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Protein-DNA Complex-Guided Discovery of an Antibacterial Lead, E1, in Restoring the Susceptibility of *Klebsiella Pneumoniae* to Polymyxin B by Targeting Response Regulator PmrA

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Supporting information

Experimental Section

Ligand library

A total of 103,000 molecules were retrieved from natural product databases, including 1,496 compounds from Specs (http://www.specs.net), 11,247 compounds from ACD (http://www.ac-discovery.com/), 144 compounds from ICC (http://indofinechemical.com/), 14,084 compounds from PNP (http://www.princetonbio.com/), 1,089 compounds from TCM (Taiwan Chinese medicine, http://www.nricm.edu.tw/bin/home.php?Lang=en), and 74,940 compounds from the InterBioScreen (IBS, http://www.ibscreen.com) diversity set. The sketch molecules and prepare ligands modules implemented in Discovery Studio 3.5 (Accelrys Software, Inc., San Diego, CA, USA) were used to create the molecular structures of all compounds, and hydrogen atoms were present in all the constructed ligands.

Receptor-ligand pharmacophore generation and ligand-pharmacophore mapping

The receptor-ligand pharmacophore generation module of Discovery Studio 3.5 was employed to build the pharmacophore models. The complex structure of PmrA-DNA (PDB ID: 4S04) was used to generate the receptor-ligand pharmacophore model. The PmrA structure is served as the "Input Receptor", and the DNA structure was utilized as the "Input Ligand". The "Minimum Features" and "Maximum Features" were set to 10 and 30, respectively, and the "Maximum Pharmacophores" was set to 10. The "fast method" was applied for conformation generation with "rigid fitting method" and the rest parameters were set as default. Subsequently, the built pharmacophores model, **Ch2pharN4** was subjected to ligand-pharmacophore mapping. For the ligand-pharmacophore mapping, all the natural products (103,000 compounds) were fit to the built pharmacophore model, **Ch2pharN4**, with the fitting method set to "flexible" and all other parameters were remained as default.

Preparation of recombinant PmrA/PmrA$_C$ and oligonucleotides

The DNA fragments which encode full-length PmrA and PmrA$_C$ (residues N121-E223 of PmrA) were cloned into a vector pET-29b (+) (Novagen) with an extra Met residue and an LEHHHHHH tag at the N- and C-termini, respectively, in *E. coli* strain BL21 (DE3). For full-length PmrA, two residues were mutated (W181 to G and I220 to D) to improve solubility.[1] The mutations were conducted according to the QuickChange site-directed mutagenesis protocol (Stratagene) and confirmed by DNA sequencing. For ^{15}N-labeled protein samples, cells were grown in H_2O containing M9 minimal medium supplemented with $^{15}NH_4Cl$ and glucose at 37 °C, and were disrupted by using an M-110S microfluidizer (Microfluidics). The protein was further purified with nickel-nitrilotriacetic acid affinity resin (Qiagen, Hilden, Germany). The purity of protein was > 95 %, examined by coomassie blue-stained sodium dodecyl sulphate (SDS) polyacrylamide gel. Full-length PmrA was activated by $BeF3^-$ (5.3 mM $BeCl_2$, 35 mM NaF and 7 mM $MgCl_2$).[2] The oligonucleotide (5'-ATTTCTTAATATTATCCTAAGCAAG-3') used in fluorescence polarization experiments was from MDBio Inc. (Taiwan). The preparation, purification and concentration determination of the double-stranded DNA were conducted with the same methods as described previously.[2]

Fluorescence polarization (FP) measurements

Compounds, **E1**-**E5**, were purchased from InterBioScreen (IBS, http://www.ibscreen.com) (**E1**: STOCK1S-95047; **E2**: STOCK1N-13224; **E3**: STOCK1N-16691; **E4**: STOCK1S-43938; **E5**: STOCK1S-55283), and the purities are all over 90% examined by NMR. The oligonucleotide (5'-ATTTCTTAATATTATCCTAAGCAAG-3'), labeled with 6-carboxyfluorescein (6-FAM) at the 5' position, was dissolved in 10 mM sodium phosphate and

