SUPPLEMENTAL DATA

Table S1: Bacterial strains used in this study

Strain	Genotype and relevant phenotype	Source or Reference
Acinetobacter baumannii 17978	Wild type; polymyxin sensitive	ATCC®17978™
Acinetobacter baumannii 17978R2	*17978 pmrB(T235I);* polymyxin resistant	1
Acinetobacter baumannii AABA1205	Clinical isolate; polymyxin resistant	2
Bacillus subtilis 168	Wild type ind^- tyr^+	ATCC®23857™
Enterobacter cloacae RS/WT	Clinical isolate; polymyxin heteroresistant	3
Enterococcus faecium 19434	Wild type	ATCC®19434™
Escherichia coli W3110	Wild type F^- λ^-; polymyxin sensitive	*E. coli* Genetic Stock Center (Yale)
Escherichia coli WD101	W3110 $pmrA^C$; polymyxin resistant	4
Escherichia coli WBBO6	W3110 Δ*(rfaC-rfaF)::tet6;* polymyxin sensitive	5
Escherichia coli W3LPTD	W3110 *lptD 4213 carB::Tn10;* polymyxin sensitive	This study
Escherichia coli WDLPXC	WD101 *lpxC101 leuB::Tn10;* polymyxin resistant	This study
Klebsiella pneumoniae Lab C-26233	Clinical isolate; polymyxin sensitive	6
Klebsiella pneumonaie ST258 RH201207	Clinical isolate; polymyxin resistant	7
Klebsiella pneumonaie NR05083	Clinical isolate; polymyxin resistant	A.C. Uhlemann
Pseudomonas aeruginosa PA14	Wild type; polymyxin sensitive	8
Pseudomonas aeruginosa PA14BNQ	PA14 *phoQ::xylE-aacC1*; polymyxin resistant	9
Staphylococcus aureus subsp. *aureus* 12600	Wild type; methicillin sensitive	ATCC®12600™
Staphylococcus aureus subsp. *aureus* MU50	Wild type; methicillin resistant	ATCC®700699™

Table S2: Lead compounds used in this study

Compound ID	Maybridge ID	Molecular Weight (g/mol)	Formula	IUPAC Nomenclature	# Chiral Centers	# Rotatable Bonds	logP	logD [pH=7]
5	HTS 03780	404.33	$C_{17}H_{10}F_6N_2OS$	3-(1-benzothiophen-2-yl)-1-[3,5-bis(trifluoromethyl)phenyl]urea	0	4	5.92	5.92
8	MGH 00136	291.69	$C_{13}H_{10}ClN_3O_3$	3-(4-chloro-2-nitrophenyl)-1-phenylurea	0	3	3.66	3.66
33	NH 00518	221.10	$C_7H_6Cl_2N_2S$	N-amino-3,5-dichlorobenzene-1-carbothioamide	0	1	2.63	2.63
43	CD 04455	383.58	$C_{14}H_8Cl_3F_3N_2O$	2,4-dichloro-6-[5-chloro-6-(trifluoromethyl)-2,3-dihydro-1H-1,3-benzodiazol-2-yl]phenol	1	2	3.12	4.88
44	JP 00319	292.16	$C_{15}H_{11}Cl_2NO$	(2E)-N-(3-chlorophenyl)-3-(4-chlorophenyl)prop-2-enamide	0	3	4.78	4.78

Table S3: Lead compounds demonstrate synergy with polymyxin B against diverse polymyxin-resistant Gram-negative bacterial species. The MIC of polymyxin was determined in various Gram-negative bacterial strains using PMB E-test strips on CAMHA plates containing 20 μM of each of the 5 lead compounds, and fold change reductions were calculated. Representative data from at least two biologic replicates are shown.

