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REVIEW

Antibiotic Hybrids: the Next Generation of Agents and Adjuvants against Gram-Negative Pathogens?

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SUMMARY 2
INTRODUCTION 2
Permeability Is an Important Consideration in Developing Antibiotics for Gram-Negative Bacteria 3
The outer membrane is efficient in restricting molecular passage 3
Membrane permeation is a limitation to most but not all agents with a high molecular weight 4
The inner membrane as a second restrictive barrier for agents with cytosolic targets 5
Intracellular drug concentrations are greatly affected by efflux 6
There is an urgent need for guidelines to develop agents that are able to penetrate both outer and inner membranes 6
Therapeutic Approaches To Overcome Antimicrobial Resistance 7
Antivirulence therapy 8
Combination therapy 8
(i) Antibiotic-adjuvant combination approach 8
(ii) Antibiotic-antibiotic combination approach 10
Challenges of combination therapy 11
ANTIBIOTIC HYBRIDS AGAINST ANTIBIOTIC-RESISTANT BACTERIA 12
Definition of an Antibiotic Hybrid 12
Conceptual Challenges in Designing Antibiotic Hybrids 13
Antibiotic Hybrid Prodrugs against Antibiotic-Resistant Gram-Negative Bacteria 14
Concept and hypothesis 14
Advantages and disadvantages 15
Examples 16
Ro 23-9424: struggle, triumph, and failure—a story from the past 17
Antibiotic Hybrid Drugs against Antibiotic-Resistant Gram-Negative Bacteria 18
Concept and hypothesis 18
Advantages and disadvantages 19
Examples 19
(i) Most hybrid drugs contain a fluoroquinolone pharmacophore 19
(ii) Neomycin B-ciprofloxacin hybrid drugs delayed development of drug resistance 21
(iii) Other hybrid drugs that are active against Gram-negative pathogens 22
(iv) Cefiderocol (S-649266): a Trojan horse strategy 23
Antibiotic Hybrids Can Adopt New Mechanistic Actions That Differ from Those of Their Constituent Pharmacophores 24
Tobramycin-based hybrids as adjuvants that potentiate legacy antibiotics against *Pseudomonas aeruginosa* 25
Structure optimization strategy for the tobramycin-based hybrid scaffold 29
(i) The tobramycin-moxifloxacin hybrid retains a new mechanism of action 29
(ii) Tobramycin-lysine peptoid conjugates resensitize MDR *P. aeruginosa* to minocycline and rifampin 29
(iii) Tobramycin-efflux pump inhibitor conjugates perturb RND efflux pumps 30
Resensitization of resistant pathogens to antibiotics may be induced by targeting the membrane 32
Proposed mechanisms of action of tobramycin-based hybrids 33
PERSPECTIVES 34
(continued)

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FUTURE OUTLOOK: ANTIBIOTIC HYBRIDS MAY BE THE NEXT GENERATION OF ANTIBIOTIC AGENTS AND ADJUVANTS 35
ACKNOWLEDGMENTS 36
REFERENCES 36
AUTHOR BIOS 44

SUMMARY The global incidence of drug-resistant Gram-negative bacillary infections has been increasing, and there is a dire need to develop novel strategies to overcome this problem. Intrinsic resistance in Gram-negative bacteria, such as their protective outer membrane and constitutively overexpressed efflux pumps, is a major survival weapon that renders them refractory to current antibiotics. Several potential avenues to overcome this problem have been at the heart of antibiotic drug discovery in the past few decades. We review some of these strategies, with emphasis on antibiotic hybrids either as stand-alone antibacterial agents or as adjuvants that potentiate a primary antibiotic in Gram-negative bacteria. Antibiotic hybrid is defined in this review as a synthetic construct of two or more pharmacophores belonging to an established agent known to elicit a desired antimicrobial effect. The concepts, advances, and challenges of antibiotic hybrids are elaborated in this article. Moreover, we discuss several antibiotic hybrids that were or are in clinical evaluation. Mechanistic insights into how tobramycin-based antibiotic hybrids are able to potentiate legacy antibiotics in multidrug-resistant Gram-negative bacilli are also highlighted. Antibiotic hybrids indeed have a promising future as a therapeutic strategy to overcome drug resistance in Gram-negative pathogens and/or expand the usefulness of our current antibiotic arsenal.

KEYWORDS antibiotic, antimicrobial, antibacterial, permeability, efflux, hybrid

INTRODUCTION

The rapid global dissemination of Gram-positive and Gram-negative bacterial pathogens that are resistant to currently available antimicrobial therapies, in both hospital and community settings, marks the onset of a possible severe worldwide health crisis (1–3). Out of all these pathogens, the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) bacteria (4) account for the majority of nosocomial infections worldwide, with an increasing incidence of drug resistance every year (3, 5). The incidence of clinical isolates belonging to the ESKAPE group that exhibit either multidrug resistance (MDR), extensive drug resistance (XDR), or pandrug resistance (PDR) is quite alarming (6–8). MDR is defined as nonsusceptibility to at least one agent in $\geq$3 chemically dissimilar antibiotic classes, XDR is defined as nonsusceptibility to at least one agent in all but $\leq$2 chemically dissimilar antibiotic classes, and PDR is defined as nonsusceptibility to all agents in all antibiotic classes (9). However, the problem is arguably more serious for Gram-negative organisms, which are more frequently MDR and for which no novel antibacterial drug entities with novel modes of action (only new drug combinations) have been approved for clinical use in 5 decades (3, 10, 11). Indeed, four out of the six ESKAPE pathogens (*K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) are Gram-negative bacilli.

Various health organizations have been vocal about the urgent need to develop new antibiotics, especially against drug-resistant Gram-negative ESKAPE bacilli. For instance, the World Health Organization (WHO) has raised its utmost concern about the possibility of a postantibiotic era where common infections and minor injuries may result in significant morbidity and mortality (12). World leaders convened in September 2016 during the 71st United Nations General Assembly (UNGA) to discuss the issue of antimicrobial resistance, an event that resulted in each governing body taking a unified stance toward preventing a postantibiotic era (13–15). The increasing frequency of bacterial infections caused by MDR pathogens and the lack of effective therapeutic

TABLE 1 FDA-approved NME antibiotics from 2010 to November 2017

Yr	NME antibiotic(s)	Class(es)	Ability to treat antibiotic-resistant Gram-negative ESKAPE bacterial infection	Route of drug administration
2010	Ceftaroline fosamil	Cephalosporin	Yes	Systemic
2011	Fidaxomicin	Macrolide	No	Nonsystemic
2014	Dalbavancin	Lipoglycopeptide	No	Systemic
2014	Oritavancin	Lipoglycopeptide	No	Systemic
2014	Tedizolid phosphate	Oxazolidinone	No	Systemic
2014	Ceftolozane-tazobactam	Cephalosporin + β-lactamase inhibitor	Yes	Systemic
2014	Finafloxacin otic suspension	Fluoroquinolone	Yes	Nonsystemic
2015	Ceftazidime-avibactam	Cephalosporin + β-lactamase inhibitor	Yes	Systemic
2017	Delafloxacin	Fluoroquinolone	Yes	Systemic
2017	Meropenem-vaborbactam	Carbapenem + β-lactamase inhibitor	Yes	Systemic
2017	Secnidazole	Nitroimidazole	Yes	Systemic

options for treatment are apparent worldwide. In response to the dwindling antibiotic pipeline, the Infectious Diseases Society of America (IDSA) launched the "10 $\times$ '20 Initiative" in 2010 that challenged stakeholders to advance 10 new U.S. Food and Drug Administration (FDA)-approved systemic agents to treat bacterial infections by 2020 (16). In a follow-up report 3 years later (2013), the IDSA noted definite but slow progress toward achieving the goal of the 10 $\times$ '20 Initiative, wherein only one systemic agent (ceftaroline fosamil) had materialized as of that time (17). As of November 2017, nine new FDA-approved systemic new molecular entity (NME) antibiotics have been developed (Table 1), with a projection that the goal of the IDSA will most likely come to fruition. However, only six (ceftaroline fosamil, ceftolozane-tazobactam, ceftazidime-avibactam, delafloxacin, meropenem-vaborbactam, and secnidazole) out of the nine systemic agents are used for the treatment of drug-resistant Gram-negative bacterial infections. Two agents (fidaxomicin and finafloxacin otic suspension) are approved as nonsystemic antibacterial agents.

The limited availability of antibiotics to treat MDR Gram-negative bacterial infections remains a serious problem. It is therefore imperative to develop new agents or new therapeutic strategies that are able to overcome drug resistance in these organisms.

Permeability Is an Important Consideration in Developing Antibiotics for Gram-Negative Bacteria

Bacteria are classified as Gram positive and Gram negative (with some exceptions, such as mycobacteria) based on their prokaryotic cell membrane structure. Gram-positive bacteria possess a thick cell wall that consists of peptidoglycan and teichoic acid layers anchored on the cytoplasmic membrane. On the other hand, Gram-negative bacteria have a thin peptidoglycan layer that is surrounded by an inner membrane (IM) and an outer membrane (OM), thus forming the periplasmic space (Fig. 1). The double layer of protection in Gram-negative bacteria, in addition to an abundance of efflux pumps and highly selective porins, makes it more difficult for an intracellularly targeting agent to elicit its antibacterial function (18).

The outer membrane is efficient in restricting molecular passage. The OM is an asymmetric bilayer (Fig. 1) with an inner leaflet consisting solely of phospholipids and an outer leaflet that contains an abundance of lipopolysaccharides (LPS). The polymeric LPS is composed of three domains: the hydrophobic lipid A, the hydrophilic core oligosaccharides, and the hydrophilic O antigen. Lipid A is responsible for forming a lipid bilayer with the inner leaflet. Core oligosaccharides and O antigen, which extend outwards to the extracellular environment, are responsible for cellular recognition and virulence (among other functions). The presence of the OM makes Gram-negative bacteria intrinsically resistant to many antibiotics, especially those with a high molecular weight and hydrophobicity. For instance, the LPS structure renders the bacterial OM more restrictive to hydrophobic antibiotics than the IM (19). It has been argued that the hydrophilic carbohydrate component of LPS creates a hydration sphere that

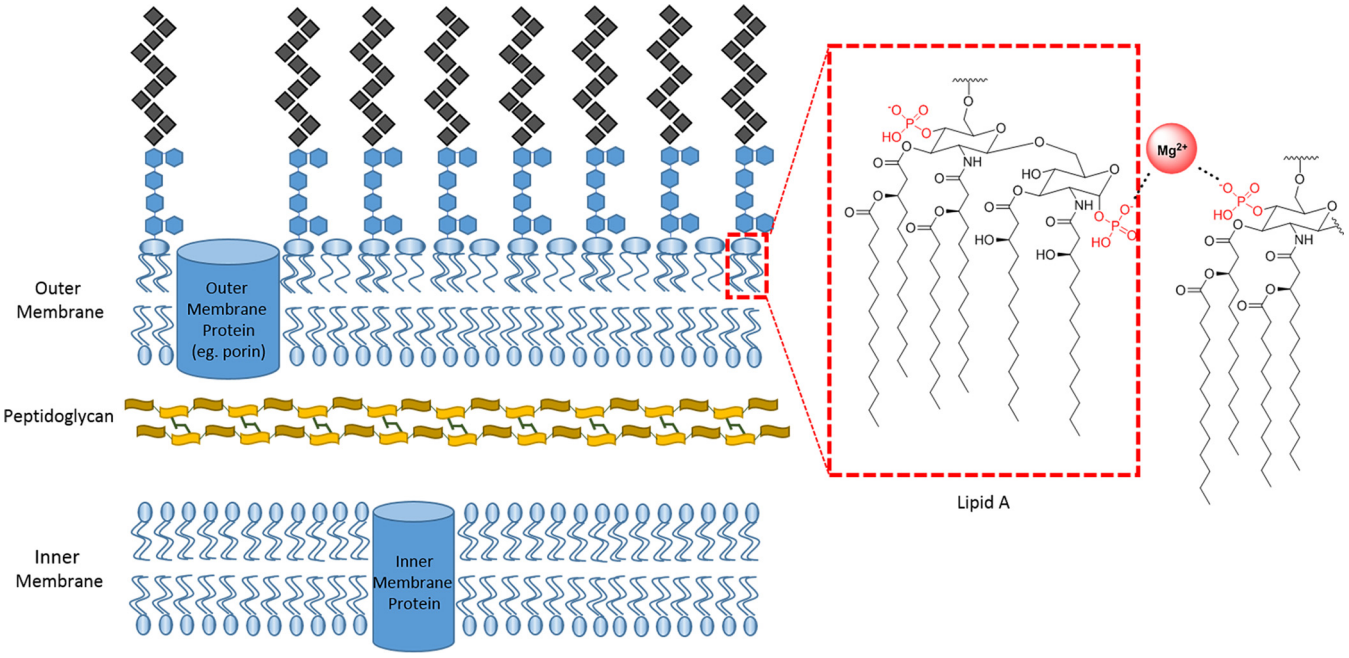


FIG 1 Dual membrane of Gram-negative bacteria. The periplasmic space that contains a thin peptidoglycan layer is enclosed by the outer membrane and the inner membrane. The asymmetric OM has an inner leaflet composed of phospholipids and an outer leaflet with abundant LPS. The LPS is sectioned mainly into hydrophobic lipid A (the structure is expanded in the dashed red box), the hydrophilic core oligosaccharides (blue hexagons), and the hydrophilic O antigen (black diamonds). However, there is some degree of variation among Gram-negative bacteria. The lipid A structure typically has two negatively charged phosphate groups stabilized by a divalent cation (such as Mg^{2+}) bridge between adjacent lipid A phosphate groups that imparts structural stability to the OM. Both the inner and outer leaflets of the IM consist mainly of hydrophobic phospholipids. The difference in molecular compositions between the OM and IM results in their orthogonal sieving properties.

restricts the movement and passage of hydrophobic molecules across the membrane (20). The efficient packing of lipid A, due to its molecular organization and lower unsaturated fatty acid content than that of a normal phospholipid bilayer, results in lower OM fluidity (21–23), thus limiting the membrane permeation of hydrophobic agents. Integral membrane proteins that interact directly with LPS, such as outer membrane protein H (OprH) in *P. aeruginosa* (24) and the Tol-Pal complex in *Escherichia coli* (25), further augment stability and, therefore, the impermeability of the membrane. Experimental evidence shows 50- to 100-fold-lower hydrophobic probe permeation rates in lipid bilayers that contain lipopolysaccharide (reflective of the OM) than in bilayers that consist of phospholipids only (reflective of the IM) (26). Structural variabilities and modifications in the LPS, especially the lipid A portion, result in significant differences in drug permeation rates among Gram-negative organisms (27). It is therefore clear that the OM constitutes a major hurdle for drug uptake in Gram-negative bacteria, especially for *P. aeruginosa*, which has 12- to 100-fold reduced outer membrane permeability relative to that of *E. coli* (28).

Membrane permeation is a limitation to most but not all agents with a high molecular weight. The glycopeptide antibiotic vancomycin, with a molecular mass of 1449.3 g/mol, lacks antibacterial activity against most clinically relevant Gram-negative bacteria. Vancomycin inhibits peptidoglycan synthesis by sequestering peptidoglycan precursors that ultimately prevent glycan cross-linking. In Gram-positive organisms, vancomycin exerts its antibacterial activity uninhibited, as its target is located at the cell membrane. However, it must traverse the OM and reach the periplasmic space to elicit its function in Gram-negative organisms, a feat which vancomycin is incapable of achieving due to the protective membrane barrier. The loss of antibacterial activity due to membrane impermeability is true for almost all clinically used glycopeptide antibiotics (29, 30). Most antibiotics and biomolecules with molecular masses of >600 g/mol are incapable of traversing the OM (31), with few exceptions, including polybasic

amphiphiles, such as polymyxins and antimicrobial peptides, and nonbasic energy sources, such as maltohexaoses (32, 33).

Charged (cationic or zwitterionic) or noncharged small hydrophilic molecules are typically able to enter the periplasmic space via nonspecific protein channels called porins. Examples of such antibiotics with porin-dependent uptake include β-lactams, fluoroquinolones, and sulfonamides. These β-barrel-structured integral protein channels allow water-soluble molecules to traverse the restrictive hydrophobic membrane through their water-filled cavity (34–36). However, porins impose molecular sieving properties, as only small molecules, typically ≤600 g/mol, are believed to pass through their narrow channels (37–39). Drug permeation through porins may also vary among Gram-negative bacteria. For instance, *P. aeruginosa* possesses lower outer membrane permeability than *E. coli*, as it expresses a more selective outer membrane protein F (OprF) porin (28, 39). The OprF porin constitutes the majority of the porins present in *P. aeruginosa* (40). Porins that are present in relatively smaller amounts in *P. aeruginosa* include OprB, OprC, OprD, OprE, OprF, OprG, OprH, and others (41, 42). The OprF porin has been shown to also allow solute diffusion much more slowly than classical porins as a consequence of its structural conformation (39, 43). For example, the monosaccharide L-arabinose was found to diffuse 50 times slower in the OprF porin in *P. aeruginosa* than in the OmpF porin channel of *E. coli* (44). Low intracellular drug concentrations due to slow porin-mediated influx in *P. aeruginosa* are exacerbated by the abundance of multidrug efflux pumps and resistance-encoding genes (28, 43, 45).

However, several antibiotics with a high molecular mass (>600 g/mol) are able to pass through the OM in a mode of uptake independent of porins or passive diffusion. These compounds are mostly cationic and are hydrophilic (such as aminoglycosides [46]) or amphiphilic (such as polymyxins) in nature (46). They are able to transit the OM via a "self-promoted" uptake mechanism, which is characterized by the initial displacement of divalent cations (Ca^{2+} or Mg^{2+}) that results in OM destabilization (47). Electrostatic interactions between the positively charged divalent cations and the negatively charged phosphate groups on lipid A stabilize the LPS structure (48–50). It is perceived that the subsequent localized OM disruption from divalent cation displacement facilitates the penetration of the antibiotic into the periplasmic space (51, 52). It was widely documented in early years that the antibacterial activities of aminoglycosides and polymyxins are antagonized by the exogenous addition of Mg^{2+} and Ca^{2+} cations (53–56). This observation was later attributed as being a hallmark of the self-promoted uptake mechanism, that it entails the displacement of divalent cation LPS bridges and that exogenous supplementation of the divalent cations immediately arrests the process (57). The physicochemical requirements for a molecule to display self-promoted uptake are yet to be fully understood, although the propensities of a molecule to be protonated (to effectively carry one or more positive charges) under physiological conditions and to strongly interact with LPS may be necessary characteristics.