15 mM NaCl pH 7.0 for fluorescence polarization experiments. About 10 µl activated PmrA (prepared in 10 mM sodium phosphate and 15 mM NaCl at pH 7.0) was firstly added into the well of ELISA plate. After that, 1 µl of serious diluted inhibitors (prepared in the same buffer of activated PmrA) were mixed with the PmrA (final concentration 30 µM) to reach the interested concentrations and incubated at 25 °C for 10 mins. Subsequently, 9 µl of 6-FAM-labeled DNA (final concentration = 10 nM) was added into the reaction, incubating at 25 °C for another 10 mins. Reactions were measured 3 times by use of a SpectraMax Paradigm plate reader (Molecular Devices, CA, USA) with excitation wavelength 485 nm and emission wavelength 535 nm. The inhibition % was derived according to the following equation:

$$Inhibition\ \% = \frac{\{[(P+D)-(D)]-[(P+I+D)-(D)]\}}{[(P+D)-(D)]} \times 100,$$

in which (D), (P+D), and (P+I+D) are the polarization intensity of DNA alone, PmrA with DNA, and PmrA mixed with inhibitor then incubated with DNA, respectively.

NMR spectroscopy and compound titrations

The preparations of NMR samples were as described previously.[2] In brief, the activated and inactivated PmrA (0.3 ml, 0.25 mM) were prepared in pH 8.0 buffer (20 mM Tris and 100 mM NaCl) with and without $BeF3^-$ activation, respectively. $PmrA_C$ (0.3 ml, 0.12 mM) was prepared in pH 8.0 buffer (20 mM Tris and 100 mM NaCl). All the protein solutions were further loaded in Shigemi NMR tubes (Shigemi Inc.). NMR experiments for activated/inactivated PmrA and $PmrA_C$ were conducted at 310 and 298 K on Brucker AVANCE 600 or 800 NMR spectrometers (Bruker, Karlsruhe, Germany) equipped with a triple-resonance cryprobe including a shielded z-gradient. The hetero-nuclear NMR experiments for backbone assignments were performed as described in a review article[3]. All the acquired NMR spectra were processed by using NMRPipe[4] and analyzed with NMRViewJ8.0a.22.[5] Compound titration experiments were carried out by adding increasing amounts of compound to ^{15}N-labled

protein at pH 8.0 to reach the interested protein/compound molar ratio and acquiring a serious of 2D-^{1}H-^{15}N TROSY-HSQC spectra. The inactivated and activated PmrA titrated with compounds were used to probe the binding site. The titrations of compound toward $PmrA_C$ were employed to determine the dissociation constant (K_D). The chemical shift changes were calculated through the equation $\Delta\delta = [(\Delta\delta_{NH})^2+(0.17\times \Delta\delta_N)^2]s^{0.5}$, in which $\Delta\delta_{NH}$ and $\Delta\delta_N$ are the chemical shift differences for $^{1}H_N$ and ^{15}N, respectively. To determine the dissociation constant (K_D), the NMR titration curves of $PmrA_C$ were fitted with the following equation valid for a 1 : 1 complex in fast exchange: $\Delta\nu(x) = 0.5 \times \Delta\nu_{max} \times \{(x+1+K-[(x+1+K)^2-4x]^{0.5}$, where x is [compound]/[$PmrA_C$], $\Delta\nu(x)$ is $[(\Delta\nu_H)^2+(\Delta\nu_N)^2]^{0.5}$ at molar ratio x, and K corresponds to K_D/[$PmrA_C$].[6] The titration curves were fitted and analyzed with the "Titration Analysis" function of NMRViewJ8.0.a22.[5]

Isothermal Titration Calorimetry (ITC)

The binding affinity of $PmrA_C$ with compound, **E1**, was estimated by ITC using a MicroCal iTC200 microcalorimeter. The sample of $PmrA_C$ was prepared in reaction buffer (20 mM sodium phosphate, 100 mM NaCl, pH 6.0). Compounds were also prepared in reaction buffer (**E1** = 0.4 mM) and titrated into $PmrA_C$ (45 μM) at 25 °C. The background heat generated from compound to buffer titration was subtracted and the corrected heat from the binding reactions of $PmrA_C$ to **E1** was used to derive the association constant *(K_a)*, the enthalpy of binding (ΔH), the change in Gibbs free energy (ΔG), and the entropy change (ΔS = ΔH-ΔG) at 298 K. All the data were analyzed by using Origin ITC Analysis (MicroCal Software, Northampton, MA, USA).

