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Discovery of Macrolide Antibiotics Effective against Multi-Drug Resistant Gram-Negative Pathogens

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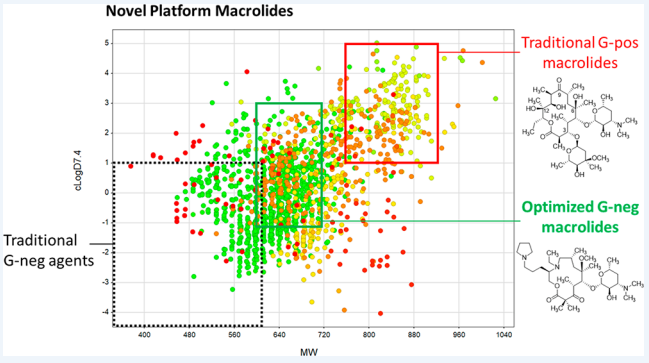
CONSPECTUS: Macrolides are among the most widely prescribed antibiotics, particularly for bacterial lung infections, due to their favorable safety, oral bioavailability, and spectrum of activity against Gram-positive pathogens such as *Streptococcus pneumoniae*, the most common cause of bacterial pneumonia. Their utility against Gram-negative bacteria is extremely limited and does not include the Enterobacteriaceae or other ESKAPE pathogens. With the increasing development of resistance to current therapies and the lack of safe, oral options to treat Gram-negative infections, extended-spectrum macrolides have the potential to provide valuable treatment options. While the bacterial ribosome, the target of macrolides, is highly conserved across Gram-positive and Gram-negative bacteria, traditional macrolides do not possess the proper physicochemical properties to cross the polar Gram-negative outer membrane and are highly susceptible to efflux. As with most natural product-derived compounds, macrolides are generally prepared through semisynthesis, which is limited in scope and lacks the ability to make the drastic physicochemical property changes necessary to overcome these hurdles. By using a fully synthetic platform technology to greatly expand structural diversity, novel macrolides were prepared with a focus on lowering the MW and increasing the polarity to achieve a physicochemical property profile more similar to that of traditional Gram-negative drug classes. In addition to the removal of lipophilic groups, a critical structural feature for obtaining Gram-negative activity in the macrolide class proved to be the introduction of small secondary or tertiary amines to yield polycationic species potentially capable of self-promoted uptake. Within the azithromycin-like 15-membered azalides, potent activity was seen when small alkyl amines were introduced at the 6′-position of desosamine. The biggest gains, however, were made by replacing the entire C10−C13 fragment of the macrolactone ring with commercially available or readily synthesized 1,2-aminoalcohols, leading to 13-membered azalides. The introduction of a tethered basic amine at the C10-position and systematic optimization of substitution and tether length and flexibility ultimately provided new macrolides that for the first time exhibit clinically relevant antibacterial activity against multi-drug resistant Gram-negative bacteria. A retrospective computational analysis of >1800 fully synthetic macrolides prepared during this effort identified key drivers and optimum ranges for improving permeability and avoiding efflux. In contrast to standard Gram-negative drugs which generally have MWs below 600 and clog$D_{7.4}$ values below 0, we found that the ideal ranges for Gram-negative macrolides were MW between 600 and 720 and c$LogD_{7.4}$ between −1 and 3. A total charge of between 2.5 and 3 was also required to provide optimal permeability and efflux avoidance. Thus, Gram-negative macrolides occupy a unique physicochemical property space that lies between traditional Gram-negative drug classes and Gram-positive macrolides.



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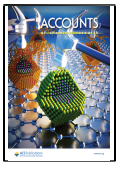

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INTRODUCTION

Most antibacterial agents in use today have their basis in natural products synthesized by bacteria or fungi to defend against bacterial competitors. As the inevitable result of natural selection, bacterial resistance has developed to most of these agents and is now widespread in the community and in nosocomial settings.[5] There is a clear need for new antibiotics to combat these resistant strains, particularly for Gram-negative bacteria which possess a second polar outer membrane and numerous efflux pumps that render these pathogens less susceptible to drug intervention.[6] Despite significant screening efforts using more traditional drug-like libraries, very few non-natural product-derived antibacterial agents have been identified.[7] It has become increasingly clear that the physicochemical properties necessary to evade these defenses are different for antibacterials vs traditional drugs, as outlined by O'Shea and Moser in their classic treatise showing that antibiotics are generally more polar and larger than drugs targeting other indications.[8] Having evolved over millennia to target bacteria, antibiotic natural products already possess those attributes necessary to overcome bacterial defenses and as such have been successfully used for decades as starting points for semisynthetically derived next-generation antibiotics.

Erythromycin[9] (**1**), originally isolated from the soil bacterium *Saccharopolyspora erythraea*, is the archetypical macrolide antibiotic (Figure 1), a class characterized by a macrolactone ring with one or more appended sugars.[10] Erythromycin and its semisynthetic derivatives are 14- or 15-membered macrolactones with a desosamine sugar at the 5-position. The earliest marketed macrolides [erythromycin, clarithromycin (**2**),[11] and azithromycin (**3**)[12]] have a cladinose sugar at the 3-position, while later-generation ketolides such as telithromycin[13] (**4**) and solithromycin[14] (**5**) have a 3-keto group. Macrolides are protein synthesis inhibitors, binding in the nascent peptide exit tunnel of the 50S subunit of the bacterial ribosome.[15] While macrolides have been widely used to treat Gram-positive lung and ear infections, their use against Gram-negative pathogens is limited to organisms such as *Haemophilus influenzae* and *Moraxella catarrhalis* among lung infections and *Neisseria gonorrheae* in

