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Evaluation and Target Validation of Indole Derivatives as Inhibitors of the AcrAB-TolC Efflux Pump

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Indole derivatives 3-amino-6-carboxyl-indole and 3-nitro-6-amino-indole were designed and synthesized based on the TolC structure. They proved to have potent synergistic antibacterial effects on chloramphenicol, tetracycline, erythromycin, and ciprofloxacin against *Escherichia coli* YD2 and FJ307 with decreased minimal inhibitory concentrations (MICs) at 2–64 folds. To research its functional site, *Escherichia coli* BL21(DE3)−3 expressing a target-site mutated TolC was constructed by red homologous recombination and the site-directed mutagenesis technique. They did not noticeably affect antimicrobial activity against BL21(DE3)−3. All the results indicate that these compounds match our design and can be developed as efflux pump inhibitors for the AcrAB-TolC efflux pump.

Key words: antibacterial activity; efflux pump inhibitor; indole derivatives; TolC

In China, colibacillosis in pigs and chicken is still prevalent, and multi-drug resistance in *Escherichia coli* is also severe. The AcrAB-TloC efflux pump as the major drug efflux system of *E. coli* plays an important role in this. Hence efflux pump inhibitors (EPIs), especially those active against multi-drug resistance pumps, are of great interest. Small molecules with conjugated aromatic rings, such as phenyls, naphthalenes, and indoles are of high potential in EPI research.[1,2] Indolenine and its derivatives have been generally developed as protein inhibitors of key targets antivirus, antitumor, and antibacterial efforts.[3,4] Especially, 2-aryl-5-nitro-1*H*-indoles have been reported as NorA EPI in *Staphylococcus aureus*.[5] The crystal structures of TolC have now been solved. The allosteric mechanism of TolC from a closed state to an open state has been analyzed. The tunnel helices of TolC are constrained at the entrance by a circular network of intra- and inter-monomer hydrogen bonds and salt bridges. Aromatic residues, particularly tyrosine and phenylalanine, cluster in a ring around the base of the TolC β-barrel, playing an important role in the closed states of TolC due to hydrogen bonds.[6] If the aperture dilatation of TolC is inhibited by strengthening the hydrogen bonding between the essential sites, the TolC channel is forced to close, and antibiotic susceptibility is increased.

Functional genomics techniques are valuable tools to evaluate the pool of targets, and can exploited for target validation and the determination of molecule action. Knockout analyses and mutation studies to create mutant strains with precise genetic alteration aid in the selection and validation of novel potential targets.[7] The application of targeted gene knockout in identifying and validating new drug targets and vaccine candidates in *M. tuberculosis* is practiced.[8] Hence, to further validate the target of indole derivatives in our study, the strain expressing mutant TolC had to be constructed by knockout and site-directed mutation.

The aim of the present study was to design and synthesize indole derivatives in order to restore the antibiotic susceptibility of resistant *E. coli* strains and to identify the functional target of indole derivatives to improve the validity of molecular designing.

Materials and Methods

Bacterial strains and antibiotics. *E. coli* strain Fj307 (chosen from 180 clinical multi-drug resistant isolates and found to be a highly drug efflux strain) served as positive control. *E. coli* strain TW1b with the *tolC* gene partly knocked out (donated by the Coli Genetic Stock Center at Yale University) was used as negative control. ATCC25922 (conserved by our laboratory), was used as quality control. The antibiotics chloramphenicol (CHL), erythromycin (ERY), ciprofloxacin (CIP), gentamicin (GM), ampicillin (AMP), and tetracycline (TET) were purchased from Sigma-Aldrich (Shanghai, China).

Design and synthesis of indole derivatives. There are four major links that establish a circular network constraining six helical pairs of TolC monomers to form a closed state. Hydrogen bonds Asp^{153}-Tyr^{362} and Gln^{136}-Glu^{359} connect the inner coiled coils to the outer coils of the same monomer, and a salt bridge between Asp^{153}-Arg^{367} and hydrogen bonds Arg^{367}-Thr^{152} connects each inner coil to the outer coil of the monomer. When the AcrAB-TolC efflux system is active, the inner coils move outward to enlarge the entrance diameter of TolC, and these links are broken.[9] Indole derivatives were designed to contain two chemical groups that can bind to TolC at the O@Asp^{153} and H@Tyr^{362} sites with strong hydrogen bonds and are hard to break, so, with its strong connection, TolC is hard to open naturally and the function of the AcrAB-TolC system is inhibited.

