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Compound Uptake into *E. coli* Can Be Facilitated by *N*-Alkyl Guanidiniums and Pyridiniums

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Abstract

Multidrug-resistant Gram-negative bacterial infections are on the rise, and with no FDA approvals for new classes of broad-spectrum antibiotics in over 50 years, these infections constitute a major threat to human health. A significant challenge is the inability of most compounds to accumulate in Gram-negative bacteria. Recently developed predictive guidelines show that appending a primary amine to an appropriately shaped compound can enhance Gram-negative accumulation. Here, we report that other positively charged nitrogen functional groups, namely, *N*-alkyl guanidiniums and pyridiniums, can also facilitate compound uptake into Gram-negative bacteria. The accumulation of a set of 60 nonantibiotic compounds, consisting of 20 primary amines and their corresponding guanidiniums and pyridiniums, was assessed in *Escherichia coli*. We also installed these alternate functional groups onto antibiotic scaffolds and assessed their accumulation and antibacterial activity in Gram-negative bacteria. The results suggest that other positively-charged, nitrogen-containing functional groups should be considered when designing antibiotics with Gram-negative activity.

Graphical Abstract

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Supporting Information

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Supporting figures and tables, materials and methods, experimental procedures, and characterization of chemical products including spectra (PDF)

Source Data, Figures 5 and S2 (XLSX)

Source Data, Figures 7 and S3 (XLSX)

The authors declare no competing financial interest.

correct shape and flexibility (**6DNM**, glob = 0.04 and RB = 0; **Debio-1452**, glob = 0.09 and RB = 4; **Ribocil C**, glob = 0.06 and RB = 5) but have no appreciable accumulation in *E. coli* and no activity against Gram-negative pathogens. Appending a primary amine at locations that do not disturb target engagement results in **6DNM-NH3**, **Debio-1452-NH3**, and **Ribocil C-PA** (Figure 1), compounds that now accumulate in *E. coli* and exhibit antibacterial activity against a variety of Gram-negative pathogens. In addition, others have constructed compounds, guided by the eNTRY rules, which have enhanced the accumulation and/or activity in Gram-negative bacteria (Figure 1, selected examples), including inhibitors of CpxA phosphatase,¹⁶ DNA gyrase,¹⁷ microbial ribosomes,¹⁸ LpxC,¹⁹ Topo IV,²⁰ and biotin carboxylase.²¹

The addition of a primary amine to a small molecule represents a large change in polarity through the increase in the number of hydrogen-bond donors/acceptors (HBDs/HBAs), and amines can provide advantages beyond Gram-negative bacterial accumulation including enhanced solubility and reduced protein binding.²² However, the addition of this ionizable nitrogen can be deleterious to target engagement (depending on the particular target and binding site), resulting in the loss of antibacterial activity. In addition, while there are over two dozen antibiotics that are safe in humans that contain primary amines,²³ like most functional groups, primary amines can have toxic liabilities in certain contexts.²² As detailed herein, we now investigate the ability of other positively charged nitrogen species to facilitate accumulation in Gram-negative bacteria. In particular, *N*-alkyl guanidiniums and pyridiniums were chosen for evaluation due to their varied lipophilicity and charge distribution compared to the parent amines, shown by the electrostatic potential maps in Figure 2A. In addition, both modifications are easily accessible directly from primary amines in 1–2 steps, allowing for direct head-to-head comparisons, and multiple FDA approved drugs contain these motifs (Figure 2B, selected examples), most notably the β -lactam ceftazidime. The demonstration that these functional groups, and perhaps other positively charged nitrogen species, facilitate accumulation gives a wider array of options to consider when designing and constructing compounds with activity against Gram-negative pathogens.

RESULTS

To investigate the ability of various positively charged nitrogen species to facilitate Gram-negative accumulation, a diverse set of primary amine containing compounds was sought that could be converted into *N*-alkyl guanidiniums and pyridiniums. Compounds were chosen that comprised a range of globularity scores (glob = 0.04 to 0.35) and number of rotatable bonds (RB = 0 to 8) that stretch just beyond the eNTRY rule cutoffs. Compounds were also evaluated for their amphiphilic moment (vsurf_A, Table S1), as a higher amphiphilic moment was previously determined to be important for accumulation across structural classes.¹² Finally, descriptors pertaining to positive charge and HBD were calculated to investigate the effects of charge delocalization and hydrogen bonding on accumulation.

