ATP-competitive, marine derived natural products that target the DEAD box helicase, eIF4A

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Recombinant protein expression and purification

eIF4AI, eIF4B, GroEL, p97, HSPA1A, DDX17, and DDX39A were each amplified from cDNA clones (DNAsu) or from cDNA libraries (ATCC) and subcloned into pSpeedET, pTrc99A, or pET14B vectors.

Expression and purification of eIF4AI

E. coli BL21(DE3) cells containing pSpeedET-eIF4A1 were grown in Luria Broth (LB) containing 50 µg/mL kanamycin at 37°C to an OD_{600} of 0.8, followed by induction with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h at 37°C. Cells were collected by centrifugation (3000 × g for 10 min), resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM β-mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF)), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon metal affinity resin (Clontech). The resin and supernatant were then loaded into a 25 mL disposable column (BioRad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 10 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME), and eluted with 2 column volumes of elution buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, and titrating concentration of imidazole (25 mM, 50 mM, 75 mM, 100 mM, 250 mM). Fractions were analyzed by 12% SDS PAGE and those containing eIF4AI were pooled and dialyzed into storage buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM BME). eIF4A was then aliquoted, frozen in liquid N_2, and stored at -80°C until needed.

Expression and purification of eIF4B

E. coli BL21(DE3) cells containing pSpeedET-eIF4B were grown in LB medium containing 50 µg/mL kanamycin at 37°C to an OD_{600} of 0.6, transferred to 16°C for 1 h, followed by induction with 0.2 mM IPTG for 24 h at 16°C. Cells were collected by centrifugation (3000 × g for 10 min),

resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 1 mM PMSF), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon metal affinity resin (Clontech). The resin and supernatant were then loaded into a 25 mL disposable column (BioRad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM MgCl2, 5% glycerol, 2 mM BME, 10 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME), and eluted with 2 column volumes of elution buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, and titrating concentration of imidazole (25 mM, 50 mM, 75 mM, 100 mM, 250 mM). Fractions were analyzed by 12% SDS PAGE and those containing eIF4B were pooled and dialyzed into storage buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM BME). eIF4B was then aliquoted, frozen in liquid N_2, and stored at -80°C until needed.

Expression and purification of DDX17

E. coli BL21(DE3) cells containing pSpeedET-DDX17-A20-M312 were grown in LB medium containing 50 µg/mL kanamycin at 37°C to an OD_{600} of 0.8, followed by induction with 1 mM IPTG for 3 h at 37°C. Cells were collected by centrifugation (3000 × g for 10 min), resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 1 mM PMSF), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon metal affinity resin (Clontech). The resin and supernatant were then loaded into a 25 mL disposable column (BioRad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 10 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME), and eluted with 2 column volumes of elution buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, and titrating concentration of imidazole (25 mM, 50 mM, 75 mM, 100 mM, 250 mM). Fractions were analyzed by 12% SDS PAGE and those containing

DDX17 were pooled and dialyzed into storage buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM BME). DDX17 was then aliquoted, frozen in liquid N_2, and stored at -80°C until needed.

Expression and purification of DDX39A

E. coli BL21(Rosetta 2) cells containing pSpeedET-DDX39A were grown in LB medium containing 50 µg/mL kanamycin at 37°C to an OD_{600} of 0.8, followed by induction with 1 mM IPTG for 3 h at 37°C. Cells were collected by centrifugation (3000 × g for 10 min), resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 1 mM PMSF), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon metal affinity resin (Clontech). The resin and supernatant were then loaded into a 25 mL disposable column (BioRad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 10 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME), and eluted with 2 column volumes of elution buffer (50 mM HEPES, pH 7.4, 1 M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, and titrating concentration of imidazole (25 mM, 50 mM, 75 mM, 100 mM, 250 mM). Fractions were analyzed by 12% SDS PAGE and those containing DDX39A were pooled and dialyzed into storage buffer (20 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 2 mM BME). DDX39A was then aliquoted, frozen in liquid N2, and stored at -80°C until needed.

