J. Biochem. 2017;162(1):27–36 doi:10.1093/jb/mvw104

JB THE JOURNAL OF BIOCHEMISTRY

High dose of antibiotic colistin induces oligomerization of molecular chaperone HSP90

Received November 29, 2016; accepted December 15, 2016; published online February 14, 2017

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Colistin is an antimicrobial cationic peptide that belongs to the polymyxin family. Colistin was clinically used for the treatment of gram-negative infections but fell out of favour because of its significant side effects including neurotoxicity and nephrotoxicity. More recently, colistin has been regarded as one of the important options for nosocomial infections caused by multidrug resistant bacteria. Mechanisms of both the side effect onset of the drug and the side effect reduction are yet to be elucidated. In this study, we identified the specific binding protein of colistin using an affinity column chromatography. Colistin binds to the molecular chaperone HSP90. Although colistin slightly suppressed the chaperone activity of HSP90, there are no effects on the ATPase activity for a low concentration of colistin. Interestingly, colistin-induced aggregation of HSP90 via the N-domain. As for the cell viability of the SHSY5Y cell, the cell viability decreased to approximately 80% by the colistin 300 μM. However, the cell viability recovered to approximately 100% by adding ATP dosage. The same result was obtained by dot blot assay using anti-HSP90 antibody. Our results may help to understand the side effect mechanism of colistin.

Keywords: antibiotics; colistin; HSP90; molecular chaperone; polymixin.

Colistin is an antimicrobial cationic peptide that belongs to the polymyxin family. It interacts with lipopolysaccharide (LPS) in the bacterial outer membrane (*1*). Colistin (polymyxin E) has been widely used as a last-option antibiotic for patients with multidrug-resistant (MDR) and extensively drug resistant (XDR) bacterial infections. Colistin can effectively treat gram-negative bacteria; it is especially effective against multidrug resistant gram-negative bacterial infections. However, it is known that colistin causes neurotoxicity and nephrotoxicity (*2*). It has been reported that polymixin B binds to the endocytosis receptor megalin (*3*) and causes nephrotoxicity, in particular, induced colistin concentrates in the kidney (*4*). In addition, oxidative stress in the mitochondria (*5*) and apoptosis caused by the activation of caspase (*6–8*) are important factors related to colistin's toxicity.

The side effect of neurotoxicity manifests in the form of dizziness, lethargy, facial and peripheral paraesthesia, vertigo, vision loss, ataxia, and neuromuscular junction block, which may lead to respiratory failure or apnoea. In patients who are sensitive, inhalation administration can cause mild bronchoconstriction. Intraventricular and intrathecal administration of colistin, particularly in high dose, must be closely monitored as it can cause seizure (*9*). Other side effects that can be found during colistin treatment are hypersensitivity reaction, skin rash, urticarial, pruritus and muscle weakness of all body parts and mild gastrointestinal disorder. It has been reported that colistin-induced nephrotoxicity in mice involves the mitochondrial, death receptor and endoplasmic reticulum pathway (*10*).

Several studies suggest that neurotoxic effects are more likely found in patients with cystic fibrosis. It has been reported that the incidence of neurotoxicity during colistin treatment is approximately 7% with main symptom of paraesthesia, and the incidence of allergic reaction is about 2% (*9*). A study conducted by Tamma demonstrated that 22% out of 229 cases of infection in children treated with colistin had both nephrotoxicity and neurotoxicity, but the side effects were reversible and diminished when the treatment was stopped (*11*).

Molecular chaperone HSP90 is one of the essential proteins for maintaining cell functions. It exists as a homodimer and consists of three domains; N-terminal ATP binding domain, middle domain, and C-terminal dimerization domain (*12*, *13*). It is presumed that the number of client proteins of HSP90 approached 300 (*14*), and that the protein is involved in the stability of the substrates and signalling pathway. HSP90 is an ATP-dependent molecular chaperone, and ATP hydrolysis induced a conformational change (*15*). ATP induces conformational changes in the HSP90 from the open to close states. It has been reported that HSP90 is targeted for treatment of cancer and anti-cancer drugs, *e.g.* cisplatin and 17-AAG

(17-N-allylamino-17-demethoxygeldanamycin) bind to it as an HSP90 inhibitor (*16–18*). Folding of the client proteins by HSP90 was prevented and degraded via proteasome in the presence of 17-AAG (*18*).

In this study, we analyzed the neurotoxic effects of colistin and investigated the binding protein using a colistin-affinity column. We identified that colistin mainly interacts with HSP90 in the brain and discussed the effect of the drug on HSP90.

Materials and Methods

Colistin-affinity column chromatography

Colistin sulphate was purchased from Wako Pure Chemical Industries (Osaka, Japan). The affinity column was prepared using Epoxy-activated Sepharose 6B purchased from GE Healthcare (Piscataway, NJ, USA). Porcine brain was obtained from Tokyo Shibaura Tissues LTD (Tokyo, Japan). Porcine brain (0.5 g) was added 25 mM HEPES-KOH, pH 7.4 in 3-fold volumes. The sample was homogenized and centrifuged (20,000 × g, 25 min, 4°C). The supernatant was applied to the affinity column equilibrated by 25 mM HEPES-KOH, pH 7.4. After washing, the binding proteins were eluted using a linear gradient method from 0 to 10 mM colistin sulphate in 25 mM HEPES-KOH, pH 7.4. These fractions were analyzed by SDS-PAGE (9%) and Coomassie Brilliant Blue R-250 and immunoblotting using anti-HSP90.

Gene cloning and plasmid constructs

The cDNA of *Escherichia coli* DH5α was provided by Meiji Seika Pharma Co. Ltd. The high temperature protein G (HtpG) was amplified by PCR using the following primers: HtpG-N 5′CATAT GAAAGGACAAGAAACTCGTG 3′ and HtpG-C 5′ TCTAGATC AGGAAACCAGCAGCTG 3′. The PCR product was digested with NdeI and XbaI restriction enzymes and cloned into the pColdIvector (TAKARA BIO, Inc. Japan).

