Supplemental Material: Small molecule inhibitor of FosA expands fosfomycin activity to multidrug-resistant Gram-negative pathogens

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S1 Table. Small molecule screening data

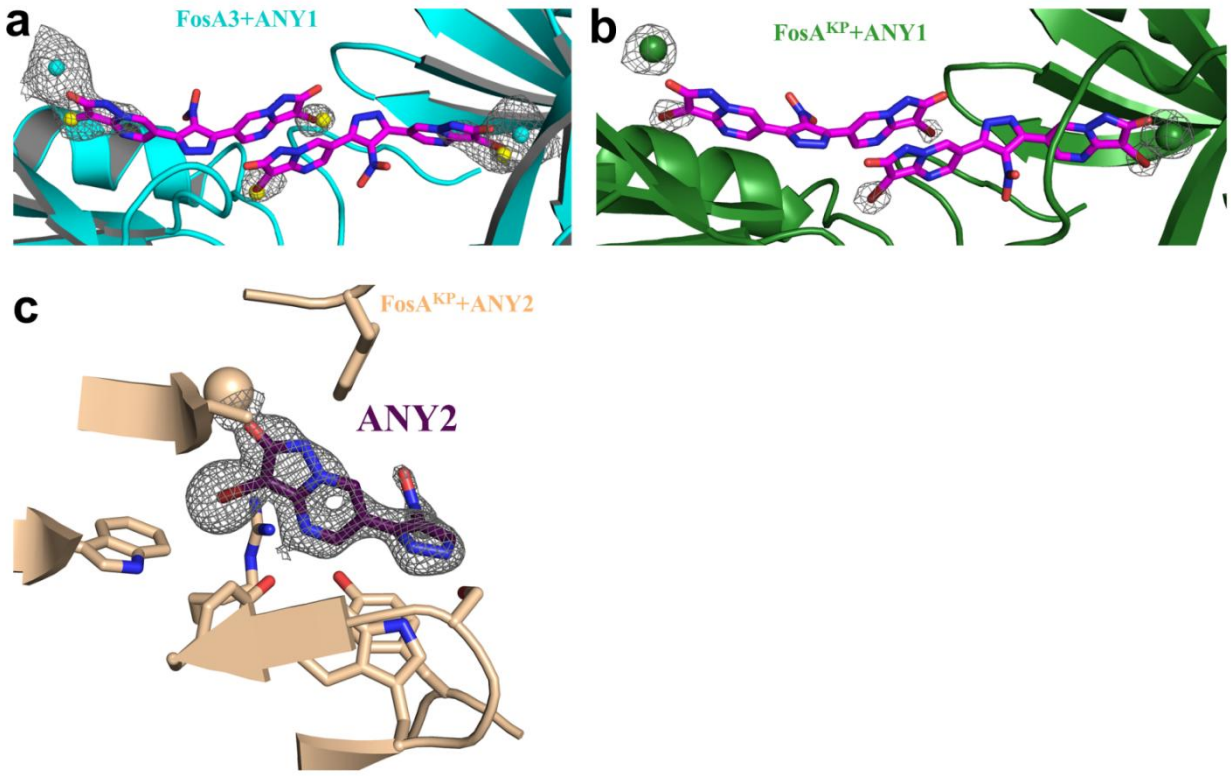
Category	Parameter	Description
Assay	Type of assay	Enzyme assay
	Target	*K. pneumoniae* $FosA^{KP}$
	Primary measurement	Fluorescence (see Figure 1c)
	Assay protocol	See Methods
Library	Library size	5,040
	Library composition	Small molecules that fall within designated MW, cLogP and Lipinski parameters
	Source	TimTec LLC (Newark, DE)
Screen	Format	96-well plate
	Concentration(s) tested	0.02 µg/µL
	Plate controls	DMSO (negative control); Foscarnet (positive control)
	Reagent/compound dispensing system	Manual
	Detection instrument and software	SpectraMax M2 (molecular Devices); Softmax Pro
	Assay validation/QC	Z'-factor of 0.52
Post-HTS analysis	Hit criteria	> 50% inhibition
	Hit rate	0.8%
	Additional assay(s)	Hits confirmed using 8-point dose response
	Confirmation of hit purity and structure	Repurchase of the hit compound; crystal structure