Isolates	PMB	20 μM Compound + PMB									
		5		8		33		43		44	
	MIC (μg/mL)	MIC (μg/mL)	Synergy (Fold Δ)	MIC (μg/mL)	Synergy (Fold Δ)	MIC (μg/mL)	Synergy (Fold Δ)	MIC (μg/mL)	Synergy (Fold Δ)	MIC (μg/mL)	Synergy (Fold Δ)
Escherichia coli W3110, PMB^S	0.125	0.125	1	0.125	1	0.125	1	0.125	1	0.125	1
Escherichia coli WD101, PMB^R	3-4	0.125	24	0.19	16	2	1.5	3	1	1.5	2
Acinetobacter baumannii 17978, PMB^S	0.19	0.125	1.5	0.125	1.52	0.125	1.5	0.19	1	0.19	1
Acinetobacter baumannii 17978R2, PMB^R	12	0.19	63	0.5	24	2	6	12	1	12	1
Acinetobacter baumannii AABA1205, PMB^R	64	1.5	43	1.5	43	32	2	64	1	64	1
Klebsiella pneumoniae Lab C-26233, PMB^S	0.19	0.125	1.5	0.19	1	0.19	1	0.19	1	0.19	1
Klebsiella pneumoniae ST258 RH201207, PMB^R	2	0.38	5.3	0.5	4	0.5	4	1	2	1	2
Klebsiella pneumoniae NR05083, PMB^R	3	0.25	12	0.25	12	0.5	6	1	3	1	3
Pseudomonas aeruginosa PA14, PMB^S	0.5	0.38	1.3	0.5	1	0.25	2	0.25	2	0.38	1.3
Pseudomonas aeruginosa PA14BNQ, PMB^R	8	0.5	16	2	4	6	1.3	3	2.7	4	2

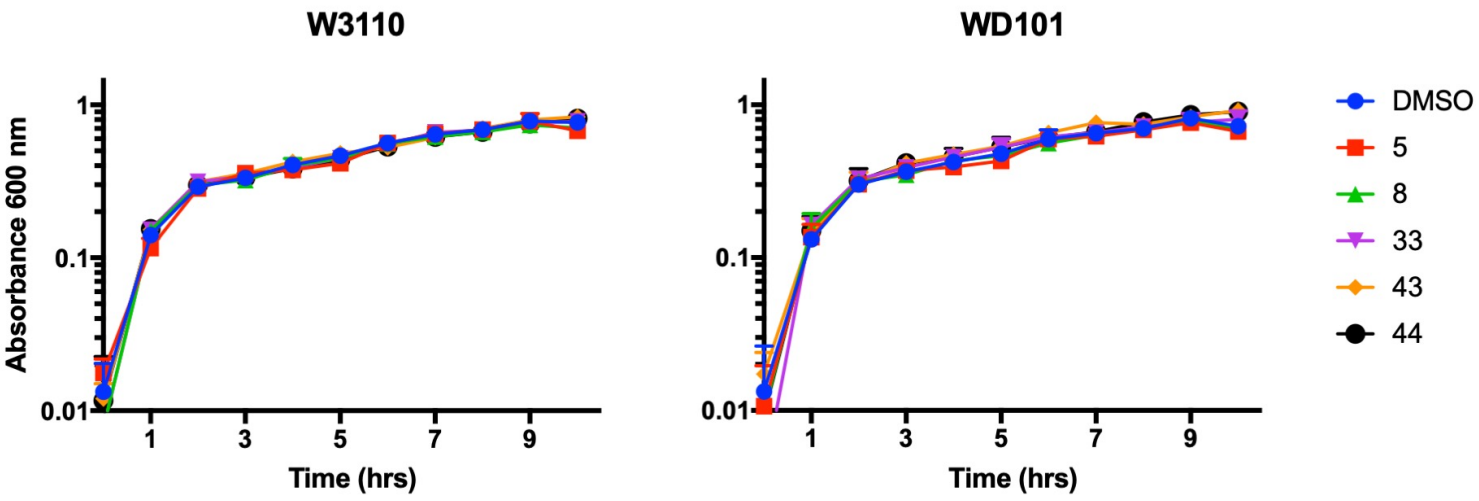

Figure S1: Lead compounds do not affect *E. coli* growth. Representative data from at least two biological replicates was generated using broth microdilution assays.

