# Discovery of antibiotics and their targets in multidrug-resistant bacteria

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# Chapter 1

# General introduction

## Golden age of antibiotic discovery

The advent of the antibiotic era in the 20th century has been transformative to the global health of humanity. Bacterial infections were a leading cause of death centuries prior,[1–3] and epidemics caused by highly pathogenic bacterial infections could decimate significant parts of human population, the 14th century plague being a key example.[4]

In 1907, arsphenamine was discovered by Paul Ehrlich as treatment for syphilis.[5] This marked the first modern medicine aimed at killing a pathogenic species, without harming the host organism: a 'magic bullet' later defined as antibiotic. In the 1930s, the sulfonamide antibacterials were discovered, and with this the first broad-spectrum antibiotics were made

available to the public.[6–8] In the years following Fleming's serendipitous discovery of penicillin, Nature itself was also found to be a prolific source of antibiotics.[9] It was found that many microorganisms protect themselves against bacterial invaders through secretion of antibiotics. A systematic antibiotic discovery platform was subsequently established, led by Selman Waksman, which involved screening soil samples for antibacterial activity, isolating the causative microorganism, followed by extraction of the antibiotic-of-interest.[10,11] This ushered in the golden age of antibiotic discovery, where between 1940 and 1970, most of the antibiotic classes still used today (Figure 1.1) were discovered.

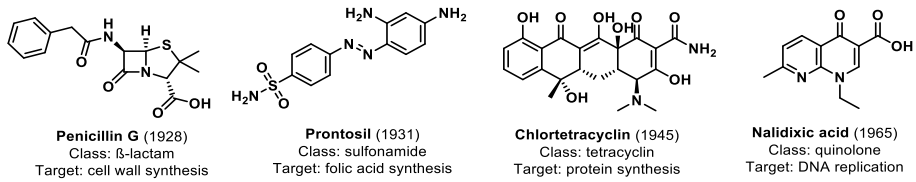



**Figure 1.1 |** Chemical structures of antibiotics from different major classes, along with the discovery year of their antibacterial activity, the class they belong to, and their targeted biological pathway.

This treasure trove of antibiotics was an important driver of the increasing health conditions experienced in the Western world in the course of the 20th century.[12] With the use of antibiotics, primary and secondary infections could be treated,[13,14] and it paved the way for safely conducting surgical procedures.[15] Life expectancy increased drastically from 47 years at the start of the century, to 77 years at the end.[16] It has been widely accepted that the ability to treat infectious diseases has had health and economic benefits all around the world.[3,17]

Unfortunately, the successful, and in some cases unregulated, use of antibiotics has also led to the rise of antimicrobial resistance (AMR).[18,19] For all the commonly administered classes of antibiotics, not long after they appeared on the market, development of resistance was observed. Case in point being penicillin. Following success in the 1940s, penicillin resistance became a widespread problem in society in the 1950s.[20,21] This was reacted to by the development and clinical application of the next-generation beta-lactam antibiotic methicillin, which was introduced in 1960 and could evade mechanisms of penicillin resistance.[22] Two years later, however, bacterial strains with resistance to methicillin were also observed, giving rise to methicillin-resistant *Staphylococcus aureus* (MRSA).[23,24] This cycle repeated itself over the following decades, inspiring the iterative development of multiple generations of beta-lactam antibiotics (penicillins, cephalosporins and carbapenems) which were all accompanied by increasingly resistant bacteria.[25,26]

## Antimicrobial resistance: a 21st century crisis

AMR development is caused by evolutionary pressure.[27] A small infectious population will consist of billions of bacteria. When introducing a potent antibacterial agent, the population will either stagnate or die out. Only bacteria that have acquired an evolutionary advantage, either through genomic mutation or acquisition of a protection gene, will grow in the presence

of the antibiotic. These are then able to thrive and seed a new population, with the advantageous gene being propagated population-wide.