S2 Table. Conservation of the amino acid residues that interact with ANY1 in FosA across different Gram-negative pathogens

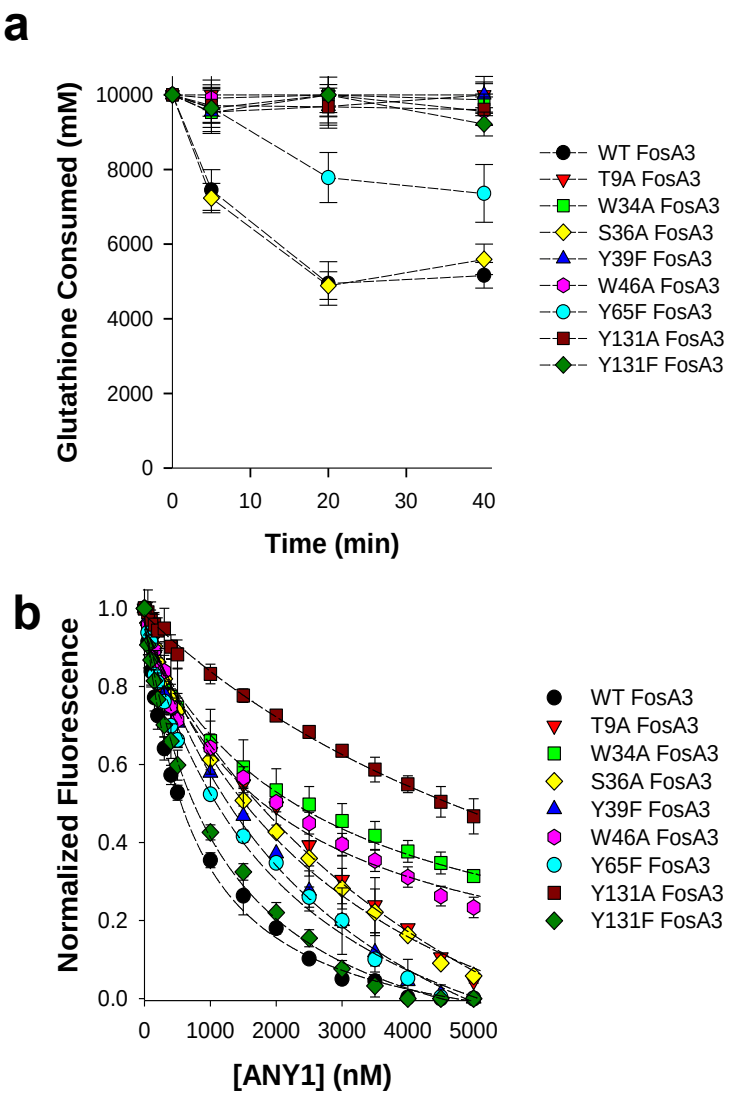
Pathogen	**Residue #**								
	9	**34**	**36**	**39**	**46**	**65**	**97**	**122**	**131**
P. stuartii	T	W	R	Y	W	Y	S	R	Y
S. marcescens	T	W	G	Y	W	Y	S	R	Y
E. aerogenes	T	W	S	Y	W	Y	S	R	Y
K. oxtoca	T	W	N	Y	W	Y	S	R	Y
M. morganii	T	W	Y	Y	W	Y	S	R	Y
K. pneumoniae	T	W	S	Y	W	Y	S	R	Y
E. cloacae	T	W	T	Y	W	Y	S	R	Y
P. aeruginosa	T	W	Q	Y	W	Y	S	R	Y
E. coli (FosA3)	T	W	S	Y	W	Y	S	R	Y

S3 Table. Minimum inhibitory concentrations for *P. aeruginosa* and *K. pneumoniae* strains with (parent) or without (transposon-mediated mutation) *fosA*. The strains listed below (MPA01, PW3042, MKP103 and KP01491) were obtained from the University of Washington.