The hydrogen bond between tyrosine and phenylalanine around the base of the TolC β-barrel was screened using software the Swiss-Pdb Viewer (v3.7). The indole derivatives were designed according to the TolC structure with the GAUSSIAN98 package of programs.[10] All the derivatives were synthesized by the College of Chemistry and Environmental Protection Engineering, Southwest University for Nationalities.[11]

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Abbreviations: CFUs, colony-forming units; EPIs, efflux pump inhibitors; MICs, minimal inhibitory concentrations; CLSI, clinical and laboratory standards institute; CHL, chloramphenicol; ERY, erythromycin; CIP, ciprofloxacin; GM, gentamicin; AMP, ampicillin; TET, tetracycline; SEM, standard error of mean

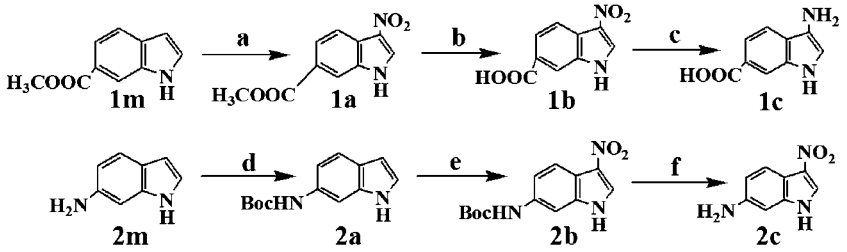

Fig. 1. Chemical Structures and Synthetic Protocol of Indole Derivatives.

Reagents and conditions: (a) HNO_3, $(CH_3CO)_2O$, −10 °C to 0 °C; (b) 40% NaOH, heat reflex, pH 1.0–2.0; (c) $SnCl_2 \cdot 2H_2O$, ethyl acetate, N_2, 60 °C reflex; (d) $(Boc)_2O$, NaOH, 1,4-dioxane, 0 °C; (e) $AgNO_3$, benzoyl chloride, acetonitrile, −10 °C to 25 °C; (f) 68% HNO_3, CH_2Cl_2, 0 °C to 25 °C.

3-Amino-6-carboxyl-indole (1c) and 3-nitro-6-amino-indole (2c) were synthesized from 6-methyl formate-indole (1m) and 6-amino-indole (2m). The molecular formula and synthetic route are shown in Fig. 1. The structures were confirmed by ^{1}H NMR, MS, and IR spectra and elemental analysis.

