Supporting information

Metformin restores tetracyclines susceptibility against multi-drug resistant bacteria

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Figures

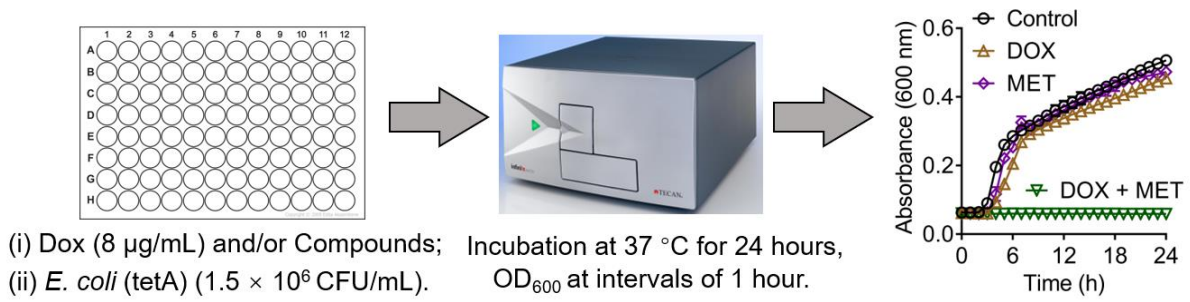

Figure S1 Scheme of screening of novel tetracyclines adjuvants from FDA-approved compounds. 158 FDA-approved compounds from Prestwick Chemical Library were screened against *E. coli* B2 (*tet*(A)) in combination with 8 μg/mL doxycycline (one quarter of the minimum inhibitory concentration). Briefly, doxycycline and/or compounds were diluted in Mueller-Hinton Broth (MHB) and mixed with an equal volume of bacterial suspensions containing approximately 1.5×10^6 colony-forming units (CFUs)/mL in a clear UV-sterilized 96-well microliter plate. Then, the real-time growth curves of *E. coli* B2 in the absence or presence of drugs were monitored during 24 hours. Compounds that significant inhibits bacterial growth (inhibition rate ≥ 50%) in combination treatment, whereas no direct antibacterial activity in monotreatment were defined as the potential tetracyclines adjuvants candidates.

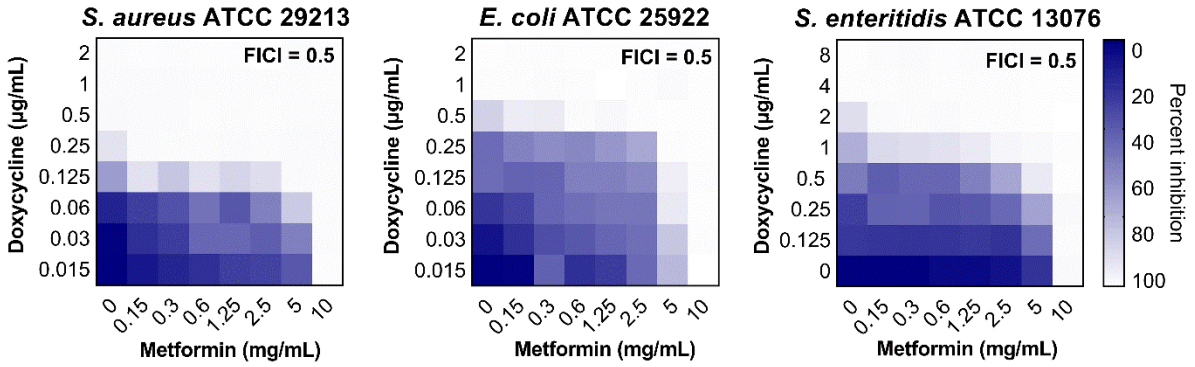

Figure S2 Weak synergistic activity between metformin and doxycycline against sensitive bacteria.

Dark blue regions represent higher cell density and lower inhibition rate. Data represent the mean OD (600 nm) of two biological replicates.

