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Growth Inhibition of Pathogenic Bacteria by Sulfonylurea Herbicides

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Emerging resistance to current antibiotics raises the need for new microbial drug targets. We show that targeting branched-chain amino acid (BCAA) biosynthesis using sulfonylurea herbicides, which inhibit the BCAA biosynthetic enzyme acetohydroxyacid synthase (AHAS), can exert bacteriostatic effects on several pathogenic bacteria, including *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Our results suggest that targeting biosynthetic enzymes like AHAS, which are lacking in humans, could represent a promising antimicrobial drug strategy.

As the prevalence of antibiotic resistance continues to rise, new therapeutic targets are urgently needed (1). One strategy for designing new antibiotics is to target essential components that are uniquely present in bacteria and not humans. Bacteria and plants, but not humans, can synthesize branched-chain amino acids (BCAA); thus, enzymes in this pathway are potential targets for antimicrobials and herbicides (2, 3). In addition, BCAA biosynthesis is essential for certain bacterial infections *in vivo*, including *Burkholderia pseudomallei* (4), the causative agent of melioidosis (5), and *Mycobacterium tuberculosis* (6).

Sulfonylurea herbicides (SHs) inhibit acetohydroxyacid synthase (AHAS) (7, 8), the first shared enzyme in the BCAA biosynthetic pathway, from plants and two of the three isozymes in bacteria; type II and type III but not type I are sensitive to SHs (3). Enterobacteria encode a type I isozyme along with a type II and/or type III isozyme and thus are resistant to inhibition by SHs (9, 10). Alternatively, soil bacteria typically contain only a type III AHAS and thus are often sensitive to inhibition by SHs (11–13). The antibacterial activity of SHs on soil microbes has also been noted to explain the potential toxic environmental effects of these compounds (14, 15). The distribution of AHAS isozymes in a variety of pathogenic bacteria is not known.

MATERIALS AND METHODS

Phylogenic analysis. Bacterial isozymes of AHAS are composed of a catalytic and a regulatory subunit that are encoded by adjacent genes. Catalytic subunits of AHAS were identified by searching the genomes of select organisms using the Enzyme Commission number 2.2.1.6 (2, 16). In most genomes, multiple genes have been annotated as AHAS catalytic subunits. To ensure that only actual catalytic subunits were analyzed, only those genes adjacent to a regulatory subunit were included. Nucleotide sequences of 50 AHAS catalytic subunits from 38 species (Table 1) were translated into protein sequences and aligned (ClustalW2), and gaps were stripped from the alignment (BioEdit). A phylogenetic tree was derived using maximum likelihood methods and subjected to bootstrap analysis 100 times (PHYLIP).

Determination of MICs. All SHs were purchased from Sigma-Aldrich although manufactured by Chem Service, Inc. (analytical standards; purity > 99.0%). SH stocks were prepared in dimethyl sulfoxide (DMSO) (200 μM), stored at −20°C, and discarded after 2 months. Antibiotics and other chemicals were also purchased from Sigma-Aldrich unless stated otherwise.

MICs were determined based on the 2-fold microdilution broth technique described in the Clinical and Laboratory Standards Institute guidelines (M7-A6). In brief, exponential-phase starter cultures in LB broth were washed twice in M9 salts (17) before being suspended in growth medium. For experiments with *B. pseudomallei*, *Burkholderia thailandensis*, or *Pseudomonas aeruginosa*, cultures were grown in M9 medium (17) that contained glucose (0.2%) and thiamine (0.5 μg/ml). For experiments with *Bacillus subtilis*, cultures were grown in Spizizen's minimal medium (18) containing glucose (1%) and tryptophan (50 μg/ml) along with glutamate (2 mg/ml), KNO_3 (0.2%), and trace elements as described by Nakano et al. (19). The MIC values reported for each strain are the median values from three to six independent experiments. For the *P. aeruginosa* clinical isolates, each MIC assay was typically performed once.

