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REVIEW

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Antibiotic–cell-penetrating peptide conjugates targeting challenging drug-resistant and intracellular pathogenic bacteria

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Abstract

The failure to treat everyday bacterial infections is a current threat as pathogens are finding new ways to thwart antibiotics through mechanisms of resistance and intracellular refuge, thus rendering current antibiotic strategies ineffective. Cell-penetrating peptides (CPPs) are providing a means to improve antibiotics that are already approved for use. Through coadministration and conjugation of antibiotics with CPPs, improved accumulation and selectivity with alternative and/or additional modes of action against infections have been observed. Herein, we review the recent progress of this antibiotic–cell-penetrating peptide strategy in combatting sensitive and drug-resistant pathogens. We take a closer look into the specific antibiotics that have been enhanced, and in some cases repurposed as broad-spectrum drugs. Through the addition and conjugation of cell-penetrating peptides to antibiotics, increased permeation across mammalian and/or bacterial membranes and a broader range in bacterial selectivity have been achieved.

KEYWORDS

antibacterials, antimicrobial peptides, cell-penetrating peptides, conjugates, drug resistance

1 | INTRODUCTION

Antibiotics have transformed medicine across the globe since the first antibiotic was discovered nearly a century ago. However, almost as quickly as each antibiotic became available for widespread usage, resistance has eclipsed the clinical usefulness of many of these antimicrobial drugs (Aslam et al., 2018; Zaman et al., 2017). The identification of pathogens resistant to most, if not all, available antibiotics is occurring routinely, such as drug-resistant *N. gonorrhoeae*, *A. baumannii*, and *C. auris*, each listed as an urgent threat by the US Centers for Disease Control (CDC 2019). Over time, the development of new antibiotics has slowed. Pharmaceutical companies traditionally dominated antibiotic research and development, but many withdrew their efforts due to the lack of long-term success and low financial return of bringing new drugs to market (Jackson et al., 2018). There is currently an urgent need to address resistance, and only two of the eight antibiotics approved since 2017 represent a new chemical scaffold (WHO, 2019). As of December 2019, approximately a quarter of the 41 antibiotics in development represented a novel drug class or mechanism of action; however, none have targeted activity against Gram-negative ESKAPE pathogens or critical threat pathogens identified by the World Health Organization (PEW, 2019).

In addition to small molecule antibiotics, antimicrobial peptides (AMPs) have been extensively examined and are a promising class of antibiotics. Their antimicrobial activities target a range of Gram-negative and Gram-positive bacteria, fungi, parasites, and viruses through a variety of mechanisms including membrane disruption and intracellular targeting via cellular penetration (Fjell et al., 2012; Koo and Seo 2019). Currently, there are ten peptide-based antibacterials, including two glycopeptides, three lipoglycopeptides, and one lipopeptide, approved for use, with just over 40 more in the clinical pipeline (Browne et al., 2020). Barriers to AMP clinical use have driven a push

toward designed peptides and peptide mimics that offer greater flexibility in structure. These designed peptides may overcome toxicity and stability disadvantages of AMPs and improve antimicrobial efficacy and selectivity (Li and Brimble 2019).

Not only is the development of resistance an impediment in treating bacterial infections, but mammalian membrane penetration is also an obstacle for antibiotics to reach their full therapeutic potential. For instance, options for treating intracellular *Staphylococcus* infections are limited due to the poor mammalian membrane permeability of many hydrophilic antibiotics, including aminoglycosides and glycopeptides (al-Nawas et al., 1998; Baltch et al., 2007; Darouiche and Hamill 1994; Maurin and Raoult 2001; Seral et al., 2003). Therefore, even if an antibiotic is effective against extracellular bacteria, it may be ineffective in clearing intracellular pathogens that may proliferate and trigger reinfection. Routes to contest both antimicrobial resistance (AMR) and therapeutic evading infections that focus on modifying existing antibiotics are promising (Cohen et al., 2019). Cell-penetrating peptides (CPPs) may provide such a tool for use in tandem with antibiotics to combat challenging bacterial infections. Where some CPP classes may improve the potency of antibiotics, others may improve their delivery to target intracellular locations.

2 | CELL-PENETRATING PEPTIDES

Accessing intracellular target sites in mammalian cells is a major challenge for many therapeutic agents. The use of cell-penetrating peptides (CPPs; protein transduction domains, PDTs) over the past few decades has proved to be a powerful tool in overcoming this obstacle. CPPs enable the delivery of cargo such as proteins, nucleic acids, and small molecule drugs at therapeutic concentrations to intracellular target sites (Chen and Harrison 2007; Koren and Torchilin 2012; Temsamani and Vidal 2004). CPPs provide advantages for cell delivery, such as versatility, efficiency, and low toxicity. These short peptides are typically cationic with multiple arginine or lysine residues and may also be amphiphilic. Classic examples of CPPs include the Tat peptide derived from the HIV-1 Tat protein, penetratin derived from the third helix of the *Antennapedia* homeodomain protein, and oligomers of arginine (Table 1) (Fonseca et al., 2009; Kaplan et al., 2005). Over a thousand CPPs have been isolated or synthetically designed, and a CPP delivery database is available to meet a range of therapeutic needs (Gautam et al. 2012).

2.1 | Antibacterial CPPs

AMPs and cell-penetrating peptides share similar physiochemical properties, such as short peptide length, cationic character, and at times amphiphilicity (Zorko and Langel 2005). This structural redundancy has led to a small number of peptides behaving as both AMPs and CPPs, leading to enhanced efficacy. The ability of these dual-function peptides to penetrate and disrupt bacterial membranes, yet non-destructively translocate across mammalian phospholipid bilayers has been reasoned to be a result of membrane potential differences among bacterial and mammalian membranes (Henriques et al., 2006; Rodriguez Plaza et al., 2014). Therefore, membrane-interacting peptides may exhibit dual activities as a few CPPs display antimicrobial activity and several AMPs can cross eukaryotic cell membranes (Splith and Neundorf 2011).

TABLE 1 Examples of common cell-penetrating peptides

Peptide	Sequence
Tat	YGRKKRRQRRR
Penetratin	RQIKIWFQNRRMKWKK
TP10	AGYLLGKINLKALAALAKKIL
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL
Poly-arginine	$(Arg)_n$ $n = 6–9$

Recently, the CPP Tat has demonstrated broad-spectrum antimicrobial activity with MICs ranging from 2 to 8 μM, including activity against *E. coli*, *S. aureus*, and *B. subtilis* (Zhu and Shin 2009b). In an attempt to improve potency, Tat was dimerized; however, the authors found only a modest increase in antibacterial activity through bacterial membrane disruption, while there was a decrease in mammalian cell membrane translocation. In this instance, there seems to be a trade-off in AMP and CPP activities for the Tat peptide. Similarly, the well-known penetratin CPP has also been examined for antimicrobial activity. Penetratin also displays broad-spectrum antibacterial activity through bacterial membrane disruption with MICs ranging from 0.5 to 4 μM, including activity against *E. coli*, *S. aureus*, and *P. aeruginosa* (Zhu and Shin 2009a). In this case, dimerization of penetratin increased cytotoxicity with no enhancement of antimicrobial activity. In another example, the CPPs TP10 and *p*VEC demonstrated activity against *M. smegmatis* and *C. albicans*, with *p*VEC displaying MIC values of 6 and 10 μM, respectively. TP10 displayed antibacterial activity against a range of Gram-positive bacteria including *S. aureus* with MIC values between 4 and 10 μM. Interestingly, TP10 eliminated intracellular *S. aureus* at 15 μM, although cytotoxicity emerged at higher concentrations (Nekhotiaeva et al., 2004). Additionally, modification of the CPP Pep-1 with lysine residues yielded Pep-1-K, which provided antimicrobial activity against a broad-spectrum of bacteria with MIC values between 1 and 8 μM. However, the cell-penetrating activity decreased with lysine mutations (Zhu et al., 2006). The sC18 peptide was also developed as a CPP by the Neundorf group, and antimicrobial activity was observed with MIC values of

5 μM against *E. coli K12* and *Mycobacterium phlei* (Reinhardt et al., 2014; Splith et al., 2010).