Primary amines (denoted as the “A” series) were obtained either via the “complexity-to-diversity”^{24,25} strategy in 5 steps (**1A–15A**) or from commercial vendors (**16A–20A**),

resulting in a range of natural-product-like and drug-like compounds (Figure 3A). The majority of guanidiniums (denoted as the “G” series) were prepared from the primary amine via a two-step procedure using diprotected guanidine triflates (Figure 3B, **1G–20G**). Pyridiniums (denoted as the “P” series) were typically synthesized directly from the primary amine using the Zincke reaction followed by purification through reverse-phase chromatography (Figure 3B, **1P–20P**). While the Zincke reaction requires long reaction times by conventional heating (refluxing in butanol for 24 h), reaction times were dramatically shortened (< 1 h) by subjecting substrates to microwave irradiation at 130 °C.²⁶ For the construction of a handful of compounds, if the corresponding alcohol was more accessible, alternate synthetic routes were followed: guanidiniums via Mitsunobu displacement with diprotected guanidines (**4G**) and pyridiniums via *in situ* activation of the corresponding alcohol followed by nucleophilic displacement with pyridine (**8P** and **9P**) (see the Supporting Information for synthetic schemes and details).

All amines, guanidiniums, and pyridiniums were assessed for accumulation in wild-type (wT) *E. coli* MG1655 (Figure 3C) using the standard liquid chromatography with tandem mass spectrometry (LC-MS/MS) assay previously reported from our laboratory^{12,14,15} and also utilized by others;^{27–32} see Table S1 for all accumulation values. The majority of primary amines (80%) and guanidiniums (85%) accumulate in WT *E. coli* to a statistically significant degree, while a markedly lower number of pyridiniums accumulate (25%). Consistent with the eNTRY rules, primary amines that possess a lower globularity and/or number of rotatable bonds accumulated while those with higher values did not. For example, **1A** (glob = 0.13, RB = 3) accumulates while **10A** (glob = 0.20, RB = 7) and **11A** (glob = 0.30, RB = 4) do not. It should be noted that the accumulation measured in WT *E. coli* is net accumulation. Further studies would be necessary to investigate the relationship between positive charge and efflux, for example.

The accumulation of guanidinium derivatives closely mirrors that of the parent amines, with the degree of accumulation generally correlating with the change in amphiphilic moment (Figure 4A). For example, accumulation increases from biphenyl amine **18A** (Accum. = 2842 ± 815 , vsurf_A = 5.38) to guanidinium **18G** (Accum. = 4736 ± 300 , vsurf_A = 6.48), while decreasing from isomannide amine **15A** (Accum. = 1647 ± 108 , vsurf_A = 5.00) to guanidinium **15G** (Accum. = 1045 ± 6 , vsurf_A = 4.12). In addition, in contrast to what is observed with primary amine accumulation, the addition of two rotatable bonds associated with the replacement of a primary amine with a guanidinium moiety did not adversely affect compound uptake. In fact, 80% of all guanidiniums tested with ≥ 6 rotatable bonds accumulate to a statistically significant degree (Figure 4B, selected examples), including multiple examples with eight rotatable bonds.

In contrast, far fewer of the alkylpyridinium derivatives (5/20) show statistically significant levels of accumulation (Figure 4C, selected examples). For the five accumulators, the degree to which they accumulate is lower as compared to their primary amine and guanidinium counterparts, with none greater than 1000 nmol/10¹² colony forming units (CFUs), much less approaching some of the values observed with the amines and guanidiniums; see Table S1 for all values. With only five pyridiniums accumulating, conclusions as to what

physicochemical properties govern their degree of accumulation compared to the parent amines cannot be drawn.

The overall accumulation trends indicate that a large increase in positive charge delocalization, such as in *N*-alkylpyridiniums, is detrimental to compound accumulation in WT *E. coli*. This is shown empirically moving from the high accumulating amines and guanidiums to the low accumulating pyridiniums. To quantify this phenomenon, the electrostatic potential energy maximum (ESPMAX) descriptor, developed by the Denmark laboratory to probe through-bond electronic effects of substituents on a core molecule containing a single cationic species,^{33,34} was calculated for all 60 compounds from Figure 3. Originally developed to correlate with Hammett parameters, the trend in ESPMAX values corresponds to the expected charge delocalization within this set of 60 compounds (Figure 5A). The overlap of the amine and guanidinium ESPMAX values mimics the small difference in delocalization between the two functional groups and is reflected in their similar overall accumulation percentages (80% and 85%, respectively). In contrast, the highly delocalized charge of the pyridiniums shows much lower ESPMAX values, and the pyridiniums are completely separated from their amine and guanidinium counterparts. This trend is also observed within structural classes; for example (Figure 5C), accumulation decreases from amine **5A** (Accum. = 1696 ± 203 , ESPMAX = 154.5) to guanidinium **5G** (Accum. = 830 ± 95 , ESPMAX = 148.8) to pyridinium **5P** (Accum. = 227 ± 13 , ESPMAX = 124.4). Indeed, the full data set shows a positive correlation between ESPMAX and accumulation (Pearson's correlation coefficient, $r = 0.47$, $p = 0.0002$; see the Source Data File for Figures 5 and S2 for full details).