Bio-layer interferometry (BLI)

The kinetics estimates were obtained by using the ForteBio Octet RED96 instrument (ForteBio, Pall Life Sciences). Recombinant $PmrA_C$ carrying C-terminal 6 × His tag was immobilized on the HIS1K biosensor in the reaction buffer (20 mM sodium phosphate, 100 mM NaCl, pH 6.0) at 25 °C for 180 s prior to kinetic experiment. For the association, the preloading biosensor was exposed to different concentration of **E1** (0-37.5 μM) for the period of 300 s. Following the dissociation step, the sensor was subjected to the reaction buffer for 300 s. Kinetic parameters, including K_{on} ($M^{-1}s^{-1}$) and K_{off} (s^{-1}), were obtained by fitting the reaction model 1 : 1 kinetics to the reference-subtracted data. The equilibrium dissociation (K_D) was calculated as the ratio of K_{off} to K_{on}. All calculation was determined using Data Analysis 7 software (ForteBio, Pall Life Sciences).

Molecular modeling of PmrA-E1 complex

We performed molecular modeling to build up the complex structure of PmrA-**E1**. The perturbed residues (S167, V169, H170, R171-Y175, R207, V209, and F212-A217) of activated PmrA upon **E1** titration (NMR) were defined as the binding site for protein-ligand flexible docking. The GOLD docking program (Cambridge Crystallographic Data Center (CCDC), version 5.1) with the GoldScore scoring function was employed for the protein-ligand flexible docking. The side chains of the binding site residues were set to be flexible for distinct rotamers during docking analysis. The constructed and energy-minimized compound, **E1**, was subsequently docked into the defined binding site with modified docking parameter settings (number of operations and population size values were 1,600,000 and 1000, respectively; default settings were used for the other parameters). The most possible orientation and position with the most favorable free energy was examined and presented.

Microbial viability assay

Relative survival rate of *Klebsiella pneumoniae* CG43S3 under antibiotic treatment is determined as previously reported[7] with some modification. Briefly, the overnight-grown bacterial was subculture to LB until OD_{600} reached 0.7. Bacterial culture was washed twice with distilled PBS and then 6.7×10^4 CFU/ml in LB was prepared. 100 μl of the suspension alone or supplemented with **E1** (0, 0.25, 2.5, and 25 μM) was incubated at 37 ˚C and then placed in each well of a 96-well micro-titer plate where contained 100 μl PBS or PBS-diluted antibiotics to final concentrations of polymyxin B (0, 0.002, 0.01, and 0.1 μg/ml), colistin (0.1 μg/ml), imipenem (80 μg/ml), and (kanamycin 64 μg/ml). The plate was incubated at 37 ˚C for 10, 30 and 60 mins with shaking. Subsequently, 100 μl of the suspension was directly plated on LB agar plates and incubated at 37 ˚C overnight to determine the number of viable bacteria. The relative survival rates were expressed as colony counts divided by the number of the same culture treated with PBS and multiplied by 100. The assays were performed thrice, and the results were shown as the average ± standard deviation from triplicate samples.

