Bioorg. Med. Chem. Lett. 87 (2023) 129282

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Siderophores: Chemical tools for precise antibiotic delivery☆

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ARTICLE INFO

Keywords:
Iron scavenger
Precise delivery
Antibiotics
Siderophore-antibiotic conjugate
Iron transport-mediated delivery

ABSTRACT

The success of precision medicine coupled with the disappointing impact of broad-spectrum antibiotic use on microbiome stability and bacterial resistance, has triggered a shift in antibiotic design strategies toward precision antibiotics. This also includes the implementation of novel vectorization approaches directed to improve the internalization of antibacterial agents into deadly gram-negative pathogens through precise and well-defined mechanisms. The conjugation of antibiotics to siderophores (iron scavengers), which are compounds that are able to afford stable iron-complexes that facilitate the internalization into the cell by using bacterial iron uptake pathways as gateways, is a strategy that has begun to show excellent results with the commercialization of the first antibiotic based on this principle, cefiderocol. This digests review provides an overview of the molecular basis for this antibiotic-siderophore conjugation approach, along with recent successful examples and highlights future challenges facing this booming research area.

Antibiotics are essential weapons to fight against bacterial infections. However, their ability to cure these diseases is now at serious risk due to the consequences of broad-spectrum antibiotic use on microbiome stability and pathogen resistance.[1-3] The disappointing returns from this approach have triggered a big shift in the antibiotic discovery perspective in recent years, to turn the attention on precision antibiotics, thus compounds with focused and well-defined activities, as well as precise delivery to the bacterial target, to address infections without damaging microbiomes or incentivizing resistance.[4] Of particular concern are infections caused by life-threatening pathogens highlighted by the World Health Organization (WHO), specifically the gram-negative bacteria *Acinetobacter baumannii, Pseudomonas aeruginosa* and Enterobacteriaceae. The increasing impact of these deadly pathogens in healthcare systems is worrisome, since the compromised immune system of these patients facilitates their pathogenicity.

One of the bottlenecks in the development of novel antibiotics is the efficient internalization of the drug in the bacteria, especially for the treatment of infections caused by gram-negative pathogens.[5,6] The latter is mainly due to: (i) the structural complexity of the bacterial cell wall composed by moieties of different polarities, charges and compositions that the drug must cross; and (ii) the difficulties in combining negatively charged functional groups often required by the therapeutic target to achieve optimal recognition (reversible) and/or transformation (irreversible/covalent) with the features of the cell wall into a single chemical entity. A good example of the latter are those inhibitors that require a free carboxylate group in the scaffold for recognition and anchoring to the target, whose unfavorable PK/PD profiles hinders, or even prevents, its efficient internalization. Hence, in recent years, the development of precise transport methods for efficient antibiotic delivery has become a growing research area for antibiotic drug development. Among them, stand out: (i) the use of antibiotic proforms that temporarily mask its disappointing profiles to enter inside the cell, where are biotransformed to the active free form and accumulated;[7-10] and (ii) the introduction of amino substituents, which are positive charged at physiological pH, in certain positions of the antibiotic core not directly involved in the recognition that facilitate permeabilization.[6,11] An alternative approach that have recently begun to bear fruit with the approval of the first antibiotic based on this principle, cefiderocol,[12] is the introduction into the scaffold of functional groups with high affinity and specificity for Fe(III), also known as *siderophores* (iron chelators produced by bacteria), to ensure effective and precise assimilation into the microorganisms (Figure 1). These compounds are capable to form stable iron-complexes facilitating its internalization into the cell by using bacterial iron uptake pathways as gateways.[13-15] Vectorization of antibacterial agents by siderophores is a promising strategy able to increase the efficacy of drugs, especially for gram-negative

☆ Dedicated to the memory of Prof. Javier Benavente.

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https://doi.org/10.1016/j.bmcl.2023.129282
Received 3 March 2023; Received in revised form 2 April 2023; Accepted 6 April 2023
Available online 7 April 2023

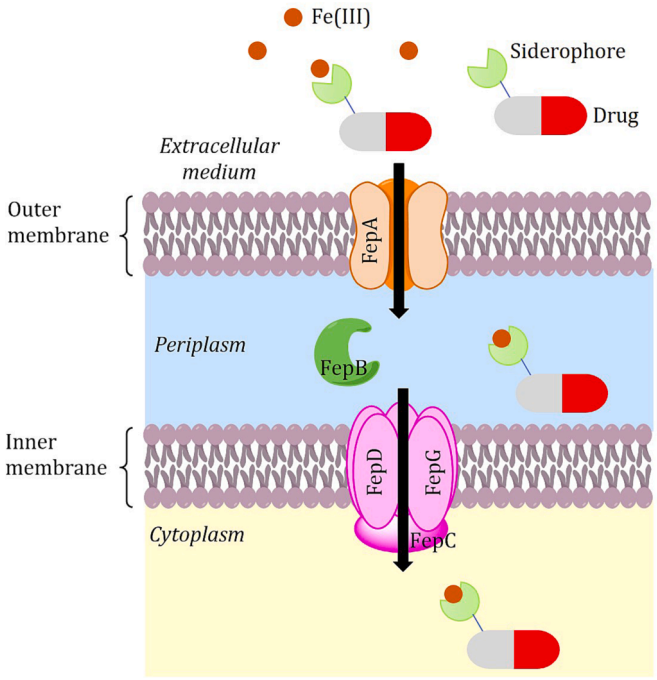

Figure 1. Schematic representation of the antibiotic-siderophore conjugate transport for precise delivery into the cell.

pathogens. This approach has proven to be particularly useful for precise delivery of β-lactam antibiotics, which represent about 70% of antibiotics prescribed today. In this digest review, an overview of the molecular basis of this approach and recent successful examples are discussed.