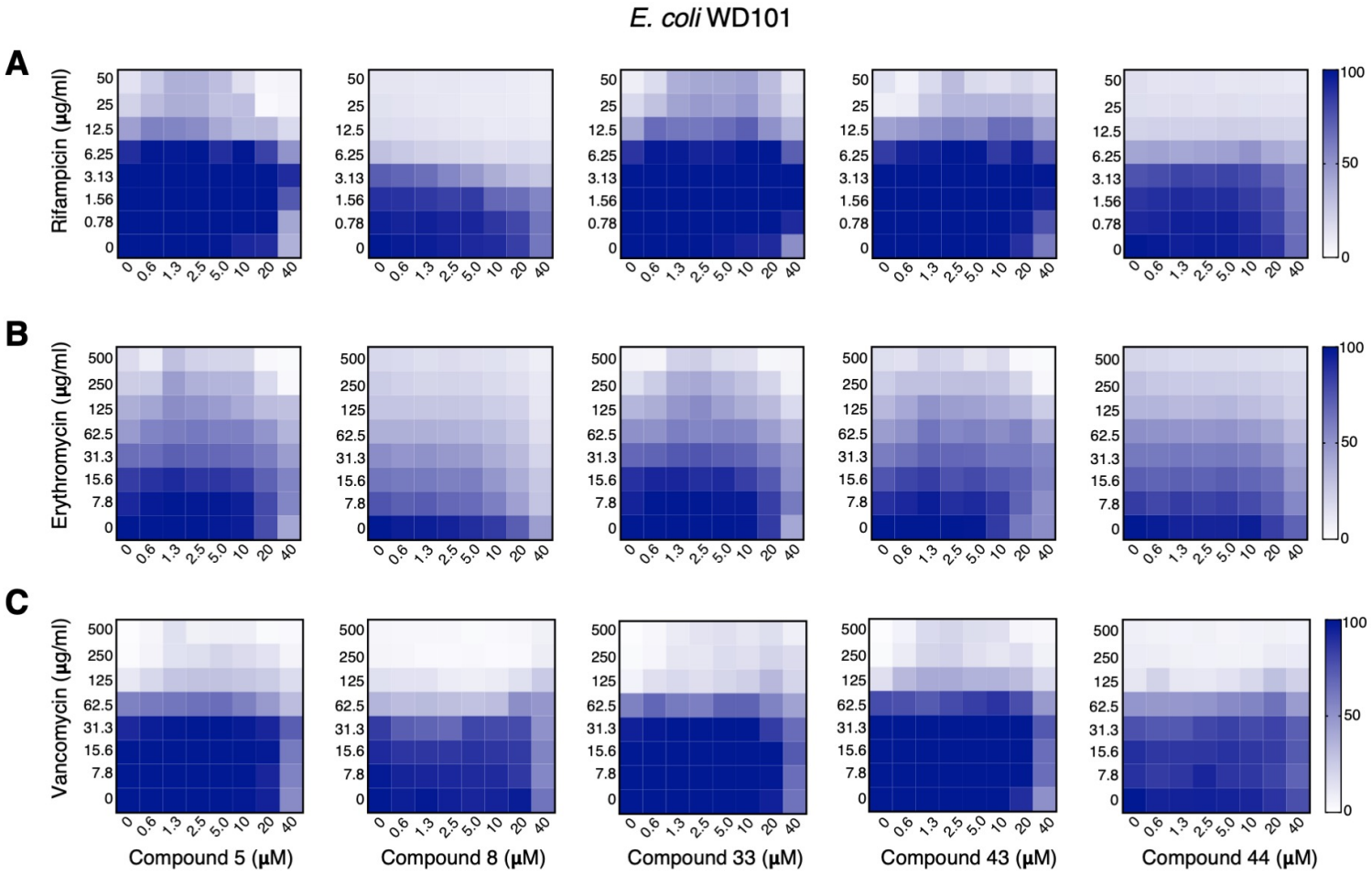

Figure S2. The synergistic activity of lead compounds is specific to polymyxin. Checkerboard broth microdilution assays performed with *E. coli* WD101 (*pmrA* constitutive mutant) to examine the synergy potential of the 5 lead compounds and **A)** rifampicin, **B)** erythromycin, and **C)** vancomycin. Percent bacterial growth is demonstrated via heat maps. Representative data from at least two biologic replicates are presented.