HSP90, HSP90 N, M and the C-domains were amplified by PCR using the following primers: HSP90 and HSP90 N-domain N 5′ GGATCCATGCCTGAGGAAACCCAGACC 3′, HSP90 and HSP90 C-domain C 5′ TCTAGATTAGTCTACTTCCATGC GTGA 3′, HSP90 N-domain C 5′ TCTAGATTCAGCCTCATC ATCG 3′, HSP90 C-domain N 5′ GGATCCGGTTACATGGCA G 3′, HSP90 M-domain N 5′ CATATGCTCAACAAAACAAAG CCCATC 3′ and HSP90 M-domain C 5′ CTCGAGTTCCAGGCCT TCTTTGGT 3′. The PCR product of HSP90, HSP90 N and C-domains was digested with BamHI and XbaI restriction enzymes and cloned into the pColdIvector (TAKARA BIO, Inc. Japan). The PCR product of HSP90 M-domain was digested with NdeI and XhoI restriction enzymes and cloned into the pET15b vector (Novagen, Inc. Japan).

Expression and purification of recombinant proteins

HtpG was expressed as a 6 × His fusion protein from the expression vector pColdI in BL21 *E. coli* cells. *E. coli* cells were grown at 37°C until the OD_{600} reached 0.5. After incubation at 4°C for 30 min, the expression was induced by 0.1 mM isopropyl β-D-1 thiogalactopyranoside (IPTG) and *E. coli* cells were grown at 15°C for 24 h. The cells were collected and suspended in 10 mM Tris-HCl, pH 7.4. This solution was sonicated and centrifuged. 2 × Apply Buffer (40 mMM Imidazole, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4) was added to the collected supernatant and applied to the Ni-NTA column. The column was washed with wash buffer (50 mM Imidazole, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4) and eluted using the linear gradient method from 0.1 to 0.5 M Imidazole, 0.3 NaCl, 10 mM Tris-HCl, pH 7.4. The HtpG-rich fractions were dialyzed with 10 mM Tris-HCl, pH 7.4. After dialysis, the solution was applied to a Q-Sepharose column, washed with 10 mM Tris-HCl, pH 7.4 and eluted using a linear gradient method from 0 to 0.8 M NaCl, 10 mM Tris-HCl, pH 7.4. The protein sample was concentrated by ultrafiltration after gel filtration chromatography using Hi prep 16/60 Sephacryl-300 HR (GE Healthcare, Piscataway, NJ, USA).

HSP90 was expressed as a 6 × His fusion protein from the expression vector pColdI in BL21 *E. coli* cells. An expression was induced by 0.5 mM IPTG. The cells were collected and cell extracts were applied Ni-NTA column, washed and eluted the same as HtpG. The protein rich fractions were loaded onto a Q-Sepharose column washed with 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4 and eluted using the linear gradient method from 0.3 to 0.6 M NaCl, 10 mM Tris-HCl, pH 7.4. The eluted fraction was dialyzed for 10 mM Tris-HCl, pH 7.4. After dialysis, the solution was applied to a Heparin sepharose column, washed with 10 mM Tris-HCl, pH 7.4 and eluted using the linear gradient method from 0 to 0.7 M NaCl, 10 mM Tris-HCl, pH 7.4. The protein sample was concentrated by ultrafiltration after gel filtration chromatography using Hi prep 16/60 sephacryl-300 HR followed by a Heparin sepharose column.

The HSP90 N- and C-domains were expressed as a 6 × His fusion protein from the expression vector pColdI in the BL21 *E. coli* cells. The HSP90 M-domain was expressed as a 6 × His fusion protein from the expression vector pET15b in the Arctic express (DE3) *E. coli* cells. The expression of the HSP90 N, M and C-domains was induced by 0.5 mM IPTG. The cells were collected and each cell extracts was applied to the Ni-NTA column, washed and eluted the same as the other chaperones.

Measurement of ATPase activity

The assay buffers (50 mM KCl, 5mM $MgCl_2$, 1mM DTT, 0.5 mM ATP, 25 mM HEPES-KOH, pH 7.4) containing 5 μM HSP90 or HtpG in the absence or presence of colistin sulphate (5, 10, 30 and 50 μM) were incubated at 37°C for 120 min. The solutions were prepared every 30 min. After incubation, the solutions were transferred to a 96-well plate and 100 μL BIOMOL GREEN was added to each well and incubated at room temperature for 30 min. The amount of free-phosphate was measured at the wavelength of 650 nm using a plate reader (Infinite F200, TECAN).

Measurement of chaperone activity

The thermal aggregation assay of citrate synthase (CS) was performed according to previous report (*16*,*17*). The solutions (0.1 μM CS, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES-KOH, pH 7.4) containing 0.5 μM HSP90 in the absence or presence of colistin sulphate (10, 30, 100 μM) were incubated at 50°C for 20 min. The HtpG chaperone activity was also measured the same as HSP90 in the presence of colistin (1, 2.5 and 5 μM). The sample absorbances were observed using an Ultrospec 3000 UV/visible spectrophotometer (Pharmacia, Uppsala, Sweden) at 500 nm.

Measurement aggregation of HSP90 – The aggregation of HSP90 was measured by three ways. One was measuring the light scattering at 37°C. The assay buffer (50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES-KOH, pH 7.4) containing 0.5 μM HSP90 in the absence or presence of colistin sulphate (0.1, 0.3, 0.5, 1 and 5 mM) and/or 0.5 mM ATP and/or 0.1 μM CS were incubated at 37°C for 20 min and the absorbance was observed using the Ultrospec 3000 UV/visible spectrophotometer at 500 nm. The aggregation of HSP90 was analyzed by SDS-PAGE. The assay buffer containing HSP90 and colistin were incubated by the same light scattering method. After incubation, the solutions were centrifuged at 20,000 × g for 10 min. The collected supernatant and suspended precipitate using 8 M urea were analyzed by SDS-PAGE. The third method was Blue native-PAGE. The reaction conditions were the same with above experiments.

Trypsin resistance of HSP90

The digestion of HSP90 was performed using trypsin. HSP90 was pre-incubated in the absence or presence of colistin (0.1, 0.3, 0.5, 1.0, 50 mM) and 0.5 mM ATP at 37°C for 20 min. After incubation, 2.5 μg trypsin was added to each solution and incubated at 37°C for 20 min. These were analyzed by SDS-PAGE.

