Simple method for studying *in vitro* protein-protein interactions based on protein complementation and its application in drug screening targeting bacterial NusB-NusE interaction

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ABSTRACT

Protein–protein interactions (PPIs) are present in essential cellular processes of all organisms, and are increasingly considered as drug targets. A number of techniques have been established to study PPIs; however, development of a simple and cost-effective method for *in vitro* high throughput screening of PPI inhibitors is still in desire. We report herein a simple method based on protein complementation for the *in vitro* study of PPIs, as well as screening of inhibitors against the PPI of interest. We have validated this system utilizing bacterial transcription factors NusB and NusE. Three derivatives of an inhibitor targeting the NusB-NusE interaction were synthesized and characterized with the system, which showed specific inhibition and antimicrobial activities. This system is expected to be suitable for more extensive high throughput screening of large chemical libraries. Additionally, our vector system can be easily adapted to study other PPI pairs, followed by inhibitor screening for hit identification in the application of early stage drug discovery.

KEYWORDS:

protein–protein interactions, protein complementation assay, in vitro drug screening, bacterial transcription, antimicrobial agent

INTRODUCTION

The re-emerging epidemic of antibiotic-resistant bacterial infections are becoming a threat to health and economics of the society. The desire for antibiotics with novel mode of action has been drawing urgent attention worldwide (1). Protein-protein interactions (PPIs) are responsible for a number of essential cellular processes, such as cell division, DNA replication, transcription and translation. Novel antibiotics, if were to be discovered to disrupt the essential PPIs in pathogenic bacteria, would offer considerable therapeutic potentials (2).

High-throughput screening (HTS) is a common approach to identify new therapeutic agents in modern drug discovery. However, HTS assays are more readily available to screen or examine

enzyme inhibitors or receptor agonists/antagonists rather than PPI inhibitors (3). There are a number of techniques established to study PPI *in vitro*, such as yeast 2 hybrid (Y2H), fluorescence resonance excitation transfer (FRET), tandem affinity purification (TAP), native PAGE, gel filtration, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and ELISA-based assays (4). Among them protein-fragment complementation assay (PCA) is one of the most widely used methods to study PPI in cell biology (5). This method uses a sensor protein or enzyme, which can be split into two or three polypeptides tagged to each of the PPI partners of interest through recombinant DNA technologies. Upon interaction of the PPI partners, a native-like protein can be reconstructed from the fragments. Several systems have been established including split-ubiquitin, inteins, dihydrofolate reductase (DHFR), β-galactosidase, β-lactamase, green fluorescent protein (GFP), RNA polymerase and luciferase (6). The PCA systems have been used to monitor PPIs in live cells or to identify new PPI partner protein, which require relatively long incubation time for cell growth and protein biosynthesis in cells, therefore unsuitable for screening large numbers of compounds. Cell permeability, efflux system and metabolic enzymes can also affect the detection of bioactive PPI inhibitors, causing false negative results. A simple system to screen PPI inhibitors is in high demand to facilitate the drug discovery process.

RESULTS AND DISCUSSION

We have developed a system for the *in vitro* study of PPI as well as screening of PPI inhibitors using the split-luciferase system. A small (19 kDa) monomeric luciferase, NanoLuc (Promega) was chosen as the senor enzyme (7). NanoLuc emits a bright stable light by converting furimazine to furimamide with light emission brighter than the traditional luminescent reporters (7). The NanoLuc PCA system involves splitting the luciferase into two peptide fragments called SmBiT (11 amino acids) and LgBiT (18kD), which has previously been used for studying PPIs in live cells (7). In our system, each of the PPI partners was tagged with one of the NanoLuc complementation fragment and overproduced in *Escherichia coli*. As designed, interaction between the PPI partners, or presence of the PPI inhibitors will be measured by luminescence released from the re-formation of native NanoLuc *in vitro* (Fig. 1).