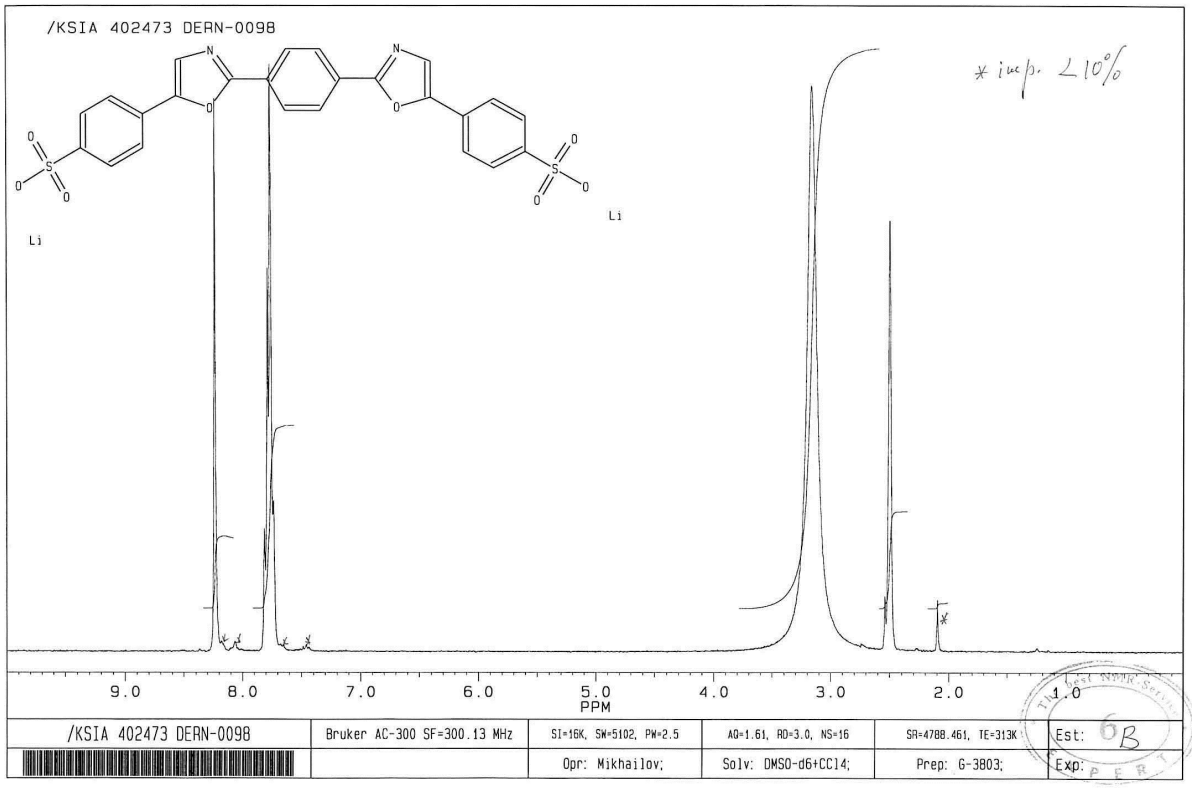

Figure S1. The purity of compound, **E1**, examined by NMR 1D spectrum, is over 90 %. This information is supplied by InterBioScreen (IBS, http://www.ibscreen.com).