Influence of colistin on antimicrobial activity – E. coli: DH5α was grown in LB media containing no antibiotics, at 37°C until a 1.0 of OD_{600}. The cells were diluted 5-fold or 2-fold and the colistin solutions were added to each culture (final concentration of 0, 0.1, 0.5, 1, 2.5, 5 and 10 μM). These samples were incubated at 250 rpm and 37°C for 16 h. After incubation, these were streaked on an LB plate containing no antibiotics and grown at 37°C for 16 h.

Cell culture

SHSY5Y (human neuroblastoma) cells were grown in Dullbecco's modified Eagle's medium (SIGMA) supplemented with 10% fetal bovine serum (Equitech-Bio, Inc. Kerrville, TX), 20 units/ml

penicillin and 20 μg/ml streptomycin (Invitrogen, Calsbad, CA). The cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2.

Cell viability assay

SHSY5Y cells were cultured in 96-well tissue culture plates at a density of 1.5×10^3 cells per well. After the 24-h incubation, the cells were treated in two ways. The cells were treated with both 500 μM colistin and the indicated concentration of ATP. On the other hand, the cells were treated with the indicated concentration of ATP then and 24 h later with 500 μM colistin. To measure the cell viability, an MTT assay (Promega) was used.

Filter trap assay

SHSY5Y cells were seeded on 100-mm tissue culture plates at a density of 2.0×10^3 cells. After a 24-h incubation, the cells were treated with the indicated concentration of ATP. After 24 h of ATP treatment, 500 μM colistin was applied to the cells. Twenty-four hours after the colistin treatment, the cells were harvested and homogenized in lysis buffer. The samples were diluted to 200 μl in lysis buffer and applied to a cellulose acetate membrane. The membrane was washed with wash buffer, and the aggregates remaining on the membrane were incubated with the anti-HSP90 antibody (*16*), then an alkaline-phosphate conjugated anti-rabbit secondary antibody (Sigma).

Results

Colsitin binds HSP90 in the brain

To investigate the side effect mechanisms of colistin, we examined the colistin binding proteins using a colistin-affinity column. The structure of colistin was shown in Fig. 1A. As shown in Fig. 1B, the protein having a 90-kDa molecular mass has been eluted at a high concentration of colistin. We investigated whether or not the protein is HSP90 by immunoblotting using an anti-HSP90 antibody (Fig. 1C). We identified that the colistin binding protein is HSP90. On the contrary, we analyzed polymixin B binding protein as control. The structure of polymixin B was shown in Fig. 1D. Some proteins were eluted from polymixin B-affinity column, but the eluted proteins were different from colistin binding proteins (Fig. 1E). We could not detect HSP90 in the eluent from polymixin B-affinity column (Fig. 1F).

Colistin does not affect the ATPase activity of HSP90

We analyzed the effects of colistin on the ATPase activities of HSP90. The ATPase activity is one of the important functions of HSP90. We purified HSP90 from porcine brain and analyzed the ATPase activity in the absence or presence of colistin (5, 10, 30 and 50 μM). As shown in Fig. 2, the HSP90 ATPase activity has no affect by colistin. The result suggested that a low concentration of colistin has no effect on the ATPase activity of HSP90.

Low concentration of colistin does not influence the HSP90 chaperone activity

Next, we examined the effect of colistin on the chaperon activity of HSP90. We used CS, which is very unstable to heat, for the client protein of these chaperones. To investigate the effect of colistin on the chaperone activity, we used a light scattering assay. The thermal aggregation of CS (0.1 μM) was completely protected by HSP90 (0.5 μM; Fig. 3). The HSP90 chaperone activity was slightly suppressed or had almost no affect by the low concentration of colistin that did not affect the ATPase activity (Fig. 2).

Colistin induces HSP90 aggregation

We speculated that neurotoxicity can be induced by the high concentration of colistin in the brain. Therefore, we analyzed the effect of a high concentration of colistin on the HSP90 stability. The HSP90 solution containing 0.5 μM in the absence or presence of colistin and/or 0.5 mM ATP, and 0.1 μM CS were incubated at 37°C (Fig. 4A). Surprisingly, the absorbance is increased in a colistin dose-dependent manner. The oligomerization or aggregation is decreased by the addition of ATP and/or CS. These results suggested that protein aggregation is not only CS but also HSP90 at a high concentration of colistin.

We detected the oligomerization or aggregation of HSP90 by other methods. As shown in Fig. 4B, we could detect the changes in the molecular mass of HSP90 by Blue native-PAGE. When in the presence of both HSP90 and colistin, we could detect oligomers of HSP90 at low concentration of colistin (start from 0.025 mM colistin). HSP90 completely aggregates from 0.5 mM colistin (Fig. 4B, left panels). As a result, we could not detect HSP90 in the presence of high concentration of colistin (1 and 5 mM). Completely aggregated HSP90 could not enter into the gels and the aggregated proteins in the lane were removed during gel stain and destain process. The addition of ATP suppressed the aggregation of the protein. We can detect HSP90 proteins band until at 5 mM colistin in the gels (Fig. 4B, right panels). We investigated the trypsin digestion of HSP90 in the presence of colistin and in the absence or presence of ATP (Fig. 4C). Because colistin-induced aggregation of HSP90 in a dose-dependent manner in the absence of ATP, the amount of the partially digested HSP90 fragments is increased (Fig. 4C, left panels). ATP is effective for the suppression of the aggregation, so HSP90 was completely digested by trypsin (Fig. 4C, right panels).

Colistin binds to HSP90 N-domain

To identify the colistin binding domain of HSP90, we prepared each HSP90 domain (N-, M- and C-domains; Fig. 5A). We performed a light scattering assay and SDS-PAGE using each domain and full-length HSP90 (Fig. 5B). When each domain of HSP90 was incubated with 5 mM colistin, HSP90 M- and C domains showed no changes in the absorbance. However, the N-domain with colistin significantly reacted and the absorbance increased. We could detect decreasing of HSP90 in the supernatant and increasing of the protein in the precipitate as colistin concentration-dependent manner on SDS-PAGE (Fig. 5C). This aggregation was caused via the HSP90 N-domain by colistin (Fig. 5C). Taken together, these results suggested that a high concentration of colistin-induced aggregation via the HSP90 N-domain, and the physiological functions of HSP90 were suppressed. We further studied the aggregation using ATPγS (Fig. 5D). ATPγS effectively suppressed the aggregation the same as ATP.