High-resolution growth curves. Glycerol stocks of *Acinetobacter baumannii* were streaked onto LB agar plates and incubated overnight at 37°C. The next day, a single colony was used to inoculate a starter culture of 3 ml of M9 medium (17) containing acetate (0.2%) and thiamine (0.5 μg/ml). After 4 h of aerobic growth at 37°C, the starter culture was then diluted to an optical density at 600 nm (OD_{600}) of 0.01 in M9 salts, and 5 μl was used to inoculate M9 medium (250 μl) containing acetate (0.2%) and thiamine (0.5 μg/ml). High-resolution growth curves were obtained using a Bioscreen C MBR (Oy Growth Curves Ab Ltd.) integrated incubator and microplate reader. All cultures (250 μl) were grown aerobically at 37°C with DMSO or SHs included as indicated, and OD_{600} readings were collected at regular intervals. The reported OD_{600} readings are the average values across three wells in one experiment, and the data shown are representative of three independent experiments.

Mouse infections. *B. pseudomallei* experiments were performed in accordance with the Animal Care and Use Committee's Guidelines, DSO National Laboratories, Singapore, using protocols approved by the DSO National Laboratories Biosafety Committee. Female 6-week-old BALB/c mice were intranasally challenged as previously described (20) with 10^2

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TABLE 1 Sequences used in the construction of the AHAS phylogeny

Organism (strain)	NCBI GeneID	Isozyme
Acinetobacter baumannii (5377)	4917404	III
Acinetobacter baumannii (AYE)	6003386	III
Actinobacillus pleuropneumoniae (L20)	4848528	II
Actinobacillus pleuropneumoniae (L20)	4848759	III
Arabidopsis thaliana (Columbia)	824015	N/A
Bacillus anthracis (Ames)	1086383	III
Bacillus subtilis (168)	936792	III
Bordetella pertussis (Tohama I)	2664081	III
Brucella suis (1330)	1167071	III
Burkholderia cenocepacia (AU1054)	4093524	III
Burkholderia cenocepacia (J2315)	6933755	III
Burkholderia multivorans (ATCC 17616)	5765837	III
Burkholderia pseudomallei (K96243)	3093947	III
Burkholderia thailandensis (ATCC 700388)	3847786	III
Campylobacter jejuni (NCTC 11168)	904899	III
Caulobacter crescentus (CB15)	943022	III
Enterobacter (638)	5110480	II
Enterobacter (638)	5113032	I
Enterobacter (638)	5113663	III
Escherichia coli (K-12 MG1655)	948182	I
Escherichia coli (K-12 MG1655)	948793	III
Haemophilus influenzae (Rd KW20)	950449	III
Klebsiella pneumoniae (MGH 78578)	5339104	III
Klebsiella pneumoniae (MGH 78578)	5339386	II
Klebsiella pneumoniae (MGH 78578)	5341318	I
Listeria innocua (Clip11262)	1130811	III
Listeria monocytogenes (EGD-e)	984805	III
Mycobacterium bovis (BCG Pasteur 1173P2)	4698974	III
Mycobacterium marinum (M)	6225967	III
Mycobacterium tuberculosis (H37Rv)	887286	III
Neisseria gonorrhoeae (FA 1090)	3282600	III
Neisseria lactamica (020–06)	10007328	III
Neisseria meningitidis (Z2491)	907658	III
Pseudomonas aeruginosa (PA14)	4384635	III
Pseudomonas aeruginosa (PAO1)	881496	III
Ralstonia solanacearum (GMI1000)	1220918	III
Salmonella enterica (Typhimurium LT2)	1251634	III
Salmonella enterica (Typhimurium LT2)	1255320	I
Salmonella enterica (Typhimurium LT2)	1255427	II
Staphylococcus aureus (Mu50)	1122066	III
Staphylococcus epidermidis (ATCC 12228)	1057224	III
Stenotrophomonas maltophilia (K279a)	6394810	II
Streptococcus pneumoniae (TIGR4)	930383	III
Vibrio cholerae (O1 El Tor N16961)	2613025	III
Vibrio cholerae (O1 El Tor N16961)	2614464	II
Vibrio vulnificus (CMCP6)	1177596	III
Vibrio vulnificus (CMCP6)	1177966	II
Yersinia pestis (CO92)	1173384	III
Yersinia pestis (CO92)	1175125	I
Yersinia pestis (CO92)	1176738	II