(a): 6-methyl formate-indole (1m, 1.75 g, 10 mM) was dissolved with 50 ml of $(CH_3CO)_2O$ (acetic anhydride) in an erlenmeyer flask; peroxyacetyl nitrate was dropped into the flask constantly with stirring at −10 °C, then a 0 °C reaction for 6 h; product was extracted with ethyl acetate, and washed with saturated NaCl solution, and 1a was gained after chromatography (red solid, 90% yield). (b): 1a (2.19 g, 10 mM) and NaOH solution (40%) was added to the flask (containing reflex condenser), heat reaction for 6 h; adjustment of solution into PH 1.0–2.0 by HCl, and subsidence 1b was collected (yellow solid, 92% yield). (c): Ethyl acetate (20 ml), 1b (2.05 g, 10 mM), $SnCl_2 \cdot 2H_2O$ (1.128 g, 5 mM) was added to the flask (containing reflex condenser), 60 °C reaction for 4 h in the presence of protective agent N_2; then the reaction solution was mixed with 100 ml of saturated $NaHCO_3$ solution with constant stirring, yellow substance was extracted with ethyl acetate, and dried with Na_2SO_4, 1c was gained after purification by column chromatography. (orange solid, 95% yield, m.p. 118–121). (d): 6-amino-indole (2m, 1.36 g, 0.01 M), 1,4-dioxane (65 ml) were mixed in the flask at 0 °C; then NaOH (1.0 ml, 2 mol/l) and $(Boc)_2O$ (2.4 g, 11 mM) was dropped into the flask separately, reaction for 2–3 h; Product 2a was extracted from solution with ethyl acetate, and dried with Na_2SO_4. (e): Solution I (acetonitrile, 5 ml; benzoyl chloride, 1.6 ml), and solution II (acetonitrile, 10 ml; $AgNO_3$, 2.55 g, 15 mM) were prepared; then solution I was poured into solution II very slowly at 0 °C to form solution III (milk-like solution). 2a (1.2 g, 4 mM) and acetonitrile (20 ml) were added to the flask, reaction at −10 °C for 15 min, and then solution III (6.18 ml) was added and this was mechanically stirred for 1 h at −10 °C, then continued stirred at 25 °C for another 30 min, and 50 ml 0 °C H_2O was added in at last. Product 2b was extracted from the solution with ethyl acetate, dried with Na_2SO_4. (f): CH_2Cl_2 (25 ml) and 2b (3 mM, 0.8 g) were added to the flask, and HNO_3 (0.65 ml, 68%) was added slowly at 0 °C, and the solution was set at 0 °C for 1 h and again at 25 °C for 3 h to finish the reaction. Sediment 2c was collected and purified by column chromatography (red solid, 75% yield, m.p. 119–122).

3-Amino-6-carboxyl-indolenine (1c), IR (KBr) ν: 3445 (NH2), 3422 (NH), 2923 (Ar–H), 3651 (–COOH), 1710 (C=O), 1620 (C=C), 1468 (C–N). ^{1}H NMR (DMSO) δ: 12.98 (br, 1H, –COOH), 11.61 (s, 1H, NH), 8.85 (s, 1H), 8.16–8.18 (m, 2H, ArH), 7.93–7.96 (m, 1H, ArH), 4.65 (br, 2H, NH2). ESI-MS (70 eV) m/z (%): 177.19 ($M^+ + 1$, 65). 3-Nitro-6-amino-indole (2c), IR (KBr) ν: 3403 (NH), 3132 (Ar–H), 1620 (C=C), 1468 (C–N), 1383 (NO_2). ^{1}H NMR (DMSO) δ: 12.82 (m, 1H, NH), 8.56 (s, 1H), 8.05–8.27 (m, 3H, ArH), 4.60 (s, 2H, –NH2). ESI-MS (70 eV) m/z (%): 178.01 ($M^+ + 1$, 100).

In the analysis, the interaction energy between the indole derivatives and TolC reduced −16.51 and −40.51 kcal/mol respectively, as compared with the natural binding energy. In the binding state, the TolC diameter was approximately 17 Å, smaller than the diameter of the opening state, 54 Å.

Bacterial inducing. Construction of a highly efflux strain by artificial induction: *E. coli* ATCC25922 was cultured in MH broth. The bacterial concentration was manually adjusted by testing the optical density ($[OD_{600}] \approx 0.5$) and by centrifugation, then approximately 10^9 colony-forming units (CFU) bacteria was inoculated into 50 ml MH broth containing CHL every time. The induced concentration of CHL started from 1/4 fold to 1/2 fold, 1 fold, 2 fold... of the MIC. We stabilized the inducing bacteria by inoculating it in the same drug concentration repeatedly. The MIC of the resistant *E. coli* against CHL, CIP, GM, TET, and AMP was tested after inducing. The final induced strain was designated *E. coli* YD02, and it served as a positive control.

Evaluation of reversal activities by MIC test. Minimal inhibitory concentrations (MICs) of CHL, ERY, CIP, and TET co-adminstrated with indole derivatives were conducted by the microdilution broth method following the CLSI (Clinical and Laboratory Standards Institute). Four different *E. coli* strains (Fj307, YD02, TW1b, and ATCC25922) were cultured in MH broth, and inoculated in tubes at a concentration of 10^5 CFU/ml. The concentration of indole derivatives was diluted to 0.5 mM in every tube. All tubes were cultured for 16 h at 37 °C.