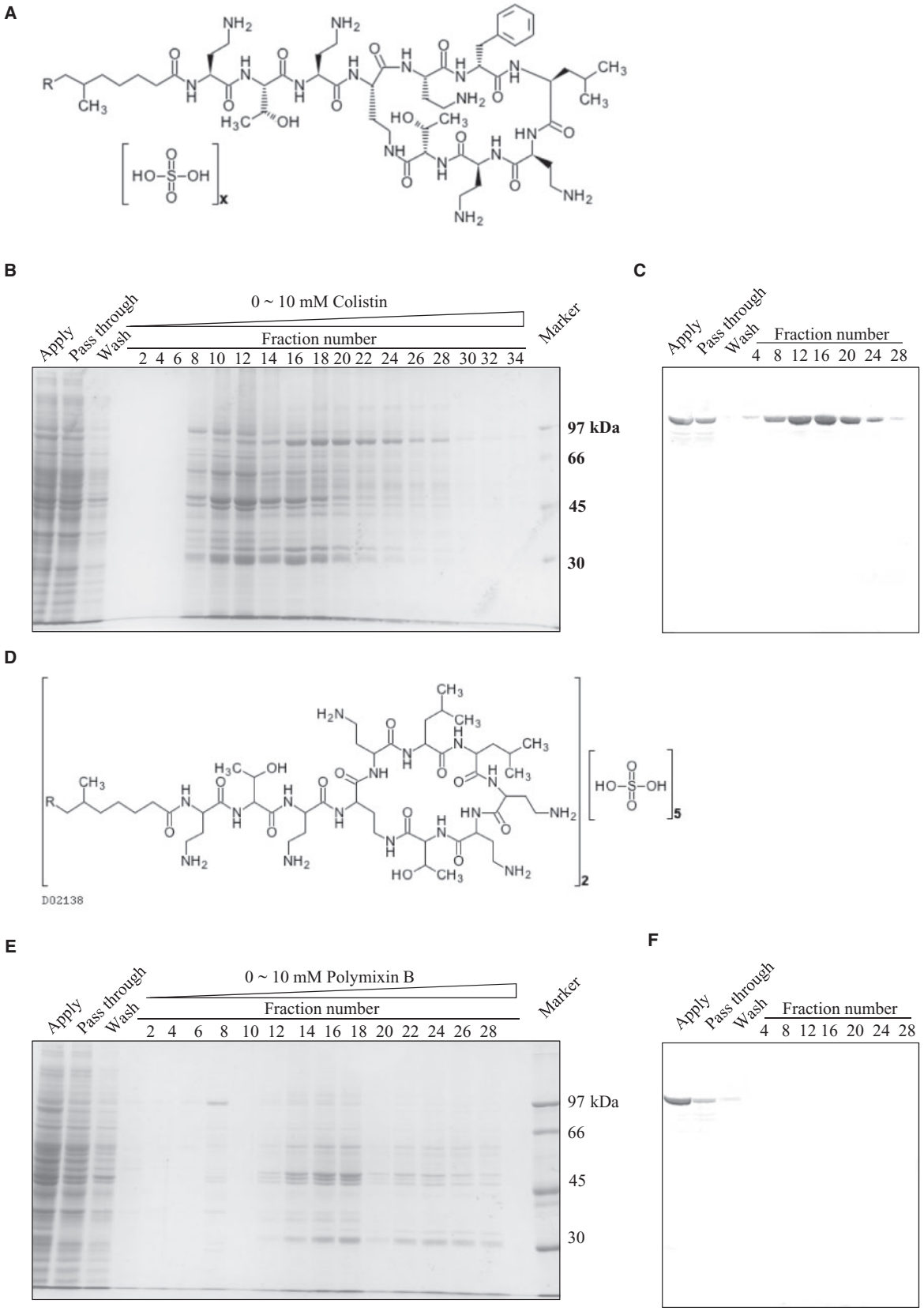

Fig. 1 Identification of colistin binding protein. Porcin brains were applied to a colistin-affinity column or polymixin B-affinity column. The binding proteins were eluted with colistin or polymixin B from 0 to 10 mM in 25 mM HEPES-KOH (pH 7.4) after washing. The structures of colistin and polymixin B were shown in **(A)** and **(D)**, respectively. The eluted fractions from colistin-affinity column were analyzed by SDS-PAGE **(B)** and immunoblotting using an anti-HSP90 antibody **(C)**. The polymixin B binding proteins were analyzed by SDS-PAGE **(E)** and immunoblotting using an anti-HSP90 antibody **(F).**

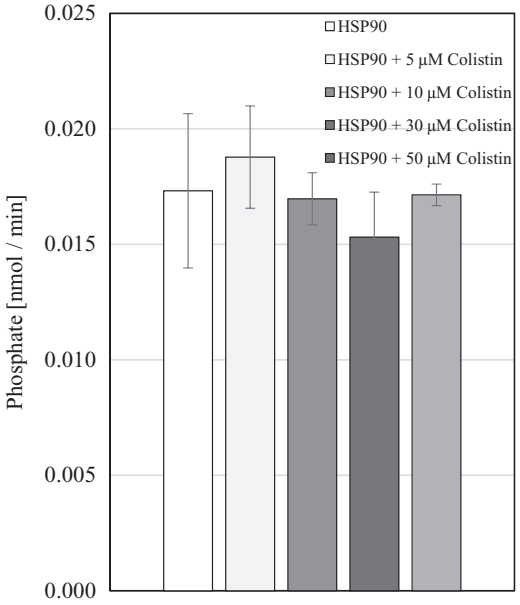

Fig. 2 Effect of colistin on ATPase activity of HSP90. The purified porcine brain HSP90 ATPase activity was measured in the absence or presence of colistin. The solutions were incubated with ATP at 37 °C for 120 min and measured at the wavelength of 650 nm. The results represent the average and standard deviations of three independent experiments. Error bars represent standard deviation ($n = 3$).

