–activity relationships of epilactaene derivatives: structural requirements for inhibition of Hsp60 chaperone activity. *Bioorg. Med. Chem. Lett.* **14**, 4425–4429
- 20 Ki, S. W., Ishigami, K., Kitahara, T., Kasahara, K., Yoshida, M. and Horinouchi, S. (2000) Radicol binds and inhibits mammalian ATP citrate lyase. *J. Biol. Chem.* **275**, 39231–39236
- 21 Miyake, Y., Kakeya, H., Kataoka, T. and Osada, H. (2003) Epoxycyclohexenone inhibits Fas-mediated apoptosis by blocking activation of pro-caspase-8 in the death-inducing signaling complex. *J. Biol. Chem.* **278**, 11213–11220
- 22 Jindal, S., Dudani, A. K., Singh, B., Harley, C. B. and Gupta, R. S. (1989) Primary structure of a human mitochondrial protein homologous to the bacterial and plant chaperonins and to the 65-kilodalton mycobacterial antigen. *Mol. Cell. Biol.* **9**, 2279–2283
- 23 Viitanen, P. V., Lorimer, G. H., Seetharam, R., Gupta, R. S., Oppenheim, J., Thomas, J. O. and Cowan, N. J. (1992) Mammalian mitochondrial chaperonin 60 functions as a single toroidal ring. *J. Biol. Chem.* **267**, 695–698
- 24 Sawano, A. and Miyawaki, A. (2000) Directed evolution of green fluorescent protein by a new versatile PCR strategy for site-directed and semi-random mutagenesis. *Nucleic Acids Res.* **28**, E78
- 25 Schmidt, M., Buchner, J., Todd, M. J., Lorimer, G. H. and Viitanen, P. V. (1994) On the role of groES in the chaperonin-assisted folding reaction: three case studies. *J. Biol. Chem.* **269**, 10304–10311
- 26 Dickson, R., Larsen, B., Viitanen, P. V., Tormey, M. B., Geske, J., Strange, R. and Bemis, L. T. (1994) Cloning, expression, and purification of a functional nonacetylated mammalian mitochondrial chaperonin 10. *J. Biol. Chem.* **269**, 26858–26864
- 27 Itoh, H., Komatsuda, A., Ohtani, H., Wakui, H., Imai, H., Sawada, K., Otaka, M., Ogura, M., Suzuki, A. and Hamada, F. (2002) Mammalian HSP60 is quickly sorted into the mitochondria under conditions of dehydration. *Eur. J. Biochem.* **269**, 5931–5938
- 28 Itoh, H., Komatsuda, A., Wakui, H., Miura, A. B. and Tashima, Y. (1999) Mammalian HSP60 is a major target for an immunosuppressant mizoribine. *J. Biol. Chem.* **274**, 35147–35151
- 29 Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J. and Schreiber, S. L. (1995) Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* **268**, 726–731
- 30 Sin, N., Meng, L., Wang, M. Q., Wen, J. J., Bornmann, W. G. and Crews, C. M. (1997) The anti-angiogenic agent fumagillin covalently binds and inhibits the methionine aminopeptidase, MetAP-2. *Proc. Natl. Acad. Sci. U.S.A.* **94**, 6099–6103
- 31 Xu, Z., Horwich, A. L. and Sigler, P. B. (1997) The crystal structure of the asymmetric GroEL–GroES–(ADP)7 chaperonin complex. *Nature (London)* **388**, 741–750
- 32 Bochkareva, E. S., Horovitz, A. and Girshovich, A. S. (1994) Direct demonstration that ATP is in contact with Cys-137 in chaperonin GroEL. *J. Biol. Chem.* **269**, 44–46