Construction of mutant variant BL21(ED3)−2. Primers shown in Table 1 were synthesized by Takara Biotechnology (Dalian, China). In order to construct an *E. coli* strain that expresses mutant TolC, we need to have its nature TolC expression to be blocked and set it be prepared to recombine the new mutant *tolC* gene. So, the *tolC* gene in the *E. coli* BL21(DE3) was partly knocked out by the red homogenous recombinant technique.[12] First, pKD46 (presented by the *Coli* Genetic Stock Center at Yale University) was transferred into BL21(DE3), and cultured in 50 ml of LB (Luria-Bertani) medium at 30 °C for 12 h. Four ml of bacteria culture was transferred into 200 ml fresh LB medium, and cultured at 30 °C until its $[OD_{600}] \approx 0.25$, and then 5 mmol/l L-arabinose was added and this was incubated for another 1 h to induce pKD46 expression (ensuring that $[OD_{600}] < 0.6$). The bacteria culture was cooled on ice for 10 min, then washed 3 times with 10% glycerin with centrifugation at 4 °C, 4,000 rpm for 10 min, and the competent BL21(DE3)/pKD46 was concentrated and prepared for recombination.

The *tet*^{r} gene as a resistance selection gene was amplified from pBR322 with primers FPQS and RPQS, which contained homologous recombination arms. Then approximately 500 ng PCR product was electro transformed into the competent BL21(DE3)/pKD46 with Gene Pulser (Xcell, Bio-Rad, USA) at 200 Ω, 25 μF, 1.8 kV. The recombined strain was then cultured on an LB plate with 34 μg/ml of tetracycline at 37 °C. BL21(ED3)/pKD46/tolC(M)tet^r was selected, and identified by PCR with primers FPJD and RPJD, and was designated BL21(DE3)−2.

Preparation of the outer membrane fraction of *E. coli* and detection of the TolC band on the SDS–PAGE gel by immunoblotting were performed as previously described.[13] Western blotting was conducted following the operation manual of Bio-Rad.

Creation of TolC mutant variant BL21(DE3)−3. To create site mutations in *tolC* gene, the wild *tolC* gene was amplified from BL21(DE3) with primers FPEC and RPEC, and then inserted into pUC18. Mutations were introduced into pUC18-tolC at Asp^{153} and Tyr^{362} of *tolC* using the site-directed mutagenesis kit from SBS Genetech (Beijing, China) with the mutant primers (Table 1), and pUC18-tolC^M was confirmed by nucleotide sequencing analysis and blasted with that of wild *E. coli* at GenBank. *tolC*^{M} was inserted into

Table 1. Primers Used in This Study

Primers	Function	Sequence
FPQS	Amplify *tet* from	5-ACAGTTTGATCGCGCTAAATACTGCTTCACCACAAGGAATGCAAGTTTGACAGCTTATCAT-
RPQS	pBR322 for	CGA-3
	recombination	5-CATTCAACACGTTGAAATAAGCGGTCGCGGTGTTGAGGATCAAGGTTTTGGTGAATCCGTT-
		AGCGAGGT-3
FPJD	Identifying mutant	5-AACGGGCAGGTTGTCTGGCTTAA-3
RPJD	isolate of *tolC*	5-CCGTTAAATCCAGAGTCGGTAAGTGA-3
FPEC	Amplifying wild	5-TCTTATTCATATGAAGAAATTGCTCCCCATTCTTATCG-3 (*Nde* I)
RPEC	*tolC* and inserting into pET30b	5-TCAATAAGAATTCAGCCCCGTCGTCGTCATCAG-3 (*EcoR* I)
$FPD^{153}A$	Introducing site-	5-GCCTGGTAGCGATCACGGCCGTGCAGAACGCCCG-3
$RPD^{153}A$	directed mutagenesis	5-CGGGCGTTCTGCACGGCCGTGATCGCTACCAGGC-3
$FPY^{362}F$	in *tolC*	5-GCGATGGAAGCGGGCTTCTCGGTCGGTACCCGTACCATTGTT-3
$RPY^{362}F$		5-AACAATGGTACGGGTACCGACCGAGAAGCCCGCTTCCATCGC-3

The sequences of the homologous recombination arms and the mutation site are underlined.