The inner membrane as a second restrictive barrier for agents with cytosolic targets. The phospholipid bilayer that comprises the IM greatly limits the diffusion of hydrophilic molecules. Compared to the OM, hydrophobic molecules easily traverse the IM through passive diffusion. However, charged solutes such as sodium cations and hydrophilic nutrients such as glucose enter freely into the cytosol once they traverse the OM. Their uptake is achieved through the use of solute-specific energy-dependent transporter proteins (58). Some weakly charged or neutral amphiphilic compounds may also enter the cytosol by utilizing proton motive force (PMF) (59, 60). Bacterial PMF is governed by the proton gradient, ΔpH, and membrane potential, $\Delta\Psi$. The ΔpH is believed to facilitate the diffusion of weakly charged molecules through charge neutralization, while the $\Delta\Psi$ is perceived to stimulate electrochemical interactions that lead to molecular uptake (61, 62). For instance, the cytoplasmic uptake of the sulfonamide (63) and tetracycline (64) classes of antibiotics is ΔpH dependent, while the uptake of the aminoglycoside class of antibiotics appears to be $\Delta\Psi$ dependent (65).

Intracellular drug concentrations are greatly affected by efflux. Once a drug makes its way intracellularly, it could be effluxed out before it mediates its antibacterial action. Efflux pumps are membrane proteins that can expel their substrates from the cytosol into the periplasm or from the periplasm into the external environment. So far, all studied Gram-negative organisms are known to express at least one multidrug efflux pump (66). Bacterial efflux systems have been extensively reviewed in the literature (see references 67–72). However, it should be noted that drug efflux affects the intracellular concentration of a therapeutic agent and that the overexpression of multidrug efflux pumps confers intrinsic antibiotic resistance on the pathogen.

There is an urgent need for guidelines to develop agents that are able to penetrate both outer and inner membranes. It is evident that drug permeability in Gram-negative bacteria is more challenging for antibiotics with cytosolic targets, as they must transit two protective lipid bilayers (73). One approach to overcome this involves appending or tweaking different functional groups on a lead structure, in the hope of generating more-amenable derivatives with enhanced biological activity and cellular permeation. "Rule-of-thumb" knowledge in structural modification is used by medicinal chemists to make rational decisions for drug optimization. Lipinski's rule of five for favorable drug oral bioavailability (74) has become a popular *in silico* guideline for the development of therapeutic agents that are able to cross intestinal epithelial cells. To possess good pharmacokinetics in the human body, Lipinski's rule proposes that an agent may not have (i) more than five hydrogen bond donors, (ii) more than 10 hydrogen bond acceptors, (iv) a molecular mass of $>$500 g/mol, and (v) a lipophilicity factor (log *P*) of $>$5, as measured by the octanol-water partition coefficient (74, 75). Unfortunately, Lipinski's metrics do not hold true for antibacterial agents that require bacterial membrane penetration. Molecular passage through the OM appears to be governed by a different set of physicochemical rules that are orthogonal to the IM (18). Compounds that are optimized solely to traverse the OM most likely would not be able to cross the IM and vice versa. A widely acceptable set of membrane permeation rules for antibacterial agents appears to be nonexistent (76). An attempt was previously made to formulate a guideline by binning all antibiotics in the pipeline and in clinical evaluation to correlate discernible physicochemical parameters with antibacterial activity (77). Compounds were binned into three categories, namely, compounds that have activity against only Gram-positive organisms, compounds that have activity against Gram-negative organisms, and compounds that are antipseudomonal. A high polarity (for porin uptake) and a reasonable level of lipophilicity (to ensure lipid membrane penetration) were observed to be ideal for agents against Gram-negative organisms (77). By exploiting the ideal physicochemical properties revealed from binning antibiotics, an effort to optimize the activity of oxazolidinones against Gram-negative bacteria was recently described (78). Members of the oxazolidinone class of antibiotics, such as linezolid, do not have potent activity against most Gram-negative organisms, presumably due to permeation impediments across the OM and/or efflux (79). The most active prepared oxazolidinone analog demonstrated only a modest enhancement of activity against *E. coli*, and those authors noted that a fully realized set of permeation guidelines is direly needed for optimizing lead compounds (78). A recent article emphasized the inherent hurdles in developing agents that are able to permeate Gram-negative membranes and suggested that the binning process should be further refined to include the route(s) of cellular entry for each antibacterial agent (18). However, the development of reliable methods that allow data mining for cellular entry and the accumulation of antibiotics is necessary to realize such a suggestion. At this point, several experimental protocols may hold the key to tackling this proposition. For instance, the elucidation of bacterial uptake mechanisms and the subsequent quantification of cytoplasmic accumulation that utilize techniques such as tandem liquid chromatography-mass spectrometry (LC-MS) (80, 81), Raman spectroscopy (82), and microspectroscopy (83) have been reported. A proof-of-concept study that utilized LC-MS for quantification and several correlation programs for analysis of 10 sulfonyladenosine-containing agents in terms of their membrane permeation in *E. coli*,

Bacillus subtilis, and *Mycobacterium smegmatis* was reported (84). This systematic approach successfully delineated the relationship between the physicochemical properties and the cytoplasmic accumulation of sulfonyladenosines. For instance, the cytoplasmic accumulation of the 10 sulfonyladenosine-containing compounds in *E. coli* was positively correlated with hydrophobicity but negatively correlated with polarity (84). This platform is envisioned by those authors (84) to be applicable to a larger diverse panel of chemical agents and other bacterial organisms and may therefore be utilized for formulating a set of antibacterial permeation rules in the future.

The impermeability of Gram-negative bacterial membranes greatly limits our ability to develop new antibiotics, and there is an apparent void in the fundamental understanding of physicochemical properties necessary for an agent to overcome the double barrier of protection in Gram-negative bacteria. However, recent advances have shed some light on this hurdle (84, 85). It was recently shown that for small molecules to accumulate in the Gram-negative bacterium *E. coli*, they must contain an amine group (a primary amine is preferred over a secondary or tertiary amine), be amphiphilic, be rigid, and have low globularity (defined as the spatial parameter of the molecule) (85). By applying these rules, the natural product deoxynybomycin, which targets DNA gyrase and which is active against Gram-positive bacteria only, was converted into an antibiotic with activity against a diverse panel of multidrug-resistant Gram-negative pathogens, excluding *P. aeruginosa* (85).

It may also be argued that instead of spending great efforts and resources on the development of intracellularly targeting antibacterials, one may focus on exploring membrane targets that are easily accessible (86). For instance, the membranolytic function of antimicrobial peptides and amphiphilic agents (87–90) or the inhibition of essential outer membrane proteins (91, 92) may be exploited. Looking forward, we foresee the materialization of the essential paradigm to predict bacterial membrane penetration. But for how long? Only time will tell.

Therapeutic Approaches To Overcome Antimicrobial Resistance

Clinicians have been saddled with the onerous task of refining medical practices and procedures to combat the spread of antibiotic resistance, but only so much can be achieved if new agents are not developed to supplement our current antibiotic arsenal. Pathogens have shown their resilience in withstanding antibiotic monotherapy due to their rapid doubling times and high mutation rates. Some pathogens, such as those of the *Mycobacterium* genus or those that form persister cells, display antibiotic tolerance due to their slow growth or dormancy (93). However, the notion of antibiotic tolerance has been observed only *in vitro*, and a recent *in vivo* experiment suggests that nonreplicating bacteria might not necessarily confer resistance (94). Acquired resistance is due mostly to the selective pressure that an agent exerts on a bacterial population. A mutation(s) that confers overall fitness under such antibiotic stress (causing the bacteria to survive) is propagated in surviving cells and therefore gives rise to a drug-resistant population. Moreover, it has been documented that some pathogens under antibiotic monotherapy may induce resistance mechanisms that confer cross-resistance to other chemically unrelated antibiotic classes (95). Conversely, resistance mechanisms that confer hypersusceptibility to other antibiotics, known as collateral susceptibility, have also been reported (96). Drug-resistant bacteria may overexpress genes that encode molecular defense mechanisms such as efflux pumps or drug-inactivating enzymes. These resistance genes can disseminate to a different organism via horizontal gene transfer of mobile genetic elements such as plasmids, transposons, and integrons (97). One way to solve this problem is to continually develop new antibiotics and/or new drug classes that delay the evolution of drug resistance. Further understanding of the molecular interplay that governs pathogenic responses during antibiotic therapy is, however, essential to guide the developmental process of overcoming drug resistance. Fundamental progress in basic science is as vital as it is in clinical science. A close rapport between clinicians and scientists is indeed critical to address the problem of antibiotic resistance development and dissemination.

Here, we discuss some therapeutic approaches that may be able to delay the development of antibiotic resistance and briefly elucidate the hypotheses behind them.

Antivirulence therapy. The development of agents that are not bactericidal but that indirectly inhibit the molecular pathway responsible for bacterial communication is a viable strategy to address the problem of antibiotic resistance (98–100). This therapeutic approach is based on the purported delayed development of bacterial resistance, as it is perceived that such agents exert reduced evolutionary selective pressure (101). On the other hand, agents that challenge bacterial survival by directly inhibiting a molecular target may result in higher rates of resistance development. For example, blocking bacterial quorum sensing may be a feasible approach. Quorum sensing is characterized by the production, release, and group-wide detection of autoinducer molecules by bacteria as a mode of communication of bacteria with their neighbors (102). This network of communication is triggered by environmental factors within the microbial community, such as differences in bacterial density or the presence of environmental challenges (either physical or chemical) (103, 104). Once these signaling molecules are detected, cascades of physiological and metabolic changes occur by orchestrated alterations in bacterial gene expression, resulting in the secretion of biomolecules needed for biofilm formation and virulence (105). Therefore, hindering quorum sensing may result in the pathogen not being able to cause harm to the host. Extensive discussions of bacterial quorum sensing and the development of agents that are able to quench this bacterial process have been reported (see references 103 and 105–108). Several agents that block quorum sensing are in preclinical development. For example, the synthetic agent *meta*-bromothiolactone (mBTL) has been reported to curb the production of the virulence factor pyocyanin and biofilm formation in *P. aeruginosa* by affecting the regulation of the Las and Rhl quorum-sensing systems (109). Moreover, *in vitro* protection of human lung epithelial cells and *in vivo* protection of *Caenorhabditis elegans* against *P. aeruginosa* by mBTL were described (109). A follow-up report detailed the optimization of mBTL for enhanced stability, as the thiolactone ring is susceptible to chemical and enzymatic hydrolysis (110). Other anti-quorum-sensing agents (111–114) have also been reported to exhibit similar promising *in vitro* and *in vivo* results. However, this paradigm was recently challenged (115, 116), and several clinical isolates have been reported to be resistant to established anti-quorum-sensing agents (117). Anti-quorum-sensing agents are yet to reach clinical trials.

Combination therapy. Combination therapy has been well received by the scientific and medical communities and has existed for more than 3 decades (118). Clinicians often prescribe two or more antibiotics concomitantly during empirical treatment to ensure the coverage of all possible bacterial pathogens and resistance profiles. It was later realized that the use of multiple antibiotic agents in a therapeutic cocktail may limit the development of resistance *in vitro* in comparison to drug monotherapy. The overall expected clinical outcome for this strategy is lower patient mortality rates. However, combination therapy is not limited to antibiotic agents but includes therapeutic interventions that may use bioactive helper molecules, also known as adjuvants, to enhance the efficacy of a primary antibiotic. In fact, it has been argued that the adjuvant-antibiotic combination approach offers a more attractive option for the treatment of drug-resistant bacterial infections than the use of multiple antibiotics (119). Here, we discuss combination therapy as (i) an antibiotic-adjuvant approach and (ii) an antibiotic-antibiotic approach.

(i) Antibiotic-adjuvant combination approach. Arguably the most successful therapeutic strategy of the 21st century, the antibiotic-adjuvant approach has resulted in several drug entities on the market. The paradigm entails the use of bioactive adjuvants that augment the antibiotic efficacy of a primary antibiotic against drug-resistant pathogens. The adjuvant may possess weak to no antibacterial activity on its own but is able to either impede antibiotic resistance mechanisms or potentiate antibiotic action. An adjuvant may be an efflux pump inhibitor (EPI) (to prevent the extrusion of drugs), a membrane permeabilizer (to increase the number of molecules that penetrate

the membrane), or an enzyme inhibitor (to prevent the degradation of drugs before they reach their targets).

(a) β-Lactam–β-lactamase inhibitor combination. Augmentin is a clinically used broad-spectrum antibiotic combination of amoxicillin and clavulanic acid (120). Clavulanic acid is a β-lactamase inhibitor that acts in synchrony with the β-lactam amoxicillin to prevent bacterial growth. β-Lactamase inhibitors such as clavulanic acid block the function of β-lactamases or β-lactam-hydrolyzing enzymes by forming an irreversible bond with the enzyme's functional/reactive site. Clavulanic acid by itself possesses poor intrinsic activity against pathogens, but it efficiently inhibits widespread β-lactamases such as many types of the extended-spectrum β-lactamase (ESBL) family (121). Inhibition of ESBLs is especially important, as members of this group of β-lactamases are promiscuous and are able to hydrolyze penicillins, cephalosporins (first, second, and third generations), and monobactams (such as aztreonam) (122, 123). Augmentin was first introduced in 1981 by GlaxoSmithKline and is useful in the clinic even today (124, 125). It is not surprising for a β-lactam to be a cornerstone antibiotic in an antibiotic-adjuvant approach, as β-lactams are considered to be ideal drugs in terms of their efficacy and tolerability. Unfortunately, their "idealness" has been significantly threatened by the global spread of bacterial β-lactamase-encoding genes. The pursuit of adjuvants that inhibit β-lactamases is therefore crucial for retaining the clinical effectiveness of the β-lactam class of antibiotics. The recent approvals of ceftolozane-tazobactam in 2014, ceftazidime-avibactam in 2015, and meropenem-vaborbactam in 2017 by the FDA (Table 1) for the treatment of drug-resistant Gram-positive and Gram-negative bacterial infections are indicative of the continued interest in the development of combination therapies that include a β-lactam and a β-lactamase inhibitor. At least four more β-lactam-based antibiotic-adjuvant combinations are currently in clinical trials (126, 127). The popularity of the antibiotic-adjuvant strategy is apparent in the number of drug combinations under preclinical evaluation. We briefly highlight three examples, although extensive reviews were reported previously (see references 119 and 128–130).

(b) Imipenem-cilastatin-relebactam triple combination. In 1985, the combination of the carbapenem imipenem and the adjuvant cilastatin was approved for use in the United States under the trade name Primaxin (131). Imipenem is a broad-spectrum antibiotic that is rapidly degraded by the human renal enzyme dehydropeptidase 1, and the resulting metabolite poses the potential for nephrotoxicity (132). Thus, the addition of the dehydropeptidase 1 inhibitor cilastatin to imipenem prevents imipenem's degradation and nephrotoxicity. Cilastatin also blocks megalin-mediated proximal tubule uptake of cationic antibiotics (133), further lowering the risk of kidney damage. However, the recent increase in the prevalence of bacterial infections caused by carbapenemase-producing organisms that inactivate imipenem calls for an improvement in this therapy. The combination of imipenem-cilastatin with the addition of the diazabicyclooctane β-lactamase inhibitor relebactam (also known as MK-7655) is currently in a phase 3 clinical trial for the treatment of Gram-negative bacterial infections (134). The adjuvant relebactam is able to inhibit the activity of ESBLs and class A (e.g., KPC) and class C (e.g., AmpC) β-lactamases against imipenem by irreversibly blocking their functional/reactive site (135). The triple combination was found to be generally well tolerated in patients, with commonly reported adverse effects being nausea, vomiting, and diarrhea (136). Recently, a phase 3, randomized, double-blind, noninferiority study of imipenem-cilastatin-relebactam in comparison to imipenem-cilastatin-colistimethate sodium for the treatment of hospital-acquired bacterial pneumonia (HABP), ventilator-associated bacterial pneumonia (VABP), complicated intra-abdominal infection (cIAI), and complicated urinary tract infection (cUTI) (https://clinicaltrials.gov/ct2/show/study/NCT02452047) was completed. Results are yet to be disclosed. Another phase 3, randomized, double-blind, noninferiority study of imipenem-cilastatin-relebactam versus piperacillin-tazobactam for the treatment of HABP or VABP is currently recruiting volunteers (https://clinicaltrials.gov/ct2/show/NCT02493764). Moreover, a phase 3, nonrandomized, open-label study of the effi-

cacy and safety of imipenem-cilastatin-relebactam for the treatment of cIAI and cUTI is currently ongoing in Japan (https://clinicaltrials.gov/ct2/show/NCT03293485). The activity of the triple combination, unfortunately, is very limited against organisms that harbor metallo-β-lactamases such as New Delhi metallo-β-lactamase 1 (NDM-1), imipenemase (IMP), and Verona integron-encoded metallo-β-lactamase (VIM) (134, 135).

(c) Aspergillomarasmine A. The adjuvant aspergillomarasmine A (AMA) was recently discovered to resuscitate the biocidal activity of the carbapenem drug meropenem against metallo-β-lactamase-producing organisms (137). The fungal metabolite AMA was first isolated in the 1960s (138) and was later evaluated for its antihypertensive properties (139, 140). In an antibiotic era where enzymes that are capable of degrading even the most powerful β-lactam (e.g., carbapenems) are abundant, it is promising to find that AMA is able to inhibit metallo-β-lactamases such as the NDM-1 enzyme. AMA was found to sequester zinc cations (137), which are essential for the hydrolytic activity of metallo-β-lactamases (141, 142). In a mouse model of NDM-1-positive *K. pneumoniae* infection, a single dose of the combination of meropenem (10 mg/kg of body weight) and AMA (30 mg/kg) led to >95% survival after 5 days postinfection (137). The use of meropenem alone (10 mg/kg) or AMA alone (30 mg/kg) resulted in 0% survival (137). These promising results stimulate the need for an optimized dosing regimen of AMA in combination with carbapenems for the treatment of metallo-β-lactamase-producing pathogens. Currently, medicinal chemists are looking into optimizing the chemical structure of this adjuvant. The total synthesis (143), structure-activity relationship (144), and structural reassignment (145) of AMA have all been recently reported.