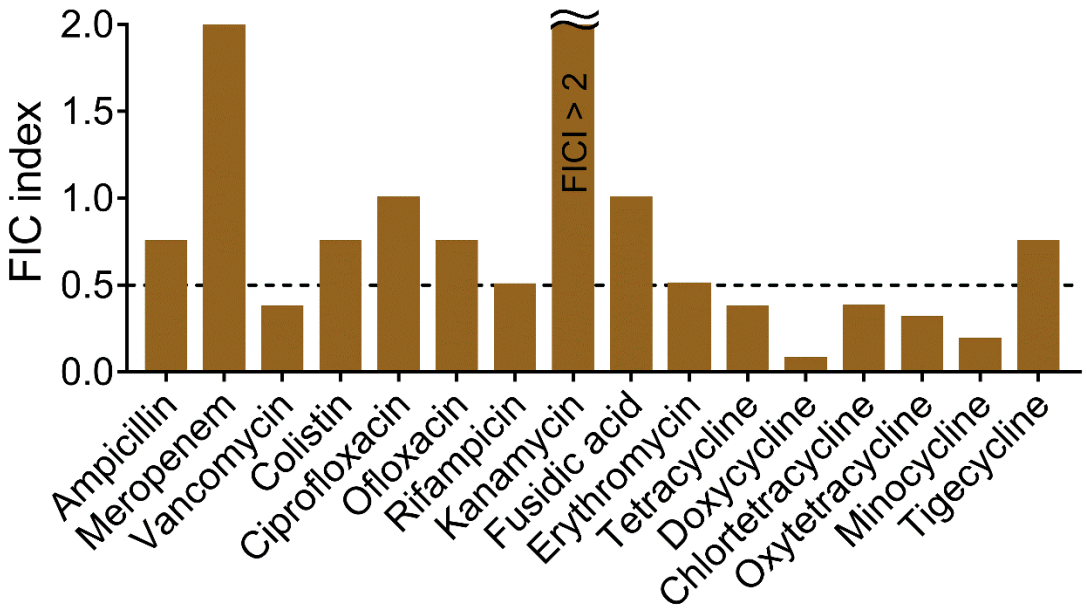

Figure S3 FIC indices of metformin and different classes of antibiotics against *E. coli* B2.

FIC indices were calculated based on chequerboard microdilution assays at 0.25 or 0.125 × MIC (only for kanamycin) of metformin. Synergy is defined as an FIC index of ≤ 0.5.

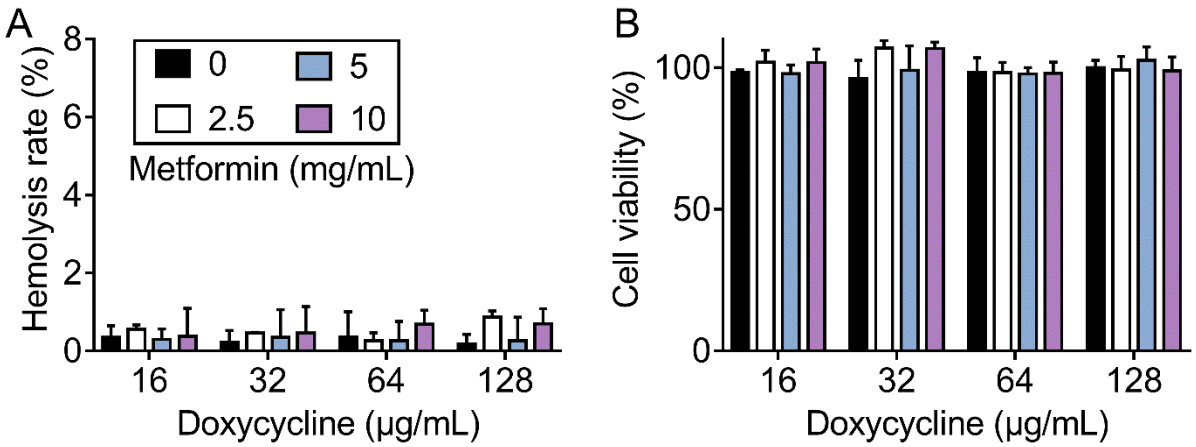

Figure S4 Effect of metformin on the safety of doxycycline.
(A) Hemolytic activity of doxycycline to the RBCs in the absence or presence of metformin. **(B)** Addition of metformin exerts negligible effect on the cytotoxicity of doxycycline in Chinese hamster ovary (CHO) cells.