Having observed the importance of through-bond electrostatic properties on accumulation as shown by ESPMAX, we also investigated through-space interactions by assessing the HBD capabilities of the 60-member collection. The sum of the HBD strengths descriptor, h_emd (proportional to the sum of the neighbor-corrected hydrogen partial atomic charges),³⁵ shows a similar, though slightly lower, positive correlation ($r = 0.32$, $p = 0.01$) with accumulation compared to ESPMAX (Figure 5B), with the high accumulating amine/guanidinium group separated from the low accumulating pyridinium group. This trend is also observed in a similar manner to ESPMAX across structural classes (Figure 5C). Accumulation decreases from amine **5A** (Accum. = 1696 ± 203 , $h_emd = 26.1$) to guanidinium **5G** (Accum. = 830 ± 95 , $h_emd = 22.8$) to pyridinium **5P** (Accum. = 227 ± 13 , $h_emd = 12.1$). The ESPMAX and h_emd results suggest that delocalizing the charge too much and removing HBDs are detrimental to accumulation. This result is consistent with previous steered molecular dynamic simulations of compound translocation through the OmpF porin in *E. coli*, which suggested that key interactions of positively charged primary amines with Asp113 enable passage through the constriction site.^{12,36}

With promising accumulation trends for guanidiniums and pyridiniums within a collection of nonantibiotic small molecules, an assessment was made to determine if these functional groups could facilitate conversions of Gram-positive antibiotics to broad-spectrum agents in a manner analogous to primary amines (e.g., Figure 1). Six structurally diverse Gram-positive-only antibiotics were chosen (Figures 6 and S1). These compounds have different biological targets and range from preclinical investigative compounds (e.g., **6-azaindazole**)

to those in clinical trials (e.g., **Debio-1452**). Of particular interest were scaffolds in which the addition of a primary amine increased accumulation in Gram-negative bacteria, allowing valuable side-by-side accumulation comparisons even in the event of low biological activity due to the loss of target engagement. The synthesis of all antibiotics and derivative primary amines, guanidiniums, and pyridiniums is described in the Supporting Information. Antibacterial activity was assessed in three strains, WT *S. aureus*, WT *E. coli*, and *E. coli tolC* (an efflux deficient mutant that is porin proficient), with WT *S. aureus* and *E. coli tolC* acting as surrogates for ligand–target engagement.

Debio-1452 (21), Figure 6A) is an inhibitor of the enoyl–acyl carrier reductase enzyme FabI, and a pro-drug version is currently in Phase 2 clinical trials for the treatment of Gram-positive bacterial infections.⁷ As demonstrated recently (Figure 1),¹⁴ the addition of a primary amine to the 3-position of the naphthyridinone ring successfully imparts Gram-negative antibacterial activity against strains of *E. coli*, *E. cloacae*, *K. pneumoniae*, and *A. baumannii*. To avoid aromatization of the naphthyridinone resulting from the elimination of a better leaving group (e.g., pyridinium), the ethyl-linked compound (**22**, Figure 6A), which also shows antibacterial activity against *E. coli* (minimum inhibitory concentration, MIC = 8 $\mu\text{g/mL}$),¹⁴ was chosen as a starting point, and guanidinium **23** and pyridinium **24** were synthesized in the manner described in Figure 3. Compounds **23** and **24** showed statistically significant accumulation in *E. coli*, with guanidinium **23** accumulating to higher levels than amine **22** (Figure 6A). Both retained potent activity in *S. aureus* and some activity in the efflux deficient strain of *E. coli tolC*. When assessed for antibacterial activity in WT *E. coli*, compounds **23** and **24** showed 8-fold lower activity (MIC = 64 $\mu\text{g/mL}$, Figure 6A) relative to the ethylamine compound.