Bacterial membrane permeation and/or disruption with AMPs is a conventional bactericidal mode of action (Mahlapuu et al., 2016). However, in some cases CPPs display antimicrobial activity without bacterial membrane disruption. For instance, Chmielewski and coworkers have developed cationic amphiphilic polyproline helices (CAPHs) consisting of a pre-organized polyproline scaffold functionalized with cationic and hydrophobic moieties with controlled amphiphilicity (Figure 3a—representative structure within the blue box). First-generation CAPHs were significantly more effective CPPs as compared to Tat (~10 to 35-fold), entered a range of mammalian cell types, including MCF-7, J774A.1, and KB-3-1 cell lines, and had specific intracellular localizations (Fillon et al., 2005; Geisler and Chmielewski 2007, 2009; Kalafut et al., 2012). Elongation of the CAPHs, and sidechain and N-terminal modifications demonstrated that the CAPH CPPs alone also display broad-spectrum antibacterial activity, including activity against *E. coli*, *S. aureus*, and *S. typhimurium*, without perturbation of bacterial membranes or hemolysis (Hernandez-Gordillo et al., 2014; Kuriakose et al., 2013; Nepal et al., 2015). Importantly, the subcellular localization of these CAPHs has been tailored to co-localize with intracellular pathogenic bacteria, such as *Listeria* in the cytosol and *Salmonella* within vacuoles (Dietsche et al., 2020; Nepal et al., 2018). Therefore, coupled with cell penetration, these CPPs proved effective against intracellular bacteria with minimal mammalian cytotoxicity and minimal lysis of bacterial membranes. These dual-function peptides were also successful in clearing preformed biofilms, enhancing wound closure, reducing *in vivo* bacterial load, and lowering inflammatory cytokines (Thangamani et al., 2015). These examples demonstrate that merging antibacterial activity with cell penetration can expand the treatment options for difficult intracellular infections.

Within the past decade, cyclic cell-penetrating peptides (cCPPs) have been comprehensively studied as an improved method of reaching intracellular targets (Dougherty et al., 2019; Park et al., 2019). Oh and coworkers showed that a library of amphiphilic cCPPs, rich in tryptophan and arginine residues, displayed moderate broad-spectrum antibacterial activity, with some displaying MICs as low as 3 μM. These peptides also showed synergistic effects with tetracycline in time-kill experiments against pathogens including *E. coli* and multidrug-resistant *S. aureus* (Oh et al., 2014). Experiments with intracellular bacteria were not explored, however.

Just as some CPPs also function as AMPs, some AMPs have been used as CPPs for the intracellular delivery of various cargos (Splith and Neundorf 2011). For instance, the SynB1 peptide, derived from the AMP protegrin-1 (PG-1), functioned as a CPP to deliver covalently conjugated doxorubicin across the blood–brain barrier (BBB) as efficiently as an analogous doxorubicin–penetratin conjugate (Pärn et al., 2015; Rousselle et al., 2000). Another AMP, bactenecin 7, was used as a non-covalent delivery vehicle for the transport of the NeutrAvidin protein into murine monocyte cells (Sadler et al., 2002). Cell penetration with two AMPs, magainin II and buforin II, was also observed as both peptides, carrying a covalently linked fluorescent protein, were internalized in HeLa and TM12 cells (Takeshima et al., 2003). In another example, Otvos and coworkers have explored analogues of AMP pyrrhocoricin (Otvos et al., 2004). This AMP demonstrated CPP activity in transporting an antigen into bacterial and mammalian cells, including dendritic cells and fibroblasts. A truncated peptide sequence from the antibacterial protein lactoferricin penetrated HeLa cells and delivered a covalently conjugated Alexa 488-labeled streptavidin protein (Duchardt et al., 2009). Also, the cationic AMP LL-37 has been used as a CPP to deliver non-covalently complexed DNA into mammalian cells (Kahlenberg and Kaplan 2013; Sandgren et al., 2004; Zhang et al., 2010). An additional class of AMPs, Iztli peptides, has been shown to penetrate mammalian cells (Rodriguez Plaza et al., 2014), and more recently, the AMP TPk was observed to possess cell-penetrating activity in HeLa cells (Bahnsen et al., 2015). Interestingly, the intracellular clearance of bacteria with these CPP AMPs has not been explored.

2.2 | Coadministration of antibiotics and CPPs

The effectiveness of the combination of antibiotics with ancillary drugs has been verified at the clinical level and is gaining support in designing new strategies to overcome microbial resistance. For instance, it is common to see the use of β-lactam antibiotics with β-lactamase inhibitors to suppress the emergence of resistance (Brown and Wright 2016), as well as the well-known combination therapy of isoniazid (INH), rifampin (RIF), ethambutol (EMB), and pyrazinamide (PZA) in treating tuberculosis (CDC 2019; Kerantzas and Jacobs 2017). There is also evidence beginning to surface that antibiotic efficacy may be improved through coadministration with cell-penetrating peptides (CPPs).

In one example, Randhawa and coworkers have shown that two arginine-rich CPPs, P3 and P8, that have limited antibiotic activity alone synergistically improved the efficacy of antibiotics against *S. aureus* (Randhawa et al., 2016). The MIC values of oxacillin, norfloxacin, and vancomycin were lowered in some cases over a thousand-fold against sensitive and resistant *S. aureus* strains by combining them with the P3 and P8 CPPs. Recently, a non-conjugated combination of curcumin and octa-arginine, R8 (molar ratio of 1:5 peptide:curcumin), showed ~twofold enhanced antibacterial

activity against Gram-negative and Gram-positive bacterial isolates. This combination also showed faster killing kinetics; *E. coli* was eradicated within 30 min, while curcumin was ineffective after 8 hr (Ratrey et al., 2020). There are a few additional examples of coadministration of antibiotics and CPPs that will be discussed below along with their corresponding covalent complexes.

There is great interest in using antibiotic–CPP conjugates. Enhanced activity against resistant bacterial strains and reduced side-effects or toxicity are objectives in the use of antibiotics with CPPs (Sheard et al., 2019). This review will highlight the recent progress on antibiotic-CPP covalent conjugates to effectively target and treat drug-resistant bacterial infections, while minimizing mammalian cell toxicity, and efforts to treat intracellular pathogenic bacteria.

3 | CELL PENETRATING PEPTIDE CONJUGATES WITH ANTIBIOTICS

3.1 | Glycopeptide–CPP conjugates

Vancomycin is a glycopeptide antibiotic used to treat drug-resistant Gram-positive infections, such as methicillin-resistant *S. aureus* (MRSA). Although vancomycin resistance has emerged in *Enterococcus* and *Staphylococcus* species, many strategies to overcome vancomycin resistance have been studied. Recent examples include functionalizing vancomycin with positively charged groups and lipophilic components. For instance, the Haldar group has explored the conjugation of vancomycin to cationic lipids (Sarkar et al., 2020; Yarlagadda et al., 2014, 2015; Yarlagadda, Manjunath, et al., 2016; Yarlagadda, Samaddar, et al., 2016) and bacterial cell wall pyrophosphate binding moieties (Yarlagadda, Sarkar, et al., 2016; Yarlagadda et al., 2018). The incorporation of sulfonium-based cationic lipophilic components to vancomycin led to enhanced interaction with the negatively charged bacterial cell membrane and increased bactericidal activity through membrane disruption (Guan et al., 2019). Boger and coworkers have built upon the chlorobiphenyl (CBP) modification used in oritavancin to incorporate a quaternary ammonium ion that resulted in better bactericidal activity, presumably due to enhanced membrane interactions (Okano et al., 2017; Wu et al., 2020). Other lipophilic and cell surface-interacting vancomycin derivatives have shown increased activity in treating vancomycin-resistant bacteria (Crane et al., 2010; Guan et al., 2018; McComas et al., 2003; Nakama et al., 2010; Printsevskaya et al., 2013; Yoganathan and Miller 2015).