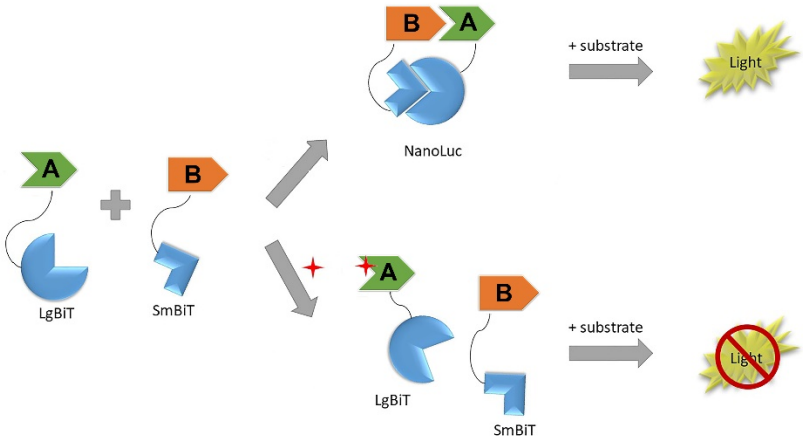

Figure 1. NanoLuc System for studying the PPI partners (A and B) respectively tagged to LgBiT and SmBiT. Interaction between the PPI pairs leads to complementation of the LgBiT and SmBiT fragments. The subsequent formation of an intact NanoLuc enzyme is measured with a bright luminescent signal. Presence

of an inhibitor (read asterisk) disrupts the interaction between the PPI pairs and thus generation of the functional reporter enzyme.

A series of vectors were designed and constructed to obtain fusion PPI pairs with the NanoLuc complementation fragments (Table S1; Fig. 2). The *lgbit*/*smbit* fragments were subcloned to create plasmids for tagging to the PPI partners at both N- and C-terminals (Fig. 2), which will allow optimization of the NanoLuc PCA system for particular PPIs of interest. Plasmids pETMCSIII and pNG209 were used as the parent vectors for overproduction of the recombinant proteins in bacteria (8-9). These vectors were used for the production of N- and C-terminal His-tagged recombinant proteins for affinity chromatography purification. Depending on the protein of interest and/or choice of overproduction host strain, there may exist a significant level of undesired overproduction of the N-terminal LgBiT tag, or the protein of interest without the C-terminal LgBiT tag, potentially due to protease activities (data not shown). Therefore, the C-terminal His-tagging allows affinity purification only for the full length recombinant proteins. The choice of unique restriction enzymes in the multiple cloning sites of each vector are the same despite differences in sequence order (Fig. 2). This is designed to simplify the optimization process so that similar sets of restriction enzymes could be used in cloning of the protein of interest with all the vectors. In all vectors, the *Xho*I, *Sac*I, and *EcoR*I sites are at the upstream of a stop codon in-frame with the start codon (Fig. 2). Therefore, at least one of these three sites must be used for directional cloning to remove the stop codon for successful overproduction of the recombinant proteins.