CFU of *B. pseudomallei* strain 22 (also known as KHW), five times the established 50% lethal dose (LD_{50}) (20). Solutions of metsulfuron methyl were prepared fresh each day by dissolving the powder in DMSO (500 mg/ml) and then diluting into phosphate-buffered saline (PBS), pH 8.0 (5 mg/ml). Groups of 6 mice were fed 200 μl of either PBS, pH 8.0 (also containing 1% DMSO), or metsulfuron methyl twice daily using a gavage tube. The treatment was initiated 24 h before challenge and continued for 10 days after infection. All experimental procedures were carried out in a biosafety level 3 (BSL3) laboratory. The data shown are representative of three independent experiments.

P. aeruginosa experiments were based on guidelines from the National Advisory Committee for Laboratory Animal Research, Singapore, and were approved by the Institutional Animal Care and Use Committee, A*STAR, Singapore. Intratracheal instillation was carried out using a modification of a previously described method (21). FVB/N mice (8 to 10 weeks old) were anesthetized with a mixture of Hypnorm/Dormicum, after which the trachea was exposed through an anterior midline incision, and the inoculum of *P. aeruginosa* strain PAO1 (3×10^5 CFU in 50 μl of PBS for survival curves; 2×10^5 CFU in 50 μl of PBS for lung CFU) was delivered just beneath the cricoid cartilage. Metsulfuron methyl (5 mg/ml) or PBS was injected through the tail vein immediately after intratracheal infection. The survival data are representative of four independent experiments. To determine CFU present in the lungs, lung homogenates were spread on LB agar plates and incubated at 37°C overnight. These experiments were performed first with 12 mice per group and a second time with 10 mice per group. Separately or combined, the results showed a statistically significant difference ($P < 0.01$; Mann-Whitney rank-sum test).

RESULTS AND DISCUSSION

To determine the prevalence of AHAS isozymes across a sample of clinically relevant bacteria, we performed a phylogenetic analysis on the AHAS catalytic subunit, which contains the SH binding site (2, 16). As seen previously (13, 22, 23), type I AHAS was exclusively present in *Enterobacteriaceae* (Fig. 1). Notably, almost all the other bacteria in our analysis, including pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Staphylococcus aureus*, encoded only a type III AHAS (Fig. 1). These findings suggest that BCAA biosynthesis in certain human pathogens may be sensitive to SHs.

MIC assays using SHs were carried out in chemically defined media lacking BCAAs to test *B. pseudomallei* (20, 24), the opportunistic Gram-negative pathogen *P. aeruginosa* (25), and the avirulent soil-resident bacterium *Burkholderia thailandensis* (26). Chlorimuron ethyl and metsulfuron methyl were consistently the most effective SH tested (Table 2). Both SHs were bacteriostatic. The MIC_{50} of both chlorimuron ethyl and metsulfuron methyl on a panel of *P. aeruginosa* clinical isolates was 62.5 μM (Fig. 2A). The MIC_{90} for chlorimuron ethyl was 125 μM and 250 μM for metsulfuron methyl. Addition of BCAAs to the cultures rendered SHs ineffective, consistent with SHs specifically inhibiting AHAS. As it is difficult to compare these MIC values against standard MIC values generated in rich media, chloramphenicol, a clinically effective antibiotic (5), was also tested and found to have similar levels of inhibitory activity (Table 2). Due to the atypical nature of these assay conditions though, it is difficult to predict how SHs would compare against a wider range of antibiotics under more established conditions.

Chlorimuron ethyl and metsulfuron methyl also slowed the growth of *A. baumannii* (Fig. 2B), another opportunistic Gram-negative pathogen and major source of nosocomial infections (27). To study the Gram-positive bacterium *B. subtilis*, MIC assays were performed using Spizizen's minimal medium (18) with additives as described by Nakano et al. (19). SHs again inhibited growth (Table 2), with chlorimuron ethyl being especially potent. Although testing additional bacterial species would be desirable, it is not trivial to determine the proper growth conditions, as chemically defined media lacking in BCAAs are not readily available for most species.