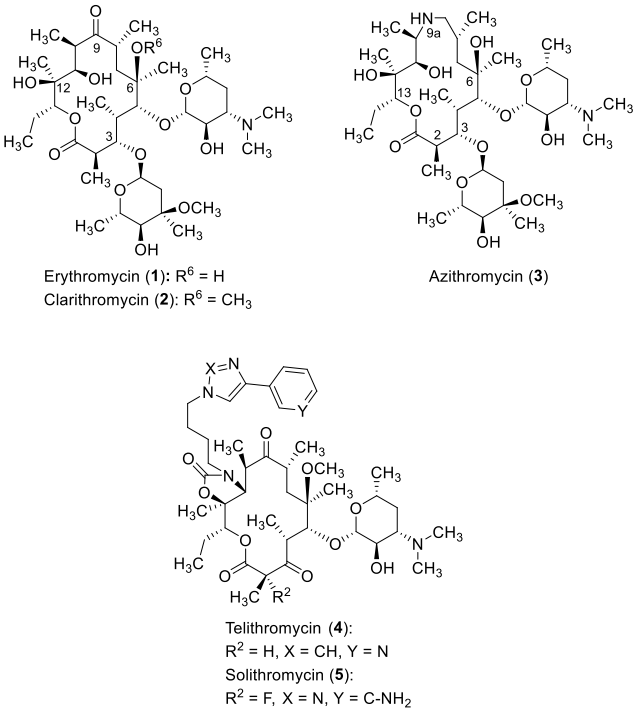

Figure 1. Erythromycin (**1**) and semisynthetically derived macrolides.

sexually transmitted diseases. They are not active against the Enterobacteriaceae, including *Escherichia coli* and *Klebsiella pneumoniae*. The reasons for this are quite clear upon analysis of their physicochemical properties: in contrast to typical Gram-negative agents such as aminoglycosides (MW = 450−620, $clogD_{7.4}$ = −10 to −6), tetracyclines (MW = 450−600, $clogD_{7.4}$ = −5 to −2), β-lactams (MW = 300−650, $clogD_{7.4}$ = −6 to −1), and fluoroquinolones (MW = 300−450, $clogD_{7.4}$ = −2 to 1), macrolides are significantly larger (MW = 730−860) and less polar ($clogD_{7.4}$ = 1.5 to 5).[8,16] Indeed, despite almost 70 years of macrolide research, we are not aware of any reports of appreciable activity in noncompromised (i.e., efflux or permeability mutants) Gram-negative strains.[17]

While it is unclear how much effort has been directed at finding Gram-negative macrolides, the limitations of semisynthesis likely play a large role in the lack of apparent progress. Because both the macrolactone core and the desosamine sugar are required for ribosomal binding and many of the ring positions are chemically inaccessible, the potential for synthetic modification is limited to a few sites and restricted to additive changes. Thus, the modifications that led to agents such as telithromycin and solithromycin produced larger molecules with increased lipophilicities (solithromycin: MW = 845.02, $clogD_{7.4}$ = 4.25). The authors of this Account have been involved in the development of practical chemistry for the convergent construction of natural product antibacterial scaffolds, broadly defined, including tetracyclines,[2−4,18] aminoglycosides,[19] lincosamides,[20] and macrolides[1] and the application of this chemistry to the discovery of novel antibiotics that address issues of bacterial resistance. In 2016, Myers and co-workers published a fully synthetic route for the general preparation of 14- and 15-membered ketolides and azalides.[1] Herein, we report the utilization and extension of this synthetic platform for the discovery of the first examples of erythromycin-type macrolides with potent activity against multi-drug resistant Gram-negative

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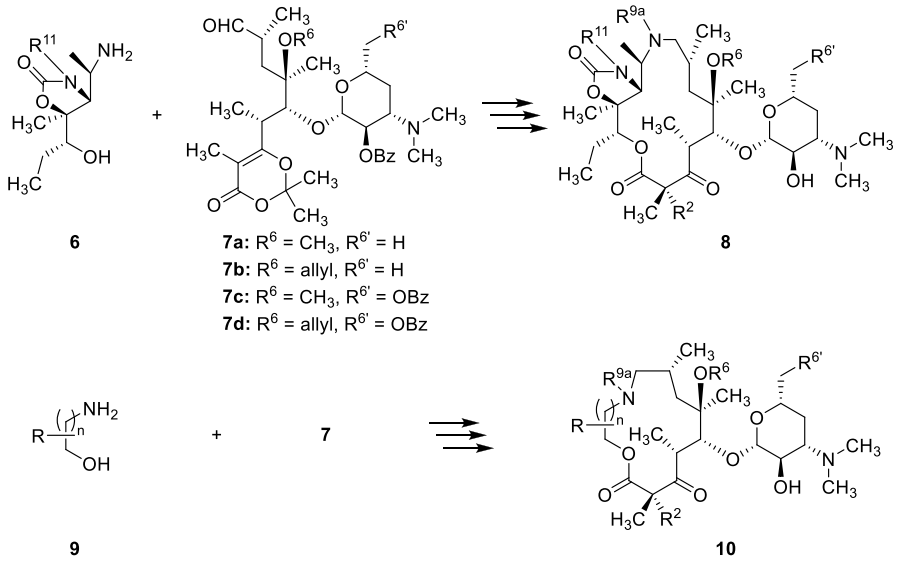
Table 1. Gram-Negative Antibacterial Activity of Macrolides Broken Down into Components

	E. coli MIC (μg/mL)						
compound	MP-4 WT[a]	MP-9 *tolC* efflux mutant	MP-74 *lptD* LPS mutant[b]	efflux ratio[c]	permeability ratio[d]	MW	clogD$_{7.4}$
1	64	4	0.063	16	64	733.9	0.99
2	64	4	0.063	16	64	748.0	1.63
3	4	0.5	0.031	8	16	749.0	−1.23
4	32	2	0.031	16	64	812.0	3.85
5	16	2	0.015	8	128	845.0	4.25

[a]ATCC 25922. [b]CGSC strain RFM795. [c]Efflux ratio = (MP-4 MIC)/(MP-9 MIC). [d]Permeability ratio = (MP-9 MIC)/(MP-74 MIC).

Scheme 1. General Synthesis of Macrolides from Left-Hand and Right-Hand Components



pathogens and define the optimal physicochemical properties necessary to achieve these results.