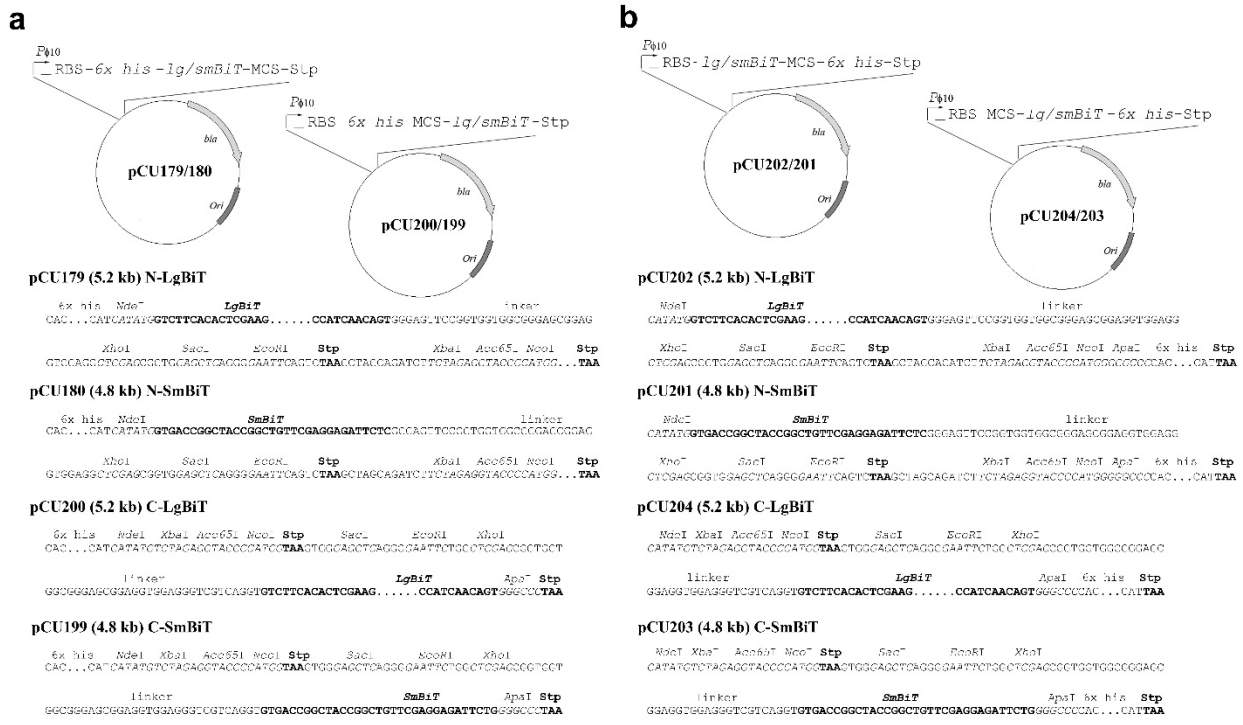

Figure 2. The plasmid vectors for overproduction of NanoLuc complementation fragment tagged recombinant proteins shown with partial sequences encompassing the 6x His tag, multiple cloning sites, linker and the *lgbit/smbit* fragments. a) Vectors for N-terminal His-tagging; b) Vectors for C-terminal his-tagging. $P_{\phi 10}$: phage T7 promoter; RBS: ribosome binding site; MCS: multiple cloning sites; Stp: stop codon; *bla*, ampicillin resistant gene; O*ri*, plasmid replication origin.

We have demonstrated our system using a PPI between the bacterial proteins called NusB and NusE. NusB and NusE are two essential transcription factors involved in ribosomal RNA transcription (10). The interaction between NusB-NusE marks the first step in the formation of the ribosomal RNA transcription antitermination complex (11). In our work, the NusB protein from the model Gram-positive organism *Bacillus subtilis* was chosen to tag with either LgBiT or SmBiT at its N-terminal domain (NTD). This design was based on the 3D spatial proximity of the NusB NTD to NusE in the co-crystal structure (Fig. 3a; 12). NusE was tagged with Lg/SmBiT fragments at both of the N- and C-terminals. All of the six fusion proteins were overproduced in *E. coli* and affinity purified. In order to find the optimum system to study the PPI, we first compared the luminescence signal generated between the SmBiT and LgBiT tagged NusB when they were mixed with their NusE fusion partners at a molar ratio of 1:3. When NusB was tagged with LgBiT, much brighter luminescence signals (~10^4 fold increase in RLU) were generated with its NusE fusion partners than when tagged with SmBiT (Fig. 3b). Therefore, the combination of N-LgBiT-NusB and C-SmBiT-NusE were chosen to perform subsequent experiments. We have then titrated N-LgBiT-NusB at a final concentration of 1 μM with C-SmBiT-NusE at series of concentrations. An increase of luminescence was observed in response to increasing level of C-SmBiT-NusE until the signal reaches its plateau (Fig. 3c). The calculated K_d for the interaction between NusB and NusE was 1.5 ± 0.3 μM, recapitulating the affinity determined with the equivalent proteins in the aqueous buffer by ITC (1.1 ± 0.1 μM; 13).

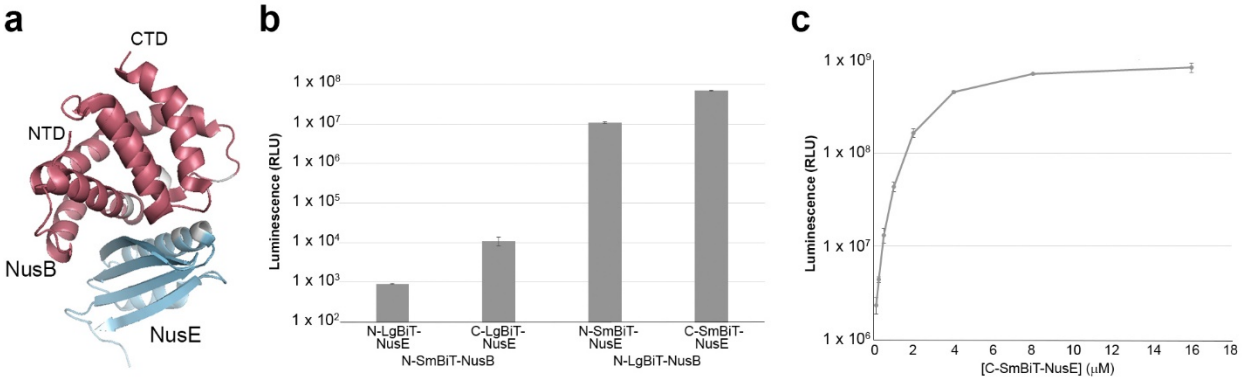

Figure 3. Design and optimization of the NanoLuc complementation system to study NusB-NusE interaction. a) The crystal structure of NusB and NusE heterodimer (pdb 3D3B); b) The luminescence signal generated by each combination; c) Titration of N-LgBiT-NusB with increasing concentration of C-SmBiT-NusE. For panels b and c: n = 3, variability displayed as ± SE of one representative experiment. Technical repeats were conducted at least twice with similar results.