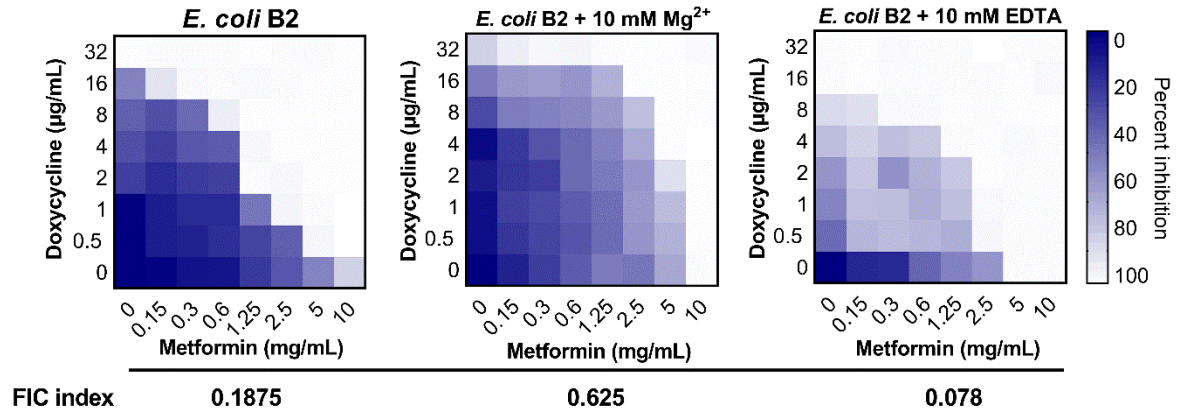

Figure S5 Effect of exogenous Mg^{2+} or EDTA on the synergistic activity between doxycycline and metformin.

The addition of Mg^{2+} (10 mM) abolished the potentiation activity of metformin to doxycycline, whereas EDTA enhanced their synergy effect. Dark blue regions represent higher cell density and lower inhibition rate. Data represent the mean OD (600 nm) of two biological replicates.

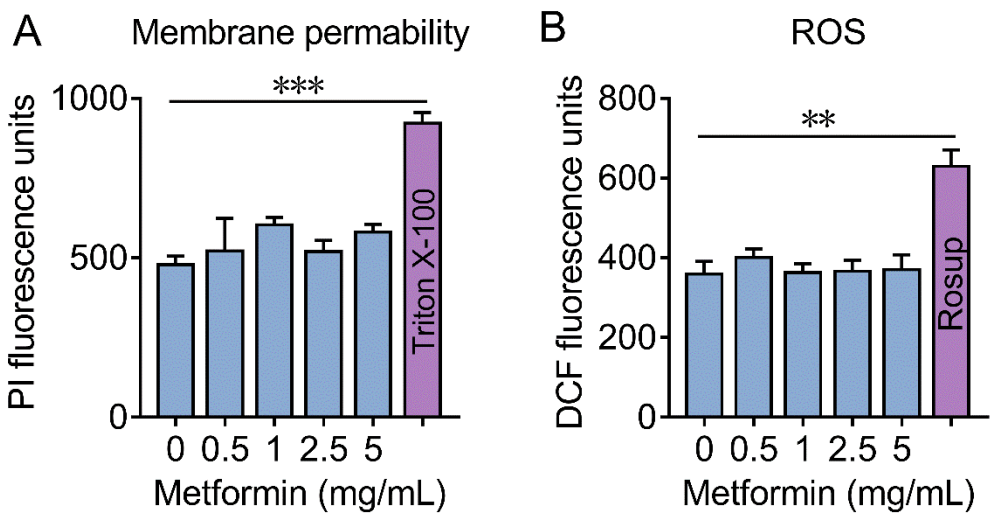

Figure S6 Metformin has no effect on the membrane permeability and ROS production of *E. coli* B2.