APPROACH

At a basic level, antibacterial activity can be viewed as the sum of three components: penetration through the Gram-negative outer membrane (permeability), the avoidance of highly promiscuous efflux pumps (efflux), and the affinity for the antibacterial target (target engagement). Each of these factors was assessed for five representative macrolides with a panel of three *E. coli* strains (Table 1). Wild-type activity (MP-4 strain) is generally poor for macrolides; only azithromycin has a single-digit MIC (4 μg/mL). An estimation of target engagement was assessed using the hyperpermeable MP-74 strain which has a mutation in the *lptD* gene, impairing outer membrane assembly.[21] The ribosomal binding site is highly conserved across Gram-positive and Gram-negative bacteria, and all five macrolides have potent activity against this strain. The improved wild-type activity of the ketolides (**4** and **5**) relative to erythromycin appears to be driven mainly by better target engagement from interactions between the aryl-alkyl side chains and the ribosome. The impact of efflux on Gram-negative activity was readily assessed through the efflux ratio, calculated by dividing a compound's MIC in the WT MP-4 strain by its MP-9 MIC in the isogenic *tolC* efflux mutant strain. All five macrolides are impacted by efflux to approximately the same extent, resulting in 8- to 16-fold reductions in antibacterial activity. Finally, permeability can be assessed through the permeability ratio, calculated by dividing a compound's MIC in the MP-9 strain by its MIC in the hyperpermeable MP-74 strain. Although these are not isogenic strains and do not give an exact measure of the impact of permeability on the WT MIC, we have found the permeability ratio to be a useful and directional estimation of a compound's ability to permeate the Gram-negative outer membrane. Physicochemical properties clearly drive permeability differences among these compounds, with the more lipophilic 14-membered compounds (**1**, **2**, **4**, and **5**) impacted the most (ratios of 64 to 128). Azithromycin, the only compound with clogD$_{7.4}$ < 0, has the best permeability ratio (16). In addition to increasing polarity, the additional basic amine within the macrolactone ring of azithromycin may facilitate self-promoted uptake, a phenomenon observed with polycationic compounds that can destabilize the lipopolysaccharide (LPS) layer of the outer membrane through the displacement of divalent cations.[22]

From this analysis, an approach to improving Gram-negative activity in the macrolide class took shape with an initial focus on the optimization of physicochemical properties driven by the removal of lipophilic groups and the addition of polarity to improve permeability. Considering the permeability gains observed by the addition of a basic amine in azithromycin and the potential for the self-promoted uptake of polycationic compounds, the introduction of a charged amine was an early priority.[23,24] We considered that some reduction in target engagement could be tolerated if it were accompanied by larger improvements in permeability and/or efflux.

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CHEMISTRY

Synthetic macrolides were prepared according to Scheme 1 by the general route previously reported.[1] Briefly, 15-membered azalides **8** were assembled from left-hand component (LHC) aminoalcohols **6** and right-hand components (RHCs) **7** via reductive amination and subsequent macrolactonization by dioxolenone thermolysis.[25] Postmacrolactonization modifications were introduced where appropriate via standard chemistry, and the methanolysis of the 2′-benzoate yielded the final compounds. These 15-membered azalides bearing the 10,11-carbamoyl group are a hybrid between the telithromycin-type ketolides and azithromycin and cannot be prepared semisynthetically. One of the greatest attributes of the fully synthetic approach is the ability to replace entire portions of the macrolide backbone. By taking advantage of the large number of commercially available or readily prepared aminoalcohols **9**, the C10−C13 portion of the semisynthetically derived macrolides could be replaced to create completely novel azalides **10**, providing access to vastly wider structural diversity and physicochemical property space than was possible via semisynthetic chemistry.

Using this methodology, we have prepared over 2500 macrolides, including the examples from 4 major classes presented in Figure 2.[1,26] Compounds **11**−**13**[26a] and **16**[26c] are 15-membered azalides with natural desosamine (15-AZAs). We first defined the minimum structural requirements necessary for activity (**11**) and then introduced polar substituents at various ring positions with a focus on basic amines. While few positions were found that tolerated polarity, we were able to successfully install amines at the 6′-position of the desosamine residue, yielding compounds such as **14**, **15**, and **17** (15-AMDs).[26c] A similar strategy was employed in the 14-membered ketolide series (14-KETs), examples of which include compounds **18** and **19**.[26b] Compounds **20** and **21** are the base unsubstituted versions of the 13-membered azalides (13-AZAs).[26d,e] We identified three issues of chemical instability caused by the C1−C3 β-keto ester functional group: (1) epimerization of position C2, (2) β-elimination of desosamine, and (3) water- or alcohol-promoted ring opening of the lactone. These issues were solved in one of two ways. In the 15-membered series, a two-carbon bridge from the C6-oxygen to the C2-carbon was installed by intramolecular alkylation as seen in compounds **16** and **17**. In the 13-membered series, a methyl group was installed at C2, simultaneously removing the stereocenter and stabilizing the compounds toward ring opening and desosamine elimination.

With the availability of many commercial 1,2-aminoalcohols or their facile synthesis from amino acids, we were able to rapidly explore the chemical space in the 13-AZA series. The C10-position was found to be ideal for the introduction of basic amines (Figure 3). An early lead in this chemotype was the C10-spirocyclic piperidine scaffold (**22**−**24**). Further exploration at C10 examined the impact on flexibility of the side chains (**25** and **26** vs **34**) and tether length to the amine (**24** and **28**). Where possible, primary, secondary, and tertiary amines were explored, as well as alkyl, aryl, and heteroaryl substitution. Nonbasic groups were also introduced (compound **30**) but were found to lack significant antibacterial activity.