Antonoplis and coworkers investigated the addition of one or two arginine residues to the C-terminus of vancomycin through an amide linkage (Antonoplis et al., 2019). This modification broadened the spectrum of vancomycin to inhibit Gram-negative bacteria with V-R and V-RR displaying MICs against *E. coli* of 8 and 12 μM, respectively. In a mouse *in vivo* infection model, V-R displayed over a 4 log unit reduction of an *E. coli* infection at 200 mg/kg, where vancomycin only reduced the infection by 10-fold at a high dose (1272 mg/kg) (Neville et al., 2021). Vancomycin has also been conjugated to a full-length CPP composed of eight arginine residues via the free carboxylic acid on vancomycin (V-r8; Figure 1a) (Antonoplis et al., 2018). Although V-r8 demonstrated potent but comparable activity as compared to vancomycin alone against susceptible and methicillin-resistant *S. aureus*, this conjugate was able to clear over 95% of preformed MRSA biofilms at 80 times its MIC and persister cells at 10 μM *in vitro*. In a mouse *in vivo* wound biofilm model, a 0.05% solution of V-r8 reduced bacterial load to the same extent as 2% fusidic acid with no toxicity. The potency of V-r8 was presumably due to enhanced cell wall binding (Figure 1b). Although the authors used a CPP, they did not explore clearance of bacteria from mammalian cells.

A selection of membrane active vancomycin conjugates was also designed by Blaskovich and coworkers (Blaskovich et al., 2018). Their design involved coupling vancomycin to a lysine-rich electrostatic effector peptide sequence (EEPS) and a lipid membrane-insertive element (Figure 1c). These cationic lipoglycopeptides (vancapticins) showed enhanced activity against MRSA and other Gram-positive bacteria, with some vancapticins showing a 100-fold improvement in MIC as compared to vancomycin alone. Mode of action studies demonstrated that membrane interaction of the vancapticins, rather than enhanced ligand binding, accounted for their activity.

Ruczyński and coworkers have explored two locations on vancomycin as options for conjugation with CPPs. The C-terminus and vancosamine of vancomycin were modified with PEG tethers of varying lengths to covalently conjugate vancomycin and the CPP transportan (TP10) (Figure 2a; Ruczyński et al., 2019). Locations on TP10 CPP were also investigated as conjugation sites, such as the N-terminus or lysine residues. All conjugates showed increased antibacterial activity against resistant *S. aureus* and most showed activity against *E. faecium*. The most potent conjugate [$Lys^7(PEG_4$-Van)]TP10 displayed about a fourfold increase in activity as compared to vancomycin. Notably, this conjugate displayed intracellular antibacterial activity against MRSA in HEK293 cells. The Van-TP10 conjugate cleared 71% of MRSA as compared to 5% and 26% clearance by vancomycin and TP10, respectively. Accumulation across the blood–brain barrier was also observed by fluorescent imaging of a fluorescein-labeled conjugate.

Recently, the CPP PP-G has been conjugated to vancomycin (Jiang et al., 2020). PP-G is a flexible guanidinium-rich polypeptide with enhanced cell penetration as compared to Tat and oligo-arginine CPPs, but with no activity against intracellular bacteria. The PP-G peptide was conjugated to

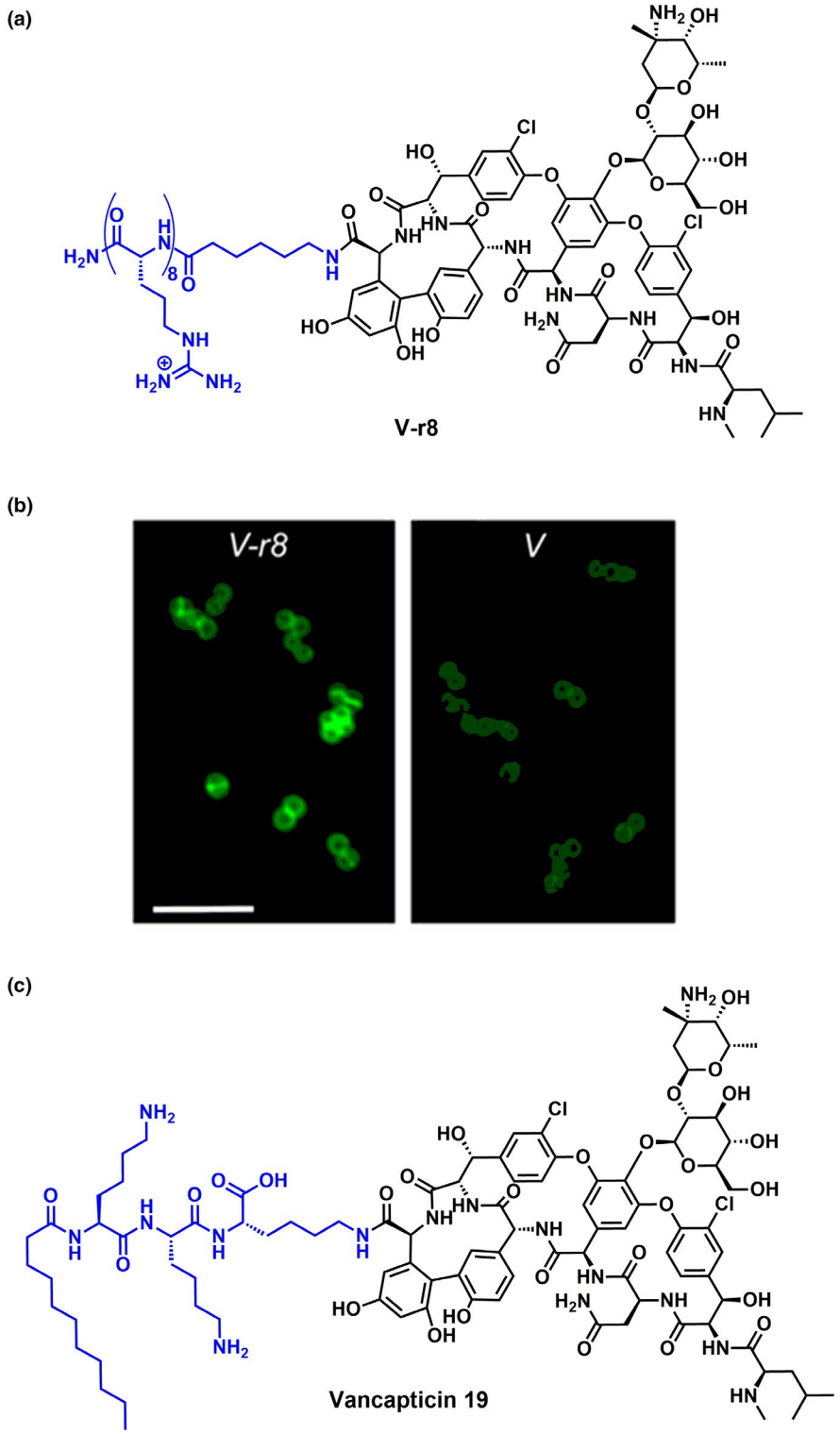

FIGURE 1 (a) Structure of vancomycin-r8 (V-r8) and (b) confocal microscopy of MRSA USA400 bacteria treated with 5 μM Fl-V-r8 and Fl-V for 5 min. Bacteria treated with Fl-V–r8 exhibit greater cell-associated and protoplast-associated fluorescence than Fl-V. Reprinted (adapted) with permission (Antonoplis et al., 2018). Copyright (2018), American Chemical Society. (c) Structure of vancapticin 19. Color scheme: antibiotic, black; CPP, blue [Colour figure can be viewed at wileyonlinelibrary.com]

vancomycin's C-terminus using click chemistry (Figure 2b). The conjugate displayed a 10-fold improvement in MIC as compared to vancomycin against vancomycin-resistant *Enterococci*, although marginally inferior activity against *S. aureus* as compared to vancomycin was observed. This conjugate showed efficient mammalian cell internalization (Figure 2c) and effectively eradicated over 99.9% intracellular MRSA and VRE at 9 and 48 μM, respectively. In a mouse intravenous MRSA infection model, the VPP-G conjugate reduced the bacterial load of MRSA about 100 times better than vancomycin.