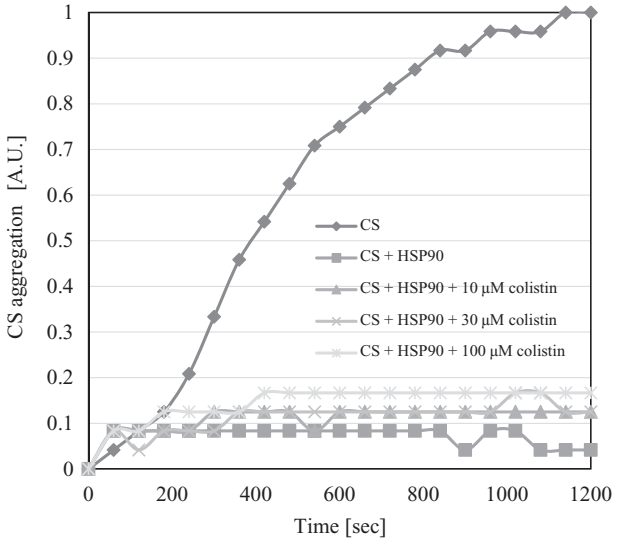

Fig. 3 Effect of colistin on chaperone activity. The HSP90 chaperone activity was measured in the absence or presence of colistin. The solutions containing CS, in the presence of HSP90 (closed gray square), HSP90 and 10 μM colistin, HSP90 and 30 μM colistin, and HSP90 and 100 μM colistin 0 °C and the absorbance monitored at 500 nm.

ATP does not influence the bacterial membrane disruption caused by colistin

We investigated the effectiveness of colistin on a bacterial membrane because it is known that colistin inhibits bacterial growth by affecting the negatively charged membrane, resulting in membrane collapse. We selected *E. coli* DH5α as gram-negative bacteria for evaluation of the antimicrobial activity. We cultured DH5α until the cell density corresponded to an OD_{600} of 1.0. The culture was diluted to a cell density corresponding to an OD_{600} of 0.1 and 0.5, then the colistin solution was added to the diluted cultures and incubated for 16 h. These cultures were streaked onto LB plates. We evaluated the limits of the bacterial growth (Fig. 6). The cultures of OD_{600} 0.5 grew to 1.0 μM, while the cultures of OD_{600} 0.1 grew to 0.1 μM in the absence of ATP. Depending on the bacterial density, the required amount of colistin to completely kill the bacteria is increased. The ATP-treatment timing, pre-treat or co-treat with colistin to *E. coli*, did not affect the bacterial growth. As a result, the microbial activity of colistin was higher, and 2.5–5 μM colistin was a sufficient concentration to disturb the cell growth during the initial growth phase.

Effects of colistin on cell viability

We investigated the effect of colistin on human cell viability. In the present study, we evaluated it using human neuroblastoma SHSY5Y cells. The cell viability was reduced to about 58% as colistin dependent manner (0–500 μM; Fig. 7A). We focused on 300 μM colistin when the cell viability is about 80%. The cell viability (at colistin 300 μM) dramatically increased about 100% with the pre-treatment of ATP (100–500 μM; Fig. 7B). We also evaluated the effect of ATP on the HSP90 aggregation induced by colistin in the cells using a filter trap assay. The SHSY5Y cell extracts, which were treated with colistin and in the absence (Fig. 7C) or presence of ATP (Fig. 7D), were filtered and the remaining proteins were reacted with the anti-HSP90 antibody. After staining the membrane, the intensity of HSP90 was measured. As shown in Fig. 7C, the intensity of HSP90 increased from 200 μM colistin. On the contrary, the intensity of HSP90 reduced from an ATP concentration of 100 μM. The ATP concentration coincided with the cell viability of the SHSY5Y cells (Fig. 7B).

Discussion

For the treatment of multi-drug resistant bacteria, the development of new antibiotics is required. Colistin is one of the antibiotics, which is desired to treat especially multi-drug resistant gram-negative bacteria. However, it has a strong nephrotoxicity and neurotoxicity during clinical use. Polymyxin B and colistin (*i.e.* Polymixin E), both from polymixin family, differ in only one amino acid and the mechanism of pharmacological effects is almost the same. The former shows almost no side effects and the latter is known to cause serious side effects. Recently, the roles of death receptors, mitochondrial and endoplasmic reticulum pathways have been reported in colistin-induced nephrotoxicity using a mouse model (*10*). All three major apoptosis pathways (*i.e.* the mitochondrial, death receptor and endoplasmic reticulum pathways) and autophagy are involved in colistin-induced