Previously through structure based pharmacophore design and *in silico* screening, an inhibitor of the NusB-NusE interaction, MC4 was identified (Fig. 4a). In this study, MC4 was used to validate our system for characterization of PPI inhibitors. The N-LgBiT-NusB and C-SmBiT-NusE combination was used at 1:1 ratio for the titration of MC4. The addition of increasing concentrations of MC4 to the PPI pairs resulted in a dose-dependent decrease of luminescence signal generation, which indicated the increase in percentage inhibition of NusB-NusE interaction (Fig. 4b). Therefore, our system is suitable for demonstration of the inhibitory role of inhibitors against the NusB-NusE interaction. The half-maximal inhibitory concentration (IC_{50}) could be

calculated for MC4 from the titration curve (Fig. 4b), which was 24.8 ± 0.3 μM, similar to the value previously obtained using the ELISA-based assay (34.7 ± 0.1 μM; 14).

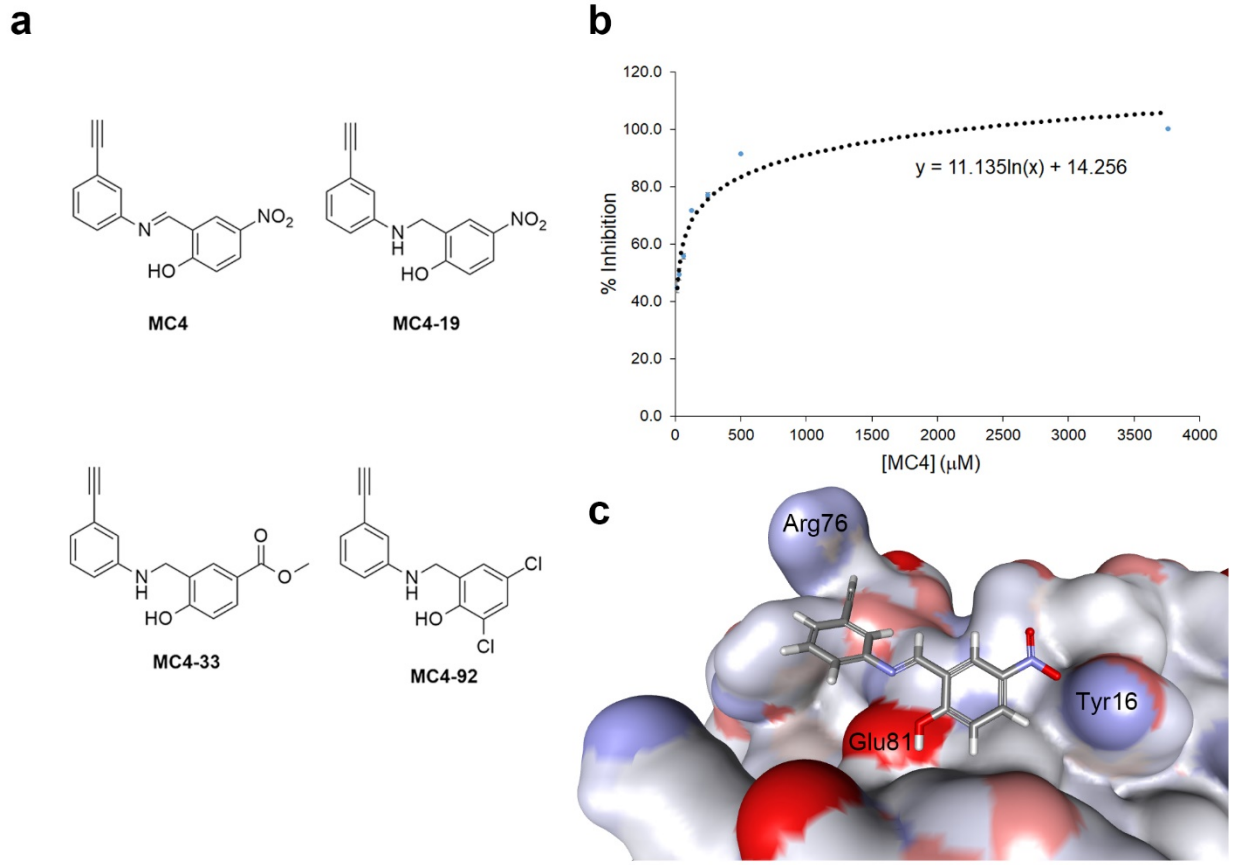

Figure 4. The NusB-NusE inhibitor, MC4. a) The chemical structures of MC4 and its derivatives; b) Inhibition of the interaction between NusB and NusE by MC4 measured using the NanoLuc PCA system. Results were presented as means ± SE (n = 3) of one representative experiment. Technical repeats were conducted at least twice with similar results. c) MC4 (shown as stick) docked with the crystal structure of NusB (shown as surface).

MC4 is an aromatic Schiff base. Aryl imine/Schiff bases are known to be stable and bioactive (15-16), and herein we intended to examine whether the antimicrobial activity of MC4 resulted from the inhibitory effect of NusB-nusE interaction rather than the chemical reactivity of imine by forming covalent bond with proteins. We synthesized three MC4 derivatives with C-N single bond by reduction of the imine group (Fig. 4a; Supporting Information), to exclude the possibility of covalent bond formation. We then tested the antimicrobial activity of the MC4 and its analogues against bacterial pathogen *S. aureus* ATCC 25923 and ATCC 29213, the commonly used strains for antibiotic susceptibility testing (Table 1). MC4 was found to demonstrate growth inhibition effects with a minimum inhibitory concentration (MIC) of 8 μg/ml against *S. aureus* ATCC 25923 (Table 1). The chemical derivatives MC4-19, MC44-33 and MC4-92 have also demonstrated antimicrobial activity against the *S. aureus* strains, in which MC4-92 even displayed the activity

to a level similar to vancomycin (Table 1). This result indicates the imine group of MC4 is not critical for bioactivity and the MC4 derivatives are very valuable to be considered as lead compounds for further development of novel antimicrobial agents.

Table 1. The Antimicrobial Activity (MIC μg/ml) of Compounds in the NusB-NusE PPI Inhibitor Derivatives (MC4 Series) against clinical *S. aureus* strains and representative Gram-positive pathogens.

	Compound	MC4	MC4-19	MC4-33	MC4-92	V[a]	O[b]	G[c]
	IC_{50} (μM)	24.8 ± 0.3	80.2 ± 2.0	83.4 ± 3.8	72.8 ± 0.9			
	%Inhibition (125 μM)	71.7 ± 0.4	50.2 ± 0.7	47.9 ± 3.4	50.6 ± 4.9			
S. aureus	ATCC 25923	8	16	32	2	1	1	1
	ATCC 29213	16	16	16	4	1	0.3	1
	ST239	16	16	>64	>64	1	>64	>64
	ATCC BAA-43	8	16	>64	>64	1	>64	>64
	ATCC BAA-44	32	16	>32	>64	2	>64	>64
	HA W-231 ST45	32	16	32	64	1	4	16
	CA W-47 ST30	16	16	32	64	2	4	0
	CA W-45 ST59	16	32	>64	64	1	8	1
	CA W-46 ST59	16	16	32	32	1	8	1
	USA 300	16	16	32	32	1	32	1
	ST22	16	16	32	16	1	64	0
	CA W-4 ST338	16	16	32	16	1	1	0
	CA W-48 ST217	8	16	32	32	1	64	1
	HA W-235 ST5	8	16	32	8	0.5	>64	>64
Gram-(+)[d]	ECAS 25788	16	64	>64	>64	4	32	4
	SEPI 12228	>64	8	32	64	2	0.3	0.1
	SSAP 15305	16	32	32	>64	1	1	0.1
	SPNE 49619	>64	16	16	16	0.5	2	32
	SPYO 19615	32	16	16	>64	0.5	0.1	8
	SAGA 12386	256	16	>64	>64	0.5	0.5	32

[a]V: vancomycin; [b]O: oxacillin; [c]G: gentamicin; [d]ECAS: *Enterococcus casseliflavus*; SEPI: *Staphylococcus epidermidis*; SSAP: *Staphylococcus saprophyticus*; SPNE: *Streptococcus pneumonia*; SPYO: *Streptococcus pyogenes*; SAGA: *Streptococcus agalactiae*