(d) SPR741. A polymyxin-based antimicrobial peptide, SPR741 (formerly NAB741), is currently being developed by Evotec AG and Spero Therapeutics as an adjuvant that potentiates antibiotics against Gram-negative pathogens (146). The recently completed, randomized, quadruple-blind, phase 1 clinical study of the safety and tolerability of this adjuvant in healthy volunteers (https://clinicaltrials.gov/ct2/show/NCT03022175) yielded favorable results. SPR741 was well tolerated by healthy adult volunteers in a single dose of up to 800 mg and at doses of up to 600 mg every 8 h for 14 days (Spero Therapeutics). In contrast to polymyxins, SPR741 has poor activity against Gram-negative pathogens on its own but can permeabilize the outer membrane to facilitate the entry of other antibiotics into the bacterial cell (147). For instance, SPR741 was reported to sensitize *Enterobacteriaceae* and *A. baumannii* but not *P. aeruginosa* to an extensive panel of antibiotics, including clarithromycin, fusidic acid, and rifampin (148–150). These three antibiotics are not classical drugs used to treat Gram-negative bacillary infections due to intrinsic resistance, notably OM impermeability. At 2 μg/ml of SPR741, the MIC_{50} and MIC_{90} of rifampin against a panel of MDR *E. coli* isolates were 0.016 and 0.06 μg/ml, respectively (149). Rifampin alone has MIC_{50} and MIC_{90} values of 16 and >128 μg/ml, respectively, against the same panel of *E. coli* strains (149). At similar concentrations of SPR741, strong rifampin potentiation against a panel of MDR *A. baumannii* isolates was also described (149). The *in vivo* efficacy of the SPR741-rifampin combination was shown in murine thigh and lung infection models (151, 152). Interestingly, the characteristic nephrotoxicity concerns usually associated with polymyxins (153, 154) were not observed with SPR741 at a dose of 60 mg/kg/day in cynomolgus monkeys after 7 days of a 1-h infusion three times daily (155).

(ii) Antibiotic-antibiotic combination approach. The use of two or more antibiotic agents that have different targets, which may or may not be for a single biochemical process, presents another attractive strategy to overcome drug resistance. The hypotheses of the antibiotic-antibiotic combination approach are (i) to achieve drug synergism between each drug component in a way that enhances treatment efficacy and (ii) to simultaneously impact multiple targets in pathogens, resulting in the suppression of antibiotic resistance development and the complete eradication of bacterial strains with intermediate susceptibility or resistance to one of two antibiotics. The assumption is that the bacterial cell will have difficulty surviving multiple "hits" at the same time. Clinicians sometimes employ this strategy during empirical treatment of infection, and

such an approach might indeed prolong the clinical utility of antibiotics. For instance, the combination of trimethoprim-sulfamethoxazole has been in use since 1968 for the treatment of bacterial infections caused by the *Enterobacteriaceae* family and nonfermentative opportunistic pathogens (156, 157). Both antibiotics work together to inhibit sequential steps in bacterial folic acid synthesis, which is detrimental, as most bacteria are obligate folate synthesizers while humans acquire folate through diet. The sulfonamide sulfamethoxazole inhibits dihydropteroate synthase that converts *para*-aminobenzoic acid to dihydrofolate, and trimethoprim inhibits dihydrofolate reductase that converts dihydrofolate to tetrahydrofolate (folic acid's bioactive form) (157). Trimethoprim-sulfamethoxazole is an efficacious antibiotic used to treat urinary tract and select gastrointestinal bacterial infections (158, 159). Sulfamethoxazole may be replaced with the sulfonamide sulfametrole in some European Union countries, although both agents, when combined with trimethoprim, exhibit the same clinical efficacy (160). However, the success of the trimethoprim-sulfamethoxazole combination has been affected by the dissemination of resistance mechanisms that prevent both antibiotics from eliciting their biological functions. The overexpression of multidrug efflux pumps that are able to expel both trimethoprim and sulfamethoxazole out of the cell and membrane modifications that limit their intracellular permeation are problematic (156). Many other antibiotic-antibiotic combinations are used in the clinic, including those of tigecycline-gentamicin, tigecycline-colistin, and carbapenem-colistin (161), to name a few.

Challenges of combination therapy. Considering the "success" of several antibiotic-antibiotic combinations in the past few decades, the strategy remains fallible, as several important pharmacological questions remain unanswered. For instance, other than for tuberculosis, there is no clinical evidence to support the notion that antibiotic resistance is suppressed by antibiotic-antibiotic combinations (162). This is a tough concept to prove, as clinical studies are usually designed not to measure the emergence of antibiotic resistance but to prevent or treat it. These data are often extrapolated from *in vitro* studies and in animal models and may be different from what is obtainable in human hosts. The clinical translatability of drug synergy *in vitro* and in animal models to humans is also debatable (162). The limited available clinical evidence suggests a statistically insignificant difference between antibiotic-antibiotic combination therapy and monotherapy for the treatment of Gram-negative bacterial infections in terms of mortality rates, which is the postulated clinical outcome for synergistic drug combinations (163–165). For instance, a systematic study reported no appreciable improvement for the combination of a β-lactam–aminoglycoside over β-lactam monotherapy for the treatment of endocarditis caused by a Gram-positive bacterium (e.g., *Staphylococcus aureus*), even though the combination shows synergism *in vitro* (166). Another study showed that the combination of a β-lactam and either an aminoglycoside or a fluoroquinolone in comparison to β-lactam monotherapy imposes no benefit for patient mortality for the treatment of infections caused by the Gram-negative organism *P. aeruginosa* (164). However, it should be noted that the supporting evidences for these studies were based on meta-analyses of a small amount of available clinical data that included those during early years when drug resistance was not as prevalent. Caution should therefore be taken in the extrapolation of data to fit the current landscape, where incidences of MDR and XDR bacterial infections are much higher. Currently, there seems to be an apparent consensus that combination therapy is preferred for the treatment of MDR pathogen infections of severely ill patients and for empirical therapy (167). A lack of pharmacokinetic complementarity between different drugs might indeed contribute to the discrepancies between *in vitro* data and clinical observations (168). Each drug component may be absorbed or distributed in the human body to different degrees. Pharmacokinetic variances, but also the patient's overall condition, would certainly impose a challenge in fine-tuning the dosages of administered drugs to replicate their observed *in vitro* synergy, as both drugs are required to be located at the site of infection at their optimal concentrations simultaneously. Noncomplementary absorption and distribution rates may therefore be cir-

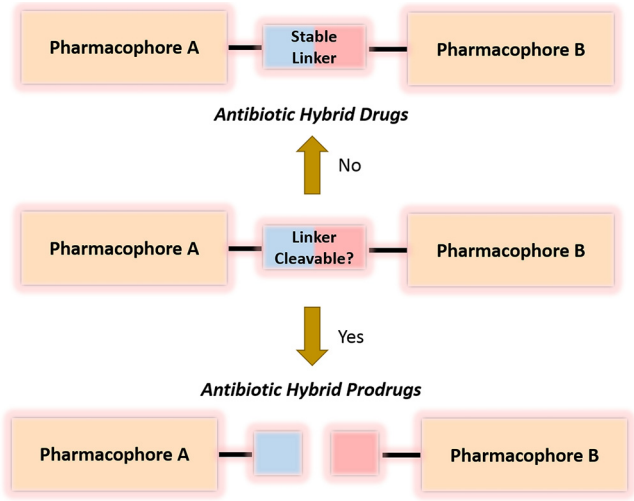


FIG 2 Two different pharmacophoric domains attached covalently by a linker domain. The lability of the linker determines the type of antibiotic hybrid generated. A linker that can be enzymatically degraded (preferably by only bacterium-specific enzymes) gives rise to two functional pharmacophoric entities that are thus used in the antibiotic hybrid prodrug strategy. A linker that is inert to enzymatic degradation is used to hold the two pharmacophoric domains together in the antibiotic hybrid drug strategy.

cumvented by fusing both pharmacophoric molecules together to make a single hybrid antibacterial agent. Here, the fundamental concept behind antibiotic hybrids is discussed.

ANTIBIOTIC HYBRIDS AGAINST ANTIBIOTIC-RESISTANT BACTERIA

The antibiotic hybrid strategy was precipitated from countless attempts to discover new synthetic scaffolds that may yield antibiotics capable of overcoming drug resistance. Scientific ingenuity led to the development of molecular hybrids (Fig. 2) by fusing different biologically active agents into one heteromeric entity with the hope of retaining the biological actions of the constituent fragments. A molecular linker/tether is often used to append the participating agents together via a covalent bond, although molecules could also be fused together directly. The mode of covalent attachment could also be designed to be either cleavable or noncleavable (Fig. 2). A cleavable linker is expected to be enzymatically biotransformed once the hybrid reaches its site of action (the bacteria), while a noncleavable linker remains unchanged throughout its time course in the body. The former constitutes a hybrid prodrug approach, while the latter constitutes a hybrid drug approach. The hypothesis of antibiotic hybrids integrates the working concept of suppressing drug resistance evolution in combination therapy into monotherapy, thus presenting a molecular agent (instead of two) with a single pharmacokinetic profile. It also eliminates the problem of noncomplementary pharmacodynamics. Hybrid drugs are also postulated to eradicate bacterial strains with intermediate susceptibility or resistance to one of the covalently linked drug fragments. Although unpredictable, retaining antibacterial potency against pathogens that possess intermediate susceptibility or resistance to both drug components is possible, as the process of hybridization may also impart additional physicochemical properties that could alter the hybrid's pharmacological spectrum. For instance, the hybridization of two therapeutic agents may enhance the efficacy or even impart a new mechanism of antibacterial action to the resulting hybrid agent.

Definition of an Antibiotic Hybrid

What defines a hybrid agent? The literature offers various subjective definitions of hybrid agents, depending on the context in which they are being used. In this account, however, we define a hybrid antibiotic as a synthetic construct of two or more pharmacophores belonging to an established agent known to elicit a desired antimi-

crobial effect. This encompasses agents described as being either dual-action antibiotic hybrids (169, 170), chimeric antibiotics (171, 172), multivalent/divalent antibiotics (173–175), or antibiotic conjugates (176). Moreover, the antibiotic hybrid approach is not confined to the covalent fusion of antibacterial agents and may also include beneficial adjuvants such as resistance enzyme inhibitors, membrane permeabilizers, siderophores, and efflux pump inhibitors. The notion of bimodality (coined "dual action" in 1994 [169]) in prospective antibiotic hybrids suggests the need for the covalently appended agents to retain their known biological actions. However, our experience reveals that it might indeed not be necessary to retain both known activities, as an unexpected third mode of action may arise from the fusion of two therapeutic agents (see below). Moreover, some antibiotic hybrids are able to "resuscitate" the antibacterial potency of legacy antibiotics against drug-resistant pathogens. The term legacy antibiotics pertains to widely used antibacterial agents that have been clinically used for decades and whose clinical efficacy is currently being challenged by the rise of antibiotic resistance mechanisms. In this review, we highlight promising antibiotic hybrids with emphasis on those that are successful in inhibiting or eradicating Gram-negative pathogens. We also discuss our recent discovery that we believe could expand the utility of the antibiotic hybrid approach.

Conceptual Challenges in Designing Antibiotic Hybrids

Several inherent problems need to be addressed before the development of an antibiotic hybrid, especially if the agent is directed at Gram-negative pathogens. Limited cellular penetration across the dual layer of protection of Gram-negative bacteria is the first major concern for hybrid agents that have a molecular mass of more than 600 g/mol. As mentioned above, there are currently no infallible permeation guidelines to assist medicinal chemists in designing therapeutic agents that are capable of traversing bacterial lipid bilayers. Antibacterial agents with a high molecular mass will not pass through nonselective porin channels, restricting cellular uptake to receptor-mediated endocytosis or passive diffusion. One way to overcome this permeability problem is to design an antibiotic hybrid that retains the porin-independent uptake mechanism of one or more of the parent antibiotic components. Periplasmic entry through a self-promoted uptake mechanism may be leveraged, as has been widely reported for amphiphilic molecules regardless of their molecular masses (28, 45, 46). Subsequent passage through the inner membrane, which may be dependent on PMF or may occur just through passive diffusion, is then required for entry into the cytosol. For instance, antibiotics of the aminoglycoside class are known to enter the OM of Gram-negative bacteria by a self-promoted uptake mechanism followed by two-step energy-dependent IM uptake to reach the cytosol and elicit their antibacterial function (177). Thus, an aminoglycoside-containing hybrid can be prepared in the hopes of retaining the aminoglycoside's inherent mode of uptake. One may also develop a hybrid that has multiple nonintracellular targets to circumvent the permeability issue altogether. Indeed, permeability impediments due to high molecular mass is a major reason why most antibiotic hybrids have limited activity against Gram-negative bacteria (178).

Another problem lies within the fundamental idea of covalently linking two pharmacological agents together. The point of attachment and the physicochemical properties of the chosen linker are crucial for the overall activity of the hybrid. Ideally, the two molecules should be attached at a nonpharmacophoric region to retain the integrity of the functional domains. According to the International Union of Pure and Applied Chemistry (IUPAC), a pharmacophore is defined as "the collective steric and electronic properties of a molecule that are essential for interaction with the biological target and to elicit response" (179). Any obstruction of the pharmacophoric region of the appended agents might be detrimental to the antibacterial potency of the resulting hybrid. Moreover, steric overlap between the two therapeutic agents, due to proximity, may also result in compromised activity. For instance, an enzyme-targeting antibiotic domain requires that its pharmacophoric region be devoid of obstruction to dock

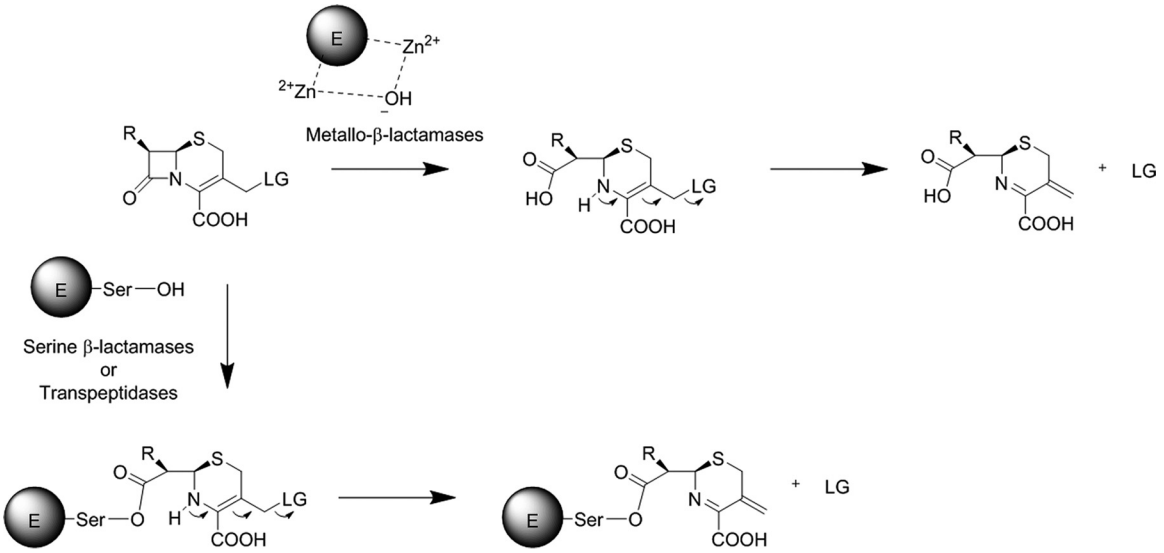


FIG 3 β-Lactam hydrolysis of cephalosporin followed by nonenzymatic release of a leaving group (LG). Enzymatic hydrolysis may be due to β-lactamases, such as serine- or metallo-based β-lactamases, or β-lactam target enzyme transpeptidases. Most hybrid prodrugs utilize this mechanism to release another antibiotic as a leaving group. R, any molecular substituent; E, enzyme.

properly on the target enzymatic pocket. The adjacent antibiotic domain may impose unwanted steric bulk that could hinder the docking sequences, thus preventing the desired enzymatic inhibition. It is therefore necessary to probe the optimal spatial length of the linker during the early stages of development by utilizing *in silico* modeling programs and/or synthesizing several analogs that differ in linker length to evaluate the optimal spatial separation. The properties of the linker/tether could also be manipulated to expand the overall chemical space of the hybrid molecule. For instance, the hydrophobicity of the tether segment may be tinkered with by substituting a polycyclic aromatic-based or a polyethylene glycol-based linker instead of an aliphatic hydrocarbon. While conceptualizing an ideal blueprint seems trivial, the molecular manipulation involved in the synthesis of antibiotic hybrids is quite demanding, and the narrow window of chemical reactions may drive a certain bias during the developmental process (180). This usually makes the development of drug candidates a matter of synthetic convenience rather than design (181).

Antibiotic Hybrid Prodrugs against Antibiotic-Resistant Gram-Negative Bacteria

Concept and hypothesis. A mutual prodrug, as defined by the IUPAC, is the covalent attachment of two drugs to form a unique molecule that undergoes biotransformation (such as bond cleavage) to exhibit its pharmacological effects (179). To achieve this for antibiotic hybrids, a prodrug strategy necessitates the use of a cleavable linker/tether with bacterium-specific lability. The linker/tether must deliver both constituent molecules to their site of action (bacterial compartment) before being degraded. The resulting antibiotic hybrid prodrug may or may not possess biological activity by itself.

Hybrid prodrugs in the literature are comprised mostly of β-lactams linked to other therapeutic agents. The foundation of antibiotic hybrid prodrugs is premised on extensive mechanistic studies of the β-lactam core structure. In 1965, it was discovered that some molecular substituents adjacent to carbon 3 of the cephalosporin (a type of an unsaturated β-lactam/penem) backbone was displaced following β-lactam ring hydrolysis (182). Mechanistically (Fig. 3), it was perceived that the liberated secondary amine (from β-lactam ring hydrolysis) would delocalize its electrons on carbon 2 to form an imine and consequently displace the unsaturated bond (carbon-carbon double bond) along carbon positions 2 and 3. Electron delocalization would ultimately release the molecular substituent or leaving group (LG) adjacent to carbon 3. This knowledge

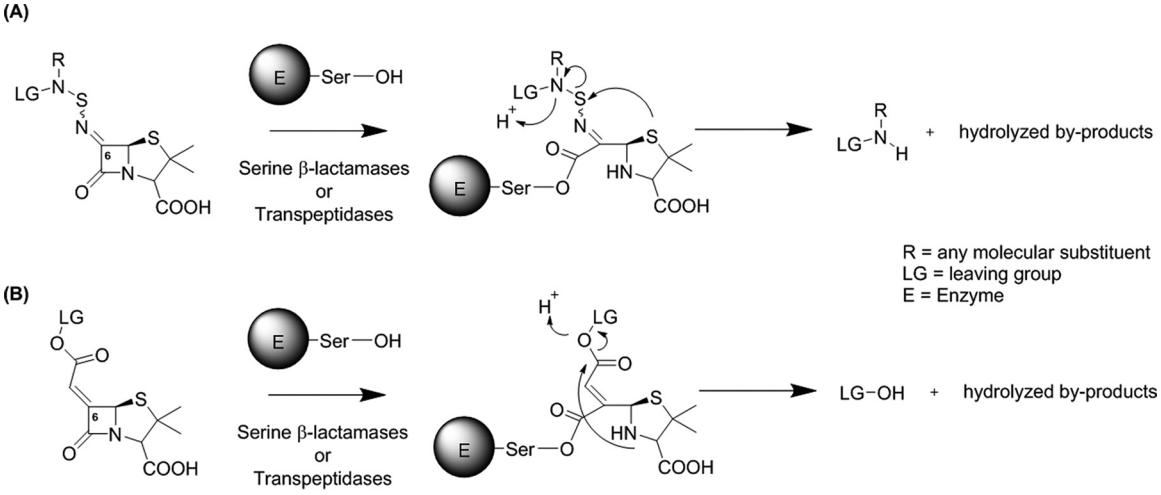


FIG 4 β-Lactam hydrolysis of penicillin that contains an installed leaving group at position 6 via an *S*-aminosulfenimine (A) or a vinyl ester (B) linkage. The mechanisms for the nonenzymatically driven release of the leaving group for panels A and B were elucidated thorough NMR experiments (185, 186). Enzymatic hydrolysis may be due to β-lactamases, such as serine or metallo-based β-lactamases (not depicted above), or the β-lactam target enzyme transpeptidase.

was then used to construct cephalosporin-based hybrid prodrugs where an antibiotic was designed to be the leaving group adjacent to carbon 3. Notably, most investigational antibiotic hybrid prodrugs are cephalosporin based (169, 183). Approximately 3 decades later, it was discovered that the installation of an *S*-aminosulfenimine moiety at carbon 6 of penicillin (a type of saturated β-lactam/penam) resulted in the rapid intramolecular displacement of the *S*-amino substituent following β-lactam ring hydrolysis (Fig. 4) (184). Nuclear magnetic resonance (NMR) studies of this phenomenon (185) led to the proposition that the release of the substituent at penicillin position 6 is due to the formation of a disulfide bond-characterized intermediate that further decomposes to yield several by-products (Fig. 4A). Interestingly, a vinyl ester linkage (186) was reported to be a viable alternative to the *S*-aminosulfenimine linkage, as it was also found to be displaced following β-lactam hydrolysis (Fig. 4B). However, we have yet to see any prospective β-lactam-based hybrid prodrug with either a cleavable *S*-aminosulfenimine or vinyl ester linkage in the literature.