(A) No significant effect on the whole membrane permability of *E. coli* B2 under the stimulation of varying metformin for 1 h, probed by propidium iodide (10 nM).

(B) None of ROS production by metformin were determined. probed with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Rosup was used as the positive control of ROS production.

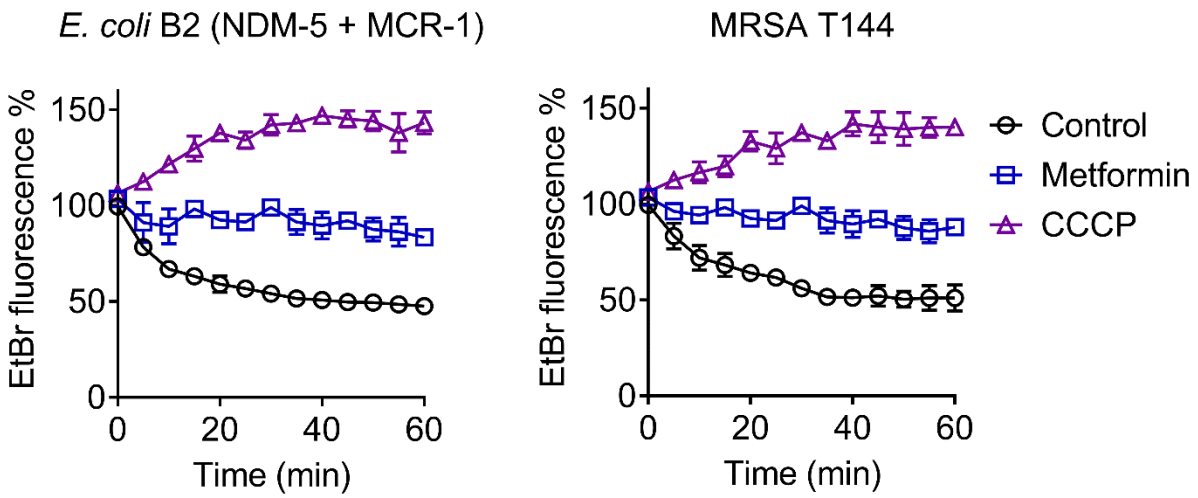

Figure S7 Inhibition of efflux pump by metformin or CCCP in *E. coli* B2 and MRSA T144.

Cells were co-incubated with 5 μM EtBr and sub-MIC of metformin (5 mg/mL), or known efflux pump inhibitor CCCP (100 μM) at 37 °C to an OD_{600} of 0.5. Then, EtBr efflux from the cells was monitored with the excitation wavelength at 530 nm and emission wavelength at 600 nm during 60 minutes.