Muhlberg and coworkers have also explored conjugating triarginine and a fatty acid to various locations on periphery of vancomycin (Figure 2d) (Mühlberg et al., 2020). The most potent vancapticin conjugate, V_V-R_3C-C12, had the peptide and lipid conjugated from the vancosamine

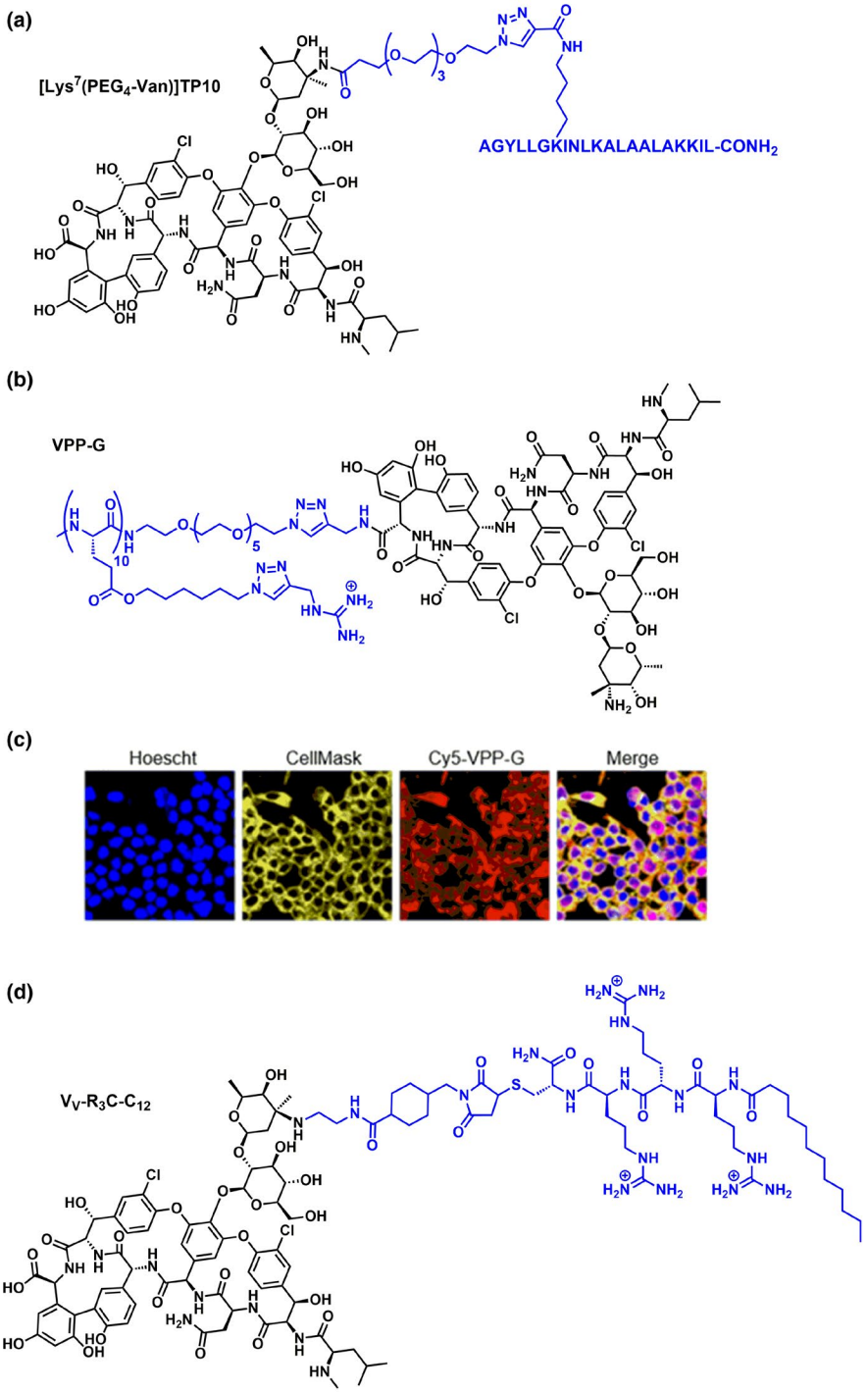

FIGURE 2 (a) Structure of [Lys^7(PEG$_4$-Van)]TP10, (b) structure of VPP-G and (c) confocal images of cellular internalization and membrane penetration of Cy5-VPP-G with RAW264.7 macrophages treated with 18 μM Cy5-VPP-G. Blue: Hoechst; yellow: CellMask; red: Cy5-VPP-G. Reprinted (adapted) with permission (Jiang et al., 2020) Copyright (2020), American Chemical Society. (d) Structure of V_V-R_3C-C_{12}. Color scheme: antibiotic, black; CPP, blue [Colour figure can be viewed at wileyonlinelibrary.com]

handle of vancomycin. This conjugate demonstrated about 100-fold better activity against VanA-, VanB-, and VanC-resistant *enterococci* than vancomycin. The Uhl group also used the same maleimide linker strategy to conjugate an Arg_6 CPP on several positions of vancomycin (Umstätter et al., 2020). In their lead conjugate, FU002, the hexaarginine was conjugated from the secondary amine of vancomycin. This conjugate displayed activity against all three types of vancomycin-resistant enterococci with MICs below 5 μM, about 100-fold more potent than vancomycin. Both examples of vancomycin conjugates showed negligible cytotoxicity at relevant concentrations, but the authors did not explore intracellular efficacy against bacteria in mammalian cells.