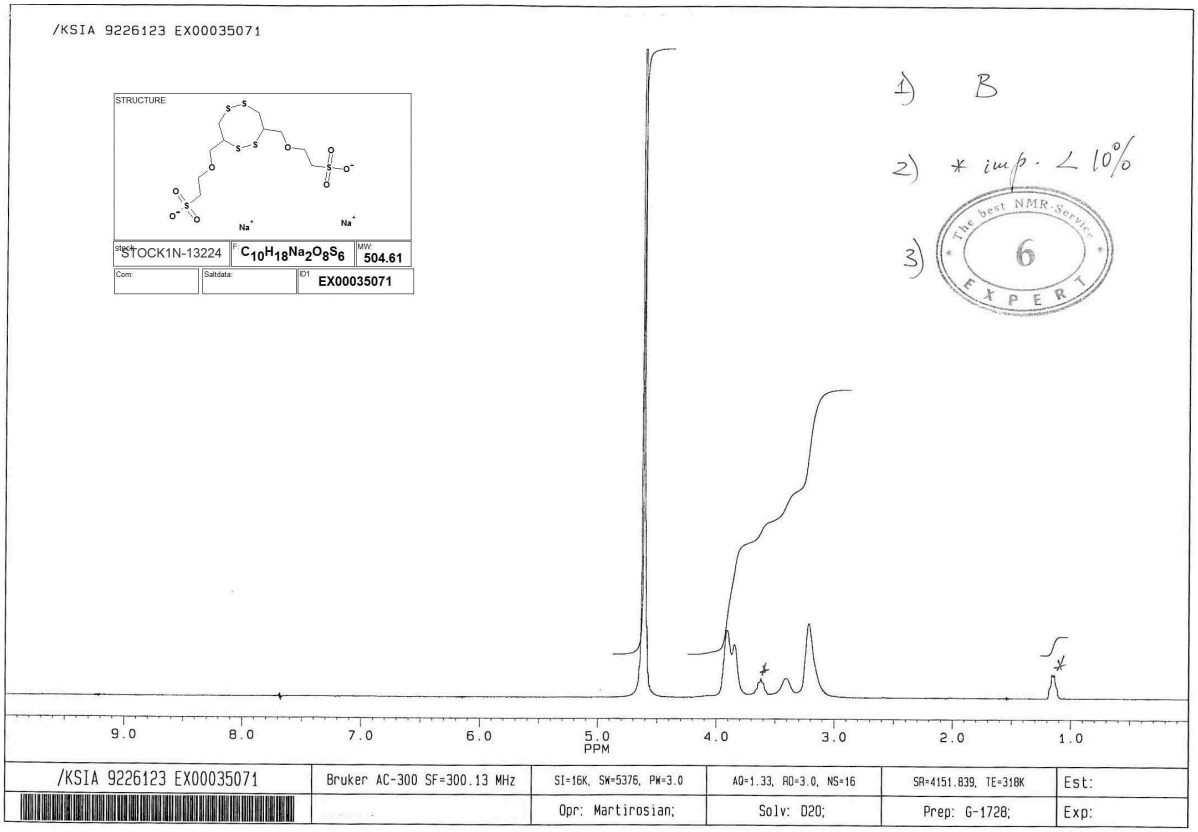

Figure S2. The NMR 1D spectrum of compound, **E2**, and the determined purity is over 90 %. This information is obtained from InterBioScreen (IBS, http://www.ibscreen.com).

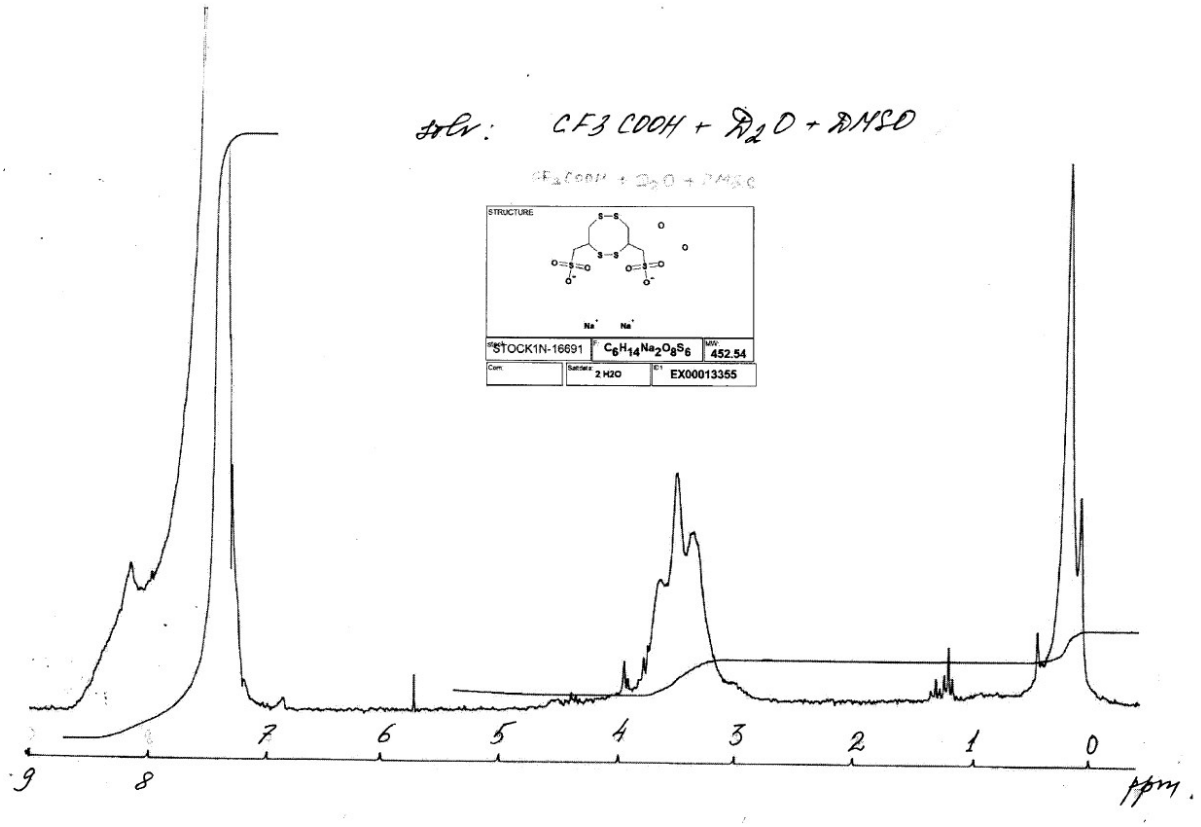

Figure S3. The purity of compound **E3**, examined by NMR 1D spectrum, is over 90 %. This data is supported by InterBioScreen (IBS, http://www.ibscreen.com).