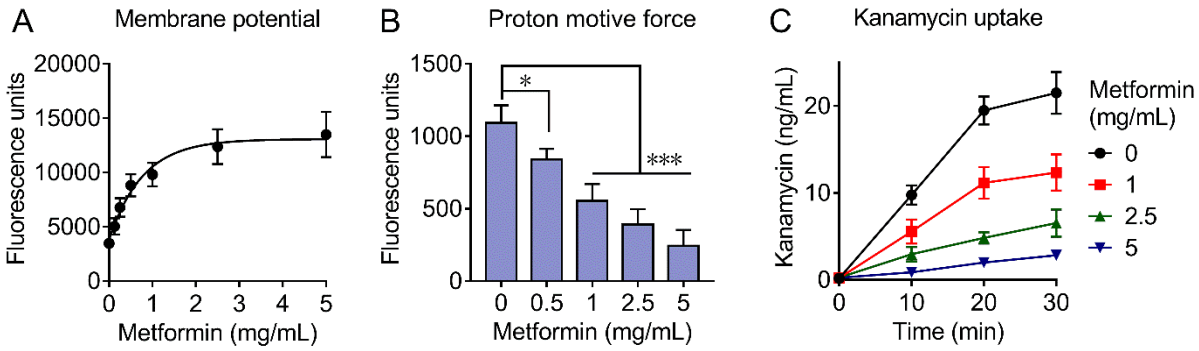

Figure S8 Metformin collapses proton motive force in *S. aureus* ATCC 29213.
(A) Metformin dissipates membrane potential of *S. aureus*. Membrane potential was determined by measuring the fluorescence of $DiSC_3(5)$ after 60 min exposure to increasing concentrations of metformin.
(B and C) Metformin decreased proton motive force (B) and thereby reduced the kanamycin uptake (C) in *S. aureus* in a dose-dependent manner. Proton motive force of *S. aureus* after exposing varying concentration of metformin were determined by monitoring BCECF fluorescence intensity. Kanamycin uptake in *S. aureus* were determined by LC-MS/MS. All data was presented as means ± SD and one-way ANOVA were used to calculate *P*-values (*$P < 0.05$, ***$P < 0.001$).

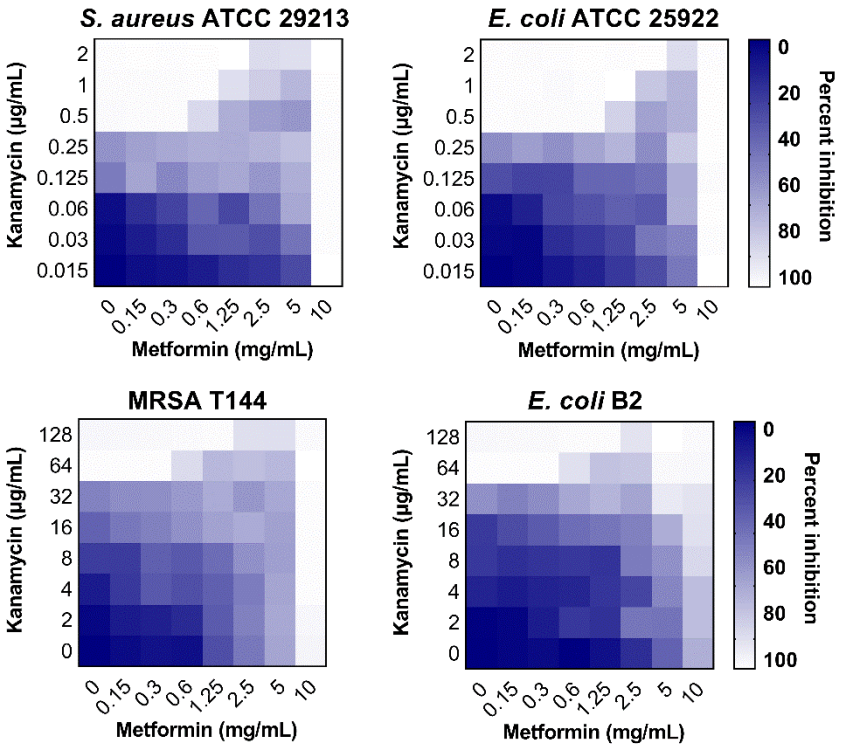

Figure S9 Combination of metformin and kanamycin leads to antagonistic interactions against sensitive bacteria (*S. aureus* ATCC 29213 and *E. coli* ATCC 25922) and resistant bacteria (MRSA T144 and *E. coli* B2).

Data were obtained by chequerboard broth microdilution assays and shown as a heat plot. Dark blue regions represent higher cell density and lower inhibition rate of combination treatment.

Tables

Table S1 Bacterial strains used in this study.