Bacteria can employ a number of mechanisms that result in AMR. These include: 1) direct modification of the bacterial target exploited by the antibiotic via genetic mutation[28]; 2) post-translational target modification[29]; 3) acquisition of an antibiotic inactivating enzyme(s)[30]; 4) active removal of the antibiotic through efflux mechanisms[31]; 5) change in composition of the membranes and cell wall of the bacteria[32]. These mechanisms are further able to be spread through general bacterial growth (vertical transfer), or through genetic jugglery between bacteria (horizontal transfer).

A recent analysis[33] found that 1.27 million deaths could directly be attributed to AMR in 2019, meaning AMR led to more deaths than malaria and HIV. The bacteria responsible for these numbers are largely composed of *Escherichia coli* (17%) and other members of the so-called "ESKAPE" family of pathogens (54%). The ESKAPE family encompasses the bacterial species *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp., and are pathogens that have an increased propensity to escape effects of antibiotic treatment.[34–36] The ESKAPE pathogens make up a large part of infections in hospital settings, which provides a hotbed for resistance development. Furthermore, the World Health Organization (WHO) categorized resistant strains of the ESKAPE pathogens, along with *E. coli*, as high and critical priority pathogens, which pose the greatest threat to human health.[37]

To highlight the urgency of the resistant ESKAPE pathogen problem, a systematic analysis of 40 studies associated resistant ESKAPE infections with a high-risk of mortality and the bulk of pathogen-related health care costs.[38] In case of *E. coli* infections, 58% of clinical isolates were found to be resistant to commonly used antibiotics.[39] Resistance against all major classes of antibiotics has been widely reported over the last few years.[40,41] In addition, and most worryingly, resistance mechanisms have even been found against drugs of last resort[42] such as colistin[43] and tigecycline[44].

If unchecked, the continuous rise in AMR, combined with a lack of proper treatment options, poses a serious threat to the future healthcare. Infections that would normally be trivial to cure could have serious health consequences, or even become fatal. Indirectly, key medical procedures would also be hampered, as surgeries that carry high-risk of infection also require antibiotic prophylaxis.[45] A 2016 report commissioned by the UK government predicts that in 2050 drug-resistant bacterial infections may cause over 10 million deaths worldwide, which would make it the leading cause of death overtaking cancer.[46]

There are two main approaches to address AMR. The first strategy involves reducing the amount of antibiotics being used as there is a clear correlation between usage of antibiotics and rise of AMR.[47] This can be achieved in different ways: more stringent prescription of antibiotics[48], tailored antibiotic regimens based on diagnostic tests, regulation of antibiotic use in agriculture and proper hygienic precautions to reduce spreading of infections. This would involve regulatory measures and cooperation on societal level, both of which go beyond the

scope of this thesis. The second approach involves increasing the supply of treatment options, and this is where the discovery and development of new antibiotics come into play.

## Current supply of antibiotics

In the 40-50 years since the golden age of antibiotic discovery, there have been relatively few original additions to the antibiotic arsenal used in the clinic. This is due in part to the widely held belief that all easily accessible antibiotics, the "low-hanging fruit", have already been discovered: 34 classes of antibiotics in use were discovered before 1980, 4 between 1980 and 1990, compared to only 1 after 1990 (bedaquiline, a diarylquinoline[49]).[50]

Almost all the antibiotics approved in the post-golden age era have been structural variations on existing antibiotics (Figure 1.2).[51] These additions have certainly been useful, as they generally increase the scope of bacteria the class is active against, increase pharmacokinetic and pharmacodynamic properties, and often even overcome existing resistance to the parent antibiotic. Furthermore, from a developmental perspective it also makes sense as there are less risks in terms of toxicity and regulation, and the microbiological and biochemical assay infrastructure are already available.[52,53]

However, in general, compounds from the same class also hit the same targets, and that means target-based cross-resistance can occur. Also, non-target resistance development, based on mechanisms like efflux or antibiotic modifications can occur over time. Furthermore, there is redundancy in the cellular processes targeted by the approved classes.[54] The largest segments of clinically used antibiotics target DNA/RNA replication/transcription and protein and cell wall biosynthesis, with more than half targeting the cell wall.[55]