As in many other occasions, the inspiration behind the vectorization of antibacterial agents through antibiotic-siderophore conjugates comes from nature, specifically how bacteria obtain iron from environmental stocks of the host.[16] Iron is an essential nutrient for bacteria and host, since both use this element as a cofactor for a large variety of enzymes involved in metabolic pathways or with a pivotal role in basic cellular processes.[17,18] This transition metal can exist in two oxidation states, Fe (III) and Fe(II). At physiological pH, the ferric form, Fe(III), is barely soluble in water limiting its concentration as free form to $\approx 10^{-12}$ μM.[14] As Fe(III) ion can be biotoxic for living organisms because it can promote the generation of reactive oxygen species, particularly hydroxyl radicals (●OH) via the Fenton and Haber-Weiss reactions, its concentration in living systems is mostly kept $\approx 10^{6}$-fold lower than solubility levels.[19,20] Only Fe(II) ion is soluble in water at physiological conditions, which is usually found in concentrations between 0.1 and 1 10^{-3} μM in the host. The host store iron coordinated to proteins, such as transferrin and lactoferrin (body fluids) or hemoglobin and ferritin (intracellular).[21,22] This low concentration of iron constitutes one of the host's first defense barriers against bacterial infections, because the level required by these microorganisms is approximately 10^{3} times higher (0.1–1 μM). To meet the needs for this nutrient, bacteria have developed efficient mechanisms to uptake insoluble Fe(III) from environmental stocks of the host, involving two main steps: chelation and precise delivery inside the cell (Figure 1).

The process starts with the secretion of small organic compounds, siderophores, that possess a high affinity and specificity for Fe(III) to afford stable and water soluble Fe(III)-complexes.[23-26] The resulting Fe (III)-siderophore complexes are then taken by ferric-chelate-specific transporters into the cell, where ferric reductase enzymes catalyze the reduction of Fe(III) into Fe(II), triggering the release of Fe(II) ion, which is the soluble and the accessible form for the microorganism.[19,27,28] The siderophore can either be degraded or released in the free form outside the cell. The biosynthesis of this secondary metabolites is regulated by the iron levels of the environment where the organism is located.

The discovery in the 1960s of sideromycins, which are naturally occurring antibiotics composed by an iron scavenger covalently bound to an antibiotic moiety actively transported into gram-positive and gram-negative bacteria by its iron uptake pathways, triggered extensive research efforts to develop synthetic sideromycins analogues for precise antibiotic delivery.[29-37] Good examples of sideromycins are albomycins that are thioribosyl pyrimidine antibiotics targeting transfer ribonucleic acid synthetase (Figure 2A). These natural compounds, which were isolated from Streptomyces spp., are covalently bound to an iron chelator via a hydrolyzable serine moiety.[38-40] Albomycins have excellent antibiotic activity against Enterobacteriaceae except Proteus and related Morganella strains, as well as against relevant gram-positive pathogens such as *S. aureus*, and *S. pneumoniae*. Salmycin is another remarkable natural compound that conjugates an aminoglycoside antibiotic with a chelating agent through a labile ester bond (Figure 2A). As albomycins, salmycins also kill relevant gram-positive pathogens, but are mainly inactive against gram-negative bacteria.

Even though all siderophore moieties perform the same function and that its affinity and specificity for Fe(III) is huge for all cases, most pathogenic bacteria secretes its specific iron scavenger and have particular transport mechanisms to actively achieve this essential nutrient from the environment.[41] The latter suggests that this bacterial machinery can be exploited for the precise internalization of other compounds, such as synthetic antibiotics, inside the cell. Despite siderophores are structurally very diverse, complex and large organic compounds,[42] only a few types of functional groups are naturally employed for the specific coordination of Fe(III) in a hexadentate fashion, particularly catechols **1**, *o*-hydroxyphenyl oxazolines **2a**, *o*-hydroxyphenyl thioazolines 2**b**, hydroxamic acids **3**, and α-hydroxycarboxylic acids **4** as containing in the natural siderophores enterobactins, tetroazolemycins, ferrimycine A and staphyloferrin A (Figure 2B).[14,43,44] It is therefore not surprising that the latter functional groups are the most common in the synthetic antibiotic-siderophore conjugates developed to date.

Antibiotic-siderophore conjugates for periplasmic space delivery: To date, the most successful antibiotic-siderophore conjugates have proven to be those in which the antibacterial agent is delivered in the periplasmic space via TonB-dependent outer membrane transporters, such as Fiu (ferric iron uptake), CirA (iron-catecholate), and/or FepA (ferric enterobactin) homologues. These conjugates involve β-lactam antibiotics (cephalosporins, monobactams), as well as non-β-lactam antibiotics, that target penicillin-binding proteins (PBPs). Aztreonam, the first monobactam antibiotic in clinical use (approved by FDA in 1986) to treat infections caused by gram-negative bacteria such as *P. aeruginosa*, was the focus of diverse siderophore conjugate studies to improve its internalization and expand its spectrum of activity against multidrug-resistant gram-negative pathogens.[45-47] Several aztreonam-siderophore conjugates in which the chelating moiety was introduced in distinct positions on the aztreonam scaffold have been reported (Figure 3A). Research efforts were mainly focused on the introduction of a 1,3-dihydroxypyridin-4-one group, which is a catechol bioisostere.