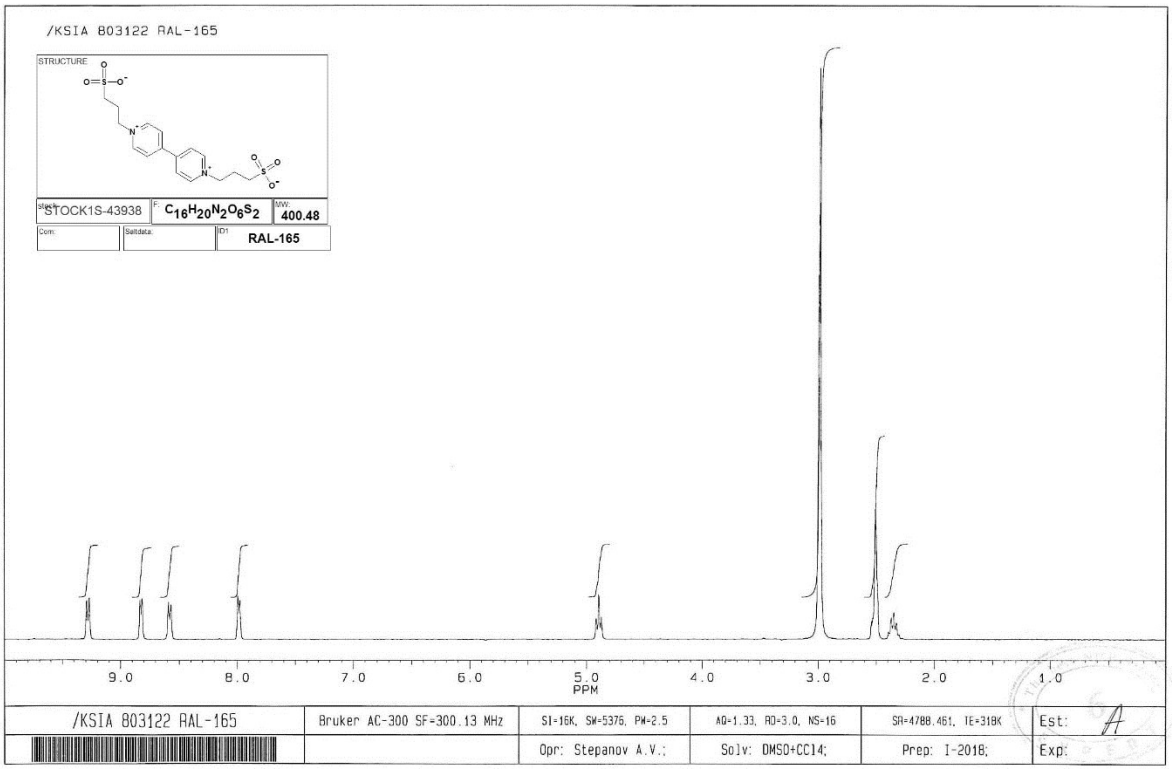

Figure S4. The 1D NMR spectrum of compound **E4**, and the purity is over 95 %. This information is available from InterBioScreen (IBS, http://www.ibscreen.com).