A number of physicochemical properties characteristic of Gram-negative agents have been reported, including the size (MW), polarity (clogD$_{7.4}$), polar surface area (TPSA), charge (average charge), rigidity (rotatable bonds), and shape (globularity).[8,24] Table 2 lists the average values and ranges for each of these properties as calculated for members of the four synthetic macrolide classes along with the reported character-

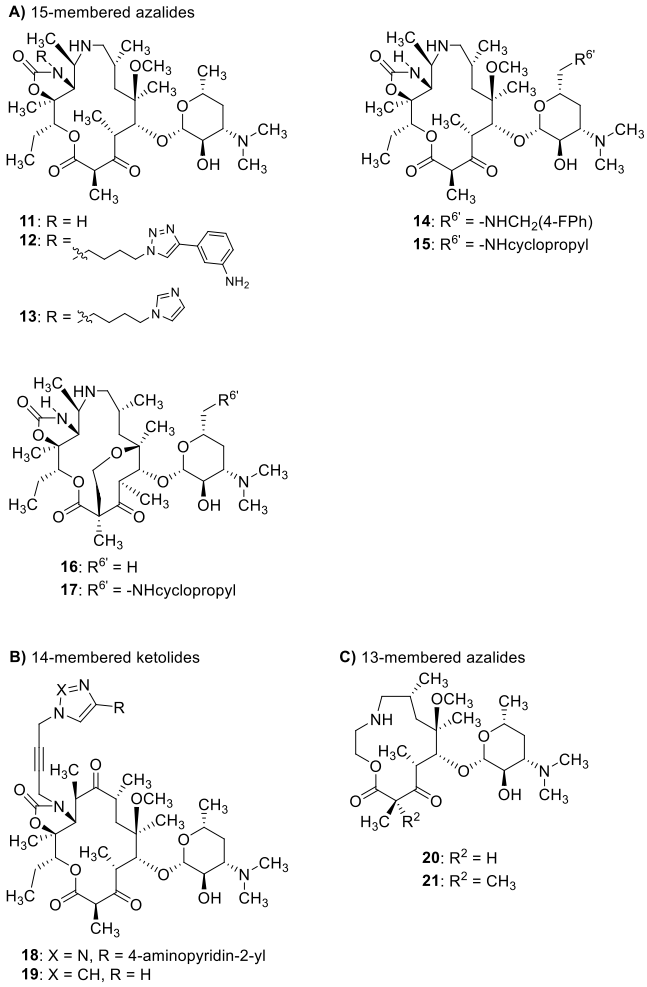

Figure 2. Four major classes of macrolides: (A) 15-membered azalides with (15-AMD) and without (15-AZA) 6′-modified desosamine, (B) 14-membered ketolides (14-KET), and (C) 13-membered azalides (13-AZA).

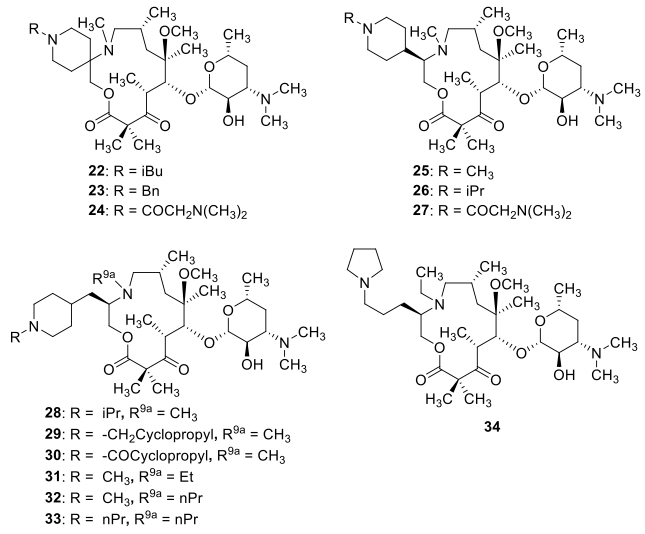

Figure 3. 13-AZAs with an amine appended from the C-10 ring position.

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Table 2. Comparison of the Physicochemical Properties of Four Major Classes of Macrolides

class (no.)	avg MW (range)	avg clogD$_{7.4}$ (range)	avg charge (range)	avg TPSA (range)	avg rotatable bonds (range)	avg globularity (range)
15-AZA (290)	715.5 (527−1002)	0.93 (−4.35 to 4.33)	1.94 (0.88−3.23)	161.6 (101−242)	7.89 (4−16)	0.13 (0.04−0.43)
15-AMD (186)	721.5 (599−951)	−0.25 (−5.04 to 3.14)	2.43 (0.85−3.65)	165.6 (139−239)	8.62 (5−16)	0.12 (0.04−0.49)
14-KET (187)	798.1 (555−939)	2.60 (−0.26 to 4.07)	1.14 (−0.05−2.00)	190.4 (128−270)	9.6 (5−15)	0.16 (0.03−0.39)
13-AZA (873)	626.4 (458−824)	0.02 (−3.81 to 5.46)	2.46 (0.89−3.50)	114.1 (94−179)	7.42 (3−17)	0.11 (0.03−0.44)
typical value for G-neg	<600	<0	charged	165 avg	≤5	≤0.25

Table 3. Antibacterial Activity of 15-AZAs and 14-KETs

	E. coli MIC (μg/mL)				*K. pneumoniae* MIC (μg/mL)			
compound	MP-4 WT	MP-9 (efflux ratio)	MP-74 (perm. ratio)	MP-538 MDR[a]	MP-546 MDR[b]	MP-648 MDR[c]	MW	clogD$_{7.4}$
3	4	0.5 (8)	0.031 (16)	8	256	128	749.0	−1.23
11	2	0.25 (8)	0.063 (4)	8	16		613.8	0.28
12	8	1 (8)	0.031 (32)	8	64		828.1	2.1
13	4	0.5 (8)	ND	8	32		736.0	0.91
14	2	0.25 (8)	0.063 (4)	4	32		736.9	0.05
15	1	0.25 (4)	0.063 (4)	2	4	4	668.9	−2.05
16	2	0.25 (8)	0.031 (8)	4	16		625.8	0.63
17	0.5	0.125 (4)	0.063 (2)	1	2	4	680.9	−3.11
18	8	1 (8)	0.031 (32)	16	>64		824.0	3.24
19	16	1 (16	0.031 (32)	16	>64		730.9	2.88
ceftazidime	0.25	0.25		>64	>64	>64		
ceftazidime-avibactam	0.25	0.125		1	0.125	2		
meropenem	0.031	0.031		2	>64	>64		
ciprofloxacin	0.008	0.008		>64	>64	>64		

[a]ESBL (CTX-M-15), FQ-R. [b]ESBL (SHV, TEM), KPC-2, *mphA*. [c]NDM, FQ-R.

istic values for Gram-negative agents.[27] Clearly the synthetic methodology allows for the wide-ranging manipulation of macrolide physicochemical properties, and in each class, compounds were constructed that fell into the characteristic ranges for Gram-negative activity. On average, the 13-AZAs had the best combination of properties, especially for MW, clogD$_{7.4}$, and average charge, while the 14-KET class, lacking a second amine in the ring, was generally well outside of the target values.