β-Lactam-based prodrugs function via a two-step process: (i) the β-lactam antibiotic elicits its activity by inhibiting the enzyme transpeptidase via acylation (ester bond formation) of the active-site serine residue at the hydroxyl side chain, which consequently results in β-lactam ring amide bond hydrolysis, followed by (ii) the release of another functional antibiotic to elicit its own antibacterial activity. In the presence of serine-based β-lactamases that confer enzymatic drug resistance to β-lactam antibiotics, the β-lactam-containing prodrug could serve as a sacrificial adjuvant to inhibit the resistance enzyme. The drug appended to the hydrolyzed sacrificial adjuvant can then be released to elicit its biological action. Inhibition of serine-based β-lactamases occurs via acylation of the active site of serine at the hydroxyl side chain. Unfortunately, β-lactam-based prodrugs do not have any benefit against metallo-β-lactamases, as this resistance enzyme utilizes zinc ions that activate water molecules to hydrolyze the β-lactam ring and therefore would not be inhibited by acylation. It would be interesting to see the future development of antibiotic hybrid prodrugs that contain zinc-sequestering agents, such as the recently reported agent aspergillomarasmine A (137), to combat metallo-β-lactamase-producing Gram-negative bacteria, as this enzyme is credited as being the main cause of pandrug resistance worldwide (187).

Advantages and disadvantages. Several advantages and disadvantages are discussed above. In addition, achieving synergistic antibacterial activity through the hybrid prodrug approach is a possibility. As the prodrug reaches its target bacterial compartment, the hybrid entity is expected to be cleaved into two functioning drugs

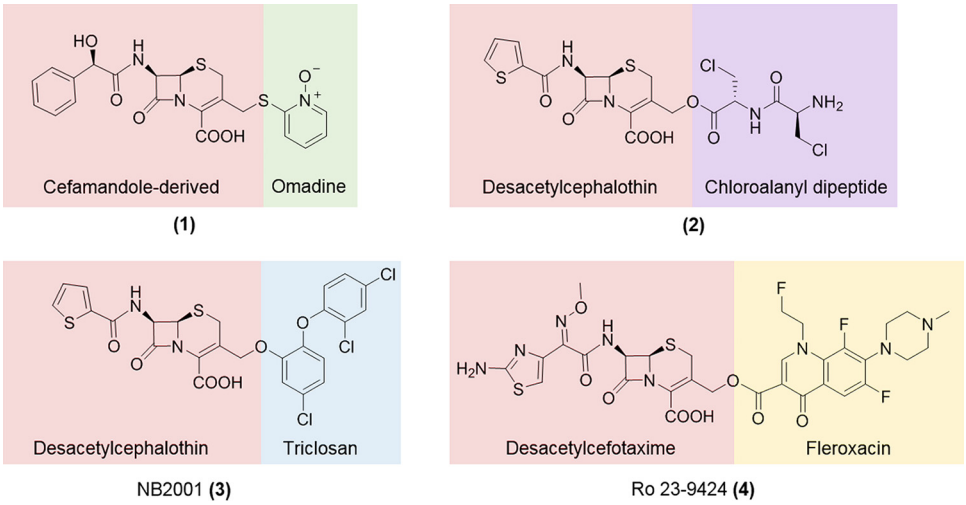


FIG 5 Examples of antibiotic hybrid prodrugs: a cefamandole derivative linked to omadine (hybrid 1), desacetylcephalothin linked to the alanine racemase inhibitor chloroalanyl dipeptide (hybrid 2), desacetylcephalothin linked to triclosan-NB2001 (hybrid 3), and desacetylcefotaxime linked to fleroxacin-Ro 23-9424 (hybrid 4). The majority of antibiotic hybrid prodrugs consist of a cephalosporin.

that may have synergistic interactions. Through the prodrug strategy, prediction of *in vivo* drug synergism from *in vitro* combination studies may be easier, as the problem of noncomplementary pharmacokinetics, a concern in combination therapy, as mentioned above, is bypassed. However, this may hold true only for drug combinations with a 1:1 optimal concentration ratio. Nonetheless, it may be ideal to consider any advantageous synergistic relationship of the antibiotics sought to be fused together.

The major challenge with this approach is the difficulty in designing a bacterium-specific cleavable linker, as the human body is a complex biological system comprising specific and nonspecific enzymes capable of degrading various covalent bonds. If the cleavable linker of the prodrug is degraded before it reaches the bacteria, then the design would no longer be different from that of combination therapy. Therefore, it is crucial to select a linker that is bacterium selective and capable of withstanding human metabolic enzymes. The stability of the cleavable linker in the human body and the drug permeability impediments in Gram-negative bacteria are the major limitations of the hybrid prodrug approach. However, like the mechanistic findings for β-lactam hydrolysis, more and more data on the bacterium-specific molecular interplay become unraveled as we progress into the future, and there is great optimism that human ingenuity will pave the way for the next generations of hybrid prodrugs.

Examples. The majority of hybrid prodrugs in the literature consist of a non-β-lactam antibiotic fused to the adjacent carbon 3 of the cephalosporin core structure (second antibiotic) (Fig. 5). The earliest hybrid prodrug, reported in 1976, was a cefamandole (cephalosporin) derivative linked to omadine at carbon 3 (hybrid prodrug 1) (188). Omadine, also known as 2-mercaptopyridine-*N*-oxide or pyrithione, inhibits bacterial ATP synthesis and is known as a metal chelator (189). Hybrid prodrug 1 was shown to be active against a panel of Gram-negative pathogens with a geometric mean MIC of 0.5 to 16 μg/ml (188). The mode of action of hybrid prodrug 1 was hinted to be mainly that of a cephalosporin, with some contributions from its omadine pharmacophore, as its MIC was reduced by 4- to 32-fold in bacterial strains that express β-lactamases. As noted by those authors, that study validated the concept of a hybrid prodrug but was of no significant therapeutic relevance, as omadine displays undesirable systemic toxicity. Ten years later, a desacetylcephalothin (cephalosporin) linked to a chloroalanyl dipeptide inhibitor of alanine racemase (hybrid 2) was reported to be active against *E. coli* (MIC of 7.05 to 14.1 μg/ml) (190, 191). The cephalosporin pharmacophore serves as an essential structural component for most hybrid prodrugs,

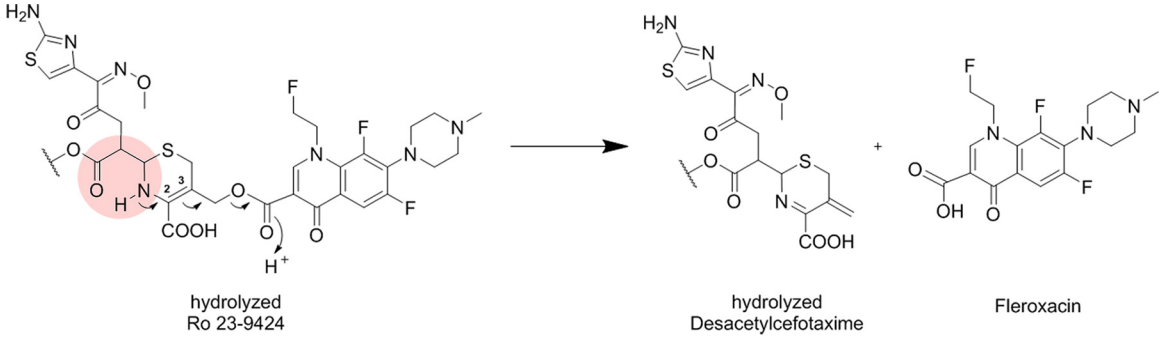


FIG 6 Electron transfer of hydrolyzed Ro 23-9424 results in the formation of hydrolyzed desacetylcefotaxime and fleroxacin by-products. Ro 23-9424 (hybrid 4) initially acts as a cephalosporin, followed by the release of a functional fluoroquinolone as a product of β-lactam hydrolysis. The highlighted red circle is the hydrolyzed β-lactam ring.

and some of the resulting hybrid entities advanced to preclinical/clinical trials. For instance, NB2001 (hybrid 3) by NewBiotics Inc., which consists of a desacetylcephalothin fused with a chlorine-containing phenolic triclosan, was reported to possess potent broad-spectrum bacteriostatic activity against Gram-positive and Gram-negative bacteria (192). However, NB2001 (hybrid 3) was inactive (MIC of $>$32 μg/ml) against *P. aeruginosa* (192), which was not surprising due to the organism's restrictive OM and abundance of multidrug efflux pumps. No data were reported for *A. baumannii*. The antibacterial activity of NB2001 was attributed to the release of its triclosan component, as the intact hybrid exhibited significantly reduced *in vitro* binding to transpeptidases relative to that of cephalothin, suggesting a diminished mode of action of cephalosporin (193). Moreover, the intact hybrid was unable to inhibit the enzyme enoyl-acyl carrier protein reductase FabI (target enzyme for triclosan) (193). NB2001 (hybrid 3) entered preclinical evaluation for bacterial and nosocomial infections but was discontinued in 2005 for undisclosed reasons. Another worthy example of a β-lactam-based hybrid prodrug is Ro 23-9424, a story of high hopes but dashed dreams.

Ro 23-9424: struggle, triumph, and failure—a story from the past. In 1989, Roche pharmaceuticals announced the development of a cephalosporin-fluoroquinolone ester hybrid prodrug called Ro 23-9424 (hybrid 4) (Fig. 5) with potent broad-spectrum bactericidal activity against Gram-positive and Gram-negative organisms (194). The hybrid prodrug contains a desacetylcefotaxime (a cephalosporin) covalently linked to a fleroxacin (a fluoroquinolone) via a cleavable ester linkage adjacent to carbon 3, which is consequently cleaved following enzymatic hydrolysis of the β-lactam ring structure (Fig. 6). Its mode of uptake was proposed to be porin mediated (195, 196) (even though it has a modestly high molecular mass of 764.7 g/mol). Ro 23-9424 (hybrid 4) displayed only limited activity against *P. aeruginosa* (194), which may be attributed to reduced OM permeability (due to the selective OprF porin that is majorly expressed) and/or an abundance of multidrug efflux pumps of the organism. Cephalosporins inhibit peptidoglycan synthesis by acetylating the active site of transpeptidases, while fluoroquinolones inhibit DNA synthesis via the inhibition of DNA gyrase and topoisomerase IV. Ro 23-9424 (hybrid 4) acts initially as a cephalosporin, where the hydrolysis of the β-lactam ring results in a fluoroquinolone secondary mode of action (197). The intact hybrid prodrug exhibits only minimal DNA synthesis inhibition (195). As conceptualized, the antibacterial activity was retained in *E. coli* strains that are resistant to either β-lactams, fluoroquinolones, or both agents (196). The *in vitro* half-life of the hybrid prodrug in human serum was reported to be 6.3 h (198), suggesting an adequate stability of the cleavable ester linkage toward nonspecific enzymatic degradation. pH-dependent stability in an aqueous phosphate buffer solution was also described, where half-lives of 6.9 and 3.0 h were observed at pH 6.5 and 7.4, respectively (198). Ro 23-9424 (hybrid prodrug 4) was also shown to enhance the induction of LPS-stimulated tumor necrosis factor alpha (TNF-α), yet it reduced the production of the proinflammatory cytokine

interleukin-1β (IL-1β) in human monocytes (199), suggesting a potential immunomodulatory benefit in reducing the possibility of LPS-induced septic shock. Promising preclinical *in vivo* pharmacokinetic parameters and tolerability were described for mouse, rat, dog, and baboon models with single- or multiple-dose intravenous administration (200). Excellent *in vivo* efficacy was reported for systemic mouse infection models of Gram-positive and Gram-negative bacterial infections, including strains that are resistant to cefotaxime and fleroxacin (198, 201). For instance, subcutaneously administered Ro 23-9424 (hybrid 4) was more active (50% effective dose [ED_{50}] of 17 mg/kg) than cefotaxime (ED_{50} of 50 mg/kg) and fleroxacin (ED_{50} of $>$100 mg/kg) in a murine meningitis model of infection by the Gram-positive organism *S. pneumoniae* (201). The hybrid prodrug resulted in an efficacy (ED_{50} of 13 mg/kg) that was similar to that of fleroxacin (ED_{50} of 9 mg/kg) but better than that of cefotaxime (ED_{50} of $>$100 mg/kg) in a murine meningitis model of infection by the Gram-negative organism *K. pneumoniae* (201). These promising preclinical data advanced the hybrid prodrug to phase 1 clinical trials for bacterial infections. Unfortunately, the trial was discontinued around the mid-1990s for undisclosed reasons. Several explanations were speculated (202), such as the fact that the *in vivo* drug stability in humans did not replicate the initial observations *in vitro* and in animal models, i.e., that the ester linkage connecting the cephalosporin and fluoroquinolone fragments was degraded by nonspecific enzymes present in humans. Moreover, in contrast to the expected multimodal suppression of drug resistance evolution, development of resistance (16- to 128-fold increase) to Ro 23-9424 (hybrid prodrug 4) was described for several bacterial strains after 2 weeks of serial passage at subinhibitory concentrations (203). Resistance in *E. coli* was attributed to two factors: (i) decreased Ro 23-9424 outer membrane permeation due to altered porin uptake and (ii) impeded fluoroquinolone activity as demonstrated by a replicative DNA biosynthesis assay in toluene-permeabilized cells (204). However, caution in interpreting the reported mechanism of resistance against Ro 23-9424 (hybrid 4) is advised, as efflux-mediated resistance was not fully recognized at the time of publication of that study. Fluoroquinolone resistance via efflux pumps was first reported in 1994 (205) and has since been known to be a major mechanism of resistance against this class. Sadly, Ro 23-9424 (hybrid 4) presented the antithesis to the idea of delayed drug resistance generation with antibiotic hybrids. This story exemplified the ingenuity of bacteria in coping with chemical assault by restricting their cellular entry. It may be beneficial to design future antibiotic hybrids as agents than can enter bacterial cells via mechanisms independent of porins, as these protein channels can be easily modified genetically by the pathogen to confer drug resistance.

Antibiotic Hybrid Drugs against Antibiotic-Resistant Gram-Negative Bacteria

Concept and hypothesis. Classical antibiotic hybrid drugs can be distinguished from prodrugs through the stability of their linker/tether. In the typical hybrid drug approach, the participating therapeutic agents are covalently linked by a robust noncleavable molecular linker that can withstand enzymatic and nonenzymatic assaults throughout its time course in the body. Upon entering the pathogen, a prototypical hybrid drug is expected to elicit its antibacterial action by utilizing either of its pharmacophoric domains or both domains simultaneously. However, it should be noted that the development of a hybrid drug that is able to simultaneously inhibit both drug targets by utilizing only a singular molecular entity at the same time is a difficult feat to achieve. Ideally, the molecular targets of the conjoined pharmacophores should be in close proximity, while the linker/tether should be of an appropriate spatial length to efficiently anchor them. This imposes a conceptual challenge that is difficult to be satisfied. For example, a single hybrid molecule that has two different pharmacophoric components—one that inhibits DNA synthesis and another that inhibits protein synthesis—will not be able to inhibit both targets at the same time due to the spatial separation of the target compartments. For now, antibiotic hybrid drugs in the literature are believed to interact with only one of the possible targets at a given time (170). However, this does not nullify the concept of multimodal mechanisms

for hybrid antibiotics as long as they retain the interactions of both individual antibacterial pharmacophores.

The antibiotic hybrid drug approach is considerably more popular in the literature than the prodrug approach. This is attributed mainly to the limited number of accessible bacterium-specific cleavable linkers for an effective prodrug delivery approach. Either way, it should be noted that both hybrid strategies require tremendous synthetic efforts, as therapeutic agents typically possess dissimilar molecular stabilities and reactivities under different preparative conditions. Designing a specific linker that is expected to be biotransformed by a bacterium-specific enzyme under specific physiological conditions therefore makes hybrid prodrugs relatively more difficult to prepare than classical hybrid drugs. Nevertheless, the characteristic high molecular weight (>600 g/mol) of hybrid drugs makes it challenging to generate agents that are able to permeate the dual membrane of Gram-negative bacteria. Emerging reports, however, project a good prognosis for this strategy, as several hybrid drugs (discussed below) that are capable of eradicating MDR Gram-negative bacteria and presumably able to delay the onset of drug resistance are already in preclinical/clinical evaluation (http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development).

Advantages and disadvantages. Similar to the advantageous pharmacokinetic properties of hybrid prodrugs, a hybrid drug is expected to remain a unimolecular entity as it travels to the site of infection and traverses the bacterial membrane into inner compartments (periplasmic and/or cytosolic space). However, differences in the two therapeutic approaches lie in how they elicit their biological function. A hybrid prodrug is subjected to enzymatic biotransformation, as it enters the bacterial cell, to yield two functional therapeutic entities, while a hybrid drug would remain a single entity throughout its time course in the body. Therefore, hybrid drugs may be advantageous in terms of their kinetics (drug metabolism and elimination), as they are expected to be cleared from the host as a single molecule. A hybrid prodrug, on the other hand, is designed to be cleaved into two separate functional molecules that may possess different pharmacokinetics after intracellular biotransformation. Drug metabolism and excretion are important factors that influence dosing regimens, as bioaccumulation above a certain threshold may result in toxicity. This, along with the above-mentioned advantages and disadvantages, should be taken into consideration when designing an antibiotic hybrid.