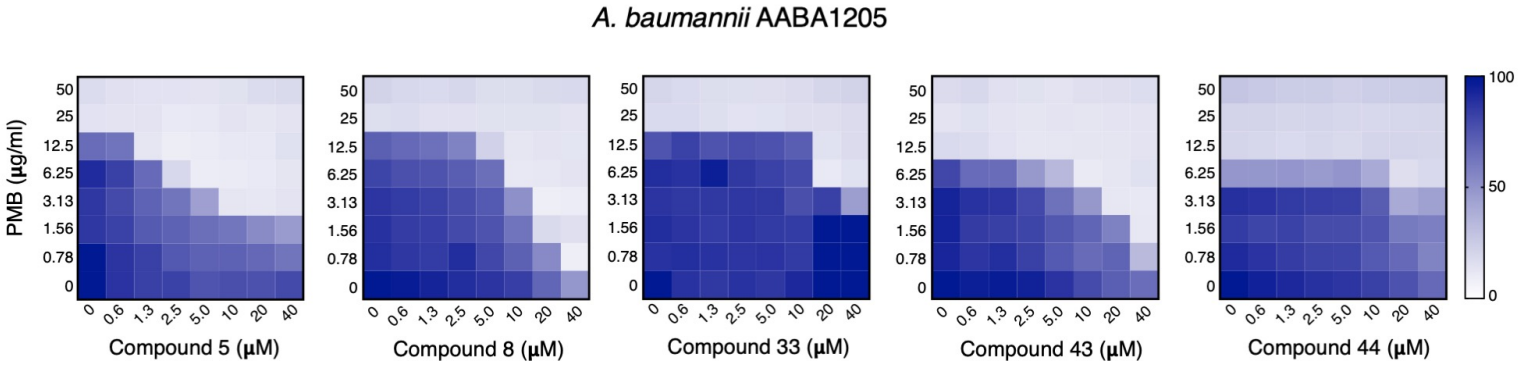

Figure S3: Compounds act synergistically with polymyxin B against a highly colistin-resistant *Acinetobacter baumannii* clinical isolate. Checkerboard broth microdilution assays performed with *A. baumannii* AABA1205 demonstrate synergy between the 5 lead compounds and PMB at varying concentrations. Representative data from at least two biologic replicates are presented, and the heat map shows percent bacterial growth.