ANTIBACTERIAL ACTIVITY

Compounds were assayed using serial 2-fold dilutions according to CLSI guidelines against a panel of Gram-negative organisms, including the *E. coli* and *K. pneumoniae* isolates in Table 3.[28] In addition to the previously described strains used to assess the baseline antibacterial activity, efflux, and permeability, activity against three multi-drug resistant (MDR) strains was determined, including those carrying extended-spectrum β-lactamases (ESBLs; MP-538, MP-546, and MP-648), *Klebsiella pneumoniae* carbapenemases (KPCs; MP-546), and fluoroquinoline-resistant mechanisms (FQ-R; MP-538 and MP-648). Notably, the MP-546 *K. pneumoniae* isolate also carries a macrolide phosphotransferase (*mph*) gene, which we have found to be highly prevalent in *E. coli* (>20%) and *K. pneumoniae* (∼10%). Two other common macrolide resistance determinants, erythromycin esterases (*ere*) and erythromycin methylases (*erm*), were found to be present in 1% or fewer strains of each species and were not included in this panel.[29] These strains were generally resistant to ceftazidime, meropenem, and ciprofloxacin, while the ceftazidime-avibactam combination was active at 2 μg/mL or lower. For macrolides, the MP-538 and MP-648 strains were found to approximate the MIC$_{90}$ values for *E. coli* and *K. pneumoniae*, respectively. In general, MICs against *K. pneumoniae* were elevated by 1 to 2 dilutions relative to those for *E. coli*. Among the 15-AZAs (**11**−**13**), antibacterial activity tracked with size and polarity, with the smallest, most polar compound (**11**) generally having the lowest MICs against the WT strains, while the largest, most lipophilic compound (**12**) had MICs that were elevated by two dilutions. Although the aryl-alkyl side chain of **12** improves target engagement (one-dilution improvement in MP-74 MIC), it leads to a significant loss in permeability (permeability ratio of 32 versus 4 for unsubstituted compound **11**). These differences result in the same MIC for *E. coli* MP-538 (8 μg/mL), while the decreased permeability is more pronounced in *K. pneumoniae*, with a three-dilution loss for the larger compound (64 μg/mL) versus a one-dilution loss for compound **11** (16 μg/mL). Compound **13**, with intermediate MW and polarity, delivered a *K. pneumoniae* MP-546 MIC in between these two compounds. The addition of a basic amine in the 15-AMDs led to a further improvement in Gram-negative activity, with *E. coli* MP-538 MICs of 4 and 2 μg/mL for compounds **14** and **15**, respectively. *K. pneumoniae* was again more sensitive to size and polarity, with a three-dilution elevation in MIC for compound **14** (32 μg/mL) versus a one-dilution loss in activity for smaller compound **15** (4 μg/mL). As mentioned above, these 15-membered compounds could be stabilized with the O6−C2 bridging group in compounds **16** and **17**, resulting in a one-dilution improvement in MICs across most strains (**11** vs **16** and **15** vs **17**). The large, lipophilic 14-KETs (**18** and **19**) were again found to have poor permeability, leading to elevated MICs despite good target engagement. In general, the improvements in Gram-negative activity observed for the 15-membered compounds relative to the traditional macrolides were driven by enhanced permeability, while the efflux ratios were largely unchanged.

Having demonstrated clinically relevant MICs against MDR Gram-negative bacteria but facing increasing synthetic complex-

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Table 4. Antibacterial Activity of 13-AZAs

	E. coli MIC (μg/mL)				*K. pneumoniae* MIC (μg/mL)			
compound	MP-4 WT	MP-9 (efflux ratio)	MP-74 (perm. ratio)	MP-538 MDR	MP-546 MDR	MP-648 MDR	MW	clogD$_{7.4}$
20	32	2 (16)	1 (2)	>64	>64		472.6	−0.6
21	4	0.5 (8)	0.125 (4)	16	8		486.7	−0.04
22	8	0.5 (16)	0.25 (2)	32	16		625.9	0.4
23	8	0.5 (16)	0.125 (4)	32	32		659.9	1.63
24	2	0.5 (4)	0.25 (2)	4	8		654.9	−0.43
25	1	0.5 (2)	0.125 (4)	2	4		597.8	−0.39
26	0.25	0.125 (2)	0.063 (2)	1	1	4	625.9	−0.11
27	2	0.5 (4)	0.25 (2)	8	8		668.9	−0.16
28	1	0.25 (4)	0.125 (2)	2	4	8	639.9	0.05
29	0.125	0.125 (1)	0.063 (2)	0.5	0.5	2	651.9	−0.09
30	>64	4 (>16)	1 (4)	>64	>64		665.9	1.35
31	0.5	0.125 (4)	0.063 (2)	1	1	2	625.9	−0.07
32	0.5	0.125 (4)	0.063 (2)	1	2	2	639.9	0.17
33	1	0.25 (4)	0.063 (4)	2	4	8	668.0	0.51
34	0.125	0.063 (2)	0.063 (1)	0.5	0.5	1	625.9	0.07