Compound MC4 was docked into the electron density map of NusB, and appropriately fitted into a basin-like semi pocket formed by the binding site on NusB (Fig. 4c). The terminal alkyne group of MC4 exhibited a hydrophobic interaction with the residue chain on Arg76 of NusB, the phenol group of MC4 bonded to Glu81 of NusB by hydrogen bond, and the nitrate group of MC4 accepted a hydrogen bond donation from Tyr16 of NusB (Fig. 4c). When we reduced the imine to amine to obtain MC4-19, as the two benzyl rings of MC4 were released from the same planar conformation, an increase in entropy penalty can be expected. As a result, the IC_{50} and MIC values of MC4-19 were higher than MC4, indicating the lowered inhibitory activity against the NusB-NusE PPI and antimicrobial activity. Based on the result of MC4-19, when we modified the nitrate group to methyl carboxylate, an alternative hydrogen bond acceptor and electron-withdrawing

group, the IC_{50} and MIC values of MC4-33 were similar to MC4-19. While MC4-92 contains a chloride group at the same position of nitrate in MC4-33, and an additional chloride group at the *ortho*-position of phenol, it seemed these doubly enhanced electron-withdrawing effect to phenol could significantly improve the antimicrobial activity (MIC 2 μg/ml against *S. aureus* ATCC 25923) and slightly decreased IC_{50} value compared to MC4-19 and MC4-33. This result indicated that this minor change in structure did not significantly change the binding affinity to NusB, but may improve the antimicrobial activity by other factors such as better cell permeability, ineffective efflux or slow metabolism. In conclusion, the structure modification of MC4 revealed the inhibition of NusB-NusE interaction could be improved, and associate to the antimicrobial activity. Additionally, the inhibitory curves of MC4 series are standard and highly repeatable. The percentage inhibitions of these compounds at a single concentration (125 μM, Table 1) correlated perfectly with their IC_{50} values, which suggested the drug screening process can be further simplified for NusB-NusE inhibition using one appropriate concentration to perform the assay described in this paper.

We have tested the compounds against several clinical *S. aureus* strains and Gram-positive species (Table 1). The four MC4 compounds demonstrated consistent antimicrobial activity against all of the tested community- or hospital-acquired (CA, HA) methicillin-resistant *S. aureus* (MRSA) strains. While several *S. aureus* strains such as ST239, ATCC BAA-43, ATCC BAA-44 have already developed resistant to oxacillin, the first-line antibiotic drug in the US for MRSA treatment, and gentamicin, another first-line aminoglycoside antibiotic. Additionally, these compounds displayed antimicrobial activity against other Gram-positive bacteria, such as *Enterococcus casseliflavus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus pneumonia*, *Streptococcus pyogenes* and *Streptococcus agalactiae*, suggesting the potentials as a broad-spectrum antimicrobial agent.

CONCLUSION

Comparing with our previously established ELISA-based assay for PPI characterization and inhibitor testing (17), this *in vitro* NanoLuc PCA system offers unique advantages of time and resources saving, which makes this system readily adaptable for HTS. The PCA system also bypassed the use of multiple antibodies in the ELISA-based assay, and thus antibody related non-specific signal was expected to be eliminated. Nevertheless, efficient reconstitution of the complemented proteins such as Nanoluc luciferase relies on the physical contact of its complement fragments during the binding of two proteins of interest. Therefore, the tagging of complementation fragments to the termini of the PPI partners need to be rationally designed to seek spatial proximity for optimum complementation, through examination of the binding mode of the PPI partners (Fig. S1). In case that the structural information is not available, all the possible protein fusions built from combinations of complementation fragments and the proteins of interest need to be considered for ideal experimental results.

In summary, we have modified a previously established cell-based split-luciferase complementation reporter system to study *in vitro* PPIs in a rapid and cost-effective manner. The vector system we constructed enable simple bioengineering of fusion PPI partner proteins with NanoLuc complementation fragments. We have demonstrated the feasibility of the system by

characterizing the bacterial transcription factor NusB-NusE PPI and its inhibitors. This simple and versatile system is readily adaptable to study any PPIs *in vitro*, and to be altered by using other PCA systems if required.