6DNM and **6DNM-NH3 (25 and 26)**, Figure 6B) are previously reported DNA gyrase inhibitors.¹² While active in *S. aureus* and *E. coli tolC*, **6DNM** is not active in WT *E. coli*, consistent with its lack of accumulation (Figure 6B). **6DNM-NH3** on the other hand accumulates well in WT *E. coli* and shows good activity (MIC = 0.5 $\mu\text{g/mL}$) while maintaining activity in *S. aureus* and *E. coli tolC* (Figure 6B). The evaluation of guanidinium **27** and pyridinium **28** showed low accumulation and activity in WT *E. coli* (Figure 6B). While guanidinium **27** shows moderate activity in *S. aureus* and *E. coli tolC*, pyridinium **28** shows poor activity, presumably due to lower target engagement.

The **Zantrins** (Figure 6C) are putative inhibitors of FtsZ, an essential protein involved in bacterial cell division.³⁷ Due to the poor solubility of the parent benzoquinazolines (**Zantrin Z3**, Figure S1), we focused our attention on the truncated 6-methylquinazoline derivatives, which have been reported with comparable activities.^{37,38} The parent diethylamino **29** is moderately active in *S. aureus* (MIC = 16 $\mu\text{g/mL}$) but not in WT *E. coli* (MIC = >64 $\mu\text{g/mL}$). As expected, reduction of the number of rotatable bonds and transition to the primary amine (**31**) resulted in high accumulation in WT *E. coli* as well as an increase in activity, while acetamide **30** showed neither accumulation nor activity. Guanidinium **32** also accumulates and, excitingly, has an activity only 2-fold lower than that of primary amine **31** (Figure 6C). In contrast, pyridinium **33**, while exhibiting modest accumulation, is not active in WT *E. coli* (Figure 6C); **33** has modest activity in *S. aureus* and *E. coli tolC* (Figure 6C).

The introduction of a primary amine and the corresponding increase in charge and number of HBDs is not always beneficial to target engagement. This is exemplified by our attempt to broaden the activity of the bacterial DNA ligase (LigA) inhibitor **6-azaindazole (34)**, Figure 6D) beyond *S. aureus* (MIC = 4 $\mu\text{g/mL}$).³⁹ Using the crystal structure of **34** bound to LigA,³⁹ we postulated that the introduction of the primary amine in place of the solvent exposed terminal hydroxyl group would provide the best opportunity for new activity. Consistent with literature values and its lack of accumulation in WT *E. coli*, **34** is active in *S. aureus*, inactive in WT *E. coli*, and active in *E. coli tolC* (Figure 6). However, while amine **35** accumulates very well in WT *E. coli* (Figure 6D), it is inactive in all three strains, indicating that the primary amine is disrupting target engagement. Despite amine **35** lacking activity, we were curious if either guanidinium **36** or pyridinium **37** could rescue activity. Both **36** and **37** accumulate in WT *E. coli* to a statistically significant degree (Figure 6D), though the pyridinium only modestly. Similar to amine **35**, pyridinium **37** is inactive in all three strains. However, while exhibiting no activity in WT *E. coli*, guanidinium **36** does show activity in *S. aureus* and *E. coli tolC*, albeit 4-fold lower than the parent compound (Figure 6D). A similar trend is observed when the amine, guanidinium, and pyridinium are homologated by one carbon (**S5–S7**, Table S2). Though not active in WT *E. coli*, these results suggest that, in cases where the primary amine fails to deliver target engagement, alkyl guanidiniums, and perhaps other positively charged nitrogen functional groups, could be viable alternatives.

Ribocil C (38), Figure 6E) is an inhibitor of the flavin mononucleotide riboswitch originally identified through a cell-based screen using an *E. coli* strain with permeability defects.⁴⁰ As recently reported, appending a propargyl amine to the unadorned pyrimidine greatly enhances the accumulation and activity in WT *E. coli* (compound ($\pm$)**39**, Figure 6E) while **Ribocil C (38)** and acetamide ($\pm$)**S8** (Table S2) do not accumulate and are not active against Gram-negative pathogens.¹⁵ Guanidinium ($\pm$)**40** accumulates to a similar degree as amine ($\pm$)**39** but shows 16-fold reduced activity in *E. coli tolC* and 8-fold reduced activity in WT *E. coli* (Figure 6E). This loss in activity is likely due to diminished target engagement, similar to that of the **Debio** derivatives. The synthesis of propargylpyridinium ($\pm$)**41** was unsuccessful via both the Zincke reaction and pyridine displacement, both of which led to decomposition of the starting material. Benzylic amine derivatives were also accessed (($\pm$)**S9**–($\pm$)**S11**, Table S2); however, while all showed accumulation in WT *E. coli*, none were active. Finally, though **Ribocil C** is known to kill *S. aureus* in culture, Gram-positive bacteria are able to scavenge riboflavin from the surrounding environment, and so, **38–40** were not tested in *S. aureus* 29213.^{15,41}