The threat of AMR is underscored by the near dry pipeline of new antibacterial agents in development. Over the past few decades, the rate of approved antibiotics has drastically fallen.[56–58] There were 29 antibacterial agents approved by the FDA in the 1980s, dropping down to 23 in the 1990s, and lowering even more between 2000 and 2009 (9 new approvals).[59] Since 2010 the situation has slightly improved, with 17 new antibiotics approved,[60] along with the first antibody-based therapy against a bacterial infection.[61]

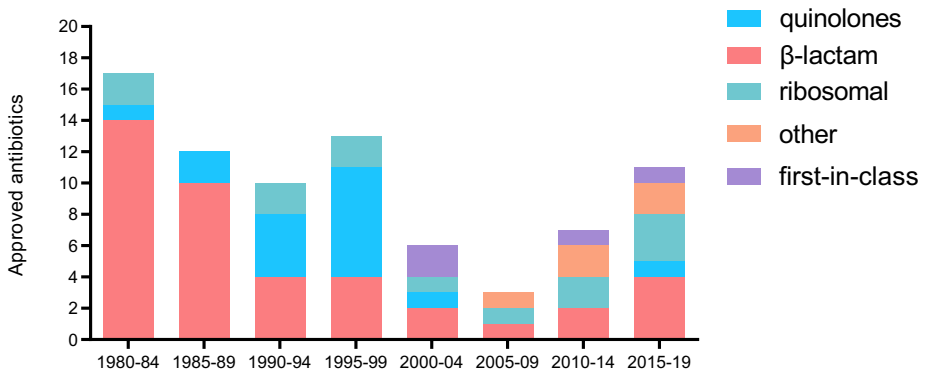



**Figure 1.2 |** Graph showing the number of systemic antibiotics that were FDA-approved from 1980 until 2019. Marked in red are approved drugs belonging to the β-lactam class (carbapenems, penicillins, cephalosporins or monobactams). Blue indicates quinolone/fluoroquinolone antibiotics. Green contains all ribosome-targeting antibiotics (macrolides, tetracyclines, aminoglycosides and others), orange contains remaining antibiotics, and purple marks first-in-class antibiotics. Data was derived from multiple sources.[59,60,62–64] Detailed information about the approved antibiotics is given in Table S1.1.

The lack of innovation in pursuing new antibacterials can be linked to the decreased interest of large pharmaceutical companies in antibiotic research. Most large pharmaceutical companies have stopped their antibiotic programs,[65] and the majority of antibiotic candidates in clinical trials are now championed by smaller enterprises.[66,67] This is for multiple reasons, the first being regulatory. Changes to clinical trial design in the last thirty years have made drug development ever more challenging.[68,69] These challenges and the associated costs mean there is very little financial incentive to develop new antibiotics. Getting new antibiotics through the pipeline costs as much resources as other drugs would, but the reward is far lower.[70,71] In contrary to some other therapeutic areas, antibiotic treatment is temporary as it is only required in case of infection and is generally curative. Also, newly discovered antibiotics would likely be kept as drugs-of-last-resort to avoid resistance development. The biggest reason, however, is that antibiotic discovery remains a major scientific challenge.

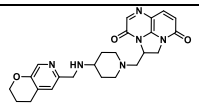
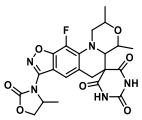
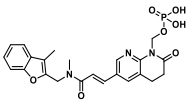
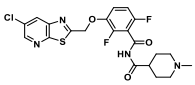
## Challenges in antibiotic drug discovery

The classical approach to drug discovery in the pharmaceutical industry is to identify a disease with unmet need and subsequently hypothesize which molecular target plays an important role in the disease mechanism (target-based approach).[72,73] Based on this target, a high-throughput screen of a large compound libraries is performed to identify hits that are, via a medicinal chemistry approach, developed into lead compounds. Unfortunately, this approach has had a low success rate in discovering new antibiotics, and voices from the pharmaceutical industry have denounced this approach for the foreseeable future.[74,75]

As of 2021, there are only four antibacterial agents in clinical development (for treating WHO priority pathogens) with a unique mode-of-action (MoA),[76] and only one of them resulted

from developing a hit found in a target-based screen (Table 1.1). This not only highlights the inefficiency of target-based screening, but also the bias for existing scaffolds.