Examples. A substantial number of hybrid drugs that are in development or have entered clinical trials possess limited antibacterial activity against Gram-negative pathogens (178, 206, 207). This is attributed mainly to impeded bacterial cellular uptake due to a high molecular mass of >600 g/mol and physicochemical properties that render the hybrid drug unable to penetrate both outer and inner membranes. Drug uptake through nonselective porin channels in bacteria is restricted to low-molecular-mass (typically <600 g/mol) and highly polar compounds. With this in mind, we performed a literature search for hybrid drugs that are potent against Gram-negative bacteria. Here, we highlight select antibiotic hybrids from recent literature (2010 to early 2017).

(i) Most hybrid drugs contain a fluoroquinolone pharmacophore. Interestingly, most of the recently reported antibiotic hybrid drugs that are active against Gram-negative bacteria possess a fluoroquinolone pharmacophore (mostly ciprofloxacin) (Fig. 7). The choice of incorporating a fluoroquinolone as a parent drug may be attributed to its robust chemical properties that are stable under many reaction conditions. Synthetically, it may be easier to append fluoroquinolones to other therapeutic agents than, for example, β-lactams with narrow windows of chemical stability. Moreover, the well-elucidated structure-activity relationship of fluoroquinolone antibiotics and their broad-spectrum of activity make them an attractive class of antibiotics (208–210).

In 2010, a patent (211) describing a series of benzyl pyrimidines linked to fluoroquinolones was filed by MerLion Pharmaceuticals Pte. Ltd. Trimethoprim was fused to ciprofloxacin and other fluoroquinolones, yielding hybrids that displayed activity against Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and

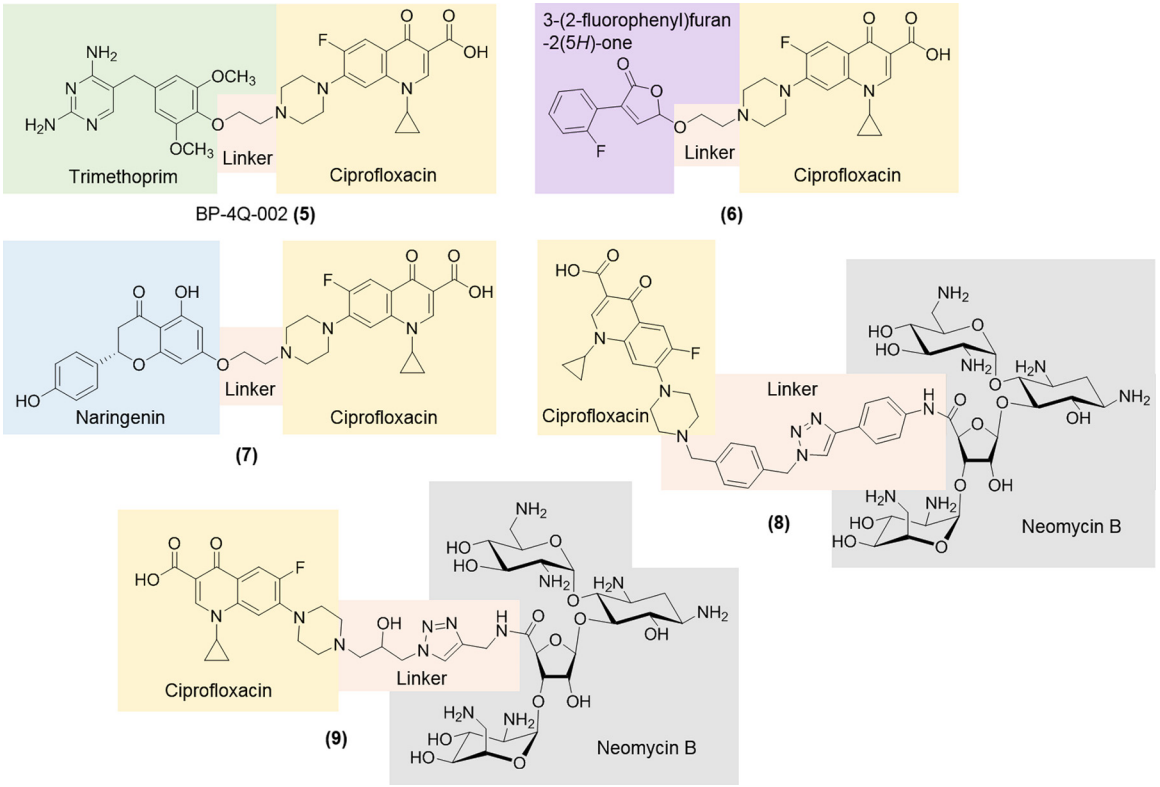


FIG 7 Examples of antibiotic hybrid drugs that are active against Gram-negative pathogens: trimethoprim linked to ciprofloxacin–BP-4Q-002 (hybrid 5), the TyrRS inhibitor 3-(2-fluorophenyl)furan-2(5*H*)-one linked to ciprofloxacin (hybrid 6), the flavonoid naringenin linked to ciprofloxacin (hybrid 7), neomycin B linked to ciprofloxacin via an aromatic triazole linker (hybrid 8), and neomycin B linked to ciprofloxacin with a hydroxyl group-containing aliphatic triazole linker (hybrid 9). Most antibiotic hybrid drugs consist of a fluoroquinolone.

Gram-negative (*E. coli*) pathogens. The antibacterial profile of the whole series was better than that of the parent drug, trimethoprim, while some entries showed activity comparable to that of ciprofloxacin. BP-4Q-002 (hybrid 5), consisting of a trimethoprim attached to the piperazine ring of ciprofloxacin, is an example that displayed potent activity against *S. aureus* (MIC of 0.5 μg/ml) and *E. coli* (MIC of 1 μg/ml) (211). This hybrid also has an MIC value of 1 μg/ml against ciprofloxacin-resistant *S. aureus* strain NRS19 (ciprofloxacin MIC of 32 μg/ml, trimethoprim MIC of 4 μg/ml, and MIC of the equimolar mixture of 8 μg/ml) (211). The observed activity of BP-4Q-002 (hybrid 5) against this drug-resistant *S. aureus* strain validates a fundamental concept in the antibiotic hybrid strategy, that hybrid agents may eradicate strains that are intermediately susceptible or resistant to one of the parent drugs. Furthermore, the 4-fold and 8-fold reductions of the MIC of BP-4Q-002 (compound 5) against *S. aureus* NRS19 compared to those of the parent drug trimethoprim and the equimolar mixture of the parent drugs, respectively, hint that the hybridization process imparted additional benefits to the hybrid's biological activity. However, there were no mode-of-action studies disclosed, and therefore, inferences of whether the hybridization of trimethoprim to ciprofloxacin influenced the resulting hybrid's mechanism of action cannot be made. A report in 2012 attempted to further fine-tune the trimethoprim-ciprofloxacin pairing by rationally synthesizing chimeric composites of both parents drug with several truncations, with the hope of improving antibacterial activity (171). Rationally guided truncations were performed on the nonpharmacophoric portions of both parent drugs. Unfortunately, none of the newly synthesized hybrids showed improved antibacterial activity relative to that of BP-4Q-002 (171).

A library of the tyrosyl-tRNA synthetase (TyrRS) inhibitor 3-arylfuran-2(5*H*)-one covalently linked to fluoroquinolones that possess broad-spectrum antibacterial activity

against Gram-positive and Gram-negative bacteria was described in 2014 (212). The most active hybrid drug comprised 3-(2-fluorophenyl)furan-2(5*H*)-one, a recently developed TyrRS inhibitor that compromises bacterial protein synthesis (213). This pharmacophore was linked to ciprofloxacin, and the resulting hybrid (hybrid 6) displayed a potent MIC_{50} of 0.11 μg/ml against MDR *E. coli* (ciprofloxacin MIC_{50} of 5.65 μg/ml) (212). It was found to inhibit DNA gyrase *in vitro* better than ciprofloxacin itself (50% inhibitory concentration [IC_{50}] of 1.15 μM, versus 5.23 μM for ciprofloxacin) and has *in vitro* TyrRS-inhibitory activity comparable to those of established 3-arylfuran-2(5*H*)-one-based TyrRS inhibitors (212). Indeed, this hybrid possesses a bimodal antibacterial mode of action *in vitro*.

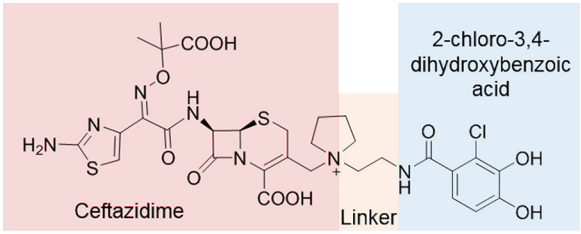
Another set of hybrids that contain phenolic flavonoids linked to fluoroquinolone has been described (214). The most promising entry was a fused naringenin-ciprofloxacin hybrid (hybrid 7) that possesses potent antibiotic activity against Gram-positive bacteria (MIC_{50} of 0.29 μg/ml against methicillin-resistant *S. aureus* [MRSA]), Gram-negative bacteria (MIC_{50} of 0.71 μg/ml against MDR *E. coli*), and fungi (MIC_{50} of 0.14 μg/ml against amphotericin B-resistant *Candida albicans*). Naturally occurring flavonoids have been reported to possess antibacterial activity, but their mode of action has been only loosely elucidated, with broadly suggested mechanisms such as membrane permeabilization and nucleic acid synthesis inhibition (215, 216). The flavonoid naringenin, however, was previously reported to have no antibacterial activity (217) against *E. coli* but is capable of selectively inhibiting drug efflux mechanisms in cancer cells (218). The naringenin-ciprofloxacin hybrid (hybrid 7) displayed 23-fold-higher inhibitory activity against DNA gyrase than that of ciprofloxacin alone. Moreover, the hybrid was found to accumulate intracellularly in MRSA at levels approximately 5-fold higher than those of the parent drug ciprofloxacin (efflux rate of 9.8% for hybrid 7, in comparison to 46.7% for ciprofloxacin) (214). The excellent antibacterial activity and high intracellular accumulation of this hybrid molecule were attributed to the possibility of the flavonoid pharmacophore serving as an efflux pump inhibitor that prevents the agent from being expelled out of the cell. The bactericidal activity of hybrid 7 was credited to the fluoroquinolone pharmacophore and the covalent attachment of naringenin directly to ciprofloxacin, which imparted an enhanced physicochemical property for a stronger DNA gyrase interaction (214).

(ii) Neomycin B-ciprofloxacin hybrid drugs delayed development of drug resistance. A series of aminoglycoside-fluoroquinolone hybrid drugs that exhibit good activity against Gram-positive and Gram-negative bacteria has been reported (219). The hybrids consist of neomycin B (aminoglycoside) and ciprofloxacin with different linkers/tethers, with the aim of probing the optimal spatial length and physicochemical property needed for optimal activity. Hybrid 8, with an aromatic triazole linker, and hybrid 9, with a hydroxyl group-containing aliphatic triazole linker, are highlighted for several reasons. The neomycin B-ciprofloxacin hybrids exhibited activity against the Gram-positive organisms *Bacillus subtilis* (MICs of 1.5 μg/ml and 3 μg/ml for hybrids 8 and 9, respectively) and MRSA (MICs of 3 μg/ml and 12 μg/ml for hybrids 8 and 9, respectively). Both compounds also showed potent activity against Gram-negative drug-resistant *E. coli* strains (MIC ranges of 0.75 to 3 μg/ml and 0.75 to 12 μg/ml for hybrids 8 and 9, respectively) that harbor several aminoglycoside-modifying enzymes (219). All the reported neomycin B-ciprofloxacin hybrids displayed significantly better antibacterial activity than neomycin B but not ciprofloxacin. Hybrid 8 inhibited protein translation *in vitro* similarly to the parent drug neomycin B. Interestingly, hybrid 8 displayed 15-fold-higher DNA gyrase and 20-fold-higher topoisomerase IV *in vitro* inhibitory activities than those of the parent drug ciprofloxacin (no data were reported for hybrid 9). It is apparent that the degree of inhibition of *in vitro* DNA synthesis for hybrid 8 is not correlated with its whole-cell activity (MIC values) in that stronger DNA gyrase and topoisomerase IV inhibition should yield a more potent activity for hybrid 8 than for ciprofloxacin. This observation strongly suggests that the neomycin B-ciprofloxacin hybrids suffer from membrane permeability issues. This may be due to the relatively high molecular masses of the neomycin B-ciprofloxacin hybrids (1,204

g/mol for hybrid 8 and 1,095 g/mol for hybrid 9). The above-described hybrid drugs BP-4Q-002 (hybrid 5), 3-(2-fluorophenyl)furan-2(5*H*)-one-ciprofloxacin (hybrid 6), and naringenin-ciprofloxacin (hybrid 7) have molecular masses of 633 g/mol, 551 g/mol, and 629 g/mol, respectively. The high molecular masses of antibiotic hybrids, as mentioned above, may become a liability, as large molecules are typically perceived to be OM impermeable, are not able to pass through nonselective porin channels, and therefore are unable to reach their intracellular targets. However, this appears to be untrue for the neomycin B-ciprofloxacin hybrids, as they still retain antibacterial activity, although it is not as potent as that of the parent drug ciprofloxacin. It could then be suggested that the hybrids somehow were able to enter the bacterial cell to reach their targets, although their level of intracellular accumulation was probably not as high as that of ciprofloxacin (which traverses the OM via porins and passively diffuses in the IM).

The reported neomycin B-ciprofloxacin hybrids 8 and 9 were further assessed for their therapeutic potential. No drug resistance was observed for the Gram-positive organism *B. subtilis* and the Gram-negative organism *E. coli* following 15 serial passages of hybrid 8 at subinhibitory (1/2 MIC) concentrations (220). Under similar experimental conditions, MIC increases of 37.5-fold, 8-fold, and 7.6-fold against *B. subtilis* were observed for ciprofloxacin, neomycin B, and an equimolar mixture of both agents, respectively (220). Similarly, MIC increases of 75-fold, 4-fold, and 20-fold against *E. coli* were found for ciprofloxacin, neomycin B, and an equimolar mixture of both agents, respectively (220). The fact that an equimolar combination of ciprofloxacin and neomycin B was not able to suppress drug resistance whereas the hybrid, which also contains equimolar components, was able to suppress resistance stimulated significant interest. This observation corroborates the hypothesized benefit of hybridizing two therapeutic agents into a unified entity, that it may impart additional properties that are otherwise absent in individual molecules (e.g., in combination therapy). The delayed resistance evolution observed with hybrid 8 supports this notion. Further molecular analysis of the mechanistic interplay between *E. coli* and hybrid 9, in a follow-up report (221), revealed that the bulk of the antibacterial activity of the neomycin B-ciprofloxacin hybrids is mediated mainly by the ciprofloxacin pharmacophore. On the other hand, the neomycin B pharmacophore was found to be mainly responsible for delaying the emergence of drug resistance that may arise from genetic mutations such as a mutation of the multiple-antibiotic-resistance (*marR*) repressor gene that leads to efflux-mediated resistance (as observed for ciprofloxacin) (221).

(iii) Other hybrid drugs that are active against Gram-negative pathogens. Other recent hybrid drugs with activity against Gram-negative pathogens include a berberine pharmacophore fused with either metronidazole (222) or benzimidazole (223), both of which displayed low MICs against *E. coli* and *P. aeruginosa*. Fusions of neomycin B with various phenolic antimicrobial agents such as chloroxylenol, triclosan, and clofoctol were also reported (224). An article (225) describes the *in vitro* and *in vivo* antibacterial evaluation of hybrids composed of the antimicrobial peptide tridecaptin linked to either rifampin, vancomycin, or erythromycin. Tridecaptins are naturally occurring lipopeptides that have potent activity against Gram-negative, but not Gram-positive, bacteria and low toxicity toward mammalian cells (226, 227). In that article, the tridecaptin utilized was either unacylated or acylated with octanoic acid at the N terminus. The reported hybrids exhibit low *in vitro* activities against *E. coli*, *K. pneumoniae*, and *A. baumannii* relative to an equimolar combination of tridecaptin and an antibiotic. Against *K. pneumoniae* ATCC 13883, the unacylated tridecaptin-erythromycin hybrid displayed an MIC of 50 μM, while an equimolar combination of both components showed an MIC of 0.4 μM (225). However, the unacylated tridecaptin-erythromycin hybrid displayed significantly better *in vivo* efficacy in a moribund mouse model of *K. pneumoniae* pulmonary infection than erythromycin alone or an equimolar combination of unacylated tridecaptin and erythromycin (225). The survival rates were 80%, 40%, and 40%, respectively, after 7 days (225). This report suggests that a potent *in vitro* activity of an antibiotic-antibiotic combination is not always translatable *in vivo*



Cefiderocol **(10)**

FIG 8 Structure of cefiderocol (hybrid 10), previously known as S-649266, derived by linking ceftazidime to the siderophore catechol 2-chloro-3,4-dihydroxybenzoic acid. This β-lactam–siderophore hybrid possesses potent antibacterial activity against metallo-β-lactamase-producing Gram-negative bacilli.

and that the covalent linking of two pharmacophores rather than a concoction may enhance/retain *in vivo* efficacy.

(iv) Cefiderocol (S-649266): a Trojan horse strategy. The β-lactam–siderophore subclass of antibiotic hybrids is an emerging type of hybrid drug for Gram-negative pathogens. The idea of covalently attaching a siderophore (Greek for iron carrier) pharmacophore to a biocidal pharmacophore exploits a "Trojan horse" strategy that deceives bacteria to actively transport the antibiotic into the cell. Iron, in the form of ferric ion, is essential to bacteria, especially in an iron-deficient environment such as that of the mammalian host. Bacteria scavenge iron from their environment through the production of small-molecule siderophores that efficiently form complexes with iron. These siderophore-iron complexes are then taken up via active transport systems, such as TonB-dependent transporters (228–231). Through the attachment of the iron-chelating siderophore adjuvant, significantly higher intracellular drug concentrations can be achieved by hijacking the bacterial iron transport system. Several of these β-lactam–siderophore hybrid drugs have entered preclinical/clinical trials (232–236), notably the cephalosporin-catechol hybrid cefiderocol, which has advanced to and is currently in phase 3 clinical trials.