BAL30072, developed by Basilea Pharmaceutica Ltd., is probably the most remarkable example because it reached phase I clinical studies to treat complicated urinary tract infection.[49,50] In this case, the 1,3-dihydroxypyridin-4-one group was introduced in the side chain by replacement of its carboxylate moiety. This conjugate displayed good activity against multidrug-resistant Gram-negative bacteria including Pseudomonas spp. and Klebsiella spp. and improved *in vitro* activity against *A. baumannii*, a pathogen that can cause severe pneumonia, urinary tract infections, and bacteremia.[51,52] The crystal structure of PBP3 inhibited by BAL30072 reveals that the antibiotic causes the covalent modification of the catalytic serine residue S294 to afford an ester adduct and the 1,3-dihydroxypyridin-4-one moiety interacts by hydrogen-bonding with the phenol group of residues Y532 and Y503 (PDB ID 4OOM, 2.0 Å)

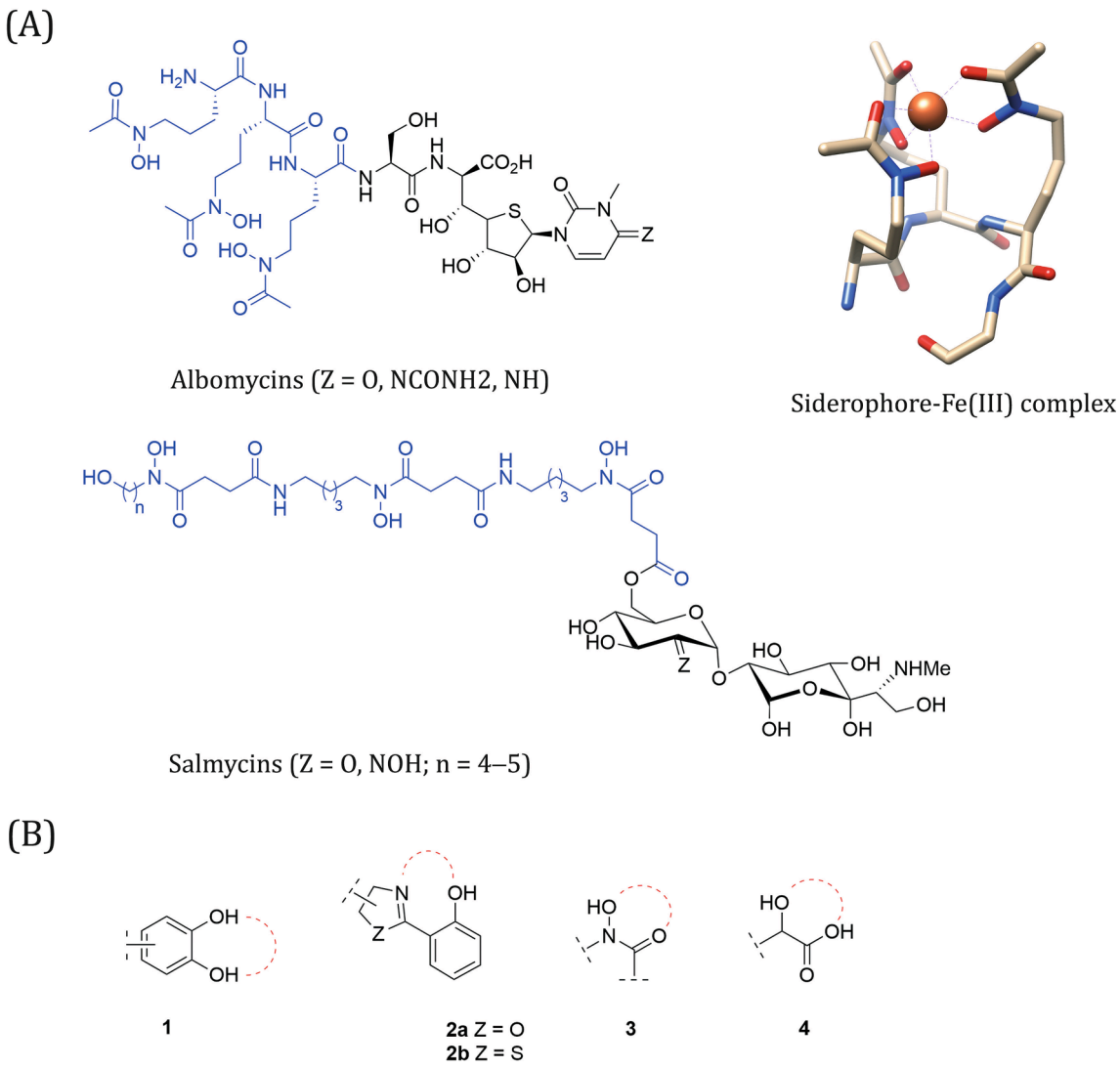

Figure 2. (A) Examples of naturally occurring siderophore-antibiotic conjugates. The siderophore moieties of these conjugates are highlighted in blue. The three-dimensional structure of the siderophore-Fe(III) complex, as observed in the crystal structure of FhuD in complexed with albomycin-δ2 (Z = $NCONH_2$; PDB ID 1K7S),[48] is also provided. (B) Main functional groups used by siderophores for Fe(III) chelation. The iron coordination positions are highlighted with a red dashed line.