Organism and genotype	Source/Reference
Gram-positive bacteria	
Enterococcus faecalis A4 (*tet*(A), *van*A, Van^{R})	[1]
Staphylococcus aureus ATCC 29213	ATCC
S. aureus 215 (LZD^{R}, *cfr*, *tet*(A))	[2]
MRSA T144 (*mecA*, *tet*(A))	[2]
Gram-negative bacteria	
Escherichia coli ATCC 25922	ATCC
E. coli B2 (*tet*(A), *bla*$_{NDM-5}$, *bla*$_{TEM-1B}$, *bla*$_{OXA-10}$, *bla*$_{CTX-M-14}$, *mcr-1*, *aadA*, *aph*(4), *oqxAB*, *mdfA*, *fosA3*)	[3]
Salmonella enteritidis ATCC 13076	ATCC
S. enteritidis H8 (*tet*(A), *bla*$_{NDM-1}$)	[3]

ATCC, American Type Culture Collection; Van^{R}, vancomycin resistance; LZD^{R}, linezolid resistance.

References

[1] Y. Liu, Y. Jia, K. Yang, R. Li, X. Xiao, Z. Wang, *ACS Infectious Diseases* **2019**, DOI: 10.1021/acsinfecdis.9b00164.

[2] Y. Liu, S. Ding, R. Dietrich, E. Märtlbauer, K. Zhu, *Angew. Chem. Int. Ed.* **2017**, *56*, 1486.

[3] Y. Liu, K. Yang, Y. Jia, Z. Wang, *ACS Infectious Diseases* **2019**, *5*, 2061.

Table S2 Interaction between 158 FDA-approved compounds with doxycycline against *E. coli* B2.

Compounds	Inhibition rate (%)[#]	Compounds	Inhibition rate (%)[#]	Compounds	Inhibition rate (%)[#]
Acetylcholine	33.58 ± 5.48	Disulfiram	69.81 ± 7.44	Oxiracetam	12.56 ± 0.13
Adenine	38.21 ± 10.96	Ebselen	26.31 ± 12.42	Oxolinic acid	24.22 ± 7.95
Amikacin	37.69 ± 7.51	EGCG	78.36 ± 1.03	Oxyfedrine	50.16 ± 6.55
Amoxicillin	-9.35 ± 0.72	Enoxacin	6.72 ± 11.88	Paraquat	35.68 ± 7.2
Anethole	38.08 ± 1.56	Enrofloxacin	20.16 ± 14.09	Paromomycin	16.09 ± 8.23
Anisindione	21.08 ± 0.53	Erythromycin	37.79 ± 13.8	Pefloxacine	1.82 ± 10.76
Apramycin	6.87 ± 7.97	Estradiol	27.92 ± 5.86	Penicillin G	-4.9 ± 14.38
Artemisinin	10.54 ± 7.72	Estriol	29.3 ± 3.97	Phenformin	38.85 ± 12.13
Artesunate	18.49 ± 13.88	Estrone	11.48 ± 1.63	Phenindione	6.15 ± 12.84
Ascorbic acid	15.91 ± 11.54	Estropipate	31.41 ± 4.75	Phleomycin	10.39 ± 3.69
Aspirin	7.33 ± 0.91	Ethacrynic acid	25.05 ± 1.21	Pipemidic acid	31.89 ± 6.3
Auranofin	37.81 ± 9.17	Fleroxacin	38.4 ± 12.83	Piperacillin	-13.59 ± 9.89
Azacytidine-5	8.91 ± 3.81	Florfenicol	36.94 ± 7.46	Piperazine	18.07 ± 13.92
Azidothymidine	13.89 ± 2.75	Floxuridine	3.92 ± 11.78	Piretanide	6.89 ± 4.04
Azithromycin	6.72 ± 8.47	Fludarabine	28.96 ± 6.83	Pivmecillinam	8.03 ± 10.39
Aztreonam	3.52 ± 6.33	Flumequine	38.08 ± 11.04	Procaine	32.24 ± 9.77
Bacitracin	4.61 ± 14.54	Fluspirilen	22.69 ± 12.89	Pseudomonic acid	-8.95 ± 6.6
Baicalein	38.81 ± 2.02	Fosfomycin	-3.52 ± 11.8	Pterostilbene	5.59 ± 14.28
Benserazide	80.75 ± 8.95	Furazolidone	3.67 ± 3.12	Pyocyanin	-15.35 ± 2.46
Benzalkonium	24.88 ± 12.24	Fusidic acid	38.45 ± 4.81	Pyrimethamine	19.8 ± 14.68
Benzydamine	73.86 ± 2.18	Gatifloxacin	30.98 ± 13.11	Reserpine	41.72 ± 5.4
Berberine	9.36 ± 4.32	Gentamicin	6.58 ± 2.04	Rifabutin	8.22 ± 2.49
Beta-thujaplicin	68.97 ± 14.86	Glutamate	6.03 ± 4.61	Rifampicin	9.14 ± 14.1
Caffeine	9.44 ± 13.08	Glycine	25.21 ± 12.1	Rifapentine	4.48 ± 0.82
Carbenicillin	-1.11 ± 2.78	Grepafloxacin	24.9 ± 8.99	Rifaximin	12.48 ± 0.54
Cefepime	-6.67 ± 8.24	Guanine	36.77 ± 10.33	Ronidazole	1.79 ± 3.26
Cefotaxime	-0.98 ± 7.22	Hexachloro-phene	23.42 ± 4.12	Roxithromycin	8.63 ± 4.83
Cefquinome	-4.69 ± 3.37	Indole	33.12 ± 4.3	Rufloxacin	20.15 ± 2.27
Ceftazidime	-6.77 ± 0.58	Kanamycin	7.99 ± 11.97	Sarafloxacin	26.85 ± 5.38
Ceftibuten	4.79 ± 4.91	Levofloxacin	3.72 ± 9.11	Spectinomycin	39.62 ± 5.13
Ceftiofur	-5.68 ± 2.67	Lomefloxacin	37.6 ± 2.65	Streptozotocin	4.13 ± 3.66
Cerulenin	-13.66 ± 8.43	Loperamide	83.65 ± 5.81	Sulfamonome-thoxine	8.19 ± 12.58
Chloramph-enicol	27.09 ± 11.21	Lovastatin	23.67 ± 4.34	Tazobactam	36.87 ± 7.52
Chlorhexidine	55.17 ± 5.34	Mecillinam	-16.79 ± 14.32	Tegaserod	66.17 ± 13.4
Chlorotropone	5.53 ± 7.97	Meclocycline sulfosalicylate	8.2 ± 11.99	Theophylline	14.32 ± 14.29