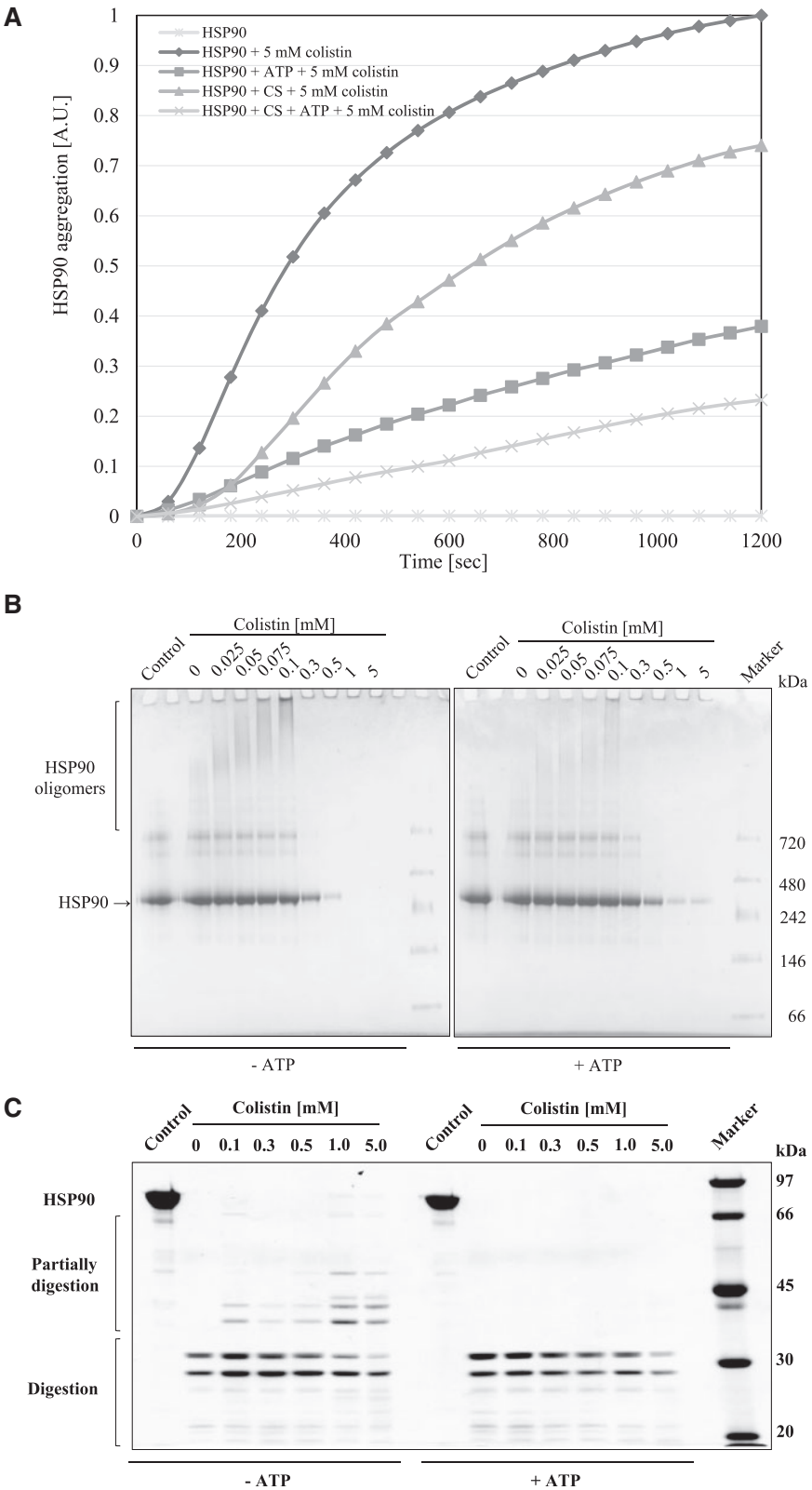

Fig. 4 Colistin induces aggregation of HSP90. **(A)** The light scattering assay was performed under high concentrated colistin conditions. The solutions containing HSP90, in the presence of 5 mM colistin, ATP and 5 mM colistin, CS and 5 mM colistin, CS and ATP and 5 mM colistin (*) were incubated at 37 °C and the absorbance monitored at the wavelength of 500 nm. **(B)** Blue native-PAGE was performed after incubation at 37 °C for 20 min in the presence of colistin. The solutions containing HSP90 in the presence or absence of ATP (0.5 mM) and colistin (0.1, 0.3, 0.5, 1 and 5 mM) were incubated at 37 °C for 20 min. Samples were analyzed by Blue native-PAGE in the absence or presence of ATP. **(C)** The solutions containing HSP90 and colistin (0.1, 0.3, 0.5, 1 and 5 mM) in the absence or presence of 0.5 mM ATP were incubated at 37 °C for 20 min. 2.5 μg/ml trypsin was added to these solutions and incubated for 20 min after the pre-incubation. These were analyzed by SDS-PAGE.