**Table 1.1 |** Clinical candidates with a unique mode-of-action for treatment against WHO high-priority pathogens.

| Name | Structure | Target | Hit found | Phase | Sources |
|---|---|---|---|---|---|
| Gepotidacin |  | Type II topoisomerases | Phenotypic screen | 3 | 77 |
| Zoliflodacin |  | Type II topoisomerases | Phenotypic screen | 3 | 78,79 |
| Afabicin |  | FabI | Target-based screen | 2 | 80–82 |
| Txa709 |  | FtsZ | Phenotypic screen | 1 | 83,84 |

There are several challenging aspects of drug discovery that are highly specific to the field of antibiotic discovery. Here, four main challenges will be discussed, along with recent examples of creative solutions.

The aspect that makes phenotypic whole-cell screens preferred to target-based screens is the difficulty of getting the drug into the bacterial cell.[85] Where mammalian cells have only a cell membrane, bacteria have an additional cell wall. Furthermore, Gram-negative bacteria also have an additional outer membrane (OM), with orthogonal permeation requirements to the inner membrane.[86,87] In addition, bacteria utilize efflux pumps that actively transport certain compounds, including many antibiotics, out of the cell.[88] In light of these difficulties, phenotypic screening can offer a more direct approach with a higher chance of success: see what works, and then figure out why it works, rather than the other way around.

Luckily, there have been considerable developments in overcoming these barriers.[89] Membrane permeation agents have been developed that can help poorly permeable antibiotics cross the OM.[90,91] Porin channels can provide an entry route,[92] and porin-permeability optimization of compounds has shown to be a valid strategy.[93] Finally, a way to evade this problem is by targeting essential proteins outside of the bacterium, as shown by the recent success of inhibition of outer membrane protein BamA.[94–96]

The second major challenge is where to find suitable chemical scaffolds. Today it is generally accepted that the Waksman platform approach towards natural product mining has been hollowed out, as exemplified by rediscoveries of existing drug types.[10] Furthermore, when searching for non-natural product leads the chemical matter found in typical synthetic

compound libraries generally does not possess the physicochemical properties necessary to get into the bacterial cell.[97] In response to such challenges, there have been developments in the search for new natural products with unique structure and antibacterial MoAs. For example, recently developed techniques have enabled the growth of microorganisms that were previously unculturable outside of their natural habitats.[98–100] This has led to the discovery of several unique natural product antibiotics.[94,101] Recent academic efforts have also identified antibiotics with unique MoAs from synthetic library screens comprising compounds with more favorable properties.[102,103] It is likely that future synthetic libraries will only get better in targeting bacteria with increased understanding of advantageous (bio)chemical properties.

Minimizing or countering the development of resistance towards new antibacterial drugs is the third challenge. Resistance has been noted against all classes of commonly used antibiotics, which seemingly makes it impossible to elude resistance completely. To reduce the propensity of AMR development, however, several strategies can be undertaken. The first being the targeting of multiple proteins or mechanisms (polypharmacology): the most successful classes of antibiotics hit more than one target (fluoroquinolones, β-lactam-based antibiotics) and recent examples adhere to this as well. [93,102,103] The second strategy is to make drugs that hit nonproteinogenic targets: not targeting proteins prevents single genetic mutations from inducing direct resistance (e.g. classes of ribosome-targeting or cell wall interfering antibiotics).[104] Virulence attenuation also provides an alternative as this involves inhibiting the mechanisms bacteria use to harm the host organism, without providing the evolutionary pressure towards resistance development associate with MoAs that directly kill the bacteria/inhibit their growth.[105,106]

The last challenge is specific to phenotypic screening: namely target/MoA identification of active compounds.[107] Fundamentally, knowing precisely how an antibiotic works is not necessary, as if it works, it works. For instance, it is still not known how salvarsan, the first antibiotic, works[108] and the mechanism of daptomycin, one of the more recently approved antibiotics, is still open to investigation.[109,110] That said, understanding how an antibiotic works can certainly provide valuable information on how to improve certain properties of the drug, or what the weak spots are of the pathogen.