Two mouse infection studies were performed. Administration of metsulfuron methyl (50 mg/kg) orally twice a day to

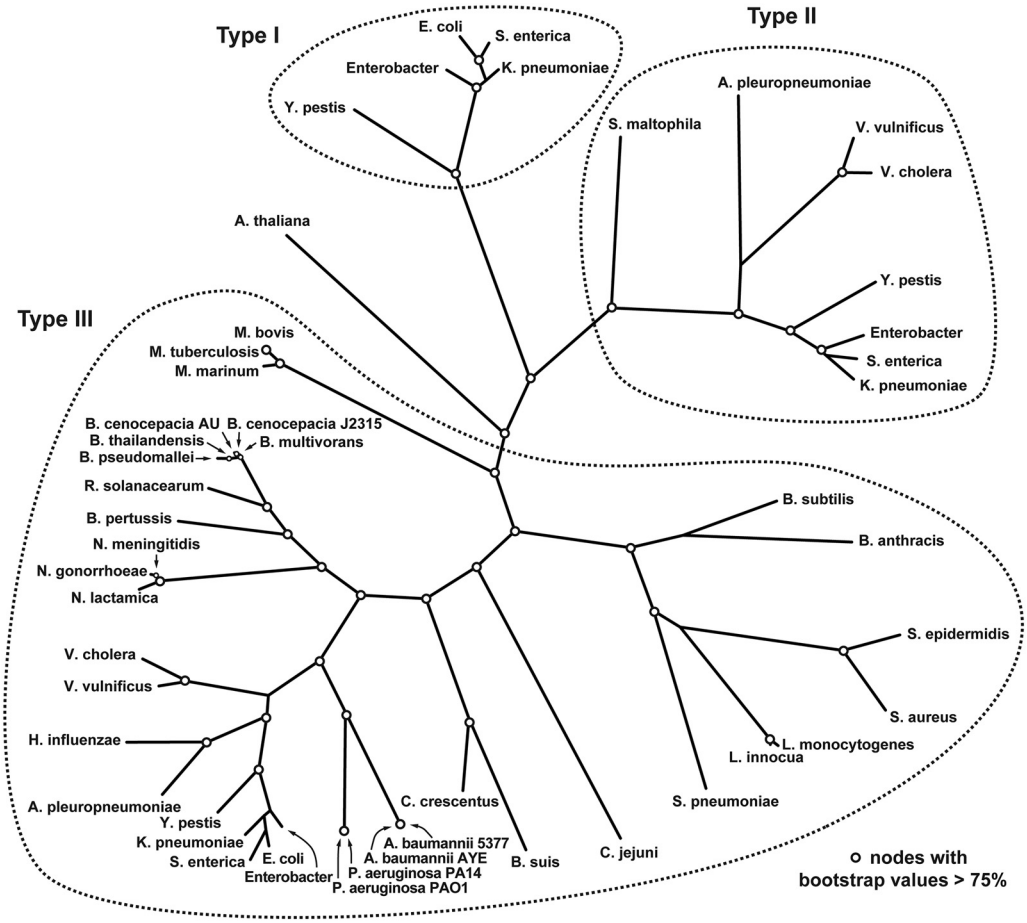

FIG 1 Type II and III AHAS are the sole AHAS isozymes present in many pathogenic bacteria. A phylogenetic tree of the catalytic subunit of AHAS was constructed using maximal likelihood methods. Bootstrap analysis was performed on 100 trees, and nodes appearing more than 75 times are indicated with a circle. The clusters formed by each isozyme are circled.

mice infected intranasally with *B. pseudomallei* led to a statistically significant increase in survival (Fig. 3A; $P < 0.01$; log rank test, Yates corrected). Upon cessation of treatment, all of the metsulfuron methyl-treated mice remained alive for the extent of follow-up, an additional 10 days. Mice infected intratracheally with *P. aeruginosa* and then given a single tail vein injection of metsulfuron methyl (200 mg/kg) survived more readily than mice injected with PBS (Fig. 3B; $P < 0.01$; log rank test, Yates corrected). Lungs harvested from the metsulfuron methyl-treated mice 24 h after infection had a reduced bacterial load (Fig. 3C; $P < 0.01$; Mann-Whitney rank-sum test). In this acute infection model, all of the mice alive on day 6 had cleared the infection, and no additional mortality was observed.