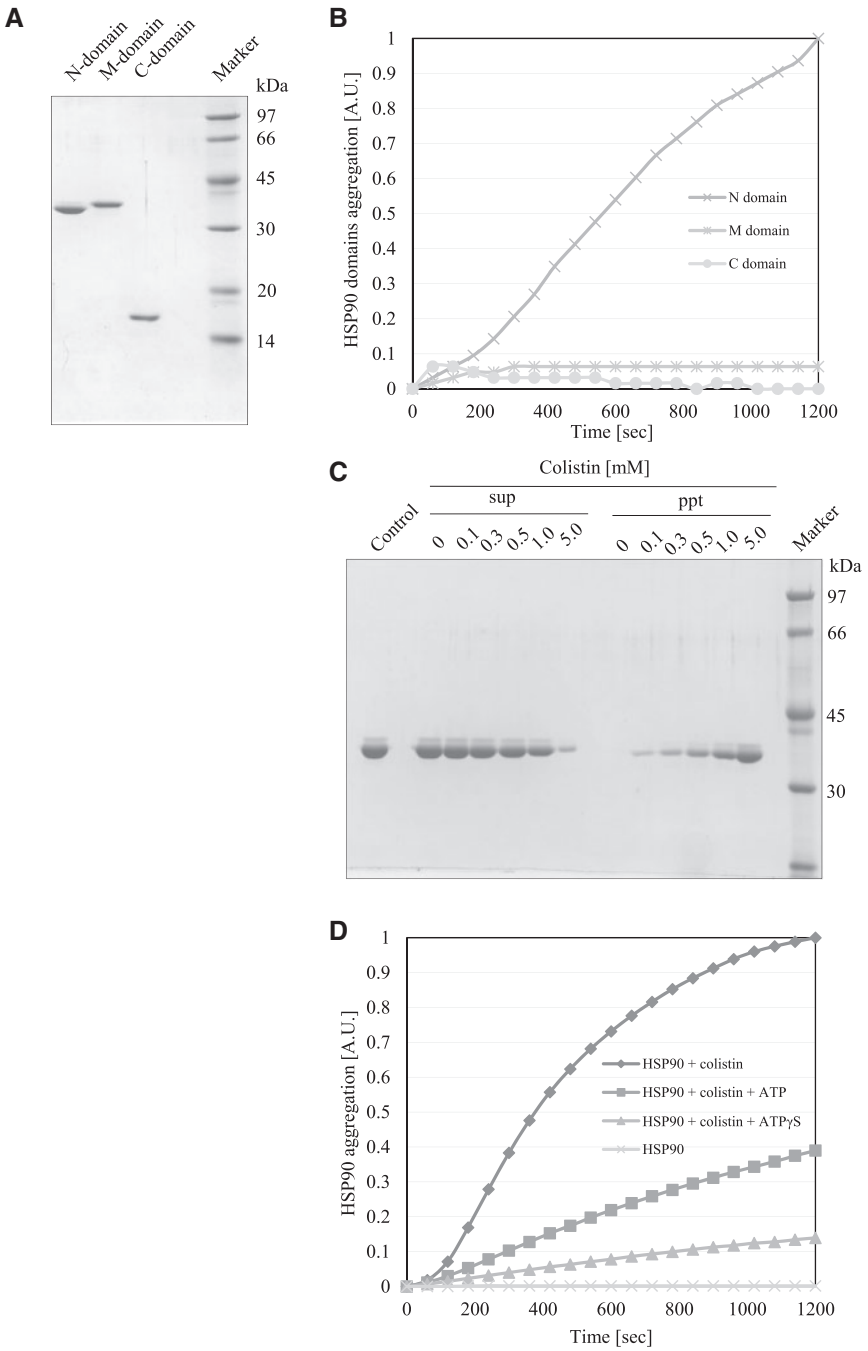

Fig. 5 Colistin binds to HSP90 N-domain and nucleotides have different effects on HSP90 aggregation. **(A)** HSP90 N-, M- and C-domains were purified and analyzed by SDS-PAGE (12% gel). **(B)** Identification of colistin binding domain was analyzed by light scattering assay. The solutions containing 5 mM colistin in the presence of HSP90 N-domain (close yellow x-mark), HSP90 M-domain, and HSP90 C-domain were incubated and monitored the same as in experiment of Fig. 4B. **(C)** Aggregation of HSP90 N-domain in the presence of colistin (0–5.0 mM) was centrifuged and separated supernatant (sup) and precipitate (ppt) followed by SDS-PAGE (9% gel). **(D)** Light scattering assay was performed in the absence of colistin, in the presence of 5 mM colistin, 5 mM colistin and 0.5 mM ATP, and 5 mM colistin and 0.5 mM ATPγS. The solutions were incubated and monitored the same as in experiment of Fig. 3B.

nephrotoxicity in mice (*10*). We tried to understand the side effects of colistin, especially regarding the neurotoxicity.

In the present study, colistin binds to HSP90 in the brain with high affinity. Interestingly, polymixin B did not bind to the protein. Colistin had little effect on the HSP90 chaperon activity at a low concentration. It has been postulated that colistin may bind to the sites that do not affect the ATPase activity. It has also been reported that a high dose of colistin was present in the kidney after dosing (*19*). We had proposed that a high concentration of colistin in the brain was a trigger for side effects. In the present study, a high concentration of colistin induced the aggregation of HSP90. We also investigated the aggregation of HSP90 in the presence of both the substrate and ATP. It has been suggested

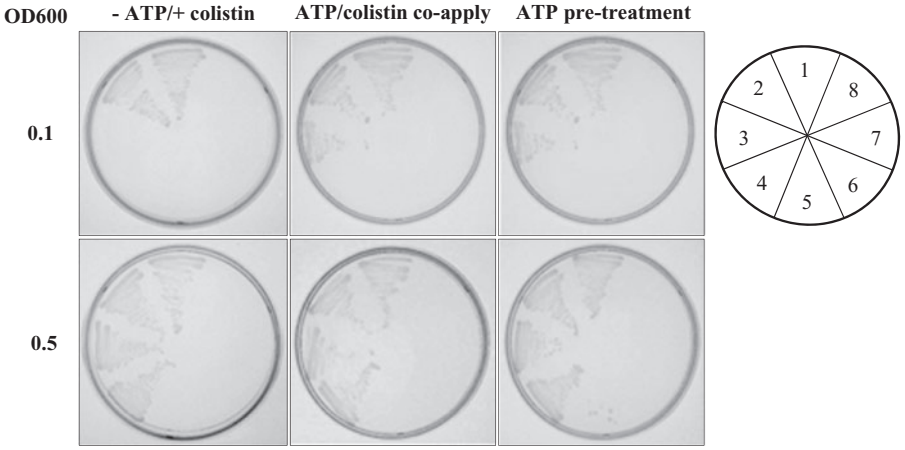

Fig. 6 Antibacterial ability of colistin. Antimicrobial ability of colistin and effect of ATP were assessed by the addition to *E. coli* DH5 α with different degrees of growth. Upper and lower panels represent OD600 = 0.1 (lag phase) and 0.5 (log phase) of DH5 α, respectively. Left side contains no ATP, middle represent co-applied of 0.4 mM ATP and colistin and right side represents pre-treatment with 0.4 mM ATP before adding colistin (left panels). Schematic view of these experiments. The solutions were streaked onto an LB plate for each numbered area (1, *E. coli* DH5α, 2, DH5 α and 0.1 μM colistin; 3, DH5 α and 0.5 μM colistin; 4, DH5 α and 1 μM colistin; 5, DH5 α and 2.5 μM colistin; 6, DH5 α and 5 μM; 7, DH5 α and 10 μM colistin; 8, no DH5 α and colistin) (right circles).