EXPERIMENTAL SECTION

Bacterial strains and chemicals

S. aureus strains ATCC ATCC 25923 and ATCC 29213 were used in this study for the assay of antimicrobial activities. *E. coli* strain DH5α (Gibco BRL) was used in this study for cloning and *E. coli* BL21 (DE3) pLysS (18) was used for protein overproduction. Compounds in the MC4-series were chemically synthesized as detailed in the SI.

Vector construction

All vectors used and created in this study were listed in Table S1. Vector construction was detailed in Supporting Information. All the plasmids constructed in this study were confirmed by sequencing.

Protein purification

Purification of the His-tagged proteins have been done as described previously (9). Briefly, *E. coli* BL21 (DE3) pLysS transformed with pCU190 and pCU194 (Table S1) was grown in 800 ml auto-induction media (AIM-LB base w/o trace elements, FORMEDIUM UK) supplemented with 0.5% (v/v) glycerol at room temperature for 24 h. Cells were pelleted by centrifugation at 5000 g for 5 min and lysed in 5 ml/g cells lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, 0.5 mg/ml lysozyme, pH 8.0). After sonication and centrifugation at 8000 g for 45 min, the supernatant was filtered and passed through a 1 ml His-trap FF column (GE) pre-equilibrated with the lysis buffer without lysozyme. The column was then washed with 10 CV of wash buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 8.0) and protein eluted with elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 200 mM imidazole, pH 8.0). Fractions containing target proteins were pooled and dialyzed into PBS + 30% (v/v) glycerol, pH 8.0 and stored at −80 °C.

NanoLuc PCA

For system optimization, 25 µl of N-LgBiT-NusB or N-SmBiT-NusB (5 µM in PBS) was added to PierceTM 96-Well White Opaque polystyrene Plates, and then mixed respectively with 25 µl N-/C-SmBiT-NusE or N-/C-LgBiT-NusE (15 µM in PBS). For the NusB-NusE titration curve, 25 µl N-LgBiT-NusB (2 µM in PBS) was added to PierceTM 96-Well White Opaque polystyrene Plates, and then mixed with 25 µl of serial two-fold diluted C-SmBiT-NusE from 0.25 µM to 32 µM in PBS. The mixture was incubated for 10 minutes at 37°C. For inhibitor testing, chemical compounds were dissolved to 10 mg/ml in DMSO and 2-fold serial diluted in PBS. 40 µl N-LgBiT-NusB (2.5 µM in PBS) was added to 96-well plates, and then mixed with 20 µl compound at desired concentrations. The mixture was incubated for 10 minutes at 37°C. 40 µl C-SmBiT-NusE (2.5 µM in PBS) was then added to each well, followed by incubation for 10 minutes at 37°C. After the final incubation step, equal volume of Promega Nano-Glo® Luciferase Assay Substrate was added to the reaction mixture. Luminescence emitted was measured using a Victor X3

Multilabel plate reader. Experiment was performed in triplicate. Technical repeats were taken to ensure consistent results were obtained.

Antimicrobial activity test

The antimicrobial activity of the compounds was determined by broth microdilution according to the CLSI guidelines (19). The test medium was cation-adjusted Mueller-Hinton broth (MH). Serial two-fold dilutions were performed for the tested chemicals starting from 256 μg/ml to 0.5 μg/ml, and the bacterial cell inoculum was adjusted to approximately 5×10^5 CFU per ml. Results were taken after 20 h of incubation at 37 °C. MIC was defined as the lowest concentration of antibiotic with no visible growth. Experiments were performed in duplicates.

Molecular docking

The X-ray structure of NusB were acquired from the Protein Database (PDB: 3R2D). The S. aureus NusB homology model was made using Phyre[2]. The molecular docking was performed in Discovery Studio 2016 (20).

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Author Contributions

X.Y. and C.M. conceived the idea. X.Y., T. F. T. and Y. Y. Q. performed the experiments. X.Y. and C.M. analyzed the data and wrote the paper. The authors thank Hoi Kiu Chan and Lin Lin for the technical assistance in antimicrobial activity testing.

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Conflict of interest

The authors claim no conflict of interest.

REFERENCES

1. O'Neill, J. (**2014**) Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. UK-AMR Review.

2. Scott, D. E., Bayly, A. R., Abell, C., and Skidmore, J. (**2016**) Small molecules, big targets: drug discovery faces the protein–protein interaction challenge. *Nat Rev Drug Discov*. 15, 533–550.

3. Wells, J. A., McClendon, C. L. (**2007**) Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature* 450, 1001–1009. doi: 10.1038/nrd.2016.29.