(Figure 3B).[53] Unfortunately, BAL30072 is no longer under study because it was found to have low activity in urine probably due to the low iron content concentration in this fluid compared with in broth, and showed to cause increased transaminase activities in healthy subjects in multiple-dose clinical studies.[54-56]

SMC-3176 is an aztreonam-siderophore conjugate in which the iron scavenger moiety is introduced in the β-lactam core (Figure 3A).[50] Despite showing high *in vitro* potency against multidrug-resistant pathogens, no clinical studies were performed due its limit efficacy against *P. aeruginosa* due to rapid adaptive resistance preventing the entry via the siderophore-mediated iron-uptake systems. Although further modifications on the side chain of this scaffold have been described, this class of antibiotics showed to have low hydrolytic stability and some off-target effects upon interaction with human plasma protein binding (PPB).[46,53]

In November 2019, the first antibiotic-siderophore conjugated, namely cefiderocol (formerly S-649266), developed by Shionogi & Co. Ltd., was approved by the FDA for the intravenous treatment of severe gram-negative bacterial infections (Figure 3A).[57,58] In September 2020, this antibiotic also received approval for treatment of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia caused by susceptible gram-negative bacteria. In contrast to BAL30072 and SMC-3176, the Shionogi's efforts were focused on the development of a catechol-based antibiotic conjugate. The chemical structure of cefiderocol merges two key features of previously developed cephalosporines in a single chemical entity: (i) the (1-carboxy-1-methylethoxy)imino group of ceftazidime (third-generation cephalosporin) to enhance stability against hydrolysis by β-lactamase enzymes, which hydrolyze the β-lactam ring to afford inactive products and are the most prevalent cause of antibiotic resistance in Gram-negative bacteria;[59] and (ii) the 1-methylpyrrolidinium-1-yl group in cefepime (fourth-generation cephalosporin) to improve antibacterial activity and stability against β-lactamases.[60] After exploring different positions on the cephalosporine scaffold, the best results were obtained by incorporation of the catechol moiety into the terminal pyrrolidinium moiety allowing the efficient delivery of the compound into the periplasmic space.

Cefiderocol is considered one of the most promising new options against most challenging gram-negative pathogens to date given its proven stability against bacteria producing: (i) extended spectrum β-lactamases (ESBL); (ii) chromosomal class C β-lactamase (AmpC), which is produced on demand by *P. aeruginosa,* thus making the bacterium intrinsically resistant to β-lactam antibiotics; and (iii)