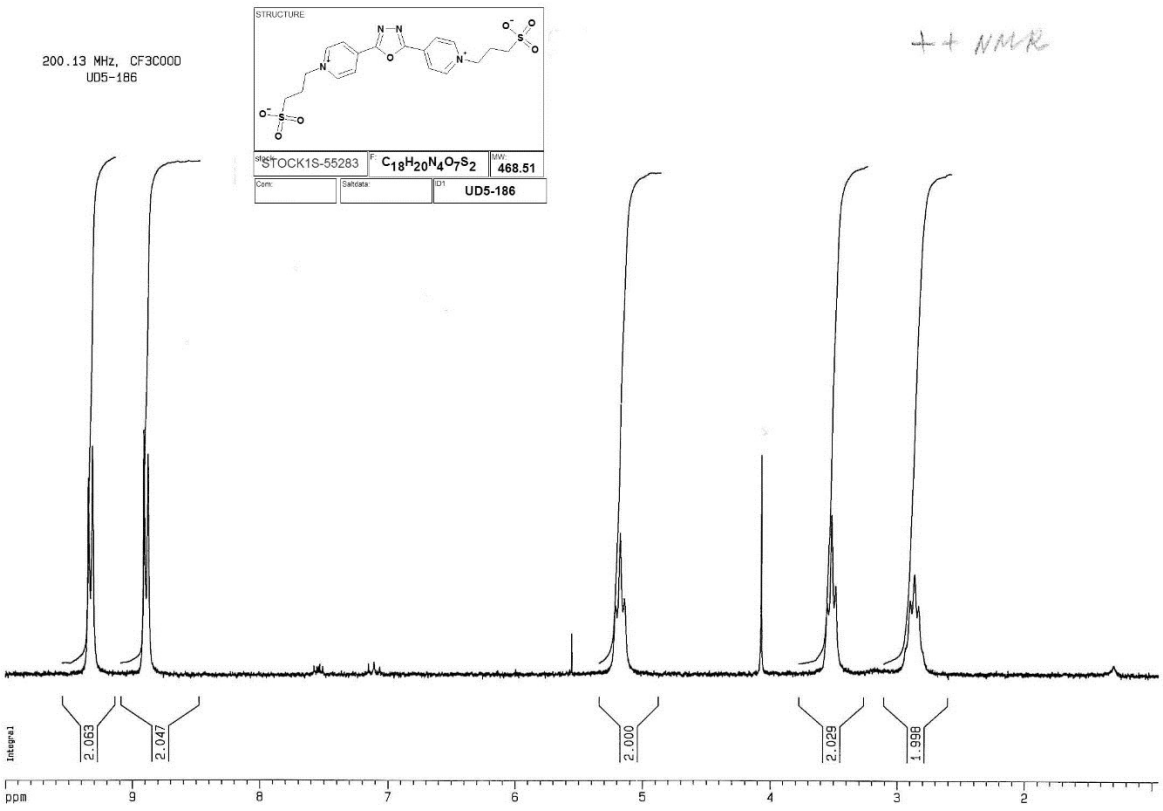

Figure S5. The purity of compound **E5,** examined by NMR 1D spectrum, is over 95 %. This data is provided by InterBioScreen (IBS, http://www.ibscreen.com).

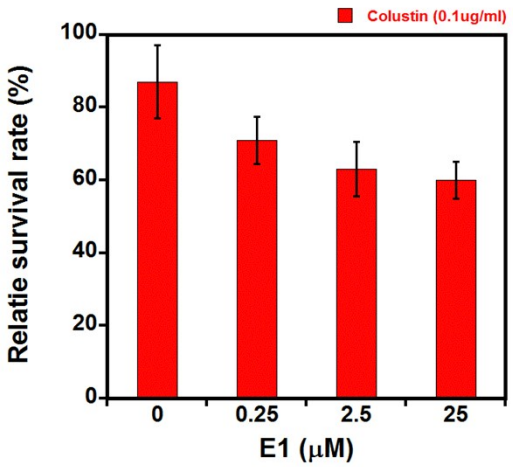

Figure S6. E1 dose-dependent effects on the inhibition of colistin against *KP*.