Expression and purification of GroEL

E. coli DH5α cells containing pTrc99A-groL were grown in LB media at 37°C to an OD_{600} of 0.8 before 0.5 mM IPTG was added. After 3 h, the cells were pelleted (3000 x g for 10 min) and resuspended in lysis buffer (50 mM Tris, 50 mM KCl, 2 mM BME, pH 7.4). An M110-T microfluidizer (Microfluidics) was used to lyse the cells and the lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C). The clarified lysate was loaded on a Fast Flow Q column using an AKTA FPLC system (GE Healthcare). The protein was eluted using a 20 column volume gradient from 0 to 1

M NaCl in buffer A (50 mM Tris pH 7.4, 2 mM BME). The presence of GroEL was confirmed using 12% SDS-PAGE and GroEL containing fractions were pooled. $(NH_4)_2SO_4$ was slowly added to the GroEL fractions to a final concentration of 1.2 M and this solutions was loaded on to a source ISO150 column (GE Healthcare) equilibrated with buffer A supplemented with 1.2 M $(NH_4)_2SO_4$. The column was washed with 2 column volumes of $(NH_4)_2SO_4$ supplemented buffer A before a gradient to buffer A over 10 column volumes was used to elute GroEL. GroEL eluted in about 1 column volume and was pooled and dialyzed against 50 mM Tris pH 7.4, 50 mM KCl, and 2 mM BME. After dialysis, the GroEL solution was supplemented with 2 mM sodium azide as a preservative and stored at 4°C.

Expression and purification of p97

E. coli BL21 (DE3) cells containing the plasmid pET14b-p97 were grown in LB medium containing 100 µg/mL ampicillin at 37°C to an OD_{600} of 0.8, followed by induction with 0.5 mM IPTG for 4 h at 37°C. Cells were collected by centrifugation (3000 × g for 10 min), resuspended in 40 mL lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM β-mercaptoethanol (BME), one complete EDTA-free protease inhibitor cocktail per 50 mL of buffer (Roche)), and lysed by single passage through an M110-T microfluidizer (Microfluidics). The lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C) and the resulting supernatant was incubated for 1 h at 4°C with Talon Metal Affinity Resin (Clontech) in 50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol. The resin and supernatant were then loaded into a 25 mL disposable column (Biorad), washed with 10 column volumes of wash buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 5 mM imidazole), followed by 10 column volumes of stringent wash buffer (50 mM HEPES, pH 7.4, 1M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 20mM imidazole), and eluted with elution buffer (50 mM HEPES, pH 7.4, 1M KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, 250mM imidazole). Fractions were analyzed by 12% SDS PAGE and those containing p97 were pooled and dialyzed into storage buffer (20 mM HEPES, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol, 2 mM BME, pH 7.4), concentrated with a 30 kDa Ultra-15 centrifugal filter (Amicon) to yield a solution of pure p97 protein, which was aliquoted, frozen in liquid N_2, and stored at -80°C until needed.

Expression and purification of HSP70

E. coli BL21 (DE3) cells containing plasmid pSpeedET-HSPA1A were grown in LB medium containing 50 µg/mL kanamycin at 37°C OD_{600} of 0.2 before being transferred to a 16°C incubator. Once the cells reached an OD_{600} of 0.8, IPTG was added to a concentration of 300 µM. After 24 h, the cells were pelleted and resuspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol). An M110-T microfluidizer (Microfluidics) was used to lyse the cells and the lysate was clarified by centrifugation (118,834 × g, 1 h, 4°C). The clarified lysate was incubated with Talon metal affinity resin (Clontech) for 1 h. The resin lysate slurry was loaded into a disposable column and washed with 10 column volumes of lysis buffer. The column was then washed with 10 column volumes of lysis buffer supplemented with 1M NaCl. The protein was eluted by washing with 5 column volumes of lysis buffer supplemented with 250 mM imidazole. The presence of HSP70 in was confirmed using SDS-PAGE and this fraction was dialyzed against storage buffer (50 mM HEPES, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 5% glycerol), flash frozen in liquid N_2, and stored at -80°C.

ATPase assay

Proteins were prepared as follows: 500 nM eIF4A1 in buffer A (20 mM MES-KOH, pH 6.0, 10 mM potassium acetate, 2.5 mM $MgCl_2$, 1% glycerol, and 1 mM DTT); 500 nM DDX3 in buffer A; 500 nM DDX17 A20-M312 in buffer A; 500 nM DDX39A in buffer A; 100 nM p97 in buffer B (50 mM Tris-HCl, pH 7.4, 150 mM KCl, 10 mM $MgCl_2$, 2 mM BME); 50 nM GroEL in buffer C (50 mM Tris-HCl, pH 7.4, 150 mM KCl, 10 mM $MgCl_2$); or 1,000 nM HSP70 in buffer C. Compounds **1** and **2** in DMSO were added to generate samples of each protein containing a gradient of compound (100 µM, 33.3 µM, 11.1 µM, 3.70 µM, 1.23 µM, 0.411 µM, 0.137 µM, and 0.0457 µM). The stock DMSO solutions of **1** and **2** were prepared so that the final DMSO concentration was 2%. A solution of 2% DMSO and 20 mM EDTA pH 8.0 were used as negative and positive controls, respectively. Following incubation at 37°C for 10 min, the assay was initiated by adding 250 µM ATP and 500 µM Poly(U) for eIF4A and associated mutants; 250 µM ATP for GroEL; 500 µM ATP for remaining DDX proteins; 200 µM ATP for p97; and 200 µM ATP for HSP70 to each well and the assay carried out at