In one common approach to target identification bacteria can be pressurized into resistance, followed by genome analysis to identify mutations responsible for resistance;[111] In addition, macromolecular assays can show if the drug interferes with the synthesis of specific biomolecules while morphology-based microscopy approaches can be used to assess phenotypic changes associated with different MoAs. More recently, advancements in mass spectrometry have enabled a whole range of proteomics-based assays, wherein direct interaction of an antibiotic with its target can be identified. Chemical proteomics assays in particular have helped in solving the MoA where classic methods were unable to.[112]

## Aim and outline

To rise to the challenge of antibiotic drug discovery, the aim of the research described in this thesis is to find unexplored pharmacophores with potential as antibiotics to counter the growing threat of AMR. This is achieved by performing a compound screen for hit finding, improving on the antibacterial profile of the initial hits, and then identifying their MoA. This was done with a focus on two high-priority pathogens, namely MRSA, which is a Gram-positive bacterium, and *E. coli*, a Gram-negative bacterium. Figure 1.3 provides a graphical summary of the content in this thesis.

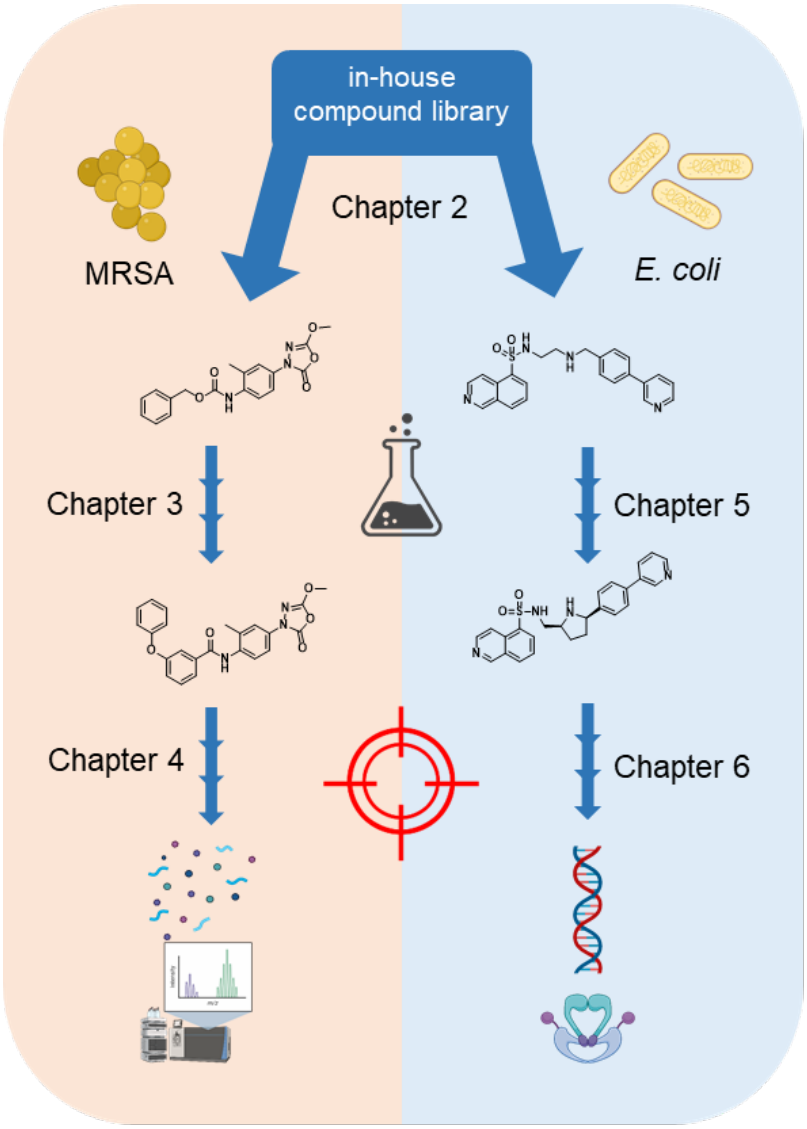



**Figure 1.3 |** Graphical summary of the content in this thesis.

**Chapter 2** starts with a phenotypic antibacterial screen. An in-house compound library was screened for antibacterial potency against MRSA, and against *E. coli*. This resulted in one clear hit against MRSA, and another hit for *E. coli*.

**Chapter 3** focuses on improving the antibacterial activity of the MRSA hit via an antibacterial activity-guided structure-activity relationship (SAR) study. Over 60 compounds were synthesized and evaluated for their antistaphylococcal properties. This resulted in one lead compound, which was further found to contain highly favorable antibacterial effects on highly-resistant clinical isolates of MRSA, as well as low propensity for resistance development.

**Chapter 4** builds on the lead compound developed in the previous chapter by focusing on the reason for antibacterial activity. The covalent binding properties of the molecule were exploited by developing a closely-related activity-based probe. Via a chemical proteomics workflow this probe was then used to identify the proteins that the lead compound binds to, and to zoom in on the most relevant proteins that contribute to its antibacterial activity.

**Chapter 5** focuses on improving the *E. coli* hit. A SAR study with over 60 tested compounds resulted in compound LEI-800 as the lead compound.

In **Chapter 6**, LEI-800 was identified as a DNA gyrase inhibitor through generating LEI-800 resistant *E. coli* mutants, of which changes in the genome were mapped. Subsequent biochemical assays showed that LEI-800 was a highly potent DNA gyrase supercoiling inhibitor. Cryogenic electron microscopy studies then revealed that LEI-800 binds to a unique allosteric pocket of the DNA gyrase complex.