Chloroxine	65.98 ± 5.75	Melatonin	20.99 ± 10.55	Thioguanosine	3.74 ± 4.49
Chlortetracy-cline	25.4 ± 7.62	Merbromin	0.67 ± 10.72	Thiostrepton	37.06 ± 2.45
Ciclopirox ethanolamine	9.62 ± 10.99	Meropenem	25.57 ± 10.37	Thonzonium bromide	37.16 ± 4.31
Ciprofloxacin	15.91 ± 5.9	Metformin	93.41 ± 2.51	Thymine	15.2 ± 12.85
Clarithromycin	62.17 ± 11.08	Methicillin	-1.06 ± 1.51	Ticarcillin sodium	29.4 ± 0.52
Clavulanate	27.68 ± 6.98	Methoxy-tropone	3.87 ± 9.05	Tobramycin	62.15 ± 1.1
Clinafloxacin	33.16 ± 9.91	Mitomycin C	42.21 ± 7.15	Tosufloxacin	5.74 ± 14.69
Clioquinol	0.44 ± 8.6	Moxifloxacin	17.39 ± 7.96	Triclosan	38.61 ± 7.42
Clofazimine	16.92 ± 2.45	Nisin	30.81 ± 15	Trimethoprim	-18.08 ± 6.53
Colistin	21.7 ± 14.33	Nitrofurantoin	21.99 ± 8.45	Tropolone	80.78 ± 11.58
Curcumin	12.68 ± 2.2	Novobiocin	28.74 ± 3.19	Tropone	28.22 ± 12.8
Cycloserine D	15.77 ± 0.57	Ofloxacin	37.42 ± 13.74	Tryptophan	6.43 ± 14.61
Cytosine	33.3 ± 13.67	Oxacillin	-0.93 ± 14.87	Uracil	6.62 ± 3.73
Dapsone	26.99 ± 1.44	Oxantel pamoate	24.88 ± 10.16	Vancomycin	21.64 ± 5.27
Demeclocycline	33.01 ± 8.57	Oxaprozin	33.14 ± 11.49	Vanillin	-17.18 ± 14.08
Didanosine	37.75 ± 7.39	Oxatomide	30.99 ± 4.25	Verapamil	66.22 ± 0.75
Dihydrostrep-tomycin	7.58 ± 6.31	Oxcarbazepine	22.88 ± 4.82	Oxiracetam	12.56 ± 0.13
Dirithromycin	37.66 ± 10.04	Oxethazaine	34.76 ± 5.34		