Another example where the addition of an amine has enhanced Gram-negative accumulation and activity is the Leucyl-tRNA synthetase inhibitor ($\pm$)**Epetraborole** (($\pm$)**42**, Figure 6F).⁴² While it is possible to append the guanidinium and pyridinium moieties to the primary amine of ($\pm$)**42** (see the Supporting Information), the examination of the crystal structure and literature SAR indicate that any bulky substituent at that position, even an α -methyl group, will disrupt the target engagement.⁴³ This was confirmed after testing of the guanidinium and pyridinium derivatives of ($\pm$)**42** revealed complete loss of activity in *S. aureus*, WT *E. coli*, and *E. coli tolC* (**S12** and **S13**, Table S2). Thus, we sought to construct derivatives at a position that might be less disruptive to target engagement. Amine **44**

(Figure 6F) accumulates well in WT *E. coli* and displays a similar activity (MIC = 2 $\mu\text{g/mL}$) to ($\pm$)**42**. The replacement of the amine with a hydroxyl group (**43**) or acetamide (**S14**, Table S2) results in slightly lower, but still appreciable, accumulation and 4-fold lower activity in WT *E. coli*; this is consistent with the known differences in activity of these compounds in lab strains vs clinical isolates.^{13,42} Guanidinium **45** accumulates to half the extent of amine **44** and shows 32-fold reduced activity, though it is slightly better in *E. coli tolC* (Figure 6F), indicating the lower activity may be partially due to the lower accumulation in addition to the disrupted target engagement. Finally, pyridinium **46** does not accumulate to a statistically significant degree and correspondingly shows no activity.

DISCUSSION

The overall trends observed within both the nonantibiotic collection and antibiotic conversions suggest that the form of positively charged nitrogen that can facilitate accumulation in Gram-negative bacteria is not limited to primary amines. Indeed, guanidiniums show similar levels of accumulation to the amines, with 87% of nonantibiotic and antibiotic derivatives showing statistically significant accumulation. In certain cases, even the decreased localization of the positive charge within pyridiniums is enough to enhance Gram-negative accumulation (40% of nonantibiotics and antibiotics), though to a lower degree than either the amines or guanidiniums. With the exception of the form of positive charge, these motifs largely fit within the eNTRY rules and in certain cases help expand them: (1) the globularity of accumulating scaffolds do not exceed 0.25, (2) up to 8 rotatable bonds are possible with the guanidiniums compared to 5 with primary amines, and (3) increased amphiphilic moment is associated with increased accumulation with the guanidiniums when compared to the corresponding amines.

The guanidiniums in particular show promise when applied to antibiotic scaffolds. Three of the six antibiotic scaffolds in this study showed 8-fold or less change in activity in WT *E. coli* when the amine is replaced with the guanidinium motif (**Debio-1452**, **Zantrín Z3**, and **Ribocil C**). In particular, **Zantrín** derivative **32** showed almost equipotent activity when compared to amine **31**. In addition, 6-azaindazole guanidinium **36** is the first example where activity is recovered after the addition of the primary amine (**35**) completely perturbed target engagement, indicating that guanidiniums could be useful in situations where a primary amine is not tractable. Finally, the potential of guanidiniums in antibacterial drug discovery has been seen with DNA gyrase inhibitor **Pyrrole 8y**¹⁷ and β -lactamase inhibitors **VNRX-5133-Guan**⁴⁴ and **ANT2681**⁴⁵ (Figure 7A). Another advantage of *N*-alkyl guanidiniums and pyridiniums could be differential toxicity profiles relative to their amine counterparts; however, additional studies need to be performed to further evaluate this potential.

The correlation of the ESPMAX descriptor with the nonantibiotic accumulation trends indicates that it should be possible to predict other positively charged functional groups that can facilitate accumulation beyond those investigated here. Using a subset of the nonantibiotic scaffolds (Figure S3), ESPMAX values were calculated for 18 additional positively charged nitrogen functional groups (Figure 7B, selected examples; see Figure S3 for the full set). As expected, the resulting ranges correlate with charge delocalization, with

both the primary amines and guanidiniums falling in the top 5 and the pyridiniums at the bottom. Excitingly, this analysis indicates that isouroniums and isothiuroniums, in particular, could facilitate accumulation in Gram-negative bacteria. In addition, cyclizing the guanidiniums has the potential to add lipophilicity while still localizing the positive charge significantly, as shown in the recently published development of the NDM inhibitor **ANT2681** (Figure 7A) where the authors demonstrated similar activity of a guanidinium derivative to its cyclized counterpart.⁴⁵ An analysis using h_emd (Figure S3) shows a similar top 5 grouping to ESPMAX.