**Chapter 7** summarizes the main discoveries of the previous chapters and puts the work in the context of the field of antibacterial research. At the end, a look will be taken at the possibilities for further research that build up on this work.

# Supplementary information

**Table S1.1 |** List of FDA-approved antibiotics from 1980-2019.

| Generic name | Approved | Class | Subclass | Note |
|---|---|---|---|---|
| bacampicillin | 1980 | β-lactam | penicillin | |
| moxalactam | 1981 | β-lactam | cephalosporin | |
| mezlocillin | 1981 | β-lactam | penicillin | |
| piperacillin | 1981 | β-lactam | penicillin | |
| cefotaxime | 1981 | β-lactam | cephalosporin | |
| cefoperazone | 1982 | β-lactam | cephalosporin | |
| azlocillin | 1982 | β-lactam | penicillin | |
| ceftizoxime | 1983 | β-lactam | cephalosporin | |
| cefuroxime | 1983 | β-lactam | cephalosporin | |
| cefonicid | 1984 | β-lactam | cephalosporin | |
| ceforanide | 1984 | β-lactam | cephalosporin | |
| amdinocillin | 1984 | β-lactam | penicillin | |
| ceftriaxone | 1984 | β-lactam | cephalosporin | |
| amoxicillin/clavulanic acid | 1984 | β-lactam | penicillin/BLI | |
| ceftazidime | 1985 | β-lactam | cephalosporin | |
| imipenem | 1985 | β-lactam | carbapenem | |
| cefotetan | 1985 | β-lactam | cephalosporin | |
| aztreonam | 1986 | β-lactam | monobactam | |
| ampicillin/sulbactam | 1986 | β-lactam | penicillin/BLI | |
| cefmenoxime | 1987 | β-lactam | cephalosporin | |
| cefotiam | 1988 | β-lactam | cephalosporin | |
| cefmetazole | 1989 | β-lactam | cephalosporin | |
| cefpiramide | 1989 | β-lactam | cephalosporin | |
| cefixime | 1989 | β-lactam | cephalosporin | |
| loracarbef | 1991 | β-lactam | cephalosporin | |
| cefprozil | 1991 | β-lactam | cephalosporin | |
| cefpodoxime | 1992 | β-lactam | cephalosporin | |
| piperacillin/tazobactam | 1993 | β-lactam | penicillin/BLI | |
| ceftibutin | 1995 | β-lactam | cephalosporin | |
| cefepime | 1996 | β-lactam | cephalosporin | |
| meropenem | 1996 | β-lactam | carbapenem | |
| cefdinir | 1997 | β-lactam | cephalosporin | |
| ertapenem | 2001 | β-lactam | carbapenem | |
| cefditoren | 2001 | β-lactam | cephalosporin | |
| doripenem | 2007 | β-lactam | carbapenem | |
| ceftaroline | 2010 | β-lactam | cephalosporin | |
| ceftolozane/tazobactam | 2014 | β-lactam | cephalosporin/BLI | |
| ceftazidime/avibactam | 2015 | β-lactam | cephalosporin/BLI | |
| meropenem/vaborbactam | 2017 | β-lactam | carbapenem/BLI | |
| imipenem/cilastatin/relebactam | 2019 | β-lactam | carbapenem/degradation inhibitor/BLI | |
| cefiderocol | 2019 | β-lactam | cephalosporin | siderophore |