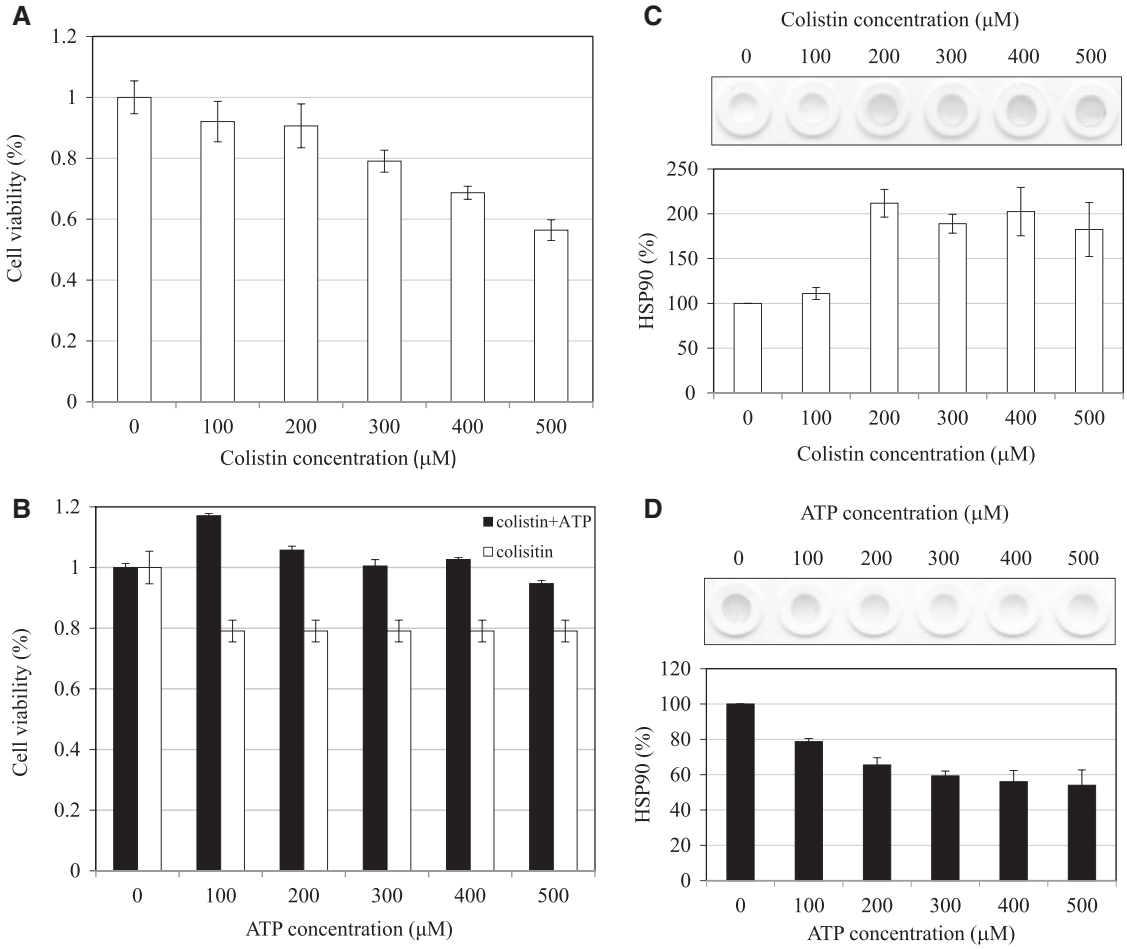

Fig. 7 Influence of ATP and colistin on SHSY5Y cells and filter trap assay of HSP90. **(A)** The cell viability of SHSY5Y cells with colistin (100, 200, 300, 400 and 500 μM). **(B)** The cell viability of SHSY5Y cells with first treatment with ATP (100, 200, 300, 400 and 500 μM), then with colistin (300 μM). Error bars of graphs A and B represent standard deviation ($n = 3$). **(C)** HSP90 aggregates were analyzed by the filter trap assay. SHSY5Y cells were treated with five different concentrations of colistin (0, 100, 200, 300, 400 and 500 μM) for 24 h. The cellular extracts were filtered and the aggregates remaining on the membrane were probed with anti-HSP90 antibody. The HSP90 aggregation based on the filter was quantified by Image J software. **(D)** HSP90 aggregates were analyzed by the filter trap assay. SHSY5Y cells were treated with five different concentrations of ATP (100, 200, 300, 400 and 500 μM) for 24 h and then treated with 300 μM colistin for 24 h. The cellular extracts were filtered and the aggregates remaining on the membrane were probed with anti-HSP90 antibody. The HSP90 aggregation based on the filter was quantified by Image J software.

that the binding of ATP and substrates to HSP90 stabilizes the conformation of the protein, resulting in the suppression of the HSP90 aggregation. We identified that colistin binds to the HSP90 N-domain and its aggregation is induced via the HSP90 N-domain. The HSP90 aggregation was suppressed by the ATP-bound form but not the GTP-bound form. ATPγS further suppressed the aggregation of HSP90. These results suggested that all of the ATP binding pockets are important to suppress the aggregation of HSP90. Colistin collapses the bacterial membrane, resulting in bacterial sterilization. For these reasons, we must consider the bad influence of the drug on HSP90. The molecular chaperone HSP90 is one of the essential proteins in living cells. Because the client proteins of HSP90 reached 300, the inactivation of HSP90 may collapse cell functions and may indirectly induce apoptosis (*20*, *21*).

We have postulated the influence of colistin on HSP90 trigger side effects. It has been reported that other compounds induce the oligomerization of molecular chaperones. For example, we reported that polaprezinc induces the oligomerization of HSP70 (*22*). It has been reported that celastrol induces the oligomerization of HSP90 via the C-domain, whereas there was no effect on the chaperone activity (*23*). We have also reported that cisplatin induced the large complexes of HSP90 (*16*).

The reason why the binding of CS to HSP90 suppressed the aggregation of HSP90 in the presence of a high concentration of colistin is that the CS-bound form of HSP90 is more stable even if colistin binds to the N-terminal of the protein. We expected that ATP will be able to suppress the aggregation of HSP90, because colistin targeted near the ATP binding site of the HSP90 N-domain. It has been reported that an optimized dosage of colistin inhibits bacterial growth (*24*). We also examined the effectiveness of colistin for *E. coli* DH5α. It is noteworthy that colistin inhibits the bacterial growth at a low concentration and it is lower than the concentration needed to aggregate HSP90. The addition of ATP does not affect the membrane disruption of *E. coli*. We examined the influence of ATP using SHSY5Y cells. The pre-treatment with ATP to the cells before the addition of colistin was very effective for cell survival. We could recognize the increase in the intact HSP90. The reason why the pre-treatment of ATP is more effective than that of both additions of ATP and colistin is as follows. When ATP first binds and then colistin to HSP90, the closed form of HSP90 may be induced by ATP. HSP90 is able to bind to the client proteins and regulate the physiological functions or assist folding of the proteins. Colistin binds to the closed form of HSP90, thus there is little influence to HSP90. With the addition of both colistin and ATP to the cells, colistin binds to the N-domain of HSP90 and ATP also binds to the ATP pocket. Although there is no influence of colistin on the ATPase activity of HSP90, the chaperone activity of the protein has been reduced or inhibited by the drug. Colistin may bind to near the ATPase domain of the HSP90 and slightly induce conformational changes in the N-domain, which maintained the ATPase activity. The structure that colistin first binds to HSP90, is not the perfect closed form, may not have a sufficient chaperone activity and tends to aggregate.

Author Contributions

T.S., H.S. and H.I. designed the experiments. S.T., K.T., A.T., I.T., S.H., A.K., I.K., E.S-K., T.O. A.H., A.M., performed the experiments. S.T., K.T., E.G. and H.I. wrote the paper.

Acknowledgements

The authors wish to thank Dr. Y.K. (Meiji Seika Pharma, Co., Ltd.) for his helpful comments about medicine safety, Dr. A.M. (Meiji Seika Pharma, Co., Ltd.) for his helpful comments on the pharmacology of medicine, and Dr. K.S. (Meiji Seika Pharma, Co., Ltd.) for his helpful suggestion and generalization of the study.

Conflict of Interest
None declared.

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