Cefiderocol (hybrid 10), also known as S-649266 (Fig. 8), is currently being developed by Shionogi & Co. Ltd. for complicated urinary tract infections and carbapenem-resistant Gram-negative bacterial infection. This hybrid is composed of the catechol 2-chloro-3,4-dihydroxybenzoic acid covalently appended via a noncleavable linker/tether at carbon 3 of the cephalosporin ceftazidime. Cefiderocol (hybrid 10) possesses potent activity against Gram-negative ESKAPE pathogens. It exhibited MIC_{90} values of 1 μg/ml and 0.125 μg/ml against randomly collected clinical isolates of the *Enterobacteriaceae E. coli* ($n = 106$) and *K. pneumoniae* ($n = 105$), respectively (237, 238). Furthermore, MIC_{90} values of 1 μg/ml and 2 μg/ml were found for *P. aeruginosa* ($n = 104$) and *A. baumannii* ($n = 104$) isolates, respectively, among which were carbapenem-resistant strains (237, 238). In a recent study, cefiderocol (hybrid 10) displayed MIC_{90} values of 4, 1, and 8 μg/ml against carbapenem-nonsusceptible *Enterobacteriaceae* ($n = 1022$), MDR *P. aeruginosa* ($n = 262$), and MDR *A. baumannii* ($n = 368$) isolates, respectively (239). These clinical isolates were randomly collected from 52 countries from 2014 to 2016 (239). In fact, cefiderocol (hybrid 10) has been described to possess higher potency (237, 238) and better β-lactamase stability (240) than cefepime, ceftazidime, and meropenem against organisms that produce carbapenemases belonging to the class A, B (metallo-β-lactamases), and D β-lactam-hydrolyzing enzymes. The superior antibacterial activity of this enhanced cephalosporin-based hybrid relative to clinically relevant β-lactams was attributed to the covalently appended catechol pharmacophore. Cefiderocol (hybrid 10) has been shown to chelate ferric irons that consequently facilitate its active transport into *P. aeruginosa* via iron transporters, besides entering through nonselective porin channels, resulting in enhanced intracellular entry (241). The hybrid cefiderocol was also described to be 10 to 100 times more stable *in vitro* against several carbapenemases than its parent antibiotic ceftazidime (240). This

enhanced stability might be credited to the steric hindrances imparted by the linker and catechol domains on the resistance enzymes.

The observed *in vitro* potency of cefiderocol (hybrid 10) against Gram-negative bacilli was well translated *in vivo*. Cefiderocol was found to display good efficacy and significantly reduced the numbers of viable bacterial cells in murine models of pulmonary infection caused by MDR carbapenem-resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* (239, 242). This hybrid drug accelerated into phase 1 clinical trials in 2012 to evaluate its safety, tolerability, and pharmacokinetics in single or multiple intravenous administrations. An intravenous dosage of 2,000 mg every 8 h was well tolerated for a duration of 10 days in healthy individuals, and the drug is eliminated mainly through renal excretion (243, 244). Currently, cefiderocol (hybrid 10) is being assessed for its efficacy in a phase 3, randomized, open-label study for the treatment of serious infections caused by carbapenem-resistant Gram-negative pathogens (https://clinicaltrials.gov/ct2/show/NCT02714595). Another phase 3, randomized, triple-blind clinical study of cefiderocol (hybrid 10) in comparison to meropenem for the treatment of nosocomial pneumonia caused by Gram-negative pathogens has recently commenced (https://clinicaltrials.gov/ct2/show/NCT03032380). The urgent need develop new antibiotics to treat MDR bacterial infections, especially infections by carbapenem-resistant Gram-negative bacilli, is apparent. With a phase 3 clinical trial under way, cefiderocol may very well be the first FDA-approved NME systemic drug to be developed in the 21st century that is able to treat infections by MDR ESKAPE pathogens.

Antibiotic Hybrids Can Adopt New Mechanistic Actions That Differ from Those of Their Constituent Pharmacophores

Although antibiotic hybrids are designed to retain the dual mechanistic functions of their constituent pharmacophores, it is clearly an unpredictable science. Most antibiotic hybrids reported in the literature either retain the activity of only one of the domains or lose the activities of both domains (206, 207, 245, 246), and their antibacterial properties often resemble those of the dominant parent drug. Rational design is particularly challenging for Gram-negative bacteria because of the requisite physicochemical properties needed to navigate the orthogonal sieving nature of the outer and inner membranes (as discussed above). Moreover, hybridization of drug scaffolds to produce a unimolecular hybrid entity often leads to a high molecular weight that negates uptake through size-exclusive OM porin channels. However, it is possible for an antibiotic hybrid, being an entirely new chemical entity, to pharmacodynamically behave differently from its parent drugs. If hybridization results in a new or additional mode of biological action, it can also, in principle, interact differently with other classes of antibiotics (as an adjuvant). An antibiotic hybrid may interfere with nonbiocidal processes that directly or indirectly aid in, potentiate, or prevent the inactivation of a primary antibiotic-adjuvant property. Antibiotic hybrids are usually designed as standalone antibacterial agents, but the idea of investigating them as adjuvants for currently used antibiotics, especially those with high incidence rates of resistance development, is a novel approach that has opened a new paradigm in drug discovery. Several tobramycin-containing hybrids (Fig. 9 and 10) have been reported to possess intrinsic physicochemical properties capable of "resuscitating" the efficacy of currently used antibiotics against multidrug-resistant Gram-negative bacteria, especially *P. aeruginosa* (247–252). These effects were determined and described as a measure of the fractional inhibitory concentration index (FICI), a numerical quantification of the interactions between antibiotics. FICIs of $\leq$0.5, 0.5 to 4, and $>$4 indicate synergism, no (or an additive) interaction, and antagonism, respectively (253). It was clear that the tobramycin-hybrid scaffold, depending on the second participating domain, exhibited a different spectrum of biological activities (247–252). These data, to the best of our knowledge, represent the only known data to date on the adjuvant properties of any hybrid antibiotics against Gram-negative bacteria.

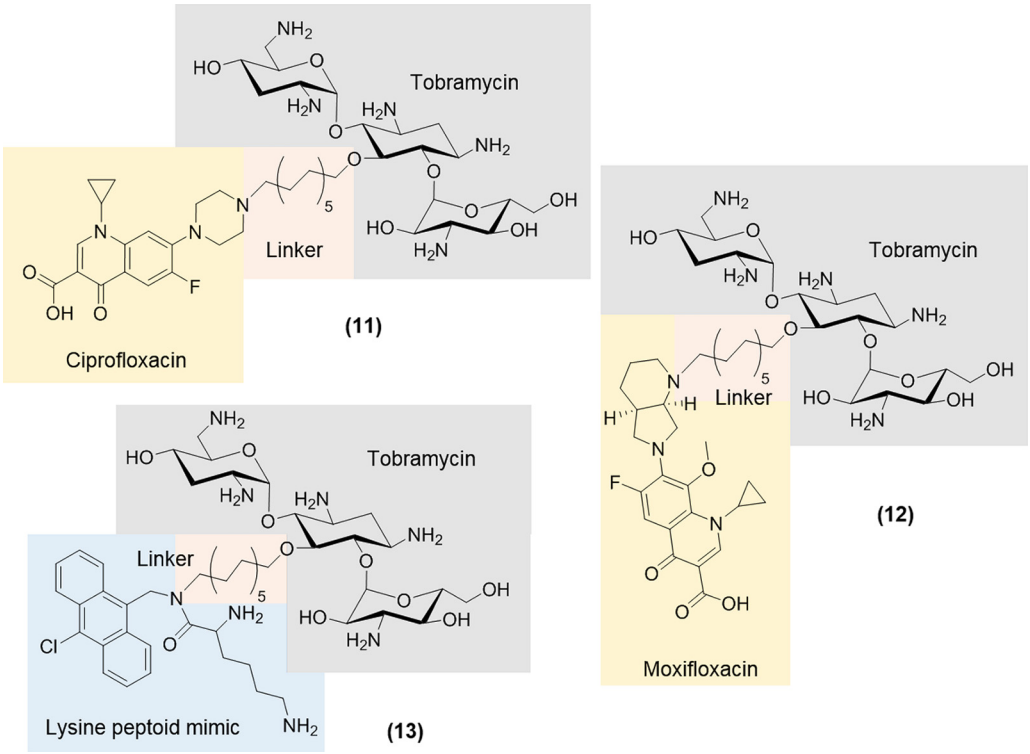


FIG 9 Examples of tobramycin-based hybrids: tobramycin linked to ciprofloxacin (hybrid 11), tobramycin linked to moxifloxacin (hybrid 12), and tobramycin linked to a lysine peptoid mimic (hybrid 13). All three tobramycin-based hybrids contain a 12-carbon-long aliphatic (C_{12}) hydrocarbon linker.

Tobramycin-based hybrids as adjuvants that potentiate legacy antibiotics against *Pseudomonas aeruginosa*. The opportunistic pathogen *P. aeruginosa* is the leading cause of nosocomial infections in immunocompromised patients and is abundantly found on many medical devices in the hospital. *P. aeruginosa*, alongside other ESKAPE pathogens, is a major threat to public health for which effective therapy is rapidly becoming elusive (3, 254, 255). In 2017, the WHO ranked carbapenem-resistant *P. aeruginosa* as critical (priority 1) in its list of the world's most dangerous superbugs that pose a serious threat to human health (http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/). Since the major impediment that antibiotics face against *P. aeruginosa* is low penetration/uptake across its impermeable outer membrane; the first tobramycin-based hybrid analog, hybrid 11, was initially conceptualized to take advantage of the self-promoted uptake mechanism of aminoglycosides to deliver a second antibiotic into the periplasm of the cell. Moreover, aminoglycosides possess a pleiotropic mechanism of antibacterial action. They interact with rRNA to inhibit protein translation at lower concentrations ($\leq$4 μg/ml), while they are known to disrupt the bacterial membrane at higher concentrations ($\geq$8 μg/ml) (256). Tobramycin is a particularly attractive aminoglycoside, as it is currently one of the most active and effective agents for the treatment of *P. aeruginosa* infections (257).

It was shown previously that tobramycin-based hybrid drugs that lose innate *in vitro* antibacterial activity (Table 2) are able to enhance the potency of other antibiotic classes (except aminoglycosides and carbapenems) against *P. aeruginosa* (Table 3 and Fig. 11) (247). These synergistic effects are exclusive to the hybrids, as their composing antibiotic fragments do not exhibit this property. For example, a tobramycin-ciprofloxacin hybrid (hybrid 11) that displayed weak antibacterial activity as a stand-alone agent (MIC of $\geq$16 μg/ml) was found to restore the efficacies of ciprofloxacin and moxifloxacin (FICI of 0.03 to 0.38) against ciprofloxacin-resistant MDR or XDR *P. aeruginosa* clinical isolates (247). It is noteworthy that some of these isolates

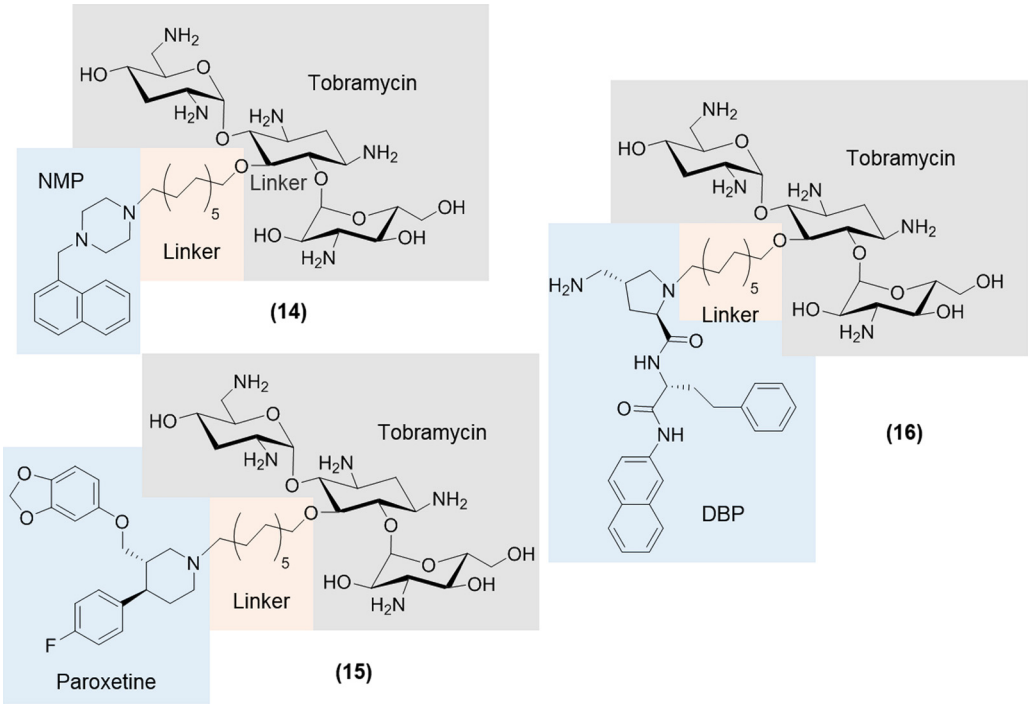


FIG 10 Examples of tobramycin-efflux pump hybrids: tobramycin linked to 1-(1-naphthylmethyl)-piperazine (NMP) (hybrid 14), tobramycin linked to paroxetine (hybrid 15), and tobramycin linked to the dibasic peptide (DBP) analog D-Ala–D-hPhe–aminoquinoline (MC-04,124) (hybrid 16). All three tobramycin-based hybrids contain a 12-carbon-long aliphatic (C_{12}) hydrocarbon linker.

were also resistant to colistin and carbapenems, our last line of defense. Importantly, ciprofloxacin-susceptible (MIC of $\leq$1 μg/ml) or -intermediate (MIC of 2 μg/ml) CLSI breakpoints were reached for most of the fluoroquinolone-resistant clinical isolates in the presence of $\leq$8 μg/ml (6 μM) of hybrid 11 (247). Measureable *in vivo* potency of this hybrid was demonstrated by using the *Galleria mellonella* larva infection model. In spite of the inherent limitations of this model, including a lack of adaptive immune responses in insects and the ease of manipulation, the remarkable similarities between the innate immune response of *G. mellonella* and those of vertebrates make this model desirable for studying the virulence of bacteria and the efficacy of antimicrobial agents (258). Upon injection of the larvae with a lethal load of XDR *P. aeruginosa* strain 101856, a 1:1 concoction of hybrid 11 and moxifloxacin (37.5 mg/kg each) administered as a single dosage at 2 h postinfection resulted in 86% survival of the larvae after 24 h. In comparison, single-dose monotherapy with either hybrid 11 or moxifloxacin alone (50 mg/kg each) was ineffective and resulted in 100% killing within the same time frame. *In vitro* biochemical assays revealed that the DNA gyrase A- and topoisomerase IV-inhibitory activities of the ciprofloxacin domain of hybrid 11 were retained (3- to 5-fold higher than those of ciprofloxacin), whereas the protein translation-inhibitory properties of the tobramycin domain were lost (156- to 1,290-fold lower than those of tobramycin) (247). These *in vitro* biochemical observations are consistent with the activities of other aminoglycoside-fluoroquinolone hybrids, e.g., neomycin B-ciprofloxacin hybrids 8 and 9, that were previously reported by another group (219). Mechanistic studies correlated the observed adjuvant effect of tobramycin-ciprofloxacin hybrid 11 to its ability to perturb the OM of *P. aeruginosa* in a dose-dependent manner, thus facilitating the influx (and bioaccumulation) of antibiotics that are typically unable to cross the OM, such as rifampin, novobiocin, vancomycin, and erythromycin (247). Since one of the main mechanisms of resistance to fluoroquinolones in *P. aeruginosa* is the overexpression of multidrug efflux pumps composed of a tripartite protein assembly spanning the inner and outer membranes (28), it is possible that the perturbation of the membrane's

TABLE 2 MICs of tobramycin-based hybrids and select antibiotics against wild-type and clinical isolates of Gram-positive and Gram-negative bacteria[a]

	MIC (μg/ml)												
	Tobramycin-based hybrid			Antibiotic									
Organism	TOB-CIP	TOB-LYS	TOB-NMP	TOB	MOX	CIP	MIN	RMP	CAZ	CAM	ERY	TMP	COL
Gram-positive bacteria													
S. aureus ATCC 29213	64	8	32	≤0.25	≤0.25	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
MRSA ATCC 33592	32	8	64	≤0.25	≤0.25	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
MSSE 81388[b,d]	16	2	16	≤0.25	≤0.25	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
MRSE 61589[b,e]	32	4	8	1	64	128	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Enterococcus faecalis ATCC 29212	128	16	128	8	≤0.25	1	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
E. faecium ATCC 27270	64	8	64	8	2	8	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
S. pneumoniae ATCC 49619	64	32	64	2	≤0.25	1	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Gram-negative bacteria													
E. coli ATCC 25922	2	32	512	0.5	≤0.25	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
E. coli 61714[b,e,f]	64	32	512	8	0.5	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
E. coli 63074[b,e,h]	64	16	512	8	1	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
E. coli 97615[b,d]	512	32	512	128	32	256	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Stenotrophomonas maltophilia 62584[b,e]	>512	>128	>512	>512	4	32	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
A. baumannii 63169[b,e]	128	>128	512	32	1	2	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
K. pneumoniae ATCC 13883	64	>128	>512	≤0.25	≤0.25	≤0.25	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
P. aeruginosa ATCC 27853	4	32	256	0.5	4	1	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
P. aeruginosa 62308[b,e,f]	32	16	64	16	16	2	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
P. aeruginosa 96846[b,d,f,g]	64	32	256	256	16	4	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
P. aeruginosa PAO1	32	32	64	0.25	1	ND	8	16	2	64	256	256	1
P. aeruginosa 100036[b]	128	32	256	64	128	64	32	16	8	>512	256	256	2
P. aeruginosa 101885[b]	256	16	256	0.25	64	32	32	16	8	512	256	>512	0.5
P. aeruginosa P259-96918[b]	128	64	>1,024	128	512	128	32	16	512	512	256	512	0.5
P. aeruginosa P262-101856[b]	64	32	64	512	128	32	256	1,024	16	1,024	1,024	>1,024	2
P. aeruginosa P264-104354[b]	32	32	128	128	128	32	64	16	64	1,024	256	256	4
P. aeruginosa 91433[b,c]	16	8	32	8	8	2	64	16	256	16	512	512	32
P. aeruginosa 101243[b,c]	16	16	64	256	4	2	4	8	64	4	1,024	1,024	>1,024

[a]See references 247–251. TOB, tobramycin; MOX, moxifloxacin; CIP, ciprofloxacin; MIN, minocycline; RMP, rifampin; CAZ, ceftazidime; CAM, chloramphenicol; ERY, erythromycin; TMP, trimethoprim; COL, colistin; ND, not determined; MRSA, methicillin-resistant *S. aureus*; MSSE; methicillin-susceptible *S. epidermidis*; MRSE, methicillin-resistant *S. epidermidis*; TOB-CYP, tobramycin-ciprofloxacin hybrid 11; TOB-LYS, tobramycin-lysine peptoid hybrid 13; TOB-NMP, tobramycin-NMP hybrid 14.
[b]Clinical isolate.
[c]Colistin-resistant strain.
[d]CANWARD (Canadian Ward surveillance).
[e]CAN-ICU (Canadian National Intensive Care Unit surveillance).
[f]Gentamicin resistant.
[g]Tobramycin resistant.
[h]The MIC of amikacin is 32 μg/ml.

lipid composition, particularly the lipids localized around the transmembrane protein, could compromise the integrity of efflux pumps and restore the potency of fluoroquinolones. This hypothesis was tested by assessing the interaction(s) between fluoroquinolones and known permeabilizers in *P. aeruginosa*. Colistin, cetrimonium bromide, benzethonium chloride, and C_{16}-$(Dab)_4$-NH_2 (259) were unable to potentiate moxifloxacin in XDR *P. aeruginosa* 96918. This suggests that tobramycin-ciprofloxacin hybrid 11 either possesses another biological mechanism aside from membrane permeabilization or exhibits an augmented membrane interaction relative to those of common permeabilizers. Indeed, fluoroquinolones are known to be rarely potentiated by classical adjuvants against MDR Gram-negative bacteria, especially *P. aeruginosa* (260). Tobramycin-ciprofloxacin hybrid 11 was relatively nontoxic to human epithelial breast (JIMT1) and prostate (DU145) cancer cell lines (the effect was comparable to those of ciprofloxacin and moxifloxacin), as determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (261), and was nonhemolytic to human erythrocytes (<10% at 1,000 μg/ml) (247). Ciprofloxacin and moxifloxacin are known to exhibit modest cytotoxicity toward these cell lines (262) and were thus used to assess the cytotoxicity of hybrid 11.