Based on this, we propose that developing inhibitors of bacterial type II and III AHAS may represent a promising strategy for new antibiotics. Just as SHs have been designed for targeted agricultural applications (2), medicinal chemists could modify

TABLE 2 MICs in minimal medium of SHs and chloramphenicol

Gram stain result	Organism	Strain	MIC (μM)[a]					
			Ce	Me	Bm	Rim	Sm	Cm
Gram-negative human pathogen	*B. pseudomallei*	K96423	31.2	31.2	125	125	250	40
	B. pseudomallei	22	31.2	62.5	62.5	125	250	80
	P. aeruginosa	PAO1	62.5	62.5	62.5	62.5	250	40
	P. aeruginosa	PA14	15.6	31.2	31.2	31.2	62.5	20
Gram negative	*B. thailandensis*	ATCC 700388	62.5	125	125	250	>500	40
Gram positive	*B. subtilis*	168	3.12	31.2	62.5	NT	NT	5

[a] Assays were repeated multiple times for each strain, and the median value is reported. Ce, chlorimuron ethyl; Me, metsulfuron methyl; Bm, bensulfuron methyl; Rim, rimsulfuron; Sm, sulfometuron methyl; Cm, chloramphenicol; NT, not tested.

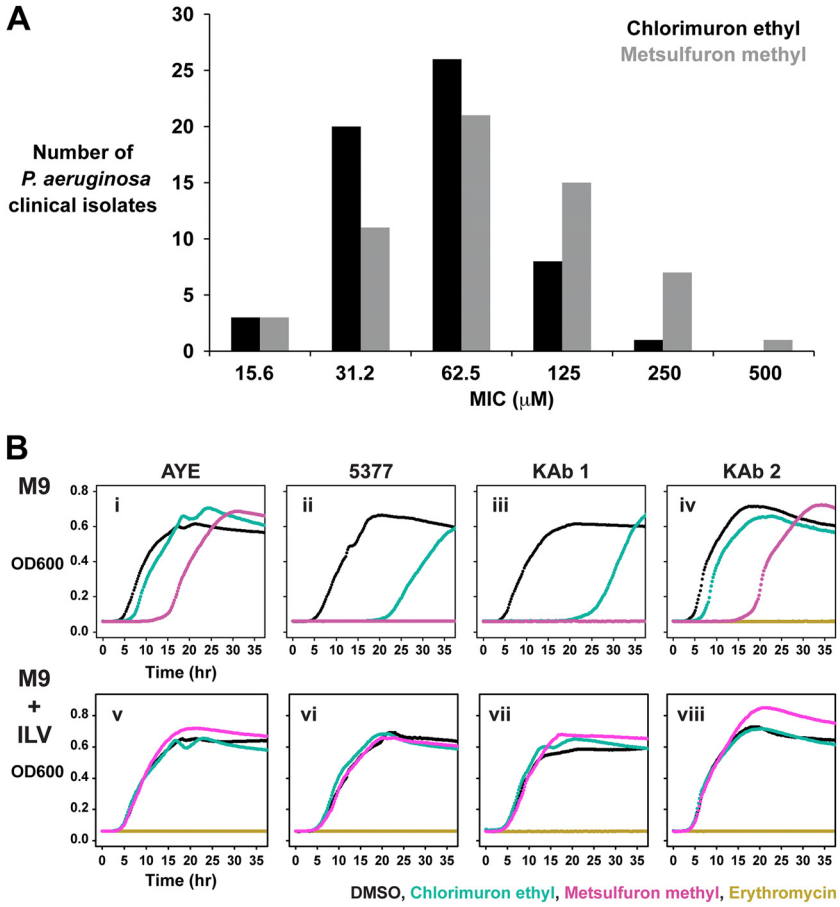

FIG 2 Clinical isolates of *P. aeruginosa* and *A. baumannii* are sensitive to SHs. (A) MIC values were determined for both chlorimuron ethyl and metsulfuron methyl against a panel of 58 local *P. aeuruginosa* clinical isolates. (B) Two reference strains, AYE (i, v) (37) and 5377 (ii, vi) (38), and two local clinical isolates, KAb 1 (iii, vii) and KAb 2 (iv, viii), of *A. baumannii* were treated with DMSO (1%), chlorimuron ethyl (2 mM), metsulfuron methyl (2 mM), or erythromycin (20 μM), and high-resolution growth curves were obtained with OD_{600} readings collected every 12 min. In panels i to iv, no BCAAs were added to the M9 medium, whereas in panels v to viii, the cultures contained leucine, isoleucine, and valine (each 75 μg/ml). AYE and KAb 2 are multidrug-resistant strains.

SHs to potentially increase potency, bioavailability, or specificity.

Some toxicity information on SHs is publically available (28), including a technical bulletin issued by DuPont on metsulfuron methyl (Escort XP Technical Bulletin, K-14796, 2007), which reports that acute oral toxicity in rats is >5,000 mg/kg and that in an 18-month dietary study in mice, no observed adverse effects were seen at 666 mg/kg/day, the highest dose tested. Uninfected mice treated with metsulfuron methyl here showed no indications of adverse effects. However, detailed safety data for intravenous administration of SHs is currently unknown.