3.2 | Aminoglycoside–CPP conjugates

Aminoglycosides are a class of antibiotics traditionally used to treat Gram-negative infections by inhibiting bacterial protein synthesis. Common antibiotics in this class include streptomycin, kanamycin, tobramycin, gentamicin, and

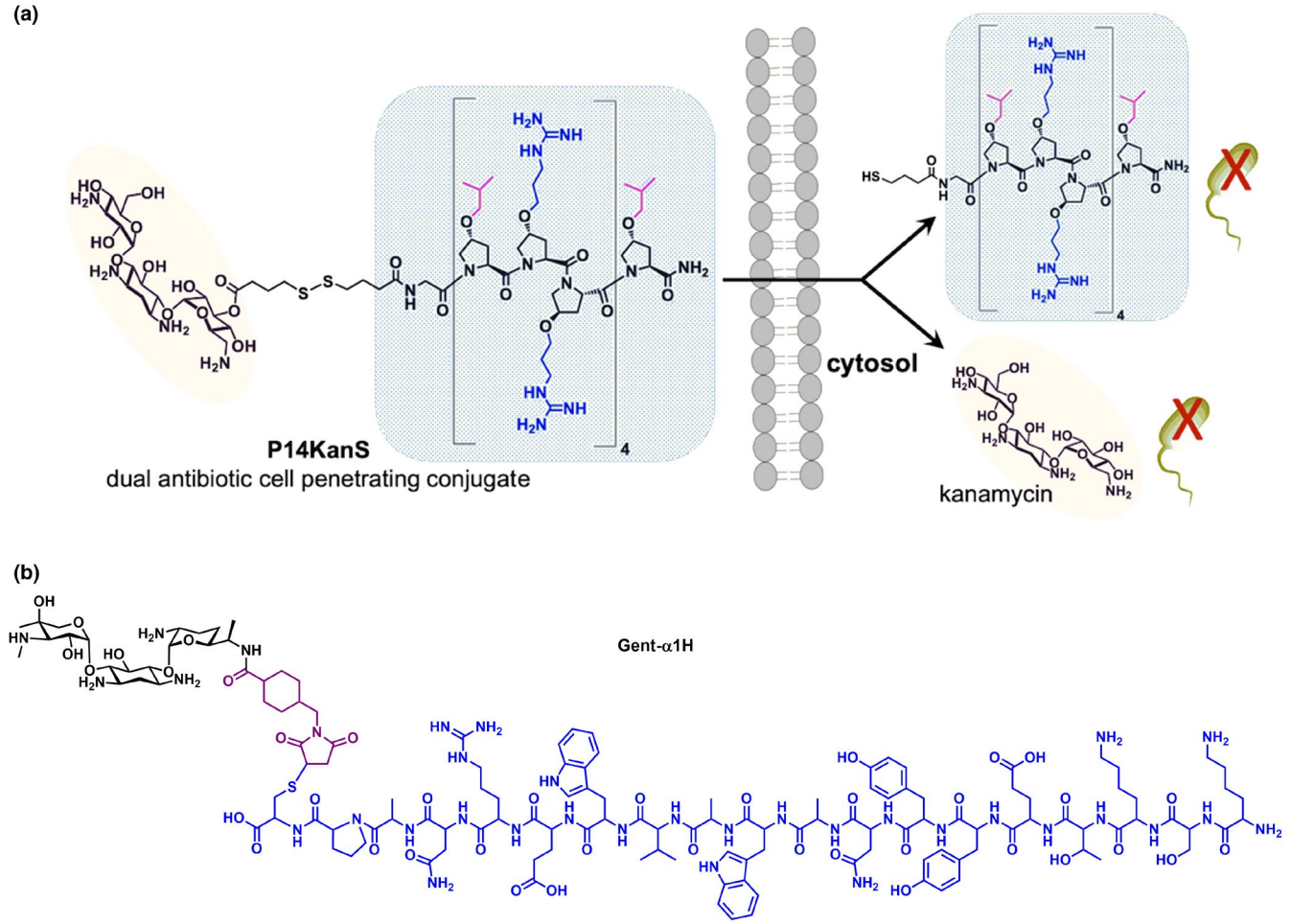

FIGURE 3 (a) Kanamycin-CAPH, P14KanS, releases two separate antibiotics under reducing conditions within cells. Reprinted (adapted) with permission (Brezden et al., 2016) Copyright (2016), American Chemical Society. (b) Structure of Gent-α1H. Color scheme: antibiotic, black; CPP, blue; linker, purple [Colour figure can be viewed at wileyonlinelibrary.com]

neomycin. The broad-spectrum aminoglycoside, kanamycin, is used to treat a range of bacterial infections and tuberculosis, but primarily used to treat Gram-negative bacteria. Along with the other aminoglycosides, the efficacy of kanamycin is limited against intracellular bacteria due to its reduced ability to traverse mammalian membranes. With this in mind, the Chmielewski group conjugated kanamycin to the antimicrobial and cell-penetrating CAPH P14LRR to produce P14KanS (Brezden et al., 2016; Mohamed et al., 2017). A disulfide linkage was used between a kanamycin ester and P14LRR in P14KanS, and both free kanamycin and P14LRR-SH were successfully released from the conjugate under reducing conditions (Figure 3a). This conjugate demonstrated excellent antimicrobial activity against a spectrum of ESKAPE pathogens with 128-fold or greater enhancement of MIC values as compared to kanamycin against drug-resistant and biofilm-producing isolates of *S. epidermidis* and *P. aeruginosa*, and drug-resistant isolates of *A. baumannii*, *K. pneumoniae,* and *E. faecium* (Mohamed et al., 2017). Strikingly, by combining the cell penetration of the CAPH and the dual antibiotic activity of the conjugate, P14KanS cleared 95% of the bacterial load of *Shigella*- and *Mycobacteria*-infected macrophage cells as compared to a ~60 and 30% reduction of these intracellular bacteria with a 1:1 mixture of kanamycin and P14LRR, respectively (Brezden et al., 2016). The conjugate also significantly reduced *Salmonella* in a *C. elegans in vivo* model (up to 90%) as compared to the 1:1 mixture of kanamycin and P14LRR (up to 50%), with no observed toxicity after 24 hr. Additionally, this conjugate also was found to clear up to 80% of preformed *S. epidermidis* biofilm mass at 64X its MIC, and cleared over 95% of preformed biofilms of *P. aeruginosa* and *A. baumannii* (16X and 32X MIC) (Brezden et al., 2016; Mohamed et al., 2017). Against challenging persister cells, P14KanS completely eradicated stationary-phase cultures of *S. aureus* after 4 hr and reduced *S. epidermidis* by 100-fold after 6 hr, whereas kanamycin was ineffective. These data demonstrate the power of combining dual antibiotic agents with cell penetration.

Another aminoglycoside antibiotic, gentamicin, has potent antibacterial activity against extracellular bacteria, but

fails to accumulate intracellularly to treat internalized bacteria within mammalian cells. The conjugation of gentamicin to CPPs was explored by Gomarasca and colleagues (Gomarasca et al., 2017). The authors used two helices of a protein transduction domain isolated from *Y. enterocolitica,* α1H and α2H (Rüter et al., 2010), as CPPs along with Tat to improve gentamicin's activity. A maleimide linker strategy was used to conjugate cysteine-modified α1H, α2H, and Tat to an amine of gentamicin (Figure 3b). These conjugates did not alter the *in vitro* bactericidal activity of gentamicin; however, the CPPs successfully delivered gentamicin within HBMEC, reducing the intracellular bacterial loads of Gram-negative pathogenic *E. coli*, *Shigella*, and *Salmonella* ~100- to 1000-fold better than gentamicin alone. Interestingly, the addition of non-conjugated α1H, α2H, and Tat with gentamicin also reduced the bacterial load five- to eightfold better than free gentamicin in *E. coli*-infected HBMEC. All three CPPs exhibited modest intracellular antibacterial activity alone, ~10-fold reduction of bacterial load compared with the negative control; thus, an additive effect of gentamicin with the CPPs is presumed.

Membrane-active antibiotic–peptide conjugates (MAAPCs) are composed of a short peptide sequence from the CPP penetratin and the broad-spectrum aminoglycoside tobramycin, traditionally used for Gram-negative bacteria (Deshayes et al., 2017). Tobramycin was linked to different penetratin analogs through its single 6′ alcohol by an ester linkage or a copper-catalyzed azide-alkyne cycloaddition linkage. These conjugates displayed selective bactericidal membrane permeation and broad-spectrum antibacterial activity that was comparable to tobramycin. However, the MAAPC01 and MAAPC05 conjugates had antibacterial activity against persister cells with a 3 log unit reduction of *E. coli* at 3 μM. Further, MAAPC01 reduced persister cells of *S. aureus* by 5 log units at 25 μM, while tobramycin displayed no activity against both *E. coli* and *S. aureus* persister cells. Although the authors reported selective bacterial membrane disruption without disrupting mammalian membranes, intracellular activity against bacteria within mammalian cells was not discussed.