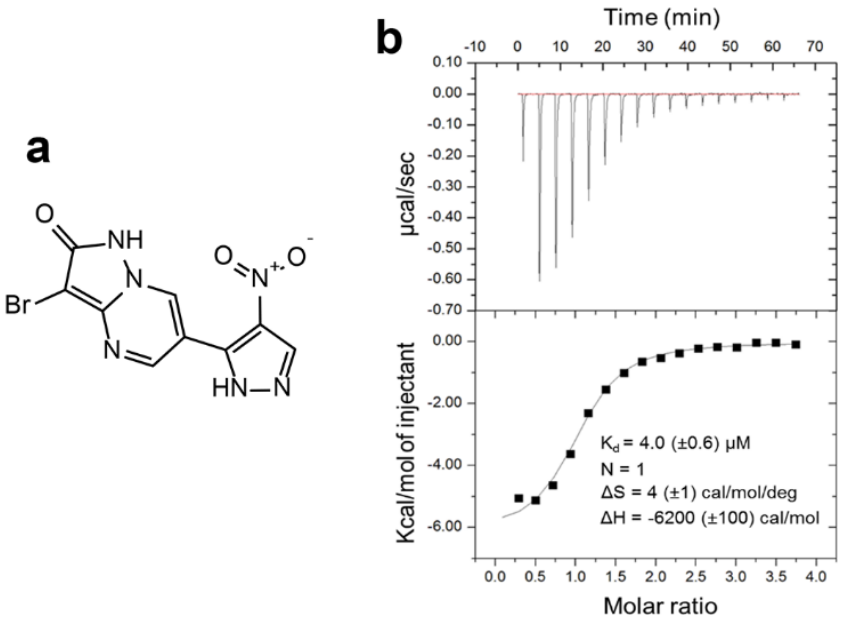
Bacterial Strain	Isolate	MIC (μg/mL)
P. aeruginosa (parent strain)	MPA01.1	64
	MPA01.2	64
	MPA01.3	64
	MPA01.4	64
P. aeruginosa (*fosA* mutant strain)	PW3042.1	4
	PW3042.2	4
	PW3042.3	4
	PW3042.4	4
K. pneumoniae (parent strain)	MKP103.1	>128
	MKP103.2	>128
	MKP103.3	>128
	MKP103.4	>128
K. pneumoniae (*fosA* mutant strain)	KP01491.1	16
	KP01491.2	16
	KP01491.3	8
	KP01491.4	16



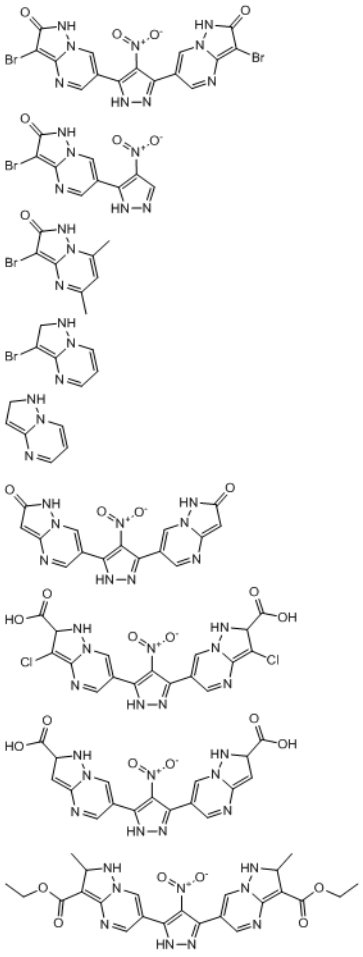
S1 Fig: Electron density surrounding ligands in FosA structures. (a) Structure of FosA3 in complex with ANY1, depicting anomalous phase map at bromine edge, contoured to 3 σ, and carved to 3.5 Å from ANY1. Yellow spheres represent center of anomalous bromine signal as determined by MR-SAD; **(b)** Structure of $FosA^{KP}$ in complex with ANY1, depicting composite omit map contoured to 8 σ, and carved to 10 Å from ANY1; **(c)** Structure of $FosA^{KP}$ in complex with ANY2, depicting composite omit map contoured to 1.1 σ, and carved to 1.7 Å from ANY2.



S2 Fig: Characterization of FosA3 mutants. (**a**) Enzyme activity of WT, T9A, W34A, S36A, Y39F, W46A, Y65F, Y131A and Y131F FosA3. Data are shown as the mean ± standard deviation from at least 3 independent biological replicates. Assays were carried out as described in the Materials and Methods using 30 mM glutathione and 50 mM FOM; (**b**) ANY1 binding isotherms to WT, T9A, W34A, S36A, Y39F, W46A, Y65F, Y131A and Y131F FosA3 as measured by protein fluorescence quenching. Data are shown as the mean ± standard deviation from at least 3 independent biological replicates.