ity, we turned our attention to the more accessible 13-azalides (Table 4). Parent 13-AZA compound **20** had weak antibacterial activity against the WT strains largely due to chemical instability that was recovered through the introduction of the C2-methyl group in compound **21**. With their favorable size (MW < 500) and polarity (clogD$_{7.4}$ < 0), these simplified macrolides demonstrated good permeability and proved to be excellent starting points for further optimization. By replacing the entire left-hand side of the molecule, we were able to rapidly explore a wide variety of changes. The C10 position proved to be particularly fruitful (**22**−**34**), where the introduction of substituted amines extended through linkers of varying flexibility and length yielded some of the most potent Gram-negative macrolides in our collection. Spirocyclic piperidine compounds **22**−**24** are the most rigid of the four variations and have moderate target engagement (MP-74 MICs of 0.125 to 0.25 μg/mL) and good permeability (ratios of 2 to 4). The isobutyl (**22**) and benzyl (**23**) substituted amines are more prone to efflux (efflux ratios = 16) than glycylamide compound **24** (efflux ratio = 4) and as a result are three dilutions less active against the MDR *E. coli* isolate and one to two dilutions weaker against the *K. pneumoniae* strain. With one additional rotatable bond, piperidine compounds **25**−**27** are slightly more flexible and position the amine further away from the macrocyclic core. These compounds are similar in target engagement and permeability to the spirocyclic compounds but have improved efflux avoidance, with ratios of 2 for alkyl-substituted compounds **25** and **26** and 4 for glycylamide compound **27**. Whereas the glycylamide compounds in both chemotypes are essentially equipotent, the improved efflux ratios in compounds **25** and **26** result in superior antibacterial activity relative to that of the spirocyclic compounds. Compound **26**, with the larger isopropyl substituent, has the best combination of target engagement (0.063 μg/mL), permeability ratio (2), and efflux ratio (2), resulting in MICs of 1 μg/mL for the MP-538 and MP-546 isolates and 4 μg/mL for the more challenging MP-648 strain. We have generally found that tertiary amines with slightly larger alkyl groups are favored over primary, secondary, or smaller tertiary amines. Adding a methylene linker between the piperidine and the macrocyclic ring (**28**−**33**) increases both the flexibility and distance between the amine and the macrocyclic core. These compounds have improved target engagement relative to the directly linked piperidines and similar permeability and efflux ratios. Although isopropyl compound **28** was one to two dilutions less active than directly linked analog **26**, cyclopropylmethyl compound **29**, with an efflux ratio of 1, was one dilution more potent than analog **26**. The relative lack of activity for compound **30**, the cyclopropylamide analog of **29**, clearly shows that the third basic amine is necessary and has the biggest impact on the target engagement (MP-74 MIC of 1 μg/mL) and efflux ratio (>16) while retaining overall permeability. The effects of larger alkyl substituents at N9a were also explored (**31**−**33**). Proper balance between size and lipophilicity is crucial, with combinations of small substituents such as methyl on the piperidine group and ethyl (**31**) or propyl (**32**) groups at N9a having good activity against all of the MDR isolates. When two larger groups are combined on N9a and piperidine, such as bis-propyl compound **33**, the antibacterial activity is reduced, particularly for the *K. pneumoniae* strains. Finally, we explored the highly flexible propyl linker between the amine and the macrocyclic ring (**34**). This compound exhibits good target engagement, permeability, and efflux avoidance, resulting in a superior MIC profile of 0.5 to 1 μg/mL across the three MDR isolates. Overall, this work demonstrates that a combination of physicochemical property optimization and chemical diversity can result in macrolides with clinically relevant MICs against MDR Gram-negative bacteria by maintaining their target engagement and improving the permeability and efflux avoidance.

DISCUSSION

The traditional semisynthetic macrolides have poor Gram-negative antibacterial activity due to both a lack of permeability through the outer membrane and significant efflux from within the bacterial cell, rather than from a lack of target engagement. The poor permeability is due to a combination of being too large (MW > 700) and too lipophilic (clogD$_{7.4}$ > 1.5) for entry either through porins or by passive diffusion through the polar outer membrane. It is generally thought that most small molecules are subject to efflux, which can be overcome through a faster rate of permeation into the bacterial cell than the rate of efflux out of the cell. Thus, efflux issues could be addressed through a structural component in reducing the affinity for efflux pumps as well as improving permeability.

As mentioned above, several physicochemical parameters have been associated with Gram-negative antibacterial activity.

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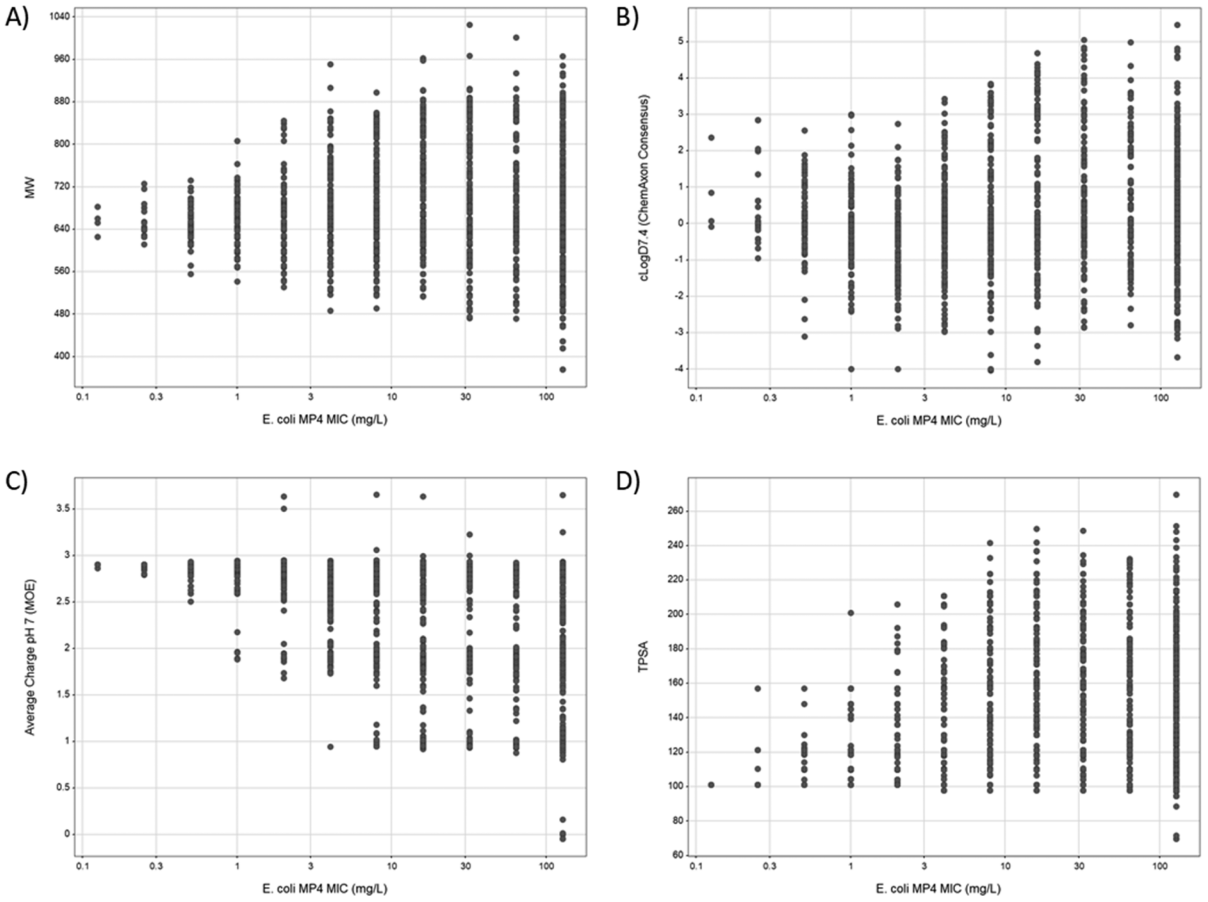