Paromomycin (PMM) is another poorly absorbed broad-spectrum aminoglycoside antibiotic used to treat infections caused by bacteria and parasites. This antibiotic was conjugated to the Tat peptide through a flexible PEG spacer and amide linkage (Defaus et al., 2017). This conjugate successfully accumulated intracellularly in *Leishmania* parasites, as visualized by confocal microscopy with a fluorescent PMM-Tat conjugate, and was hypothesized to penetrate within *Leishmania* ulcers better than the poorly penetrating paromomycin. Although intracellular accumulation into the cytoplasm of parasites was observed, killing assays and mammalian cellular accumulation were not investigated.

3.3 | Other small molecule antibiotic–CPP conjugates

As described above, a number of bacteria reside within mammalian cells where they are difficult to access. For instance, the treatment of a life-threatening intracellular *Listeria monocytogenes* infection is challenging. The Kelley group has explored using their cyclohexyl- and guanidinium-rich CPP to treat such infections through conjugation with methotrexate (Mtx) (Lei et al., 2013). This Mtx-CPP exhibited enhanced bactericidal activity compared to Mtx with MICs of 3.7 and 13.1 μM against extracellular and intracellular *L. monocytogenes*, respectively. The authors tailored the CPP and demonstrated that charge and hydrophobicity played a role in mammalian cellular uptake and intracellular location, thereby promoting an efficacious clearance of intracellular *Listeria*. For example, one conjugate displayed an intracellular MIC around 5 μM (~7X the extracellular MIC) in HeLa cells. Another fascinating pro-drug delivery strategy explored by the Kelley group used an alternate Mtx-CPP approach to treat intracellular phagocytic mycobacterial infections (Pereira et al., 2015). Their three-component drug design included the cyclohexyl- and guanidinium-rich CPP conjugated to Mtx with a β-lactamase-cleavable linkage to a negatively charged shielding peptide (Figure 4a). Once inside the cell, and in the vicinity of intracellular mycobacteria that secrete β-lactamases, they showed that the cephalosporin linkage between the shielding peptide and Mtx-CPP was cleaved. This Mtx-CPP successfully accumulated in mammalian cells and cleared over 50% of intracellular *Mycobacteria* at a concentration about 2 times the MIC in RAW264.7 cells. More recently, the Kelley group facilely conjugated the same cyclohexyl- and guanidinium-rich CPP to nalidixic acid, a quinoline-based Gram-negative antibiotic (Ahmed and Kelley 2017). In doing so, their conjugates exhibited bacterial uptake in *S. aureus* and over a 10-fold increase in antibacterial activity compared with nalidixic acid against sensitive and drug-resistant *S. aureus*. The authors determined that conjugation to the CPP allowed nalidixic acid to become effective against Gram-positive *S. aureus* through a mode of action involving DNA gyrase.

Fosmidomycin, an antibiotic used to treat Gram-negative bacterial and parasitic infections, was conjugated to the CPP octa-arginine through an amide linkage (Figure 4b) (Sparr et al., 2013). This conjugate and non-covalent mixture (1:4 R8:fosmidomycin) displayed activity against *Mycobacterium*, for which fosmidomycin is ineffective. A 50% reduction in growth rate of *M. bovis* was observed with the mixture and the covalent conjugate at 5 μM demonstrating that the conjugation is not essential for activity. This mixture was also effective against parasitic *Plasmodium* and *Toxoplasma*. For example, the pretreatment of *T. gondii*

with 1 μM of the fosmidomycin-R8 mixture resulted in over a 75% decrease in the number of tachyzoite plaques. However, the covalent conjugate was not tested in the parasitic growth experiments. Another antiparasitic drug, miltefosine (MT), was conjugated to the CPP Tat via a cleavable disulfide or non-cleavable thioether linkage (de la Torre et al., 2014; Luque-Ortega et al., 2012). Both conjugates visually accumulated within parasites and BALB/c peritoneal macrophages, and the disulfide MT-Tat conjugate reduced the growth of intracellular amastigote forms of *Leishmania* by 10-fold compared with MT.

Broad-spectrum fluoroquinolone antibiotics have been investigated for increased bactericidal activity by conjugating with a few different CPPs. For instance, Ghaffar and coworkers investigated amide-linked and ester-linked levofloxacin–Tat conjugates (Ghaffar et al., 2015). Unfortunately, neither of their conjugates, or the non-conjugated mixture of levofloxacin and Tat, cleared bacterial infections more efficiently than the antibiotic alone. Although this Tat conjugate did not show improved activity, the combination of levofloxacin and alternate CPPs has shown promise. Levofloxacin and a piperazine-truncated analog, levofloxacin Q, were conjugated to a lysine residue on the cyclic CPP R4W4 and its linear equivalent through the carboxylic acid group of the antibiotics (Figure 4c) (Riahifard et al., 2017). While levofloxacin Q alone was ineffective, it was shown that the [R4W4]–levofloxacin Q conjugate and the non-conjugated mixture of levofloxacin Q and CPP had increased antibacterial activity against MRSA (MIC 8 μM) and against *Klebsiella* (MIC 32 μM). However, the levofloxacin–[R4W4] conjugate did not show an improvement in antibacterial activity compared with levofloxacin alone. Additionally, the conjugates with the linear equivalent of the CPP were less potent than those with cCPP. Another levofloxacin–CPP conjugate was designed with an acid-cleavable thioester linkage (Figure 4d) (Taheri-Ledari and Maleki 2020). The CPP sequence GAFPHR was selected for its bacterial membrane interaction and cell-penetrating activity (Zhang et al., 2020). The N-terminus of the CPP was modified with a cysteine for conjugation. Upon exposure to acidic conditions (pH 4.5), the authors demonstrated cleavage of the conjugate and the release of both components. In regard to bactericidal activity, a slight improvement in antibacterial activity compared with levofloxacin was seen for the conjugate against *S. aureus* and *E. coli*, with a modest 80% reduction in bacterial growth at 20 μg/ml with both strains. At the same concentration, the CPP alone was ineffective, and levofloxacin alone reduced bacterial growth by 60%.

Antimicrobial photodynamic therapy (PDT) has also shown promise in treating infections by generating singlet oxygen and radical species to kill bacteria. Bourré and coauthors developed a Tat–porphyrin conjugate using a cysteine-modified Tat peptide and porphyrin with a maleimide handle (Bourré et al., 2010). This Tat–porphyrin conjugate was effective in killing both Gram-negative and Gram-positive bacteria, including activity against *E. coli*, *S. aureus*, and *S. pyogenes*, which is encouraging since PDT is typically less effective in Gram-negative bacteria. Reductions in viable bacterial counts ranged between 5 and 7 log units for all strains with 120 s of light exposure. It was concluded that the activity of this Tat conjugate was the result of the combined effects of membrane destabilization from Tat and induction of toxic reactive oxygen species from the porphyrin photosensitizer.