37°C. At 60 min (eIF4A, eIF4A mutants, DDX39A, p97) or 120 min (DDX17 A20-M312), a 20 µL aliquot of the reactions was taken and added into 40 µL of malachite green solution (9.3 µM malachite green, 53 mM $(NH_4)_2MoO_4$, 1M HCl, 0.04% Tween 20). After 5 min, the OD_{660} was read on a GEN5 plate reader (BioTek Synergy 2). The IC_{50} values were calculated by fitting the normalized percentage inhibition at a given compound concentration plotted on a semi-log scale using KaleidaGraph (Synergy Software).

Kinetic study

Assay buffer (100 µL) containing 500 nM eIF4A protein in buffer A (see ATPase assay) was dispensed into each well of a 96 well plate. Test compounds **1** and **2** at the desired concentrations (50 µM, 25 µM, and 5 µM) in DMSO were added to each well. Following incubation at 37°C for 10 min, the ATPase assay was initiated by adding 500 µM Poly(U) and a gradient of ATP (4 mM, 2 mM, 1 mM, 0.5 mM, 0.25 mM, 0.125 mM, 0.0625 mM, and 0.03125 mM) to each well followed by incubation at 37°C. A 20 µL aliquot was taken every 10 min and added into 40 µL of malachite green solution (9.3 µM malachite green, 53 mM (NH4)2MoO4, 1 M HCl, 10% tween 20). After 5 min, the OD_{660} was read on a GEN5 reader (BioTek Synergy 2). A Lineweaver-Burke plot was created by converting the concentration of ATP and the initial velocity into the reciprocals and plotted using KaleidaGraph (Synergy Software).

Helicase assay

All helicase experiments were performed using a real-time fluorescence assay protocol [30]. Solutions containing 500 nM eIF4A or 1000 nM eIF4B in buffer A (20 mM MES-KOH, pH 6.0, 10 mM potassium acetate, 2.5 mM $MgCl_2$, 1% glycerol, and 1 mM DTT) were incubated with 100 µM of **1**, **2**, or **3** at 22°C for 10 min. Compounds **1**, **2**, or **3** were added from DMSO stocks such that the final DMSO concentration was 2%. Negative controls were conducted using 2% DMSO in buffer A following incubation, the assay was initiated by adding 50 nM labeled RNA (Integrated DNA Technologies) and 1 mM ATP or water (negative control) in a quartz cuvette (Starna Cells) for 10 min at 25°C. Percent inhibition was calculated by using the equation: Percent inhibition = 100-((A-B)/(C-

B)*100); where A is the slope of the protein-compound interaction, B is the slope of water control, and C is the slope of DMSO control.

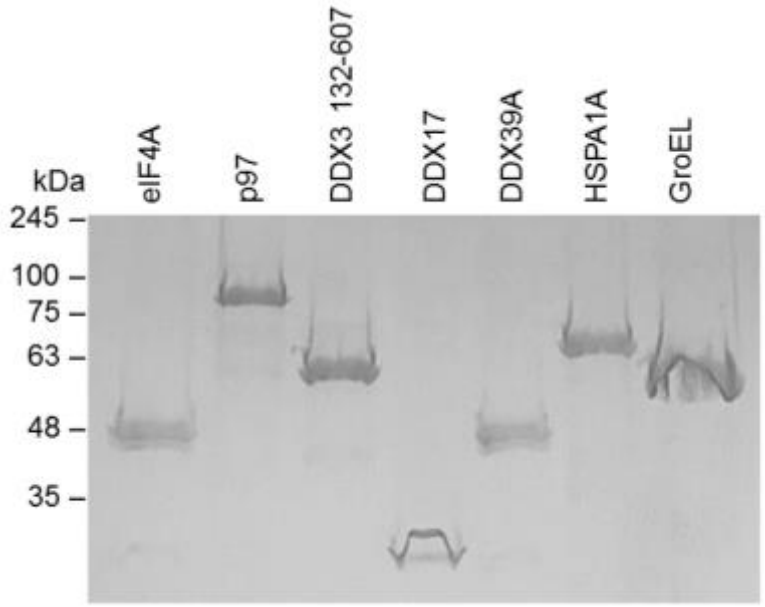

Figure S1. Expression and purification of eIF4A, p97, DDX3 132-607 truncation, DDX17, DDX39A, HSPA1A and GroEL.