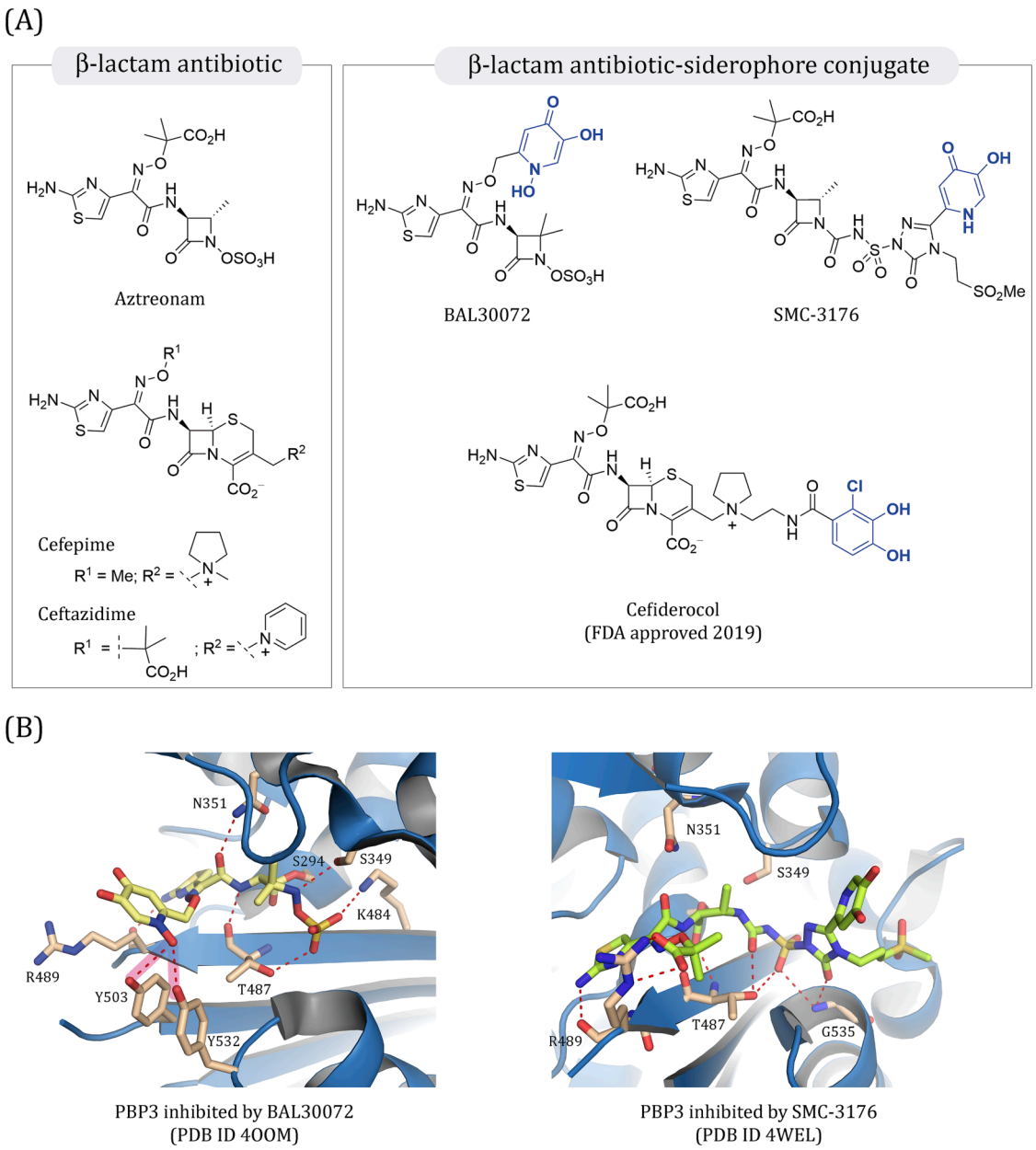

Figure 3. (A) Relevant reported examples of β-lactam-siderophore conjugates for precise delivery to the periplasmic space. The siderophore group is highlighted in blue. The parent β-lactam antibiotic is also shown. (B) Crystal structure of the catalytically active site of PBP3 inhibited by BAL30072 (PDB ID 4OOM,[61] 2.0 Å) and SMC-3176 (PDB ID 4WEL,[53] 1.99 Å).

carbapenemases, including the enzymes OXA-48 (serine-β-lactamase) and NDM-1 (metallo-β-lactamase).[60,62] Besides cefiderocol have demonstrated high *in vitro* activity against Enterobacterales (including carbapenemase producing isolates), showing MIC_{50} values of 0.12–0.25 mg/L,[63] emergence of resistance of reduced susceptibility have already been reported *in vivo* (Table S1). Unfortunately, the specific mechanisms responsible for these findings are still unclear and remain the subject of numerous studies.[64,65]

To our knowledge, compounds **6–8** are the first examples of vectorization of β-lactamase inhibitors via iron-uptake pathways (Figure 4). These compounds are resistance breakers that in conjunction with an antibiotic recover the antibacterial effect of the latter by inhibition of the most widely spread inactivation mechanism in gram-negative bacteria, in particular high-risk β-lactamases such as OXA-type carbapenemases (OXA-23, OXA-24/40, OXA-48). Thus, compound **6** revealed to have an excellent capacity to restore imipenem efficacy *in vitro* (32–128-fold) and a good *in vivo* activity in preclinical models of murine pneumonia.[67-69] The combinations imipenem/**7** and imipenem/**8** also restored almost completely the antibiotic efficacy in OXA-23 and OXA-24/40 carbapenemase-producing *A. baumannii* strains (1 μg mL^{-1}), and also provided good results for OXA-48 carbapenemase-producing *K. pneumoniae* strains (4 μg mL^{-1}) (Table S2). These derivatives have nanomolar activity against carbapenem hydrolyzing class D β-lactamases (OXA-23, OXA-24/40), and also restored the antibiotic efficacy in *E. coli* strains carrying class A (TEM-1 and CTX-M−2) and class C (CMY-2, DHA-1 and AmpC) β-lactamases.[66,69] Studies carried out with **6–8** against a collection of multidrug-resistant clinical isolates and laboratory mutant *P. aeruginosa* strains with different *ampC* gene expression levels, which regulates the production of chromosomal class C β-lactamase known as PDC (for *Pseudomonas*-derived cephalosporinase), revealed that these compounds efficiently restore the ceftazidime (cephalosporine) antibiotic activity.[66] These inhibitors have an unique

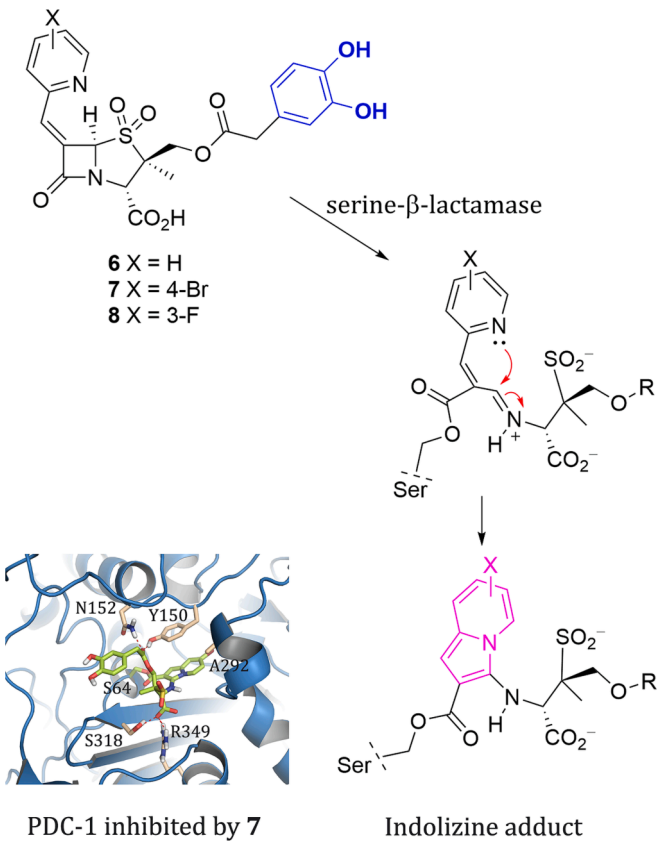

Figure 4. 6-Pyridylmethylidene penicillin-based sulfones-catechol conjugates for precise delivery into the periplasmic space. These compounds are efficient serine-β-lactamase inhibitors that promotes covalent modification of the catalytic serine to afford an indolizine adduct. As an example, a detailed view of the PDC-1 enzyme covalently modified by **7** is provided.[66.]

mechanism of action due to the formation, after nucleophilic attack on the β-lactam core, of an indolizine adduct that is highly resistant to hydrolysis.