Table 2. MIC Test Results for ATCC25922 and *E. coli* YD02 before and after Induction by CHL

Drug		CHL	TET	CIP	GM	AMP
MIC	ATCC25922	4	2	0.5	2	4
(μg/ml)	*E. coli* YD02	128	64	128	128	128

pET30b with *Nde* I and *EcoR* I (Takara), and then transformed into BL21(DE3)−2. BL21(DE3)−2/pET30b-$tolC^{M}$ was selected with Kan^{r} and designated BL21(DE3)−3. Expression of mutant TolC in BL21(DE3)−3 was induced with IPTG and confirmed by electrophoresis and western blotting.

The *tolC* gene in BL21(DE3), BL21(DE3)−2, and BL21(DE3)−3 was examined by PCR with primers FPJD and RPJD.

Target validation. In order to determine whether these indole moleculars are functional with the target site according to our design, MICs of BL21(DE3), BL21(DE3)−2, and BL21(DE3)−3 against four different antibiotics (CHL, ERY, CIP, and TET) were tested with and without indole derivatives. The method was almost the same for CLSI, but the drug concentration gradient was adjusted into 10 lower degrees (0, 0.125, 0.25, 0.5, 1, 1.5, 2, 3, 4, 6 μg/ml). The statistical data were analyzed by SPSS12.

Results and Discussion

Bacterial inducing

Multi-drug resistant strain *E. coli* YD02 was produced after induction from ATCC25922. The results are shown in Table 2.

Evaluation of reversal activities

Indole derivatives restored the activity of CHL, TET, ERY, and CIP to *E. coli* (Table 3).

The reverse ability of 1c, 2c as measured was at least 2-fold MIC decrease on TET and ERY, but at least 8-fold on CHL and CIP to resistant *E. coli*. 1m and 2m also showed a little reverse ability on ATCC25922, but had no effect on positive strains FJ307 and YD02. TW1b was sensitive to all the antibiotics, since *tolC* was partly knocked out.[14] These results are well accordant to the prediction by molecular design.

Construction of TolC mutant variants BL21(ED3)−2 and BL21(DE3)−3

In BL21(DE3), Natural *tolC* are 2,096 bp. Primers FPJD and RPJD for identification were designed to detect the tolC gene sequence between 72–1,121 bp. A 1,049 bp amplicon was detected in normal BL21(DE3). In BL21(DE3)−2, the plasmid pKD46 contained three genes, γ, β, and *exo*, whose products were Gam, Bet, and Exo. These three proteins were recombinant protein of the λ bacteriophage and all of them are vital to the red system. The recombination arms on tet^{r} were designed to be homologous to *tolC*. Hence, pKD46 can express the red system under the control of its regulated promoter, and can recombine tet^{r} into the *tolC*. The tet^{r} gene (1,305 bp) was recombined into the *tolC* between 302 bp and 723 bp, so *tolC* gene was partly knocked out, and a 1,981 bp amplicon was detected. All the amplicons were verified by PCR from bacterial chromosomes, which was consistent with the expected size (Fig. 2).

No expression of TolC in BL21(ED3)−2 was detected by SDS–PAGE or western blot (Fig. 2). The results showed that the function of *tolC* in BL21(DE3)−2 was inactive, but in BL21(DE3)−3, the site mutated

Table 3. Evaluation of the Ability of Indole Derivatives to Increase Antimicrobial Sensitivity