**Table S1.1** continued.

| Generic name | Approved | Class | Subclass | Note |
|---|---|---|---|---|
| daptomycin | 2003 | other | lipopeptide | first-in-class |
| telavancin | 2009 | other | lipoglycopeptide | |
| bedaquiline | 2012 | other | diarylquinoline | first-in-class |
| dalbavancin | 2014 | other | lipoglycopeptide | |
| oritavancin | 2014 | other | lipoglycopeptide | |
| pretomanid | 2019 | other | nitroimidazole | |
| cinoxacin | 1980 | quinolone | quinolone | |
| norfloxacin | 1986 | quinolone | fluoroquinolone | |
| ciprofloxacin | 1987 | quinolone | fluoroquinolone | |
| ofloxacin | 1990 | quinolone | fluoroquinolone | |
| enoxacin | 1991 | quinolone | fluoroquinolone | |
| lomefloxacin | 1992 | quinolone | fluoroquinolone | |
| temafloxacin | 1992 | quinolone | fluoroquinolone | |
| sparfloxacin | 1996 | quinolone | fluoroquinolone | |
| levofloxacin | 1996 | quinolone | fluoroquinolone | |
| alatrofloxacin | 1997 | quinolone | fluoroquinolone | |
| trovafloxacin | 1997 | quinolone | fluoroquinolone | |
| grepafloxacin | 1997 | quinolone | fluoroquinolone | |
| moxifloxacin | 1999 | quinolone | fluoroquinolone | |
| gatifloxacin | 1999 | quinolone | fluoroquinolone | |
| gemifloxacin | 2003 | quinolone | fluoroquinolone | |
| delafloxacin | 2017 | quinolone | fluoroquinolone | |
| sisomicin | 1980 | ribosomal | aminoglycoside | |
| netilmicin | 1983 | ribosomal | aminoglycoside | |
| azithromycin | 1991 | ribosomal | macrolide | |
| clarithromycin | 1991 | ribosomal | macrolide | |
| dirithromycin | 1995 | ribosomal | macrolide | |
| quinupristin/dalfopristin | 1999 | ribosomal | streptogramin/streptogramin | |
| linezolid | 2000 | ribosomal | oxazolidinone | first-in-class |
| telithromycin | 2004 | ribosomal | ketolide | |
| tigecycline | 2005 | ribosomal | tetracycline | |
| fidaxomycin | 2011 | ribosomal | macrolide | |
| tedizolid | 2014 | ribosomal | oxazolidinone | |
| plazomicin | 2018 | ribosomal | aminoglycoside | |
| eravacycline | 2018 | ribosomal | tetracycline | |
| omadacycline | 2018 | ribosomal | tetracycline | |
| lefamulin | 2019 | ribosomal | pleuromutilin | first-in-class |

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