Screening of drug candidates that inhibit ArfA-dependent ribosome rescue system

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Research objective

When a truncated messenger RNA (mRNA) lacking a stop codon is translated, the ribosome stalls at the 3' end of the mRNA and Peptide Chain Release Factor 1 (RF1) and RF2 do not function. Since the accumulation of stalled ribosomes is deleterious to cells, each organism should possess ribosome rescue system(s). In *Escherichia coli*, two ribosome rescue systems have been reported: (i) a tmRNA/SmpB-mediated rescue system and (ii) an ArfA-mediated rescue system. While *ssrA* (the gene for tmRNA) or *arfA* (the gene for ArfA) is not essential, disruption of *ssrA* and *arfA* results in a synthetically lethal phenotype. Although compounds that inhibit tmRNA activity have been reported, no compounds that inhibit the ArfA rescue system are currently known. In this study, we developed a screening system to identify drug candidates that could inhibit the ArfA-mediated ribosome rescue system by phenotype microarray.

Methods

Preparation of Strain

E. coli MG1655Δ*smpB*::Kan^R was created through P1 phage transduction with BW25113Δ*smpB*::Kan^R from the Keio collection as the donor strain. *E. coli* MG1655 was the recipient strain. Transductants were confirmed using colony PCR.

Phenotype Microarray

Strains MG1655 and MG1655Δ*smpB* were inoculated into wells of 96-well PM plates (Biolog) containing different antimicrobial compounds at different concentrations. The PM plates were placed on a HiTS microplate reader. Data were collected every 15 min for 48 h at 37ºC.

Preparation of ArfA

C-terminally His_6-tagged ArfA was overexpressed and purified from *E. coli* BL21(DE3). Cells harboring pQE80L arfA were grown in Luria-Bertani broth. At the mid-log phase, isopropyl

β-d-1-thiogalactopyranoside was added to a final concentration of 500 μM, and the cells were incubated for 3 h. Cells were harvested, suspended in buffer A (50 mM HEPES-KOH pH 7.5, 100 mM KCl, 7 mM 2-mercaptoethanol) and disrupted by sonication. ArfA was purified by cation exchange chromatography on an SP-Sepharose column. ArfA was further purified by affinity chromatography (Profinity IMAC) and concentrated by ultrafiltration with buffer A (containing 10% glycerol).

Ribosome bindng assay

The binding of ArfA to the 70S ribosome was assessed using a BLItz instrument. ArfA was immobilized on a Ni-NTA biosensor. Ribosomes were diluted in buffer-B (80 mM Tris-HCl pH 7.8, 7 mM $MgCl_2$, 150 mM NH_4Cl and 2.5 mM dithiothreitol). The Kd value was calculated using BLItz Pro v.1.3.0.5.

Results

Our previous studies revealed that ArfA binds to the ribosomal A-site, where many translation factors and tRNAs associate (Ref. 1, 2). Although many antibiotics that inhibit canonical translation by binding to the A-site have been reported, compounds that inhibit ribosome rescue systems are unknown. To search for drug candidates, we performed a phenotypic microarray using an *E. coli* strain lacking *smpB*. SmpB, tmRNA-binding protein, is essential for tmRNA activity. We prepared the MG1655ΔsmpB strain by P1 phage transduction. We then utilized a Biolog phenotype microarray. The doubling time was calculated by the nonlinear exponential growth equation using GraphPad Prism software (Table 1). It has been reported that *arfA* and *smpB* are genetically related to synthetic lethality (3) and that the expression of *arfA* is repressed by smpB (4). Although no significant difference was observed under many conditions, a growth defect was confirmed in the presence of erythromycin. In the future, we expect that we will be able to identify ArfA inhibitor candidates by performing this analysis on other compounds.