Figure 4. *E. coli* MP-4 MIC in μg/mL plotted vs (A) molecular weight (MW), (B) clogD$_{7.4}$, (C) average charge at pH 7, and (D) topological polar surface area (TPSA). The three MDR strains show similar trends.

We calculated these values for more than 1800 synthetic macrolides using either ChemAxon (clogD$_{7.4}$) or MOE software (MW, average charge, TPSA, globularity) and analyzed the data with respect to target engagement, permeability, and efflux in order to define the key drivers of Gram-negative activity for macrolides. In analyzing the overall Gram-negative activity, several trends are apparent (Figure 4). The ideal MW for macrolides appears to be in the range from ~600 to 720, which is significantly higher than the reported cutoff of ~600 for Gram-negative antibacterials. This can be partially explained by a trend toward higher target engagement with increasing MW (Figure 5A) but may also be due to the fact that these macrocyclic compounds have smaller effective volumes relative to their MW in contrast to more linear compounds such as the aminoglycosides or tetracyclines. Similarly, the optimal range for clogD$_{7.4}$, −1 to 3, is also somewhat higher than normally found for Gram-negatives (<0). We found that larger alkyl-substituted tertiary amines were generally favored over primary, secondary, or smaller alkyl-substituted tertiary amines, which contributes to these elevated clogD values. This contrasts with the "eNTRy rules" described by Richter et al. that state that accumulation in bacteria is favored for compounds that contain a nonsterically encumbered primary amine.[30] It is clear that a polycationic macrolide is necessary to achieve Gram-negative activity (Figure 4C), with all compounds having an MIC of <1 μg/mL against *E. coli* MP-4 bearing an average charge of >2.5. For the most challenging MDR strain, *K. pneumoniae* MP-648, only one compound with an average charge of <2.5 had an MIC of ≤8 μg/mL (data not shown). The addition of a fourth amine is not beneficial, although only a small number of compounds were analyzed. The topological polar surface area (TPSA) and relative polar surface area (not shown) both tend to be high for Gram-negative antibacterials. The opposite trend is seen for macrolides (Figure 4D), where a lower TPSA is required for activity. *E. coli* tends to tolerate compounds in the 100 to 160 range, while *K. pneumoniae* has a cutoff below 130.

The compounds have also been analyzed for correlations between the calculated physicochemical properties and the three critical aspects of antibacterial activity (Figure 5). There is a slight, positive correlation between the target engagement and MW (Figure 5A). This is driven at the high MW end by 14-KET and 15-AZA compounds with aryl-alkyl side chains that have been shown to make additional binding interactions to domain II of the bacterial ribosome[31] and at the low MW end by simplified compounds lacking some of the key lipophilic ribosomal binding elements. Nevertheless, a compound as small as compound **21** (MW = 486.7) maintains reasonable target engagement (MIC = 0.125 μg/mL). Permeability, on the other hand, is negatively correlated with MW (Figure 5B), as is clogD$_{7.4}$ (data not shown). Once again, the high MW lipophilic 14-KET and 15-AZA compounds with aryl-alkyl side chains are poorly permeable, while the smaller, more polar 13-AZA compounds show excellent cell penetration. Permeability is also dependent on average charge (Figure 5C), with the majority of the singly charged compounds (<1.5) having permeability ratios of >16 and almost all of the compounds with an average charge >1.75 having permeability ratios of ≤8. Of the three facets contributing to antibacterial activity, efflux shows the least

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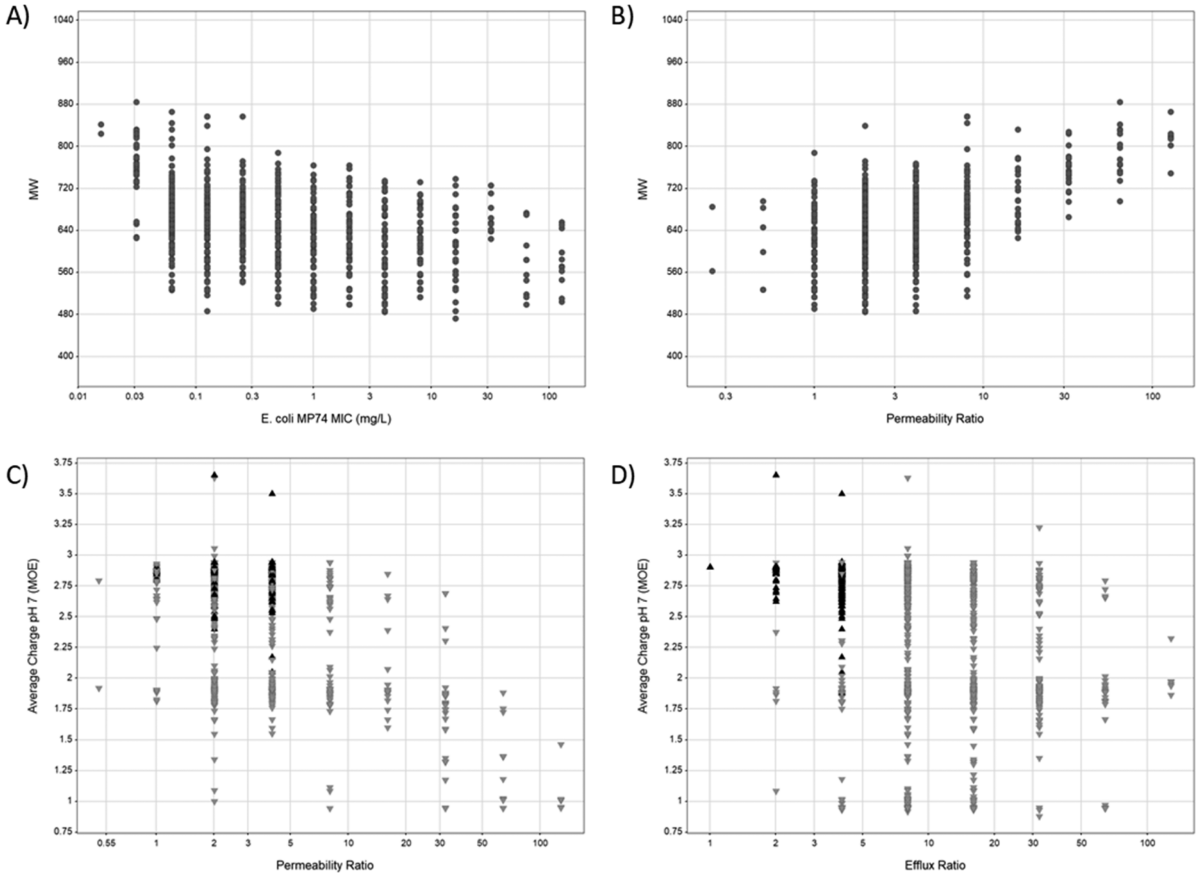