With the discovery that *N*-alkyl guanidiniums and pyridiniums can successfully facilitate compound uptake into *E. coli*, we anticipate that, in conjunction with the eNTRY rules, the addition of these functional groups (and other positively charged nitrogen species) will provide a wider array of options for consideration when building in Gram-negative antibacterial activity.

METHODS

Bacterial Strains.

S. aureus 29213 and *E. coli* MG1655 were obtained from the American Type Culture Collection (ATCC). *E. coli* BW25113 and *E. coli* JW5503 *tolC* were obtained from the KEIO Collection.

Antimicrobial Susceptibility Tests.

Susceptibility testing was performed in biological triplicate, using the microdilution broth method as outlined by the Clinical and Laboratory Standards Institute. Bacteria were cultured with cation-adjusted Mueller-Hinton broth (Sigma-Aldrich; catalogue number: 90922) or M9-MOPS media in round-bottom 96-well plates (Corning; catalogue number: 3788).

Accumulation Assay.

The accumulation assay^{12,14,15} was performed in triplicate in batches of ten samples, with each batch containing tetracycline as a positive control. *E. coli* MG1655 was used in these experiments. For each replicate, 2.5 mL of an overnight culture of *E. coli* was diluted into 250 mL of fresh Luria-Bertani (LB) broth (Lennox) and grown at 37 °C with shaking to an optical density (OD₆₀₀) of 0.55. The bacteria were pelleted at 3220 r.c.f. for 10 min at 4 °C, and the supernatant was discarded. The pellets were resuspended in 40 mL of phosphate buffered saline (PBS) and pelleted as before, and the supernatant was discarded. The pellets were resuspended in 8.8 mL of fresh PBS and aliquoted into ten, 1.5 mL Eppendorf tubes (875 μ L each). The number of colony-forming units (CFUs) was determined by a calibration curve. The samples were equilibrated at 37 °C with shaking for 5 min; compound was added (final concentration = 50 μ M), and then, the samples were incubated at 37 °C with shaking for either 10 min or 2 h (**Debio** derivatives). These time points were short enough to minimize metabolic and growth changes (no changes in OD₆₀₀ or CFU count observed). After incubation, 800 μ L of the cultures was carefully layered on 700 μ L of silicone oil (9:1 AR20/Sigma High Temperature, cooled to -78 °C). Bacteria were pelleted through the oil

by centrifuging at 13 000 r.c.f. for 2 min at room temperature (with the supernatant remaining above the oil); the supernatant and oil were then removed by pipetting. To lyse the samples, each pellet was dissolved in 200 μL of water, and then, they were subjected to three freeze–thaw cycles of 3 min in liquid nitrogen followed by 3 min in a water bath at 65 °C. The lysates were pelleted at 13 000 r.c.f. for 2 min at room temperature, and the supernatant was collected (180 μL). The debris was resuspended in 100 μL of methanol and pelleted as before. The supernatants were removed and combined with the previous supernatants collected. Finally, the remaining debris was removed by centrifuging at 20 000 r.c.f. for 10 min at room temperature. Supernatants were analyzed by LC–MS/MS.

Samples were analyzed with the 5500 QTRAP LC/MS/MS system (AB Sciex) with a 1200 series HPLC system (Agilent Technologies) including a degasser, an autosampler, and a binary pump. Liquid chromatography separation was performed on an Agilent SB-Aq column (4.6 $\times$ 50 mm, 5 μm) (Agilent Technologies) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.3 mL/min. The linear gradient was as follows: 0–3 min, 100% mobile phase A; 10–15 min, 2% mobile phase A; 15.5–21 min, 100% mobile phase A. The autosampler was set at 5 °C. The injection volume was 15 μL . Mass spectra were acquired with both positive electrospray ionization at the ion spray voltage of 5500 V and negative electrospray ionization at the ion spray voltage of –4500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 33, 50, and 65, respectively. Multiple reaction monitoring was used to quantify the metabolites. Power analysis was not used to determine the number of replicates. Error bars represent the standard error of the mean of three biological replicates. The statistical significance of the accumulation was determined using a two sample Welch's *t* test (one-tailed test, assuming unequal variance) relative to the negative controls. All compounds evaluated in the biological assays were 95% pure.