[#]Data are representative of three independent experiments ± SD. Synergy effect were defined as the inhibition rate of ≥ 50% and marked by green background.

Table S3 Antimicrobial susceptibility test of strains used in this study (MIC, μg/mL).

Organism and genotype	Ampicillin	Vancomycin	Colistin	Doxycycline	Tigecycline
Gram-positive bacteria					
S. aureus ATCC 29213	0.25	0.5	16	0.25	0.125
MRSA T144	32	2	128	16	2
S. aureus 215	64	1	64	16	1
E. faecalis A4	32	>128	128	32	0.125
Gram-negative bacteria					
E. coli ATCC 25922	8	128	0.5	1	0.125
E. coli B2	>128	128	8	32	2
S. enteritidis ATCC 13076	8	128	0.25	4	0.125
S. enteritidis H8	8	128	0.25	16	0.125

ATCC, American Type Culture Collection.

Table S4 Adjuvant potency of metformin in combination with doxycycline against multi-drug resistant and sensitive bacteria.

Pathogens	MIC[a] (μg/mL)	FIC index	MIC[b] (μg/mL)	Potentiation (fold)[c]
Resistant				
MRSA T144	16	0.091	0.5	32
S. aureus 215	16	0.310	0.5	32
VRE A4	32	0.313	1	32
E. coli B2	32	0.188	0.5	64
S. enteritidis H8	16	0.208	0.5	32
Sensitive				
S. aureus 29213	0.5	0.5	0.125	4
E. coli 25922	1	0.5	0.25	4
S. enteritidis 13076	4	0.5	1	4

[a/b]MICs of doxycycline in the absence or presence of 0.25 × MIC metformin.

[c]Degree of doxycycline potentiation in the presence of 0.25 × MIC metformin.

Table S5 Synergistic activity of metformin in combination with different classes of antibiotic against *E. coli* B2.

Targets	Antibiotics	MIC[a] (μg/mL)	FIC index	MIC[a] (μg/mL)	Potentiation (fold)[c]
Cell wall	Ampicillin	128	0.75	32	4
	Meropenem	32	2	32	–
	Vancomycin	128	0.375	64	4
Cell membrane	Colistin	8	0.75	2	4
DNA synthesis	Ciprofloxacin	32	1	16	2
	Ofloxacin	32	0.75	16	2
RNA synthesis	Rifampicin	128	0.5	32	4
Protein	Kanamycin[d]	64	>2	>128	–
	Fusidic acid	8	1	4	2
	Erythromycin	128	0.5	64	4
	Tetracycline	128	0.375	16	8
	Doxycycline	32	0.078	0.5	64
	Chlortetracycline	128	0.378	32	4
	Oxytetracycline	256	0.313	64	4
	Minocycline	16	0.188	1	16
	Tigecycline	2	0.75	1	2

[a/b]MICs of antibiotic in the absence or presence of 5 mg/mL metformin (0.25 × MIC).

[c]Degree of antibiotic potentiation in the presence of 5 mg/mL metformin (0.25 × MIC).

[d]For kanamycin, 0.125 × MIC of metformin was used to calculate its FIC index and potentiation fold in *E. coli* B2.

–, none of potentiation activity.