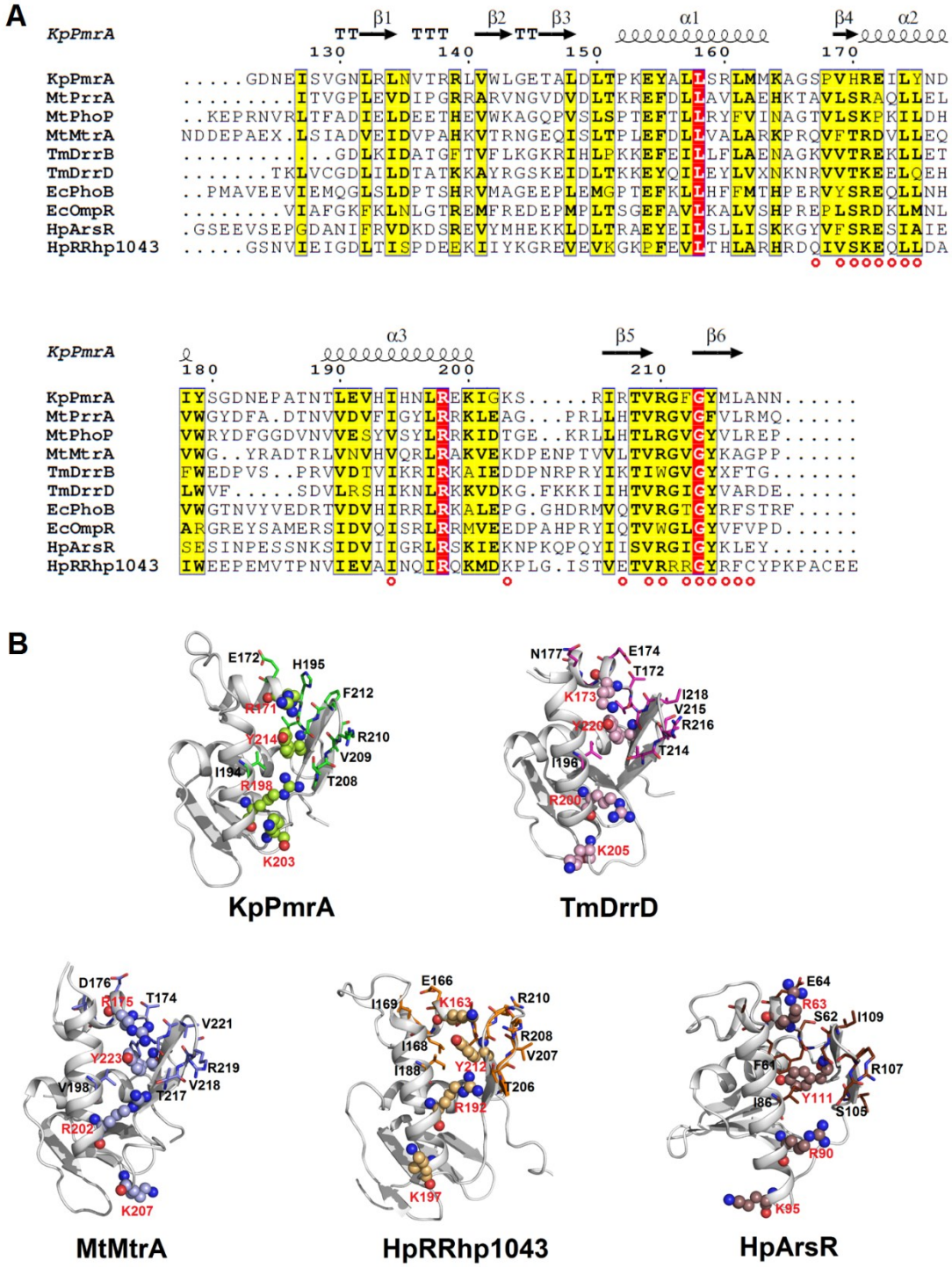

Figure S7. Structure and sequence conservations among PmrA$_C$ and RRs of other species. (**A**) The multiple sequence alignment of PmrAC and RRs of other organisms was conducted using Clustal-omega[8] and presented by ESPript - http://espript.ibcp.fr. The red circles denote the E1 binding site on PmrAC. (**B**) The E1 binding site (showed in sticks and spheres) is conserved among KpPmrA, TmDrrD, MtMtrA, HpArsR, and HpRRhp1043. (UniproKB ID: KpPmrA_A0A1S5WMZ9, MtPrrA_P9WGM1, MtPhoP_P71814, MtMtrA_P9WGM7, TmDrrB_Q9WXY0, EcPhoB_B7TYI5, EcOmpR_P0AA16, HpArsA_A0A1U9IS85, HpRRHp1043_O25684) (PDB ID: KpPmrA_4S04, TmDrrD_1KGS, MtMtrA_2GWR, HpArsR_2K4J, HpRRhp1043_2HQR)

References

1. Y. C. Lou, I. Wang, M. Rajasekaran, Y. F. Kao, M. R. Ho, S. T. Hsu, S. H. Chou, S. H. Wu and C. Chen, *Nucleic acids research*, 2014, **42**, 4080-4093.
2. Y. C. Lou, T. H. Weng, Y. C. Li, Y. F. Kao, W. F. Lin, H. L. Peng, S. H. Chou, C. D. Hsiao and C. Chen, *Nature communications*, 2015, **6**, 8838.
3. L. E. Kay, *Progress in biophysics and molecular biology*, 1995, **63**, 277-299.
4. F. Delaglio, S. Grzesiek, G. W. Vuister, G. Zhu, J. Pfeifer and A. Bax, *Journal of biomolecular NMR*, 1995, **6**, 277-293.
5. B. A. Johnson and R. A. Blevins, *Journal of biomolecular NMR*, 1994, **4**, 603-614.
6. H. K., *J. Incl. Phenom. Macrocycl. Chem.*, 2001, **39**, 193-209.
7. H. Y. Cheng, Y. F. Chen and H. L. Peng, *Journal of biomedical science*, 2010, **17**, 60.
8. F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Soding, J. D. Thompson and D. G. Higgins, *Molecular systems biology*, 2011, **7**, 539.