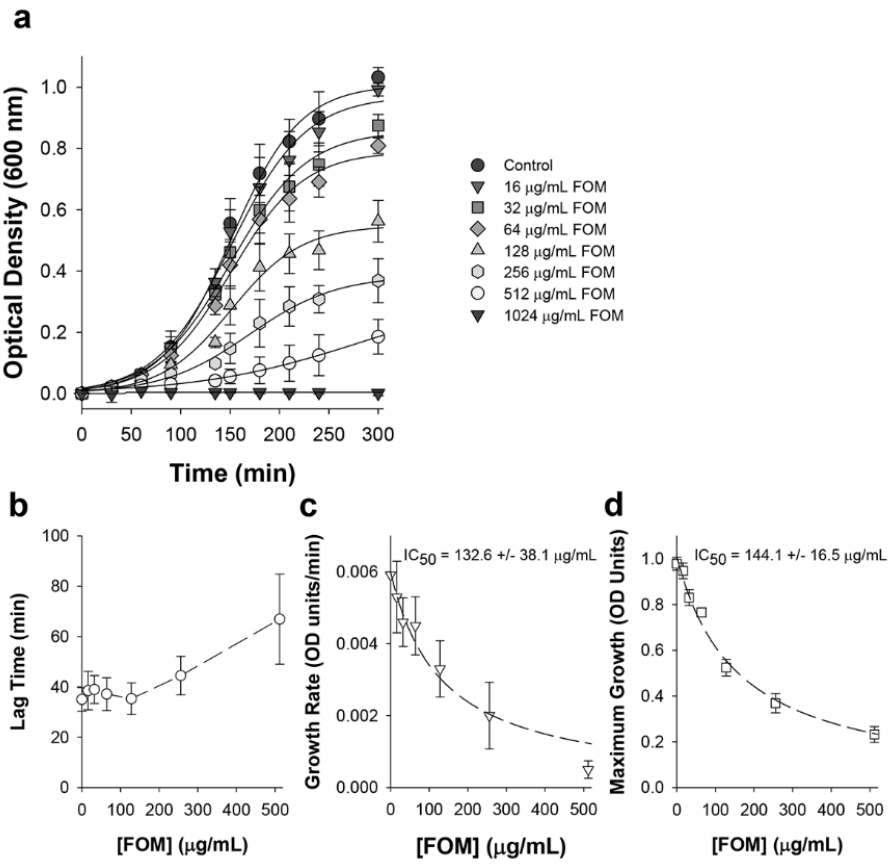


S3 Fig: Characterization of ANY2 binding to FosA^{KP}. (**a**) Chemical structure of ANY2; (**b**) Representative run of ANY2 binding to FosA^{KP} as measured by isothermal titration calorimetry. The upper panel represents the isotherms measured for 3860 seconds at 230 s injection intervals. The lower panel shows a sigmoidal curve from an individual heat flow as a function of the total molar ratio [(ANY2)/(FosA^{KP} monomer)] in the calorimeter cell. Binding isotherms were performed in triplicate and corrected for heats of dilution.



ANY1	IC_{50} = 5.1±2.2 μM
ANY2	IC_{50} = 8.4±3.1 μM
3	no inhibition
4	no inhibition
5	no inhibition
6	no inhibition
7	no inhibition
8	no inhibition
9	no inhibition

S4 Fig: Structure-activity relationships of ANY1. The concentration of drug that inhibited 50% of $FosA^{KP}$ activity (i.e., IC_{50}) was carried out as described in the Materials and Methods using 30 mM glutathione and 50 mM FOM.



S5 Fig: Analysis of bacterial growth curves of *K. pneumoniae* I1 in the absence or presence of FOM. (**a**) Growth curve of *K. pneumoniae* I1 in the absence or presence of varying concentrations of FOM (0-1024 μg/mL). Data were fit to a modified 3-parameter Gompertz equation (see Methods), which facilitated quantification of the lag time (min), growth rate (OD units/min) and maximum growth (OD units); (**b**) Lag time as a function of FOM concentration; (**c**) Growth rate as a function of FOM concentration; (**d**) Maximum growth as a function of FOM concentration. All data are shown as the mean ± standard deviation from at least 3 independent biological replicates.