MG1655	240.6	233.1	239.7	278.4	276.7	391	1000	1000	249.2	282.5	367.5	1000
	218.5	213.3	228.3	257.2	235.8	248.8	369.4	1000	258.5	278.8	322.9	1000
	187.3	190	192.2	361.1	223.6	1000	1000	1000	216.5	249.7	1000	1000
	210.6	220.6	210.5	279.3	218.1	327.9	1000	1000	202.2	213.9	244.8	1000
	226.1	218.1	204.2	205.5	205.5	208.4	1000	1000	197.9	240.5	1000	1000
	219.1	323.9	1000	1000	160.9	163.7	177.8	300.9	170.1	185.6	181.6	1000
	179.1	194.1	180.4	192.1	180.4	165	178.4	185.2	180.9	237.5	588.1	1000
	159.2	158.1	215.2	1000	166.5	167.3	171.2	186.3	131.5	137.4	216.3	1000
DsmpB	162.8	154	149.3	171.6	133.8	170.5	389.4	955.6	143.4	148.7	171	1000
	151.3	144	142.4	148.9	143	142.1	156.8	365.2	145.3	140.3	315.9	1000
	126.5	125.6	213.9	416.7	117.3	1000	1000	1000	107.3	122.3	1000	1000
	142.5	136.4	151.2	257.6	125.2	153.1	355	1000	133.4	135.6	143.6	1000
	147.1	137.4	143.9	150.6	135	136.2	1000	1000	157.7	166.3	425.2	1000
	196.4	251.4	1000	1000	131.5	143.2	196.1	1000	146	146.5	148.3	157.5
	173.4	160.1	150.4	213.5	141	146.4	141.3	199.1	229.4	341.8	574.7	1000
	171.4	172.9	187.8	843.4	163.9	174.1	157.1	163.6	160.9	177.3	256.1	1000

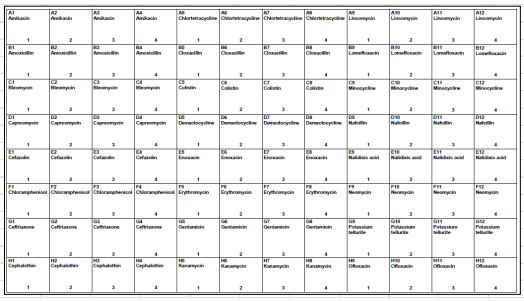

Table 1. Comparison of doubling time between wild type and smpB null strains

To assess ArfA binding to the ribosome, we performed bio-layer interferometry (BLI) using BLItz system. The association of ArfA with ribosomes was monitored in real time by analyzing the wavelength shift of white light reflected from the surface of a biosensor chip (Figure 1). An Ni-NTA biosensor was loaded with C-terminally His_6 tagged ArfA, washed in buffer, and immersed in a solution containing ribosomes for the association phase, followed by immersion in buffer for the dissociation phase. The determined k_{on} and k_{off} values were used for the Kd value calculation. In the absence of compound, the Kd value of ArfA was 1.2±0.5 nM. In future, we will examine whether ArfA inhibitor candidates affect the binding of ArfA to the ribosome.

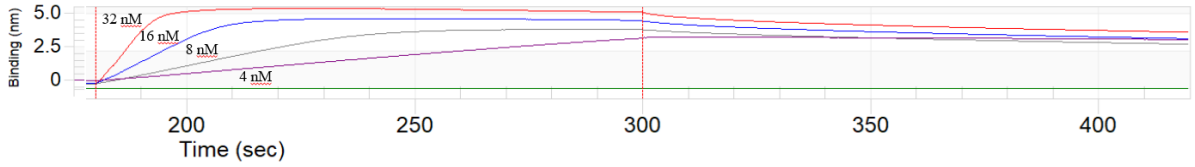

Figure 1. Bio-layer interferometry interaction analysis of ArfA binding to the ribosome

Conclusion

We succeeded in constructing experimental systems. Using these systems, we will be able to identify new drug candidates and elucidate the mechanisms of action of drugs.

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