3.4 | AMP-CPP conjugates

Antimicrobial peptides and cell-penetrating peptides can be combined to afford agents with the shared advantages of each peptide. For instance, Wang and coworkers constructed a cell-penetrating peptide and antimicrobial peptide (CPP-AMP) conjugate (Li, Wang, et al., 2018). They used N2, a cyclic AMP with excellent antimicrobial activity against Gram-negative infections such as *E. coli* and S. *typhimurium*, and directly conjugated it via an amide linkage to the C-terminus of the CPPs Tat or a CPP fragment of the protein lactoferricin ($bLFcin_6$) (Figure 5a). Intracellular uptake was visualized for the FITC-labeled analogs, and both conjugates were localized primarily in endosomes of RAW264.7 macrophage cells, whereas the N2 peptide alone did not display strong fluorescence, demonstrating that the CPP conjugate improved intracellular delivery. Both conjugates also displayed enhanced *in cyto* bactericidal activity against *Salmonella* as compared to N2 alone and 1:1 mixtures. For example, the Tat-N2 conjugate (T11N2) completely eradicated intracellular *Salmonella* at 50 μM (~25X MIC for T11N2), whereas N2 alone and 1:1 mixtures of N2 and CPPs at 50 μM showed about a 90% intracellular bacterial reduction. These authors also designed CPP-AMP conjugates using a cathepsin-cleavable linker between Tat and a cyclic AMP, N6 (Figure 5b) (Li, Teng, et al., 2018). The most potent conjugate, Tat-linker-$N6NH_2$, reduced the bacterial load of intracellular *S. typhimurium* in RAW264.7 macrophage cells by about 3 log units at 50 μM (~4X MIC). The N6 peptide and 1:1 mixture of N6 and Tat at 50 μM showed about a 90% intracellular bacterial reduction. The survival of *S. typhimurium*-infected mice also improved by over 65% as compared to N6.

The AMPs magainin and M15 have been conjugated to a polyarginine CPP, R9, as a continuous peptide sequence (Figure 5c) (Lee et al., 2019). Both CPP-AMP conjugates displayed broad-spectrum antimicrobial activity with modest increases of twofold to fourfold against Gram-positive bacteria, and a fourfold to 16-fold increase against Gram-negative bacteria as compared to the AMPs alone. The conjugates

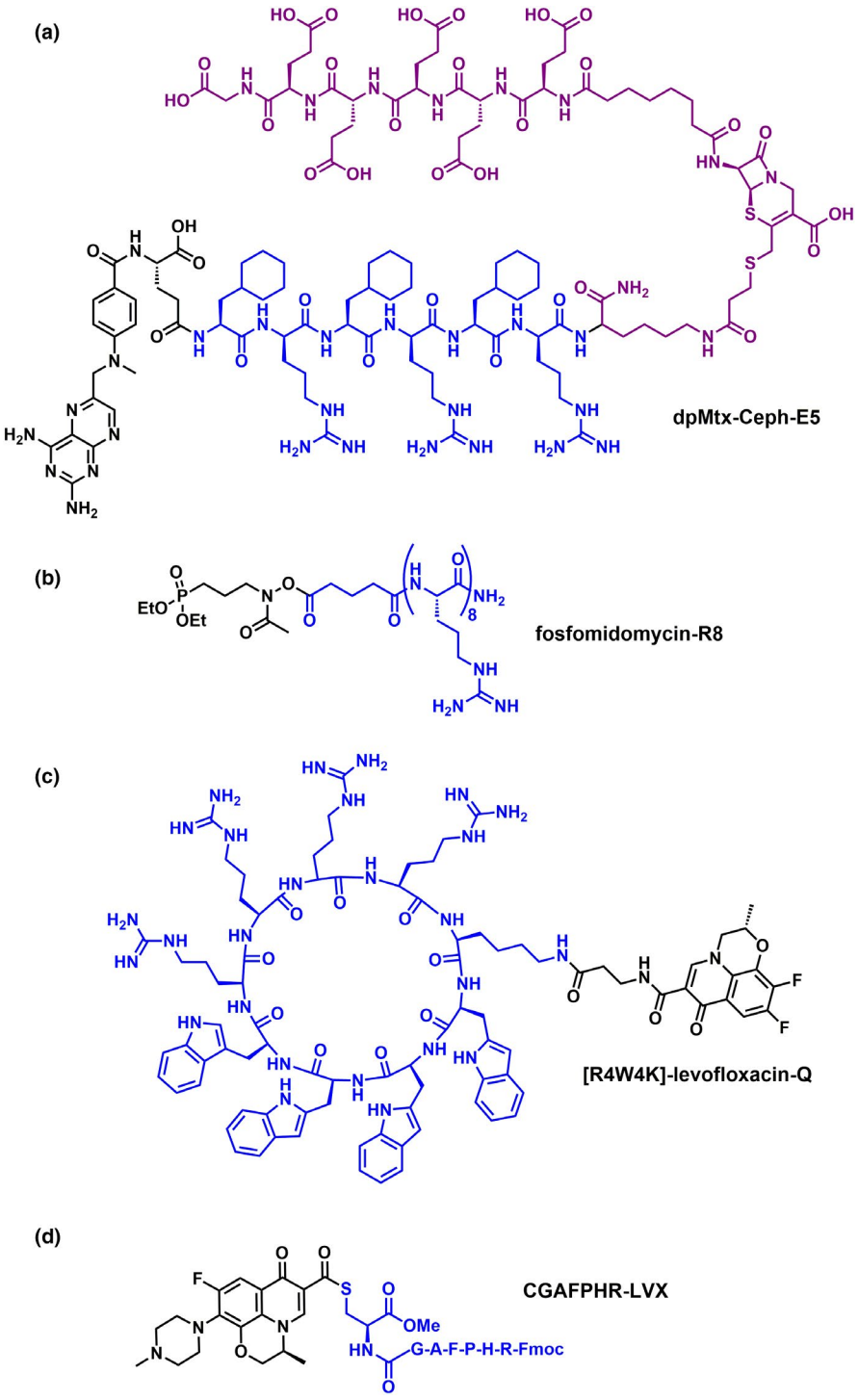

FIGURE 4 (a) Design of dpMtx-Ceph-E_5 conjugate, (b) structure of fosmidomycin-R8, (c) structure of [R4W4K]-levofloxacin Q, and (d) structure of CGAFPHR-LVX. Color scheme: antibiotic, black; CPP, blue; shielding peptide and linker, purple [Colour figure can be viewed at wileyonlinelibrary.com]

displayed similar membrane permeability and disruption compared with the AMPs, but the increase in activity was determined to be the result of an increase in bacterial internalization in *E. coli* and DNA affinity. Thus, these data suggest that there may be additional antimicrobial functions obtained by internal targeting of the AMP-CPP conjugates, in addition to AMP membrane disruption, as modes of action. Although these conjugates showed good antibacterial activity, no *in cyto* data were provided to explore intracellular penetration or clearance.

In an alternate approach, a non-antimicrobial peptide pheromone (DILIIVGG) of *Streptococcus agalactiae* and a CPP (KERKKRRR) were conjugated and investigated for antibacterial activity (Li et al., 2020). Multiple conjugates with truncated and rearranged amino acid sequences displayed good broad-spectrum antibacterial activity against *S. aureus*, *S. epidermidis*, *S. agalactiae*, *E. coli*, *P. aeruginosa,* and *S. typhimurium* with many MICs between 2 and 8 μM, whereas the 1:1 mixture did not show activity against any strains tested. The mechanism of action was determined to be disruption