Compounds 0.5 mM	Strains	MIC(μg/ml)			
		CHL	TET	ERY	CIP
None	ATCC25922	4	2	0.25	0.5
None	YD02	128	64	64	128
None	FJ307	256	64	64	128
None	TW1b[a]	—	—	—	—
1m	ATCC25922	2	2	0.25	0.25
1m	YD02	128	64	64	128
1m	FJ307	256	64	64	128
1m	TW1b[a]	—	—	—	—
2m	ATCC25922	2	1	0.25	0.5
2m	YD02	128	64	64	128
2m	FJ307	256	64	64	128
2m	TW1b[a]	—	—	—	—
1c	ATCC25922	1	1	0.25	0.25
1c	YD02	4	32	32	4
1c	FJ307	8	16	32	16
1c	TW1b[a]	—	—	—	—
2c	ATCC25922	1	1	0.25	0.25
2c	YD02	4	16	16	8
2c	FJ307	4	16	4	16
2c	TW1b[a]	—	—	—	—

1m, 6-methyl formate-indole; 2m, 6-amino-indole; 1c, 3-amino-6-carboxyl-indole; 2c, 3-nitro-6-amino-indole; YD02, Efflux *E. coli* by artificial induction; FJ307, Efflux *E. coli* from clinical isolates; TW1b[a], Mutant *E. coli*, *tolC* gene was partly knocked out; —, Bacteria were not grown at every concentration of antibiotics.

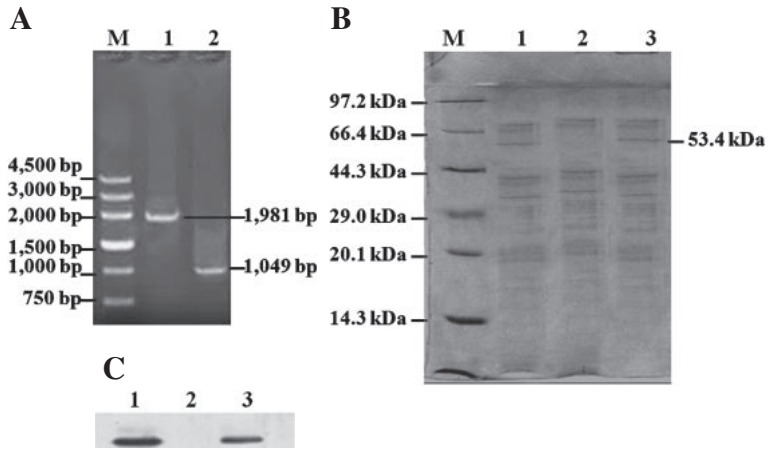

Fig. 2. Identification of TolC in Various *E. coli* Strains by PCR, SDS–PAGE, and Western Blotting.

A, PCR test of the *tolC* gene. M, DNA markers III; 1, BL21(DE3)−2; 2, BL21(DE3). B, SDS–PAGE of membrane protein expression. M, Low molecular weight protein marker; 1, BL21(DE3)−3; 2, BL21(DE3)−2; 3, BL21(DE3). Bacteria were grown to exponential phase in LB medium. Twenty ml bacteria were washed by centrifugation, and treated with lysozyme (0.2 μg/ml) in sucrose solution (12%) for 30 min. Then spheroplasts were broken down gently by sonication on ice and centrifugation at 10,000 *g* for 20 min at 4 °C to remove cell debris. The supernatants obtained were subjected to ultracentrifugation at 100,000 *g* for 1 h at 4 °C, and then the membrane fraction was suspended in Tris-Mg buffer (10 mM, pH 7.8) containing sarcosyl (2%), and incubated at 20 °C for 30 min. Then the tubes were centrifuged at 70,000 *g* for 1 h at 4 °C. Fraction sediment was collected with 200 μl ddH_2O and used for SDS–PAGE. C, Western blotting to check TolC expression. 1, BL21(DE3)−3; 2, BL21(DE3)−2; 3, BL21(DE3). Dilution of anti-TolC antiserum (presented by P. C. Tai, Georgia State University) was used to detect TolC. Bound antibody alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Boster Biological Technology, WuHan, China) was detected using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

$TolC^M$ can be expressed. The sequencing result demonstrated that Asp^{153} and Tyr^{362} were mutated into Ala and Phe. BL21(DE3)−2 and BL(DE3)-3 were constructed successfully.