Siderophore conjugation studies on non-β-lactam antibiotics have also been described. Starr et al.[61] reported the conjugation of lactivicin, a natural non-β-lactam antibiotic that inhibits the penicillin-binding protein (PBP), with various siderophores mainly introduced at the lactone moiety of the scaffold. The study allowed the identification of the lactivicin derivative **5** with improved activity against relevant gram-negative pathogens such as *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* (Figure 5A).[70] Conjugate **5** also showed low susceptibility against strains producing all four classes of β-lactamases, such as OXA-24/40, NDM-1, VIM-2, CTX-M−15, SHV-15, which would prevent potential resistance mechanism based on enzyme catalyzed hydrolysis of the drug to afford inactive species, which is the most prevalent cause of antibiotic resistance in Gram-negative bacteria.[59] The authors also suggested that this compound **5** may use a broader set of receptors to deliver the antibiotic in the periplasmic space. The mechanism of action of compound **5** involves the nucleophilic attack by the catalytic serine residue that triggers the lactam ring opening to afford an alkyloxyimino adduct serine adduct, as revealed by the X-ray crystal structure of the PBP1a enzyme inhibited by **5** (PDB ID 4OON,[61] 3.2 Å; Figure 5B).

On the other hand, Goldberg et al.[71] improved the intrinsic low activity of pyrazolidinones,[72,73] which are non-β-lactam PBP inhibitors, against Gram-negative pathogens by precise delivery into the periplasmic space (Figure 5A). The reported conjugate, YU253434, showed to have remarkable activity against the top priority pathogens accordingly to the WHO such as *P. aeruginosa* and Enterobacterales. Specifically, YU253434 proved to have MIC values of 1 μg/mL against a large number of clinical isolates of *P. aeruginosa*, *K. pneumoniae* and *E. coli*. Importantly, this conjugate showed to be stable against all four classes of β-lactamases. YU253434 caused the covalent modification of the catalytic serine residue by an addition–elimination reaction on the bicyclic lactam core, as revealed the X-ray crystal structure of the corresponding PBP3 adduct (PDB ID 6VOT, 2.4 Å) (Figure 5B).[71] It is noteworthy that the phthalimide siderophore group points towards the bulk solvent for both PBP1a/**5** and PBP3/YU253434 enzyme adducts, with little contribution to the binding. These structures suggest that the covalent modification of antibiotics with Fe(III) scavengers, in positions distant from the reactive site, targeting biological objectives with an accessible active center/recognition site as in the latter examples, results in no significant alteration of the binding of the parent antibiotic, thereby facilitating the efficiency of the conjugate. This fact might explain the general success achieved with the latter antibiotic-siderophore conjugates targeting PBP enzymes compared with those targeting with less accessible or more restricted arrangements of the active site despite the large molecular size of the conjugates.

Antibiotic-siderophore conjugates for cytosol delivery: Only few efficient examples of antibiotic-siderophore conjugates for cytosol delivery have been described. Diverse attempts to improve the activity of ciprofloxacin, a DNA gyrase inhibitor and broad-spectrum antibiotic widely used for the treatment of infections caused by *E. coli,* against infections caused by susceptible and resistant ciprofloxacin strains of *P. aeruginosa* and *E. coli* have been described.[74] The piperazine group in the scaffold was used to link the siderophore group, as compound **9**, but no relevant beneficial effects were obtained in general (Figure 6).[75-77] Remarkably, Neumann et al.[78] identified that Fes, the cytoplasmic enterobactin hydrolase generally expressed by all *E. coli* strains, is not involved in the hydrolysis of the siderophore to release the ciprofloxacin active form in the *E. coli* cytosol. Instead, this activation is catalyzed by IroD, a cytoplasmic hydrolase only expressed by strains harboring the *iroA* gene cluster. These findings might explain previous results with other *E. coli* strains and open the door to the development of narrow-spectrum antibiotics targeting this type of pathogenic *E. coli*.

Diverse catechol conjugates based on 4,5,6,7-tetrahydrobenzo[d] thiazole-2,6-diamine **10**, which are DNA gyrase B inhibitors, were described (Figure 6).[79] The most potent conjugate, compound **11** had an IC_{50} of 58 nM against *E. coli* DNA gyrase and displayed MIC of 14 μg/mL against *E. coli* ΔtolC strain (Table S3). However, for wild-type *E. coli* strains, only minor improvements in the *in vitro* activities under low-iron conditions were observed.

Kim et al.[80] modified the central methoxy group of trimethoprim, an inhibitor of the dihydrofolate reductase enzyme used for the treatment of infections caused by gram-negative bacteria such as *E. coli*, *K. pneumoniae*, and Enterobacter spp., with diverse catechol groups and 1,3-dihydroxypyridin-4-one moieties that were anchored to the scaffold through diverse linkers (Figure 6). The central methoxy group was selected because this group is located out of the plane of the aromatic ring in the available crystallographic structure of the enzyme in complex with trimethoprim (PDB ID 6XG5,[81] 1.90 Å), and is therefore the most accessible to bulk water. The authors used these analogs to study the transport into the cytosol for the first time. The most relevant compounds in this study, which have a catechol moiety as in compound **12**, proved to be primarily internalized into the outer membrane mainly via the CirA transporter. They are then delivered into the cytosol by exploiting the transporter system on the inner membrane designated for enterobactin. Thus, the conjugates bind first to FepB, and then to the FepCDG permease complex.