Interestingly, amphiphilic aminoglycosides composed of aliphatic hydrocarbons attached to tobramycin (e.g., tobramycin-C_{14} tether fragment of the hybrids) were found to possess beneficial immunomodulatory functions that may be exploited therapeutically. These amphiphilic tobramycin analogs selectively induced the chemo-

TABLE 3 MIC_{50} and MIC_{90} values of ciprofloxacin, minocycline, or rifampin alone and in combination with either tobramycin-ciprofloxacin hybrid 11, tobramycin-lysine peptoid hybrid 13, or tobramycin-NMP hybrid 14 against MDR/XDR *P. aeruginosa* isolates ($n = 10$)[a]

Antimicrobial(s)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	MIC range (μg/ml)
CIP	>32⊥	>64⊥	2–256
MIN	64⊥	256⊥	4–256
RMP	16⊥	>16⊥	8–1,024
TOB-CIP	>64⊥	>128⊥	4–256
TOB-NMP	>128⊥	>256⊥	2–1,024
TOB-LYS[b]	32⊥	64⊥	8–64
CIP + 8 μg/ml TOB-CIP	1†	4⊥	<1–4
CIP + 4 μg/ml TOB-NMP	6⊥	16⊥	0.1–16
CIP + 4 μg/ml TOB-LYS[b]	≤8⊥	32⊥	0.125–32
MIN + 4 μg/ml TOB-CIP	0.25†	4†	0.25–4
MIN + 4 μg/ml TOB-NMP	1†	4†	0.5–4
MIN + 4 μg/ml TOB-LYS[b]	0.5†	2†	0.5–2
RMP + 1 μg/ml TOB-CIP	1†	2‡	0.25–16
RMP + 4 μg/ml TOB-NMP	0.25†	8⊥	0.125–8
RMP + 4 μg/ml TOB-LYS[b]	0.25†	16⊥	0.0625–16

[a]See references 247–251. CIP, ciprofloxacin; MIN, minocycline; RMP, rifampin; TOB-CIP, tobramycin-ciprofloxacin hybrid 11; TOB-LYS, tobramycin-lysine peptoid hybrid 13; TOB-NMP, tobramycin-NMP hybrid 14. †, susceptible; ‡, intermediately resistant; ⊥, resistant.
[b]$n = 5$.

kine IL-8 in macrophages but not growth-related oncogene (Gro-α), proinflammatory cytokines (TNF-α and IL-1β), or the IL-1 antagonist IL-1RA (263). IL-8 is a potent neutrophil chemotactic factor responsible for the migration and activation of monocytes, lymphocytes, basophils, and eosinophils necessary for the resolution of infections (264). Moreover, the production of the LPS-induced proinflammatory cytokine TNF-α was abrogated by amphiphilic tobramycin via a mechanism that is independent of LPS interaction/neutralization, perhaps through an alteration of intracellular signaling downstream of pattern recognition in macrophages (263). This finding was of importance because the significant production of proinflammatory cytokines induced by endotoxins such as LPS has been implicated in septic shock (265, 266). Tobramycin by

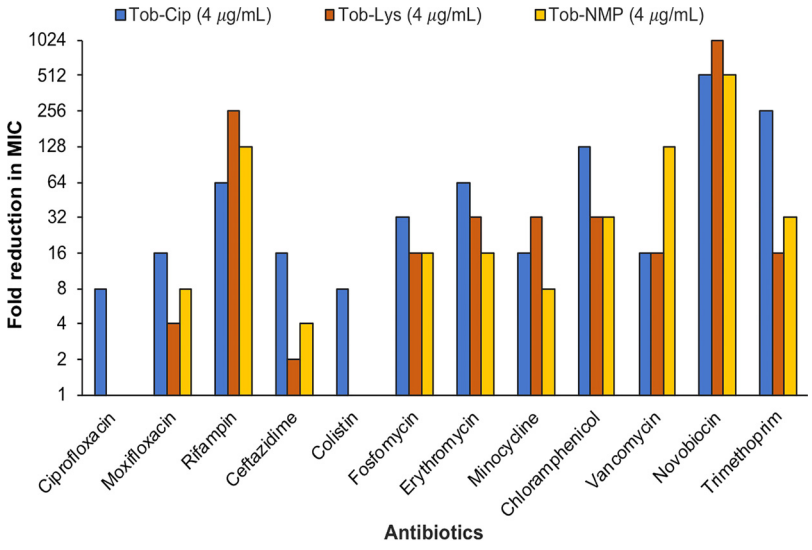


FIG 11 Potentiation of legacy antibiotics against *P. aeruginosa* PAO1 by tobramycin-based hybrids: tobramycin-ciprofloxacin hybrid 11 (Tob-Cip), tobramycin-lysine peptoid hybrid 13 (Tob-Lys), and tobramycin-NMP hybrid 14 (Tob-NMP). Tobramycin-lysine peptoid and tobramycin-NMP could not potentiate colistin and were not tested with ciprofloxacin.

itself is not known to possess these immunomodulatory properties, but a tobramycin-copper complex has been reported to possess anti-inflammatory properties (267). Host defense peptides (HDPs) such as cathelicidin LL-37 and indolicidin are also known to be potent inducers of IL-8 (268–270), but they can also induce the production of other chemokines (such as monocyte chemoattractant protein 1 [MCP-1] and Gro-α) (268, 271), suggesting a selective chemoattractant ability of amphiphilic tobramycins. Relative to tobramycin, the tested amphiphilic tobramycin analogs exhibited negligible to $<$10% cytotoxicity against human monocytic THP-1 cells (ATCC TIB-202) at working concentrations (263).

Structure optimization strategy for the tobramycin-based hybrid scaffold. Enthused by the unexpected biological properties of the tobramycin-ciprofloxacin hybrid, the core (and least possible) structural fragment necessary for the adjuvant effect of this scaffold was investigated. It was reasoned (and data later confirmed) that the tobramycin fragment might be critical to the hybrid's scaffold, due to its well-known pleiotropic mechanism of action and/or perhaps its self-promoted uptake mechanism. A nonclassical structure-activity relationship study was therefore instituted to replace the ciprofloxacin domain of tobramycin-ciprofloxacin hybrid 11 with other pharmacophoric fragments (Fig. 9 and 10).

(i) The tobramycin-moxifloxacin hybrid retains a new mechanism of action. The effect of replacing ciprofloxacin in the hybrid scaffold with another fluoroquinolone, while keeping the tobramycin fragment and the C_{12} linker/tether, was investigated. Moxifloxacin (Fig. 9) was selected because it is clinically relevant in the treatment of *P. aeruginosa* infections, is less affected by bacterial efflux systems due to its bulky C-7 substituent, and is robust enough to withstand chemical manipulations (209, 210, 272). Tobramycin-moxifloxacin hybrid 12 retained the adjuvant properties of hybrid 11 and potentiated a range of legacy antibiotics against *P. aeruginosa* in a similar fashion (248). Mechanistic studies also showed that hybrid 12 perturbed the OM of *P. aeruginosa* PAO1 and induced a dose-dependent depolarization of the cytoplasmic membrane in a manner comparable to that of membrane-acting colistin. The ability of this compound to strongly reduce the flagellum-dependent swimming motility of *P. aeruginosa* PAO1 at sub-MIC values in a concentration-dependent manner, a function that requires intact PMF (273), supports the notion that it dissipates the cytoplasmic membrane PMF. This perhaps explains the strong synergistic effects observed with efflux-susceptible antibiotics (Fig. 11) at sublethal concentrations (248), as PMF energizes RND-based efflux pumps in *P. aeruginosa* (274, 275). Moreover, the observed dissipation of PMF by hybrid 12 is consistent with its antagonistic or weakly additive effects with aminoglycosides (248), as they require the electrical component ($\Delta\Psi$) of an intact PMF for cytosolic uptake (65). Other nonbiocidal agents that dissipate bacterial PMF have also been shown to synergize the activities of some pH-dependent antibiotics (e.g., minocycline) against Gram-negative bacteria, at one-half the MIC for each strain (276). Thus, the replacement of ciprofloxacin in hybrid 11 with moxifloxacin in hybrid 12 did not alter the characteristic adjuvant properties of this scaffold, suggesting the indispensability of amphiphilic tobramycin as one of the primary domains required for their observed adjuvant properties.

(ii) Tobramycin-lysine peptoid conjugates resensitize MDR *P. aeruginosa* to minocycline and rifampin. Tobramycin-based hybrids that contain an appended fluoroquinolone pharmacophore seem to possess intrinsic properties that make them permeabilize the OM and dissipate the PMF across the inner membrane. These compounding membrane effects make membrane-impermeable or efflux-susceptible legacy antibiotics able to reach their corresponding cytosolic targets in *P. aeruginosa*. From previous studies, the tobramycin domain linked to the C_{12} tether (also considered an amphiphilic tobramycin) appears to be responsible for the observed membrane effects, while that of the fluoroquinolone domain is unclear. The necessity of having a fluoroquinolone pharmacophore and to what extent it affects the observed biological activity (adjuvant property) of the tobramycin-based hybrids were then probed. Tobramycin-ciprofloxacin hybrid 11 potentiated other classes of antibiotics but displayed poor

activity on its own (Fig. 11), even though its ability to inhibit DNA synthesis, a characteristic function of fluoroquinolones, was retained. This suggests that the contribution of the fluoroquinolone to the overall chemical nature is perhaps just a physicochemical modulation. In this light, the fluoroquinolone fragment was replaced with a lysine-based peptoid mimic (Fig. 9) that is capable of membrane permeabilization and depolarization (277). Several compounds of this peptoid scaffold were reported to exhibit wide-spectrum potency against Gram-positive and Gram-negative bacteria (277). Tobramycin was conjugated at the terminal end of the aliphatic C_{12} chain in the lysine-based peptoid, such that the amphiphilic tobramycin and the lysine peptoid domains shared a common alkyl chain. Tobramycin-lysine hybrid 13 displayed good activity (MICs of 2 to 8 μg/ml) against staphylococci but only weak activity (MIC of $\geq$16 μg/ml) against the tested Gram-negative bacilli (Table 2). Hybrid 13 displayed strong synergistic interactions with membrane-impermeable and efflux-susceptible antibiotics, consistent with other tobramycin-based hybrids (Fig. 11). For instance, 4 μg/ml of hybrid 13 lowered the MICs of minocycline and rifampin against wild-type *P. aeruginosa* PAO1 by 32- and 256-fold, respectively (Table 3) (250). Synergy between hybrid 13 and other antibiotics was also observed for MDR/XDR *P. aeruginosa*, *A. baumannii*, *Enterobacter cloacae*, and *K. pneumoniae* clinical isolates. Extrapolating the CLSI breakpoints of minocycline against *Acinetobacter* spp. (MIC of $\leq$4 μg/ml) and of rifampin against *Enterococcus* spp. ($\leq$1 μg/ml) as interpretive MIC standards (278), susceptible breakpoints for these antibiotics were reached in most MDR and XDR Gram-negative bacillus isolates by using just 4 μg/ml (3 μM) of tobramycin-lysine peptoid conjugate 13. However, it could not potentiate β-lactams, colistin, and aminoglycosides. It is noteworthy that β-lactamase inhibitors like tazobactam or avibactam require concentrations of about 12 to 15 μM to potentiate β-lactam antibiotics in *in vitro* studies (121).

Mechanistic studies revealed that tobramycin-lysine hybrid 13 is rapidly bactericidal after 2 h at an MIC value of 32 μg/ml (250). The combination of hybrid 13 with minocycline (both at 1/8$\times$ MIC) or rifampin (both at 1/4$\times$ MIC) rendered *P. aeruginosa* PAO1 cells completely nonviable after 2 h and 4 h, respectively. Tobramycin-lysine hybrid 13 was found to permeabilize the outer membrane and depolarize the inner membrane, which is reflective of the biological action of the lysine-based peptoid fragment. Indeed, these membrane effects are also inherent to amphiphilic tobramycins, suggesting that the strong membrane effects of hybrid 13 stemmed from both fragments of the hybrid structure. Moreover, hybrid 13 was found to dissipate PMF and inhibit bacterial swimming motility at 4 μg/ml. It also demonstrated significantly lower rates of ovine erythrocyte hemolysis ($<$20%) than the lysine-based peptoid (85%) at a high concentration of 512 μg/ml and was not cytotoxic to JIMT1 and DU145 epithelial cells at 20 μg/ml (250). *In vivo* efficacy studies using XDR *P. aeruginosa* P262-challenged *G. mellonella* larvae demonstrated the ability of hybrid 13 to offer protection when used in combination with minocycline or rifampin, resulting in a 77% survival rate (for both combinations) after 24 h (250).

(iii) Tobramycin-efflux pump inhibitor conjugates perturb RND efflux pumps. Since the above-mentioned tobramycin-based hybrids (Fig. 9) comprise antibacterial agents (ciprofloxacin, moxifloxacin, and lysine peptoid) that are substrates for *P. aeruginosa* RND pumps, the effects and implication of their conjugation to tobramycin on RND efflux pumps have also been examined (279). Tobramycin is known to be a poor substrate for most RND efflux pumps except for MexXY pumps (280, 281). The overall effect of attaching an efflux substrate antibiotic (such as moxifloxacin) to another nonsubstrate agent (such as tobramycin) on the ability of the resulting molecule to be effluxed was investigated. Although the substrate fragment may still be recognized by the active site of the efflux protein, its extrusion by the pump might be impeded by the hybrid's sheer steric bulk. Previous studies have shown that increasing the size or molecular weight of compounds reduces the likelihood of efflux (282). In concept, this may also alter the protein environment and incapacitate the efflux pump. The effect of efflux on tobramycin-based hybrids was explored by using a MexAB-OprM deletion strain (PAO200) and an efflux-sensitive strain (PAO750) that lacks five different clinically relevant RND pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY) and the OM protein OpmH

(283, 284). Whereas the antibacterial activity of moxifloxacin was greatly affected by the absence of RND pumps (128-fold reduction of the MIC for PAO750) (248), the MICs of tobramycin-based hybrids 11, 12, and 13 were quite similar across wild-type *P. aeruginosa* strains and efflux deletion strains, and only a marginal 2- to 4-fold reduction in the MIC was observed for PAO750 (247, 248, 250). This clearly shows that the hybridization of an efflux substrate antibiotic to a nonsubstrate antibiotic resulted in an entity that could resist efflux, perhaps due to steric bulk. In terms of their adjuvant properties, a significant potentiation of several classes of antibiotics was retained across these efflux-deficient strains (247, 248, 250), suggesting that the synergistic interactions between these hybrids and antibiotics are independent of clinically relevant RND efflux pumps. However, dissipation of the PMF, which is induced by the tobramycin-based hybrids, may also be implicated in the inactivity of PMF-dependent efflux systems (285).

Overexpressed multidrug efflux pumps that effectively reduce intracellular antibiotic concentrations remain a major problem (23, 28). Efflux pump inhibitors (EPIs) that block active efflux have been demonstrated to potentiate efflux-susceptible antibiotics in Gram-positive and some Gram-negative pathogens but in only a few *P. aeruginosa* isolates (286–289). EPIs work by competing with the antibiotic binding site and/or by perturbing the integrity of the transmembrane protein channel or the RND tripartite protein complex assembly (275). The inability of most EPIs to synergize other antibiotics in *P. aeruginosa* is perhaps attributable to their penetration impediments across the bacterial membrane. However, some EPIs may also be subject to expulsion, as the efflux pump architecture varies among different efflux systems. The possibility of inducing vector-assisted intracellular uptake of EPIs in *P. aeruginosa*, following a hybrid drug approach, by utilizing the self-promoted uptake of aminoglycosides and amphiphilic aminoglycosides was thus investigated (290). Aminoglycosides are desired since they are poor substrates of most efflux pumps in *P. aeruginosa*, especially those of the RND family (291). It was posited that the physicochemical property of the tobramycin-hybrid scaffold required to elicit a biological response (such as PMF dissipation) and the native efflux pump-inhibitory effects of EPIs would be preserved during hybridization. Thus, three EPIs [1-(1-naphthylmethyl)-piperazine (NMP), paroxetine (PAR), and dibasic peptide (DBP)] were appended to tobramycin using aliphatic hydrocarbon tethers, to give hybrids 14, 15, and 16, respectively (Fig. 10), and screened against a panel of clinically relevant pathogens (249). It should be noted that DBP is an analog of the dibasic dipeptide D-Ala–D-homophenylalanine (hPhe)–aminoquinoline (MC-04,124) (292), a former drug candidate that was able to potentiate fluoroquinolones via efflux pump inhibition and membrane-destabilizing effects (284, 292, 293). None of the resulting conjugates, hybrids 14, 15, and 16, displayed potent antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 2) (249). However, the tobramycin-EPI conjugates retained adjuvant properties similar to those of other tobramycin-based hybrids, as they enhanced the antibacterial activity of minocycline in wild-type, MDR, and XDR *P. aeruginosa* isolates (Table 3). On the other hand, neither NMP nor PAR potentiated minocycline. Minocycline is a known substrate of *P. aeruginosa* RND efflux pumps (291). At 8 μg/ml (6.1 to 7.2 μM), all the tobramycin-EPI hybrids (hybrids 14, 15, and 16) reduced the MIC of minocycline below its CLSI interpretive susceptibility breakpoint ($\leq$4 μg/ml) in all tested *P. aeruginosa* clinical isolates. Strong synergism was similarly observed with other tetracyclines, such as doxycycline and tigecycline, and potentiation was observed for other Gram-negative pathogens as well (249).