As with all antimicrobials, resistance is of great concern. Not surprisingly, the emergence of SH-resistant weeds is a significant problem in the agricultural community (29). In *P. aeruginosa*, *B. thailandensis*, and *B. subtilis*, we found that spontaneous mutants resistant to chlorimuron ethyl occurred at a frequency of approx-

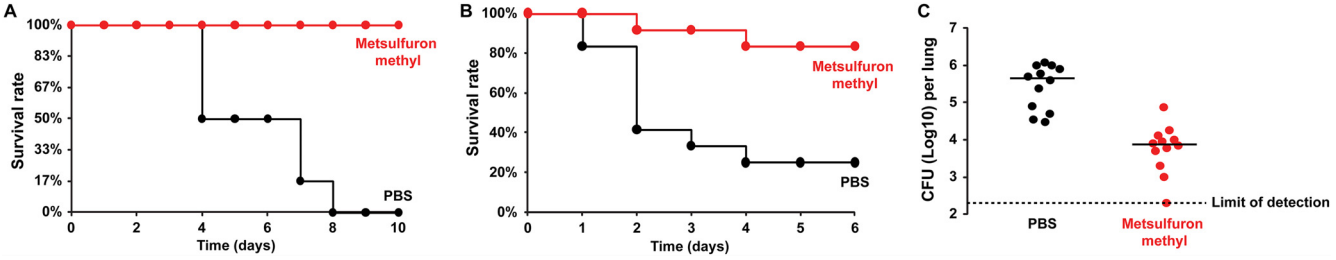

FIG 3 SHs increase mouse survival in response to an otherwise lethal challenge by *B. pseudomallei* or *P. aeruginosa*. (A) The survival curves of BALB/c mice (n = 6 per group) inoculated through the intranasal route with *B. pseudomallei* strain 22 (20) and orally treated twice daily with either PBS or metsulfuron methyl (50 mg/kg). Survival curves (B) or CFU (C) present in the lungs of FVB/N mice (n = 12 per group in both experiments) inoculated intratracheally with *P. aeruginosa* strain PAO1 and given a single tail vein injection with PBS or metsulfuron methyl (200 mg/kg).

imately 10^{-8}. Similar to plants (2, 29), a single mutation in the catalytic subunit of AHAS was sufficient to confer resistance to chlorimuron ethyl and other SHs. To counter the rapid emergence of single-step, high-level SH-resistant mutants, AHAS inhibitors could be used in combination with other antibiotics (30) or inhibitors of BCAA biosynthesis (31–33).

There are approximately 30 to 40 biosynthetic enzymes present in microbes but absent in humans that are responsible for the production of essential amino acids. Auxotrophs for some of these are attenuated (34–36), suggesting that these pathways might be fruitful targets for future small-molecule discovery.

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J.F.K. and P.T. have applied for a patent covering the medical use of sulfonylurea herbicides (Singapore patent application 201008793-0).

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