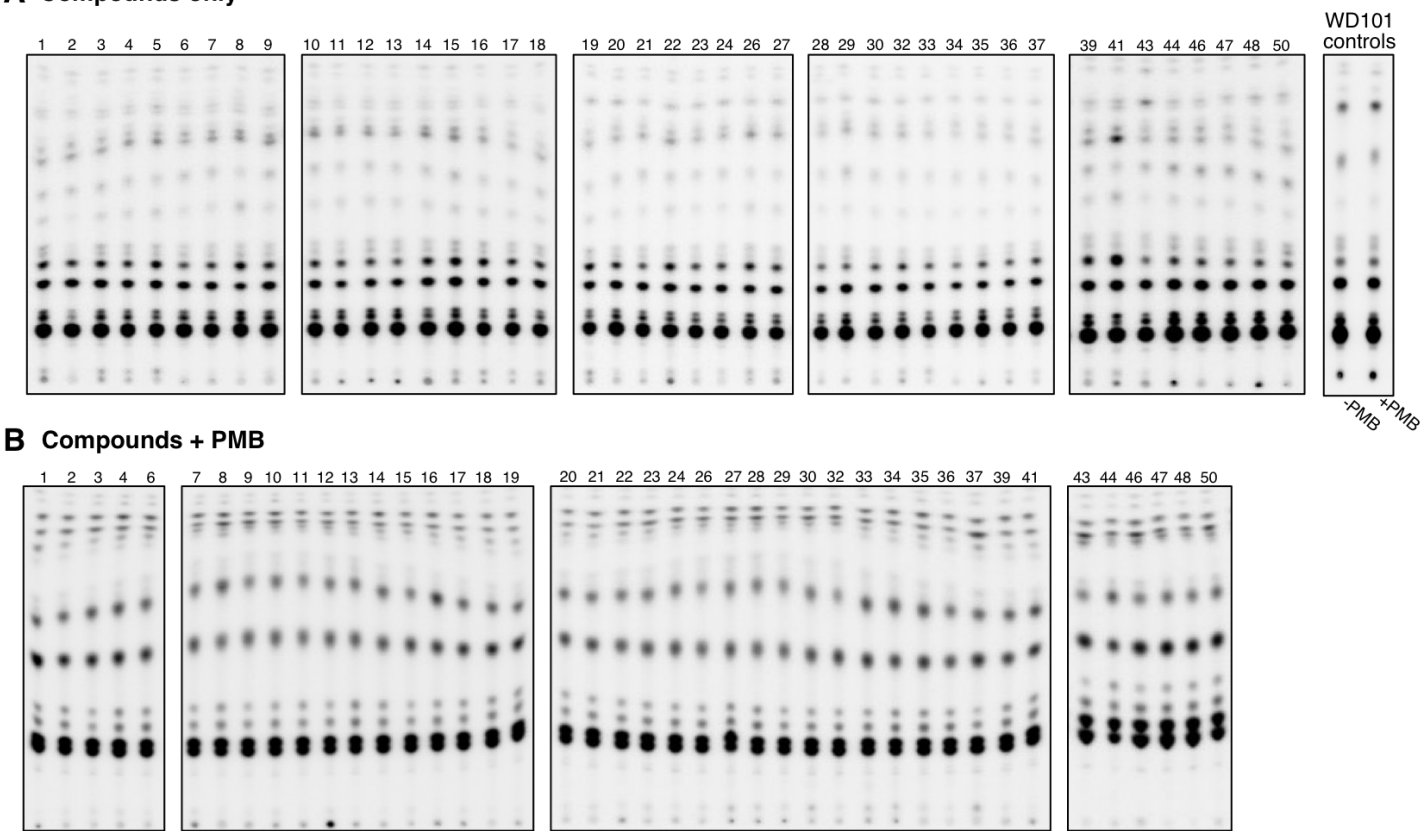

Figure S4: Lipid A modifications are not altered in *E. coli* treated alone or in combination with compounds. ^{32}P-labeled lipid A species isolated from *E. coli* grown under different conditions are shown. *E. coli* WD101 (*pmrA* constitutive mutant) is used as a control for PmrA-mediated lipid A modifications. **A)** Bacterial cells were grown in LB broth supplemented with 2% DMSO and 15 μM of each compound of interest. **B)** Bacterial cells were grown in LB broth supplemented with 2% DMSO, 0.5 μg/mL PMB, and 10 μM of each compound of interest.