The conjugation of linezolid, an oxazolidinone approved by FDA in 2002 for the treatment of multi-resistant gram-positive infections, with catechol siderophore showed to improve the activity against *P. aeruginosa*.[82] Compound **13** revealed to be 10-fold (MIC = 128 μM) more active *in vitro* against this pathogen than linezolid (MIC > 1024 μM) and this effect was increased under low-iron conditions (Figure 6, Table S4). Following a similar approach, the conjugation of daptomycin,

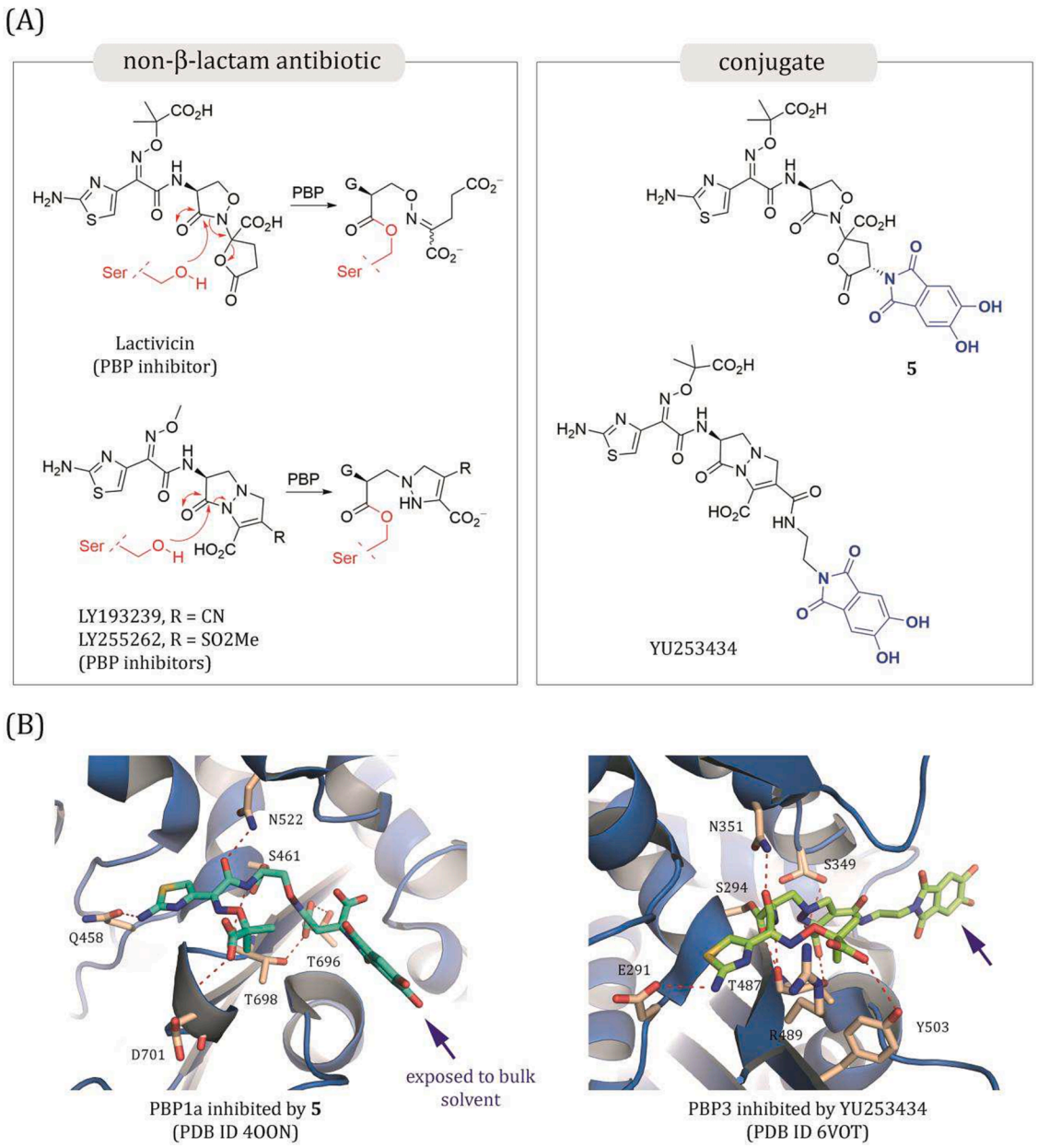

Figure 5. (A) Relevant reported examples of non-β-lactam-siderophore conjugates for precise delivery into periplasmic space. The siderophore group is highlighted in blue. The parent antibiotics and their covalent modification mechanism of PBP3 enzyme is also included. (B) Detailed view of the catalytically active sites of PBP1a and PBP3 inhibited by **5** (PDB ID 4OON,[61] 3.2 Å, B) and YU253434 (PDB ID 6VOT,[71] 2.4 Å, C), respectively, as identified by X-ray crystallography. Note how the siderophore group is exposed to bulk solvent for both enzyme adducts, with no relevant contribution to ligand binding.

a lipopeptide antibiotic only efficient for infections caused by gram-positive bacteria, with a derivative of fimsbactin A, which is a siderophore used by certain high-risk *A. baumannii* strains, provided compounds that are active against virulent strains of *A. baumannii*, including those harboring carbapenemases and cephalosporinases.[83]