Figure 5. Correlation of physicochemical properties with target engagement, permeability, and efflux: (A) *E. coli* MP-74 MIC vs MW, (B) permeability ratio vs MW, (C) permeability ratio vs average charge at pH 7, and (D) efflux ratio vs average charge at pH 7. ▲, compounds with permeability and efflux ratios ≤4; ▼, compounds with permeability or efflux ratios >4.

correlation to any of the calculated physicochemical properties. The most predictive single parameter appears to be average charge (Figure 5D), where there is a higher percentage of triply charged compounds (>2.5) with efflux ratios ≤4 than doubly or singly charged compounds. However, when examining the set of compounds that have both permeability and efflux ratios ≤4 (black triangles in Figure 5C,D), only 4 of 339 compounds have an average charge of less than 2.4. Limiting either permeability or efflux ratios to 2 eliminates these compounds. This analysis supports the hypothesis that permeability and efflux are related. Self-promoted uptake of the polycationic macrolides leads to an increased rate of diffusion into the bacterial cell, where retention is aided by tight binding to the ribosome, at least partially overcoming the rate of efflux. The net result is greater accumulation in the bacterial cell, and correspondingly lower MICs. The fact that not all highly charged, highly permeable compounds have low efflux ratios, however, indicates that there is likely a structural component that influences binding to the efflux pumps and plays a critical role in avoiding efflux. Unfortunately, we have yet to identify clear SAR to support this.

While this program focused on improving Gram-negative activity in *E. coli* and *K. pneumoniae*, activity against the ESKAPE pathogens was routinely monitored. The significant physicochemical property changes to the macrolide class required for Gram-negative activity came at the expense of activity against Gram-positive species, including *Staphylococcus aureus*. Among the Gram-negatives, *Enterobacter* spp. activity tracked well with *K. pneumoniae* activity, while *Pseudomonas aeruginosa* strains proved more resistant. *Acinetobacter baumannii* activity was also diminished relative to *K. pneumoniae* while exhibiting somewhat divergent SAR that could provide unique starting points for future work.

■ CONCLUSIONS

The ability to systematically and drastically manipulate both chemical structure and physicochemical properties through chemical synthesis has led to the first examples of clinically relevant antibacterial activity against MDR Gram-negative bacteria in the macrolide class. Critical factors in increasing permeability through the Gram-negative outer membrane and efflux avoidance were identified, including reducing the molecular weight, increasing the polarity, and including three basic amines. While the direction of these findings agrees with the recent literature, Gram-negative macrolides are larger (MW 600−720), are slightly more lipophilic ($clogD_{7.4} = -1$ to 3), and favor tertiary amines with larger alkyl substituents over primary amines. A deeper structural analysis of the macrolide library in the future may help to unlock key structural features that impact efflux. The current work adds to the growing body of literature aimed at improving Gram-negative activity that will be necessary in combatting the ever-growing resistance issues that diminish our current antibacterial arsenal.

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Notes

The authors declare the following competing financial interest(s): Although I no longer am affiliated with Macrolide Pharmaceuticals (now Zikani Therapeutics) I am a stock holder and this might be considered a conflict of interest. I disclose this in an abundance of caution.

Biographies

Andrew Myers is the Amory Houghton Professor of Chemistry & Chemical Biology at Harvard University, where he has been directing chemical research for more than 20 years.

Roger B. Clark is Vice President of Discovery Sciences at Zikani Therapeutics and has over 20 years of experience in medicinal chemistry.

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■ ABBREVIATIONS

13-AZA, 13-membered azalide; 14-KET, 14-membered ketolide; 15-AMD, 15-membered azalide with modified desosamine; 15-AZA, 15-membered azalide; ATCC, American Type Culture Collection; $clogD_{7.4}$, calculated octanol/water partition coefficient at pH 7.4; CLSI, Clinical and Laboratory Standards Institute; ESKAPE pathogens, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp; ESBL, extended-spectrum β-lactamase; FQ-R, fluoroquinolone-resistant; KPC, *Klebsiella pneumoniae* carbapenemase; LHP, left-hand piece; LPS, lipopolysaccharide; MDR, multi-drug resistant; MIC, minimum inhibitory concentration; MIC_{90}, minimum inhibitory concentration required to inhibit the growth of 90% of a panel of organisms; *mph*, macrolide phophotransferase; MW, molecular weight; NPET, nascent peptide exit tunnel; RHP, right-hand piece; SAR, structure−activity relationships; TPSA, topological polar surface area; WT, wild type

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