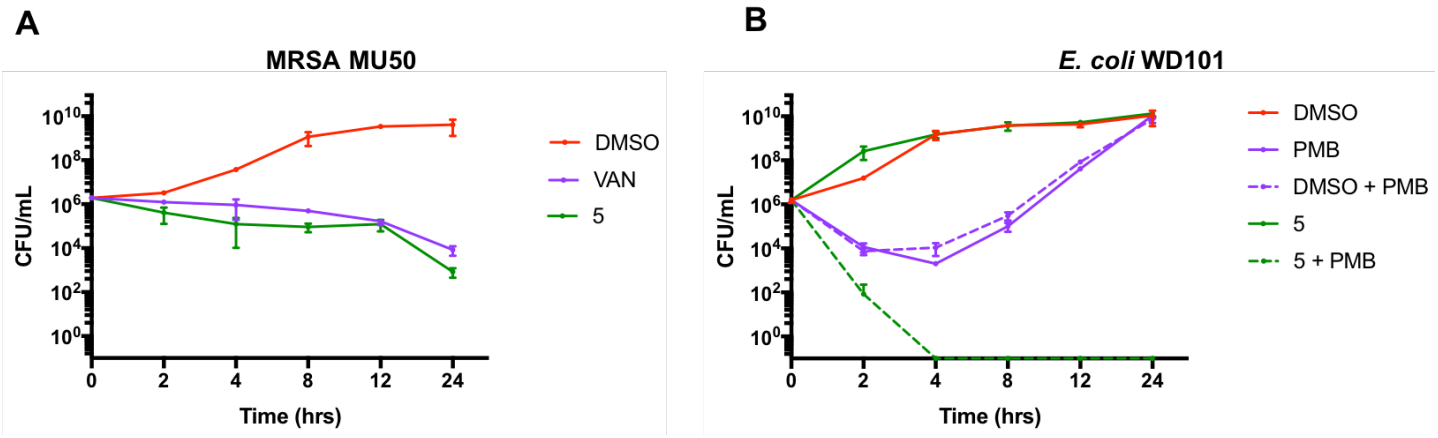

Figure S5: At low concentrations compound 5 exhibits potent bactericidal activity. A) Time-kill curves for 0.625 μM of compound 5 against vancomycin-intermediate MRSA MU50 demonstrated killing comparable to vancomycin with bactericidal activity (≥ 3 $\log_{10}$ reduction in CFU/mL) achieved at 24 hours. **B)** Time-kill curves for 2.5 μM of compound 5 with and without 2.5 μg/mL PMB against polymyxin-resistant *E. coli* WD101 (*pmrA*^{c}) demonstrates synergistic bactericidal activity and complete bacterial killing achieved at 4 hours. For all data sets, representative data from at least two biologic replicates are shown.