Calculation of Physiochemical Properties.

Data sets of chemical structures were created and managed using Maestro.⁴⁶ Initial structure preparation and 3D minimization were performed with LigPrep⁴⁷ using OPLS_2005 force fields. The generation of ensembles of conformations was performed using Macromodel,⁴⁸ using the OPLS3 force field, with solvation (water). The search method used was the mixed torsional/Lowmode sampling, and the energy cutoff was set to 10 kcal/mol. All other options were left as defaults. Physiochemical descriptors (both 2D- and 3D-based) were calculated using MOE 2015.10⁴⁹ for each conformation. Descriptors were averaged (unweighted mean) across all conformations for each molecule.

Calculation of ESPMAX.

The procedure to calculate ESPMAX was adapted from Zahrt et al.³⁴ First, Macromodel⁴⁸ was used to calculate conformer distributions for each molecule. The calculation was done with the OPLS3 force field with solvation (water), using a 10 kcal/mol window; all other options were left as defaults, and the lowest energy conformer was kept. Second, the geometry of each compound was optimized using the semiempirical method at the PM3 level of theory using Spartan[®] 18.⁵⁰ Third, an electrostatic potential interaction field (MIF) was calculated for each compound using NWChem⁵¹ with DFT (B3LYP/G-31G* level of

theory). After the energies were calculated, the minimum and maximum MIF values were saved giving ESPMIN and ESPMAX, respectively.

Materials and Methods for Chemical Synthesis.

Chemical reagents were purchased from commercial sources and used without further purification. Anhydrous solvents were either purchased from commercial suppliers or dried after being passed through columns packed with activated alumina under the positive pressure of nitrogen using a PureSolv MD-5 (Inert, previously Innovative Technology Inc.) solvent purification system. The automated purification of guanidinium and pyridinium salts was performed on a Biotage Isolera using Agela Technologies AQ C18 spherical 20–35 μ m 100A columns (12 and 40 g cartridges, 12 and 25 mL/min, respectively) with a gradient elution of H₂O/MeCN containing 0.1% formic acid or acetic acid. The final compounds were dried via lyophilization (Labconco FreeZone 2.5 L) to remove any residual solvents. Microwave reactions were performed using an Anton Paar Monowave 400 Microwave Synthesis Reactor. ¹H NMR, ¹³C NMR, and 2D NMR experiments for prepared intermediates and products were recorded on a Bruker Advance III HD 500 MHz NMR system equipped with a CryoProbe, a Varian Unity Inova 600 MHz NMR system equipped with an autoX broadband probe, or an Agilent Premium Compact Shield 600 MHz NMR system equipped with a PFG OneNMR Probe. Spectra were obtained in the following solvents with reference peaks included for ¹H and ¹³C NMRs: CDCl₃ (¹H NMR 7.26 ppm; ¹³C NMR 77.16 ppm), DMSO-*d*₆ (¹H NMR 2.50 ppm; ¹³C NMR 39.52 ppm), CD₃OD (¹H NMR: 3.30 ppm; ¹³C NMR: 49.00 ppm), D₂O (¹H NMR: 4.79 ppm). Chemical shift values are expressed in ppm (δ), coupling constants (*J*, Hz) and peak patterns are reported as broad singlet (bs), singlet (s), doublet (d), triplet (t), quartet (q), pentet (p), heptet (hept), and multiplet (m). High resolution mass spectra (HRMS) were obtained in the School of Chemical Sciences Mass Spectrometry Laboratory on a Waters Q-TOF Ultima quadrupole time-of-flight spectrometer using electrospray ionization ESI. All compounds were purified to >95% as assessed by an Agilent Technologies 1290 Infinity II UHPLC equipped with a Phenomenex Kinetex C18 column (2.1 mm ID $\times$ 50 mm, 1.7 μ m particle size, 100 Å pore size) and ¹H NMR.

General Procedure for the Synthesis of *N*-Alkyl Guanidiniums.

Primary amine (1 equiv) and triethylamine (2.2–4 equiv) were dissolved in CH₂Cl₂ (0.05 M). Guanidinylation reagent **S15** (1.5 equiv) was added, and the reaction mixture was stirred at room temperature for 16–24 h. The crude reaction mixture was passed through a plug of silica and eluted with CH₂Cl₂ followed by 5% MeOH/CH₂Cl₂, and the MeOH/CH₂Cl₂ portion was concentrated. The resulting residue was dissolved in 4 M HCl in dioxane (0.1 M) or TFA in CH₂Cl₂ and stirred at room temperature for 4–16 h. The reaction mixture was concentrated from Et₂O 3 $\times$. The resulting residue was purified by C18 chromatography (0.1% formic acid or acetic acid) to yield the desired product after lyophilization.