Outlook: Taken together, the recent results summarized herein clearly show that the antibiotic-siderophore conjugation strategy has huge therapeutic potential for the treatment of infections caused by gram-negative pathogens, which are the nightmare in healthcare-associated systems and against which more effective therapies are urgently needed. To date, this vectorization approach has only proven to be efficient for the delivery of drugs, mainly β-lactam antibiotics, into the periplasmic space where their therapeutic targets (protein-binding proteins) are located. More efforts need to be devoted for achieving the efficient delivery of non-β-lactam antibiotics into the cytosol. The lack of success might come from the difficulties in crossing distinct transport systems allocated in the outer and inner membrane of the gram-negative bacterium, having distinct recognition patterns, specificity, and overall machinery among them. The recent knowledge achieved on how this internalization occurs in certain multidrug-resistant pathogens and the role that each transport protein seems to play in the delivery of the drug as recently reported, will undoubtedly facilitate progress to achieving this long-pursued yet still unmet need. Among the distinct functional groups explored to date, siderophore moieties based on catechol clearly stand out. From the synthetic point of view, this finding represents a huge advantage since catechol groups can be easily introduced into the skeleton of the parent antibiotic through an ester or amide linkage, without the need of huge synthetic efforts in most cases. Despite the latter, the choice of the anchorage position for the siderophore and the antibiotic, as well as the linker (length and arrangement) is not a trivial

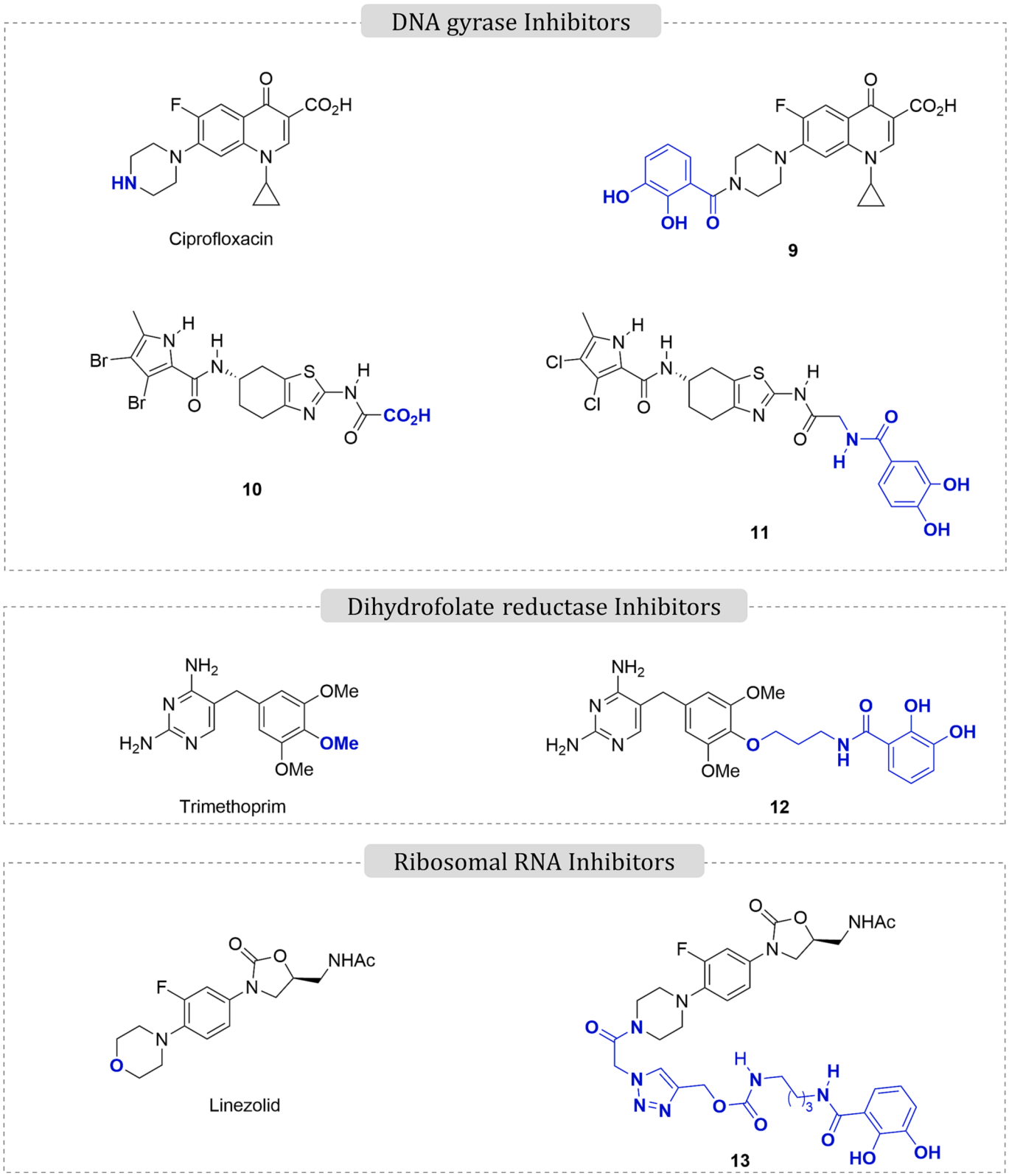

Figure 6. Relevant reported examples of antibiotic-siderophore conjugates for precise delivery into the cytosol. The parent antibiotic and the position in which the siderophore group is introduced via a linker are highlighted in blue.

task, because both factors play a vital role in the overall efficacy of the conjugate, especially for those therapeutic targets with a less accessible binding sites and/or which are exposed to the bulk environment. In any case, the future in this field looks promising.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgments

Financial support from the Spanish State Agency of Research (PID2019-105512RB-I00/AEI/10.13039/501100011033), the Xunta de Galicia [ED431C 2021/29 and Centro Singular de Investigación de Galicia Accreditation 2019-2022 (ED431G 2019/03)], and the European Regional Development Fund (ERDF) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2023.129282.

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