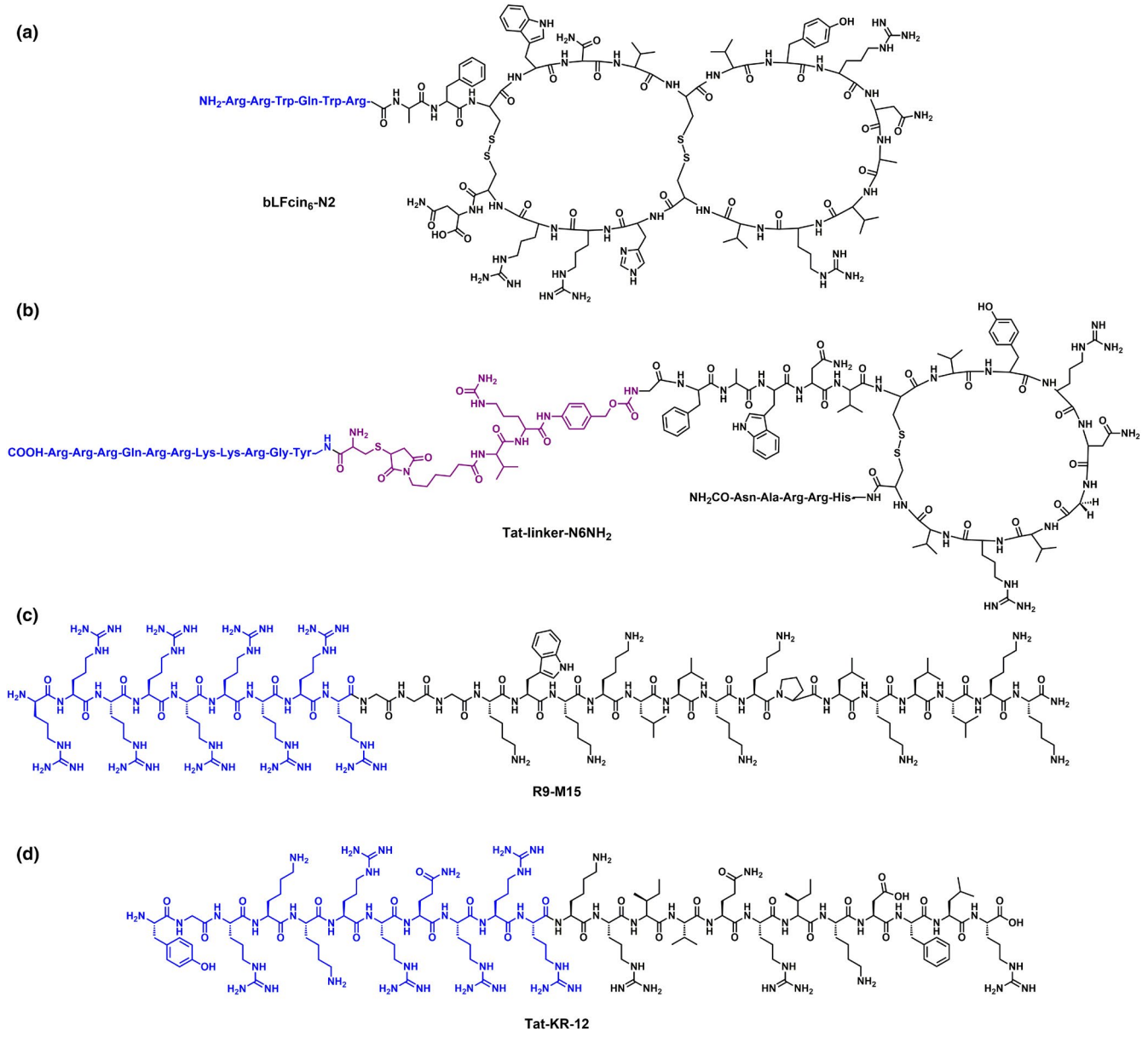

FIGURE 5 (a) Structure of bLFcin$_6$-N2 conjugate (B6N2), (b) structure of Tat-linker-N6NH$_2$, (c) structure of R9-M15, and (d) structure of Tat-KR-12. Color scheme: antibiotic, black; CPP, blue; cleavable linker, purple [Colour figure can be viewed at wileyonlinelibrary.com]

of the bacterial membrane, similar to many AMPs. Several of the peptide conjugates reduced the *S. agalactiae* load in infected RAW264.7 macrophage cells by over 95%, and two conjugates reduced the bacterial load in a mouse mammary gland *in vivo* infection model by about fivefold compared with the negative control.

Tat was also investigated as a means to deliver the KR-12 AMP to intracellular locations and kill *S. aureus* (Huo et al., 2020). KR-12 is the smallest sequence of the LL-37 AMP that maintains the antimicrobial properties of the parent AMP. The Tat-KR-12 conjugate was again synthesized as a continuous peptide (Figure 5d). This conjugate was up to 32 times more effective in killing *Staphylococcus* species than KR-12 alone. Furthermore, the conjugate penetrated RAW264.7 cells to almost the same extent as Tat with minimal cytotoxicity. At 10 times the MIC, the Tat-KR-12 conjugate reduced intracellular *S. aureus* in RAW264.7 cells by over 90% as compared to the negative control, where vancomycin was ineffective. Lastly, in a mouse *in vivo* model, this conjugate effectively lowered the bacterial CFUs by about 99% and 75% as compared to the negative control in a planktonic-infection mouse model and intracellular-infection mouse model, respectively.

Peptide nucleic acids (PNAs) contain a peptide backbone with pendent nucleic acid bases. Although PNAs do not have the charges of oligonucleotides, these peptides are limited in their efficiency of crossing cell membranes. CPPs

have potential to offer a delivery method for antimicrobial PNAs in order to reach their intracellular targets within mammalian and/or bacterial cells. The Seleem group has successfully used this strategy to silence essential genes within bacteria (Abushahba et al., 2016). For instance, they used a PNA that targets the *rpoA* gene that encodes a subunit of RNA polymerase that is essential for *L. monocytogenes* viability. The PNA was conjugated to a selection of CPPs (Tat, penetratin, (RXR)4XB, and (RFR)4XB) through a linkage from the CPP C-terminus to the PNA N-terminus. The authors demonstrated that a *L. monocytogenes* infection could be cleared in an *in vitro* culture within 20 min at 8 μM and in infected J774A.1 macrophage cells after 4 hr at 8 μM. These PNAs significantly reduced *L. monocytogenes* in a *C. elegans* bacterial infection model, and the most potent CPP-PNA, PRXR, eradicated bacteria at 32 μM (8X MIC). In a similar approach, a phosphorodiamidate morpholino oligonucleotide (PMO) was amide coupled to a CPP derived from the human protein Foxp3, YARVRRRGPRGYARVRRRGPRRC, using an aminopropanoic acid linkage from the C-terminus of the CPP. This conjugate was designed to target a highly conserved *Gyrase A* gene of *E. coli* and change gene expression to ultimately kill bacteria (Wesolowski et al., 2011, 2013). This conjugate was effective in accumulating within bacteria as observed by microscopy with a fluorescein-labeled analog. This conjugate also was successful in inactivating a broad spectrum of bacteria with conserved *gyrA* gene sequences with MIC values against *E. coli* and *S. aureus* of 0.4 and 6 μM, respectively.

4 | CONCLUSIONS AND PERSPECTIVE

Nearly a century has passed since the discovery of the first antibiotic. In this "golden era," optimism for treating infectious diseases reached its peak and quickly faded as antibiotic resistance overshadowed the practicality of permanently curing transmissible diseases. The modification of existing antibiotics through conjugation with cell-penetrating peptides shows potential in reviving treatments rendered ineffective by drug resistance mechanisms and bacterial evading treatments within mammalian cells. In this review, we highlighted many examples of antibiotic–CPP conjugates with improved antibacterial activity compared with the unaccompanied antibiotic. Many conjugates displayed activity against a range of drug-resistant bacteria and bacteria within mammalian cells. Traditional antibiotics such as aminoglycosides and glycopeptides were demonstrated to reach intracellular locations with little to no toxicity to mammalian cells. The CPPs not only play a role in accessing previously inaccessible locations, but also may interact with bacterial membranes in a synergistic fashion to improve the bactericidal action. Additional optimization and translation to clinical trials for these conjugates is highly anticipated for the future. The outlook is bright for antibiotic–CPP conjugates as these therapies provide a powerful means to address challenging bacterial infections.

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