General Procedure for the Synthesis of *N*-Alkylpyridiniums.

In a G4 or G10 MW vial under air, the primary amine (1.2–2 equiv) and Zincke reagent **S14** (1 equiv) were dissolved in a 2:1 mixture of ethanol/water (0.2 M). The reaction vessel was

sealed and irradiated at 130 °C for 1 h. The reaction was cooled to room temperature and diluted with water and ethyl acetate. The layers were separated, and the aqueous layer was washed with ethyl acetate (3×) and dichloromethane (1×) and concentrated *in vacuo*. The resulting residue was purified by trituration with CH₂Cl₂ or C18 chromatography (0.1% formic acid or acetic acid) followed by lyophilization to yield the desired product.

Please see the Supporting Information for full details of the synthetic procedures and compound characterization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

MDR	multidrug-resistant
CFUs	colony forming units
CLSI	Clinical and Laboratory Standards Institute
MIC	minimum inhibitory concentration
HBDs	hydrogen bond donors
HBAs	hydrogen bond acceptors
WT	wild-type
LC-MS/MS	liquid chromatography with tandem mass spectrometry
ESPMAX	electrostatic potential energy maximum
h_emd	sum of hydrogen bond donor strengths
LigA	DNA ligase inhibitor

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Figure 1.

eNTRY rules have been used to enhance the Gram-negative accumulation and antibacterial activity of Gram-positive only antibiotics in multiple contexts. **6DNM**,¹² **Debio-1452**,¹⁴ **Ribocil C**,¹⁵ **AZ17**,²⁰ **Pyrrole 8v**,¹⁷ and **CDCHD-FuA**.¹⁸ Accumulation measured in nmol/10¹² CFUs in *E. coli* MG1655.

Figure 2.
(A) Functional groups with ionizable nitrogens and their electrostatic representations. (B)
Some FDA approved drugs containing primary alkyl guanidiniums and alkylpyridiniums.

Figure 3.

(A) 20 primary amines evaluated for accumulation in *E. coli*, along with their guanidinium and pyridinium derivatives. (B) A two-step guanidinylation or Zincke reaction was used in most cases to construct the corresponding guanidinium and pyridinium, respectively. (C) Summary of accumulation of these 60 compounds in *E. coli* MG1655. Compounds are categorized as accumulators or non-accumulators on the basis of statistical significance relative to the negative controls; see the Methods and Supporting Information for full details.

Figure 4.

Properties affecting accumulation of alkyl guanidiniums and pyridiniums in *E. coli*. (A) Amphiphilic moment (vsurf_A) is correlated with guanidinium vs primary amine accumulation within structural classes. (B) A higher number of rotatable bonds does not prevent the accumulation of certain guanidiniums. (C) Structure and properties of accumulating pyridiniums. All experiments were performed in biological triplicate. Accumulation measured in nmol/10¹² CFUs in *E. coli* MG1655.

Figure 5.

Charge delocalization and sum of HBD strengths are important for the accumulation of amines vs guanidiniums vs pyridiniums for the 60 compounds described in Figure 3. (A) ESPMAX and (B) h_{emd} correlate with the overall accumulation trends. (C) Accumulation across a structural series decreases as ESPMAX and h_{emd} decrease.

Figure 6.

Accumulation and activity of antibiotics with appended amines, guanidiniums, and pyridiniums. All experiments were performed in biological triplicate. Accumulation measured in nmol/10¹² CFUs in *E. coli* MG1655 after a 10 min incubation with the exception of the **Debio** derivatives (**21–24**, in which accumulation was assessed after a 2 h incubation). MICs were performed in MH broth or M9-MOPS media per Clinical and Laboratory Standards Institute (CLSI) guidelines in *S. aureus* 29213, *E. coli* MG1655, and *E. coli* JW5503 *tolC* and reported in μg/mL. n/a = not applicable. n.d. = not determined

due to the lack of solubility at the assay concentrations. ^{a, b, c}Data previously reported.
12,14,15

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Figure 7.

(A) Structures of preclinical antibiotics and adjuvants bearing guanidinium motifs.^{17,44,45}

(B) Calculated ESPMAX ranges for different positively charged functional groups on nonantibiotic scaffolds.