4. Zhou, M., Li, Q., and Wang, R. (**2016**) Current Experimental Methods for Characterizing Protein-Protein Interactions. *ChemMedChem* 11(8), 738-756. doi: 10.1002/cmdc.201500495.

5. Wehr, M. C., and Rossner, M.J. (**2016**) Split protein biosensor assays in molecular pharmacological studies. *Drug Discov Today* 21(3), 415-429. doi: 10.1016/j.drudis.2015.11.004.

6. Shekhawat, S. S., and Ghosh, I. (**2011**) Split-protein systems: beyond binary protein-protein interactions. *Curr Opin Chem Biol* 15(6), 789-797. doi: 10.1016/j.cbpa.2011.10.014.

7. Dixon, A.S., Schwinn, M.K., Hall, M.P., Zimmerman, K., Otto, P., Lubben, T.H., Butler, B.L., Binkowski, B.F., Machleidt, T., Kirkland, T.A., Wood, M.G., Eggers, C.T., Encell, L.P., and Wood, K.V. (**2016**) NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. *ACS Chem Biol* 11(2), 400-408. doi: 10.1021/acschembio.5b00753.

8. Neylon, C., Brown, S.E., Kralicek, A.V., Miles, C.S., Love, C.A., and Dixon, N.E. (**2000**) Interaction of the *Escherichia coli* replication terminator protein (Tus) with DNA: a model derived from DNA-binding studies of mutant proteins by surface plasmon resonance. *Biochem* 39, 11989-11999.

9. Yang, X., and Lewis, P.J. (**2008**) Overproduction and purification of recombinant Bacillus subtilis RNA polymerase. *Protein Expr Purif* 59(1), 86-93. doi: 10.1016/j.pep.2008.01.006.

10. Richardson, J. P. and Greenblatt, J. (**1996**) In Escherichia coli and Salmonella: Cellular and molecular biology, 2nd ed., pp 822– 848, American Society for Microbiology, Washington, DC.

11. Greive, S. J., Lins, A. F., and von Hippel, P. H. (**2005**) Assembly of an RNA-protein complex. Binding of NusB and NusE (S10) proteins to boxA RNA nucleates the formation of the antitermination complex involved in controlling rRNA transcription in *Escherichia coli*. *J. Biol. Chem* 280, 36397– 36408.

12. Luo. X., Hsiao, H.H., Bubunenko, M., Weber, G., Court, D.L., Gottesman, M.E., Urlaub, H., and Wahl, M.C. (**2008**) Structural and functional analysis of the *E. coli* NusB-S10 transcription antitermination complex. *Mol Cell* 32(6), 791-802. doi: 10.1016/j.molcel.2008.10.028.

13. Das, R., Loss, S., Li, J., Waugh, D.S., Tarasov, S., Wingfield, P.T., Byrd, R.A., and Altieri, A.S. (**2008**) Structural biophysics of the NusB:NusE antitermination complex. *J Mol Biol* 376(3), 705-720.

14. Yang, X., Luo, M.J., Yeung, A.C.M., Lewis, P.J., Chan, P.K.S., Ip, M. and Ma, C. (**2017**) First-In-Class Inhibitor of Ribosomal RNA Synthesis with Antimicrobial Activity against *Staphylococcus aureus. Biochemistry* 56(38), 5049-5052. doi: 10.1021/acs.biochem.7b00349.

15. Shi, L., Ge, H.M., Tan, S.H., Li, H.Q., Song, Y.C., Zhu, H.L., and Tan, R.X. (**2007**) Synthesis and antimicrobial activities of Schiff bases derived from 5-chloro-salicylaldehyde. *Eur J Med Chem* 42, 558-564.

16. da Silva, C.M., da Silva, D.L., Modolo, L.V., Alves, R.B., de Resende, M.A., Martins, C.V.B., and de Fátima, A. (**2011**) Schiff bases: A short review of their antimicrobial activities. *J. Adv. Res*. 2, 1-8.

17. Yang, X., Ma, C., and Lewis, P.J. (**2015**) Identification of inhibitors of bacterial RNA polymerase. *Methods* 86, 45-50. doi: 10.1016/j.ymeth.2015.05.005.

18. Studier, F.W., and Moffatt B.A. (**1986**) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113-130.

19. CLSI, Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fifth Informational Supplement (M100S25). Clinical and Laboratory Standards Institute, Wayne, PA, 2015.

20. Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N., and Sternberg, M.J. (**2015**) The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* 10(6), 845-8i58. doi: 10.1038/nprot.2015.053.