Target validation

Statistical results: Means with SEM (standard error of mean) of MICs in the testing groups are shown in Fig. 3. In BL21(DE3) control groups, indole derivatives 1c and 2c could significantly potentiate the antibiotic activity of CHL, CIP, TET, and ERY, especially in CHL: BL21(DE3) 4 μg/ml, BL21(DE3)+1c 1 μg/ml, BL21(DE3)+2c 0.83 ± 0.17 μg/ml, and CIP: BL21(DE3) 0.83 ± 0.17 μg/ml, BL21(DE3)+1c 0.125 μg/ml, BL21(DE3)+2c 0.125 μg/ml ($p < 0.01$) but in TolC mutant BL21(DE3)−3 groups, 1c and 2c could not significantly restore the activity of all the antibiotics, CHL: BL21(DE3) 4 μg/ml, BL21(DE3)+1c 3.83 ± 0.17 μg/ml, BL21(DE3)+2c 3.33 ± 0.17 μg/ml, CIP: BL21(DE3) 0.83 ± 0.17 μg/ml, BL21(DE3)+1c 0.83 ± 0.17 μg/ml, BL21(DE3)+2c 0.67 ± 0.17 μg/ml ($p > 0.05$). Similarly to TW1b, BL21(DE3)−2 could not be cultured in MH broth containing any antibiotics, while *tolC* was not expressed.[9)]

In BL21(DE3)−3, the site-mutated $tolC^M$ gene was inserted into pET30b with the natural signal peptide of TolC, the signal peptide was used as a conductor of TolC located at the natural membrane position. Compared to the MIC results for BL21(DE3)−3 to BL21(DE3) without indole derivatives (Fig. 3), the data were almost same, indicating that the natural function of mutant strain BL21(DE3)−3 was not affected by experimental manipulation and that the signal peptide itself can lead TolC to the right location in BL21(DE3)−3. It also reflects that the site mutation on TolC itself can not reverse the antibiotic sensitivity of BL21(DE3)−3. This conforms to a study by Koronakis (2004), in which the efflux function of TolC was not

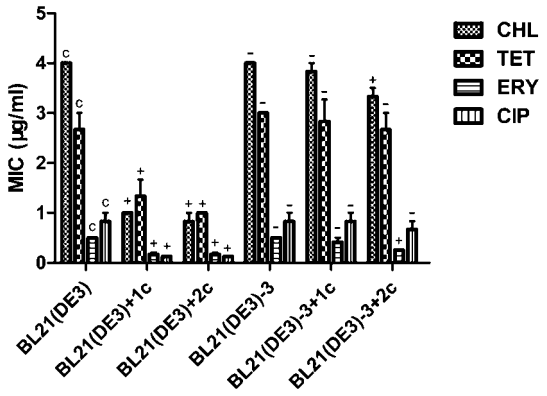

Fig. 3. MIC Test of Mutant *E. coli* against Different Antibiotics with and without Indole Derivatives.

C, Controls of each antibiotic group. +, MICs showed significant difference from control group statistically. −, MICs showed no difference from control group statistically. Error bars, SEM (standard error of mean). BL21(DE3), BL21(DE3)−2, and BL21(DE3)−3 were cultured in MH broth and inoculated in tubes at a concentration of 10^5 CFU/ml. Concentrations of 1c and 2c were diluted to 0.5 mM in every tube. Antibiotics and indole derivatives were added to tubes separately, and three repeating groups were tested at every concentration degree. All the tubes were cultured for 16 h at 37 °C.

been affected when Asp^{153} and Tyr^{362} were mutated into Ala and Phe.[6)] Compared to the MIC results for BL21(DE3)−3 and BL21(DE3) plus 1c and 2c, the major difference was the change in the target site in TolC according to our design. Therefore, due to that indole derivatives interact with the Asp^{153} and Tyr^{362} on the TolC and inhibit its aperture expanding to restore the antimicrobial sensitivity of antibiotics.

Conclusions

3-Amino-6-carboxyl-indolenin and 3-nitro-6-amino-indole can be used as efflux pump inhibitors of TolC and can be co-administrated with antibiotics to potentiate

antibiotic susceptibility. TolC can be developed as a suitable target of efflux pump inhibitors. The conception of designing EPIs targeting TolC is worth further research.

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