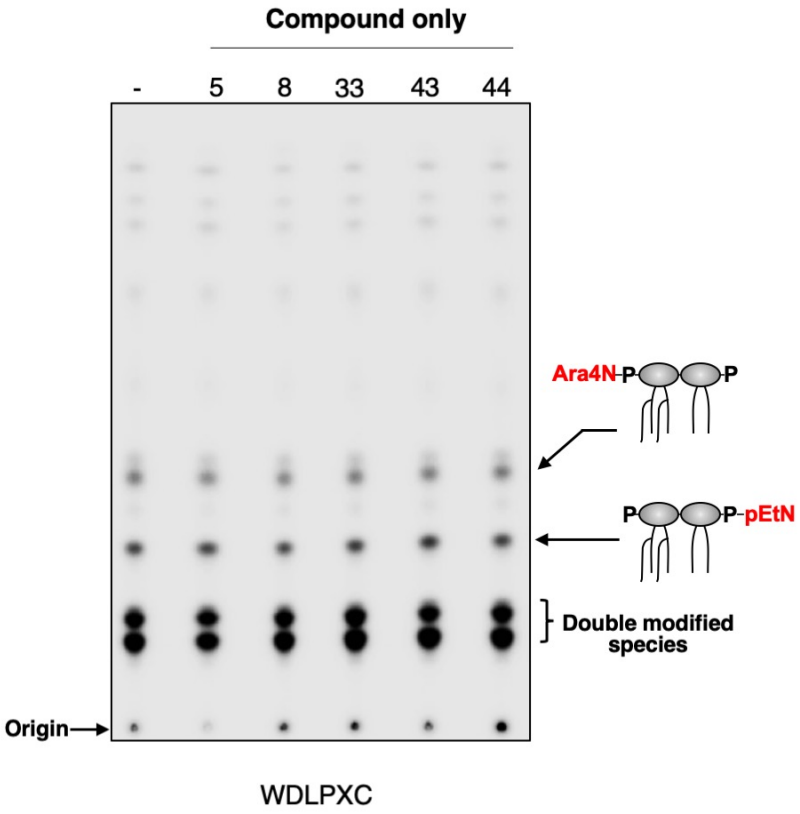

Figure S6. Lead compounds do not alter existing lipid A modifications in *E. coli* with outer membrane defects. ^{32}P-labeled lipid A species isolated from *E. coli* WDLPXC (*pmrA*^{c} *lpxC101*) grown under different conditions are shown. Bacterial cells were grown in LB broth supplemented with 2% DMSO and 1.0 μM of each compound.

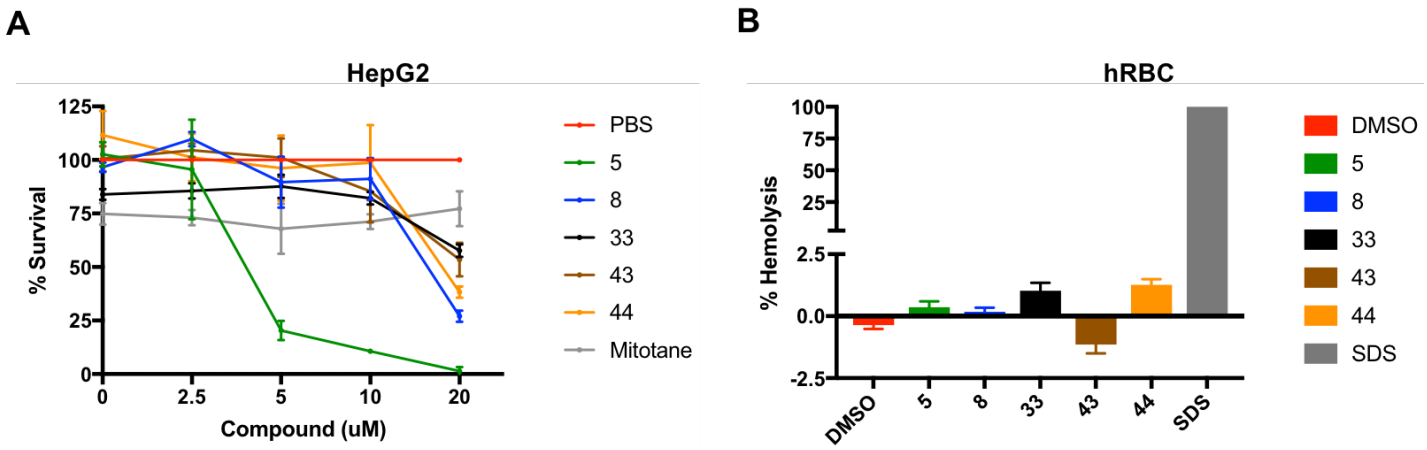

Figure S7: Lead compounds only exhibit hepatotoxicity at high concentrations and are not hemolytic. A) MTT cytotoxicity assay with washed HepG2 cells demonstrates that at ≤ 2.5 μM, all of the lead compounds are less toxic to human hepatocytes than mitotane, with some compounds (8, 33, 43, and 44) demonstrating minimal toxicity up to 10 μM. **B)** Hemolysis assay with washed human red blood cells (hRBC) and 40 μM of compound demonstrates that these compounds have little, if any, hemolytic activity after 3 hrs. These assays were performed at multiple concentrations (up to 40 μM) and at both 45 min (not shown) and 3 hrs (shown). For all data sets, representative data from at least two biologic replicates are shown.

References

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