Mechanistic validation for hybrid 14 showed modes of action similar to those of other tobramycin-based hybrids. It permeabilizes the OM in a dose-dependent manner, induces a dose-dependent depolarization of the cytoplasmic membrane, and inhibits the PMF-driven flagellum-dependent motility of *P. aeruginosa* PAO1 at sub-MIC values (249). The combination of hybrid 14, but also other tobramycin-EPI combinations, with minocycline at a 1:1 mass ratio delayed the emergence of resistance in PAO1 after 25 serial passages. However, minocycline or tobramycin alone resulted in a 16-fold or 256-fold increase in the MIC, respectively. Tobramycin-NMP conjugate 14 displayed *in vivo* potency in XDR *P. aeruginosa* 101856-challenged *G. mellonella* larvae. A single dose

of the minocycline-hybrid 14 combination (75 mg/kg each) resulted in 77% survival after 24 h (249). On the other hand, minocycline (75 mg/kg) or hybrid 14 alone (75 mg/kg) resulted in 0% survival after 24 h. Tobramycin-NMP hybrid 14 was shown to be nonhemolytic ($\leq$5% hemolysis of ovine erythrocytes at 1,000 μg/ml) and displayed low cytotoxicity to human epithelial cancer cell lines (50% cytotoxic concentration [CC_{50}] of $>$30 μM). CC_{50} is the concentration of a drug required to reduce the viability of a cell population by 50% relative to untreated controls (294, 295). This rules out the suspicion of a nonspecific mode of action (296), as the hybrid molecule could discriminate prokaryotic from eukaryotic cells. The synergism of minocycline and tobramycin-EPI conjugates was more pronounced in *P. aeruginosa* strains that express RND efflux pumps but was less pronounced in efflux deletion strains (249). This subtle observation may suggest that, in addition to dissipating the PMF, the tobramycin-EPI conjugates may also disrupt active efflux via RND pumps, particularly that of the minocycline-relevant MexAB-OprM RND pump. The ability of tobramycin-EPI conjugates to enhance the uptake of tetracycline in *P. aeruginosa* PAO1 was reversed in the presence of the oxidative phosphorylation uncoupler CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), an agent that disrupts the proton gradient of Gram-negative bacteria (297). This observation is consistent with the ability of the hybrids to interfere with bacterial PMF.

More recently, a polymyxin B_3-tobramycin hybrid was also reported to potentiate rifampin, minocycline, and vancomycin against MDR and XDR *P. aeruginosa* isolates, in a fashion similar to those of other tobramycin-based hybrids (252).

In summary, tobramycin-based hybrids appear to have an intrinsic physicochemical property that enables the potentiation of legacy antibiotics (Fig. 11). The pairing of a pharmacophore to tobramycin may impart an additional biological action to the scaffold, and/or hybridization may result in a new (third) mechanism of antibacterial action independent of the composing parent drugs. Table 2 summarizes the MICs of tobramycin-based hybrids and some select antibiotics against a panel of Gram-positive and Gram-negative bacteria. Tobramycin-based hybrids, in combination with ciprofloxacin, minocycline, or rifampin, resensitized a panel of MDR/XDR *P. aeruginosa* isolates and decreased their respective MICs from resistant to susceptible or intermediately resistant values (Table 3).

Resensitization of resistant pathogens to antibiotics may be induced by targeting the membrane. A recent review aptly described the role of proton-dependent processes in the propagation of bacterial infections (298). Membrane-active agents are known to disrupt vital bacterial processes by perturbing the well-ordered membrane constituents, resulting in the loss of transmembrane protein integrity and function but also the loss of transmembrane potential (ion/proton balance) (86, 299). The mechanistic action of membrane disruption may stem from membrane perforation via the formation of transient pores (300), lipid disintegration leading to lysis, and/or the segregation of phospholipids (301). These concentration-dependent effects are usually driven by the amphiphilic nature of the compounds and could range from mild to fatal (302–307). Since the bacterial membrane is central to a host of its vital, survival, and adaptive mechanisms such as energy production via the respiratory chain, quorum sensing, and efflux pumps, etc., agents that alter the transmembrane protein environment (such as membrane charge, fluidity, and thickness) and/or steric hindrance of membrane-embedded proteins can theoretically lead to cell death via the inhibition of signaling cascades. For instance, swimming motility is a flagellum-dependent bacterial movement that is governed by the respiratory chain on the cytoplasmic membrane (273). When cytoplasmic membrane potential or PMF is disrupted, electron transfer across the respiratory chain is inhibited, resulting in a reduction of ATP synthesis, which is essential for flagellar function (308). These flagella (and pili) are surface appendages that serve as the major means of motility (chemotaxis) as well as the anchors that facilitate initial binding to the asialylated glycolipid (asialoGM1) receptor on the host's epithelial cells prior to the destruction the protective glycocalyx (309).

Membrane depolarization can therefore result in the resensitization of MDR pathogens to antibiotics, as the inhibition of signal transduction coordinated by ATP ma-

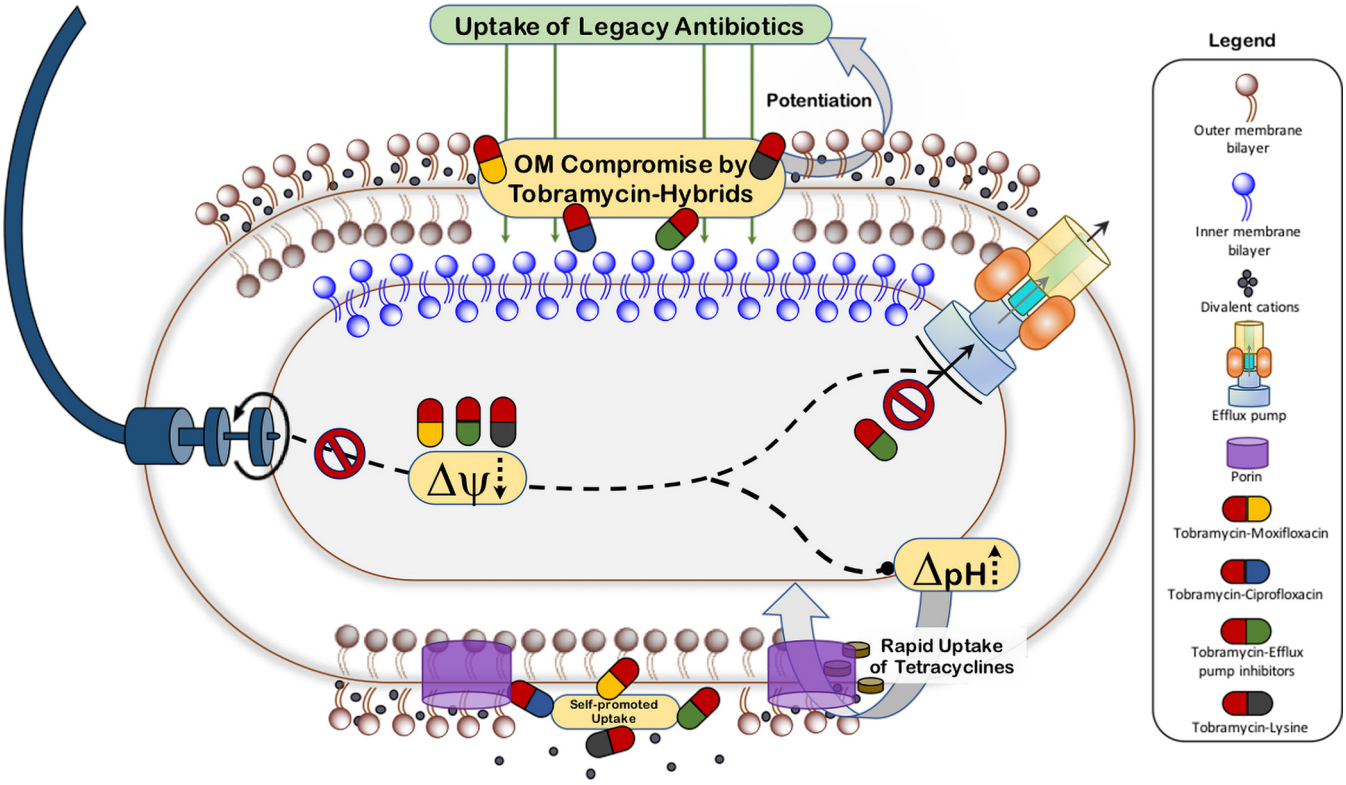


FIG 12 Proposed mechanism(s) of action of tobramycin-based antibiotic hybrids.

chinery of the respiratory chain could inflict a crippling fitness cost on the bacteria. Some HDPs are endowed with excellent membrane-disruptive capabilities for selectively interacting with bacterial membranes (310) but have severe limitations of protease cleavage and systemic cytotoxicity, etc. (311). Other polycationic agents such as the pentabasic polymyxin analogs that increase OM permeability in Gram-negative bacteria are also relatively toxic (312). Reducing the number of basic groups in polymyxins may represent a path forward to reduce potential nephro- and/or ototoxicity, as evident in preliminary studies with tribasic SPR741 (150). The reduction in basic groups could, however, compromise the ability of the adjuvant to sensitize certain pathogens, such as the inability of SPR741 to synergize antibiotics in *P. aeruginosa* (149).

Proposed mechanisms of action of tobramycin-based hybrids. The mechanism of action of tobramycin-based hybrids is quite consistent with that of membrane-active agents that target cellular energetics of prokaryotes. The cellular uptake and mode of action of this scaffold, particularly in but not limited to *P. aeruginosa*, involve the following (Fig. 12).

Competitive displacement of divalent cation cross-bridges that stabilize the LPS structure on the outer leaflet of the OM, in a fashion analogous to that of the self-promoted uptake mechanism of aminoglycosides and amphiphilic aminoglycosides (90, 224, 312–322), results in OM destabilization. This destabilization facilitates the permeation of other antibiotics into the periplasm and may explain the observed sensitization of MDR Gram-negative bacteria to membrane-impermeable antibiotics such as rifampin, novobiocin, and vancomycin, etc.

Insertion into the OM, presumably promoted by the hydrophobic aliphatic hydrocarbon linker, allows tobramycin-based hybrids to reach the periplasm. This may further augment the entry of other membrane-impermeable antibiotics, as reflected by their synergistic relationship with the hybrids.

Perturbation of transmembrane efflux protein domains prevents the relay of signaling cascades required to elicit conformational changes necessary to extrude substrate

molecules (such as antibiotics). The loss of protein integrity may be induced by the hybrid directly, via hydrogen bonding with exposed residues, or indirectly, by altering the lipid composition surrounding the protein. This may in part explain the observed enhancement of the intracellular concentration (synergy) of efflux-susceptible antibiotics such as fluoroquinolones.

Dissipation of the membrane potential ($\Delta\Psi$) component of PMF leads to compensatory transmembrane pH gradient (ΔpH) adjustments to maintain a constant PMF. PMF is composed of an electrical component ($\Delta\Psi$) and a proton component (ΔpH), which are complementary to one another. If one component is altered, the bacteria compensate for and ensure a stable PMF by adjusting the other complementary component (59). This phenomenon was previously described as being the basis for the potentiating effects of the antidiarrheal drug loperamide (276). Tobramycin hybrids dissipate $\Delta\Psi$ (at sub-MIC values), thereby prompting the pathogen to raise its transmembrane ΔpH. This view is supported by the following findings: (i) the swimming motility (a $\Delta\Psi$-controlled process) of *P. aeruginosa* was severely constrained in the presence of the hybrids; (ii) the hybrids could not potentiate the aminoglycoside class of antibiotics, as they require an optimal $\Delta\Psi$ component for cytoplasmic uptake; and (iii) the strong synergy between tobramycin-based hybrids and minocycline (and other tetracyclines) is indicative of an increased ΔpH component. Tetracyclines are known to penetrate bacterial cells in a ΔpH-dependent fashion (64). Moreover, the inability of tobramycin-based hybrids to potentiate meropenem, which is exclusively taken up by active transport, may indirectly suggest that their effects on PMF not only affect efflux but also may reduce active energy-dependent transport.

A combination of any or all of the above-described mechanisms, simultaneously or sequentially, is most likely responsible for the adjuvant effects of tobramycin-based hybrids. Although these hybrids target bacterial membrane energetics, some FDA-approved nonantibiotics have also been shown to target and disrupt the physical integrity of this membrane (59, 274, 276). This suggests that these membrane-active compounds may similarly be tolerated in humans, as evident in pilot studies with *G. mellonella* larvae and human epithelial cancer cells.

Indeed, the characteristic mechanism of action of this tobramycin hybrid scaffold is what sets it apart from those of other hybrid antibiotics discussed above, and we encourage everyone involved in antimicrobial drug discovery to partake in this exciting area of investigation.

PERSPECTIVES

Our viewpoint of the current landscape of antibiotic development is simple: there is an urgent need to develop new therapeutics that are able to treat antibiotic-resistant infections, especially those caused by MDR Gram-negative pathogens. However, coming up with a magic bullet to address this problem has proven to be elusive over the years. We highlight several strategies to potentially develop new antibiotics, yet these are only a few of the possible solutions to this ever-worsening global issue. Imagination is perhaps the greatest limitation to drug discovery.

The concept of antibiotic hybrids is attractive considering their apparent advantages, but they are not without an Achilles' heel. Molecular complexity, intractable chemical synthesis, and the rigorous work needed to establish the mode of action and benefit of hybrids over conventional drugs make the hybrid approach daunting. Nonetheless, we argue that drug discovery should be driven not only by synthetic convenience but also by scientific exploration to solve an important problem. Despite its associated challenges, the antibiotic hybrid strategy remains a viable approach to expand the chemotherapeutic space of our current antimicrobial arsenal. Our experience in this research field has broadened our views to rethink the principles of antibacterial drug discovery, that (i) an inactive agent, in terms of its antibacterial activity (MIC), is not necessarily biologically irrelevant, and (ii) molecular fusion/hybridization of pharmacophores could in fact present a new scaffold with a completely different biological activity and pharmacological profile. Our explora-

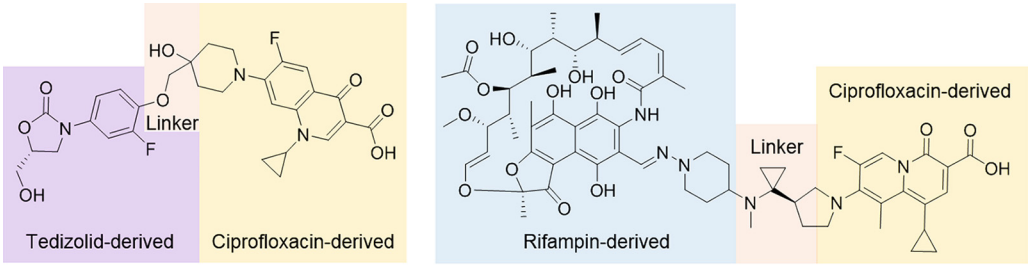


FIG 13 Examples of advanced hybrid antibiotics undergoing clinical trials: cadazolid (hybrid 17) and TNP-2092, also known as CBR-2092 (hybrid 18). Cadazolid was derived by fusing ciprofloxacin and tedizolid (with overlapping pharmacophores), while TNP-2092 comprises ciprofloxacin and rifampin-derived pharmacophores. These hybrids display limited antibacterial activity against Gram-negative bacilli.

tion with tobramycin-based hybrids yielded a core scaffold that appears to have intrinsic adjuvant properties and is amenable to modifications.

FUTURE OUTLOOK: ANTIBIOTIC HYBRIDS MAY BE THE NEXT GENERATION OF ANTIBIOTIC AGENTS AND ADJUVANTS

With the globally escalating incidences of MDR, XDR, and PDR infections in both inpatients and outpatients, especially those caused by the carbapenem-resistant ESKAPE superbugs, there is an obvious need to restock our antibiotic arsenal and stay ahead of these pathogens. Hybridization of legacy antibiotics to generate new drug scaffolds might be the future of antibacterial drug discovery. There are at least two hybrid drug candidates (Fig. 13) currently in consideration for the treatment of Gram-positive bacterial infections, while only one (Fig. 8) is under consideration for Gram-negative bacterial infections.

Cadazolid (hybrid 17) (323–327), a noncleavable heterodimer consisting of fused pharmacophoric portions derived from ciprofloxacin and tedizolid (Fig. 13), has completed phase 3, randomized, double-blind clinical trials for the treatment of *Clostridium difficile*-associated diarrhea in comparison to vancomycin (https://clinicaltrials.gov/show/NCT01987895). According to Actelion Pharmaceuticals (now acquired by Johnson & Johnson), the study yielded mixed results: cadazolid met its primary endpoint of clinical cure for its pivotal IMPACT 1 study but failed to do so for its follow-up IMPACT 2 study, raising concerns about its efficacy. CBR-2092 (hybrid 18) (328, 329), a rifamycin-quinolone hybrid (Fig. 13), is also in clinical development for acute bacterial skin and skin structure infections but also for hospital-acquired pneumonia, ventilator-associated bacterial pneumonia, and bacteremia. Hybrid 18 was renamed TNP-2092 after Cumbre Pharmaceuticals was acquired by TenNor Therapeutics in 2009 and has successfully completed phase 1 clinical trials. Other hybrid drug candidates in clinical trials for the treatment of Gram-positive bacterial infections include the glycopeptide-cephalosporin hybrid cefilavancin (completed phase 2 trials [https://clinicaltrials.gov/ct2/show/NCT00442832]) and the oxazolidinone-quinolone hybrid MCB3837 (currently in phase 1) (http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development).

As elaborated above, the cephalosporin-siderophore hybrid cefiderocol (hybrid 10) (241, 243, 244) is currently in phase 3 clinical trials for the treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria (https://clinicaltrials.gov/ct2/show/NCT02714595) as well as nosocomial pneumonia caused by Gram-negative pathogens (https://clinicaltrials.gov/ct2/show/NCT03032380). The fact that cefiderocol is in late stages of clinical trials is very encouraging, as it is one of the very few antibiotic hybrids that retain potency against recalcitrant carbapenem-resistant Gram-negative bacilli.

We envisage an increase in the number of antibiotic hybrids that progress into clinical studies in the near future, either as stand-alone antibacterial agents or as adjuvants. The use of adjuvants to rescue the efficacy of currently used antibiotics is

increasingly gaining attention, especially for use against Gram-negative pathogens (11, 119, 127, 146, 330), and antibiotic hybrids certainly have a role to play in this going forward. With the recent examples of experimental hybrid agents in the literature (245), we are optimistic that a few of these drugs will make it all the way to the clinic for patient treatment. It is our hope that this article and others (169, 207, 245, 246) will stimulate curiosity and interest in the antibiotic-hybrid strategy as a viable way to develop agents that are capable of combating drug-resistant pathogens.

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