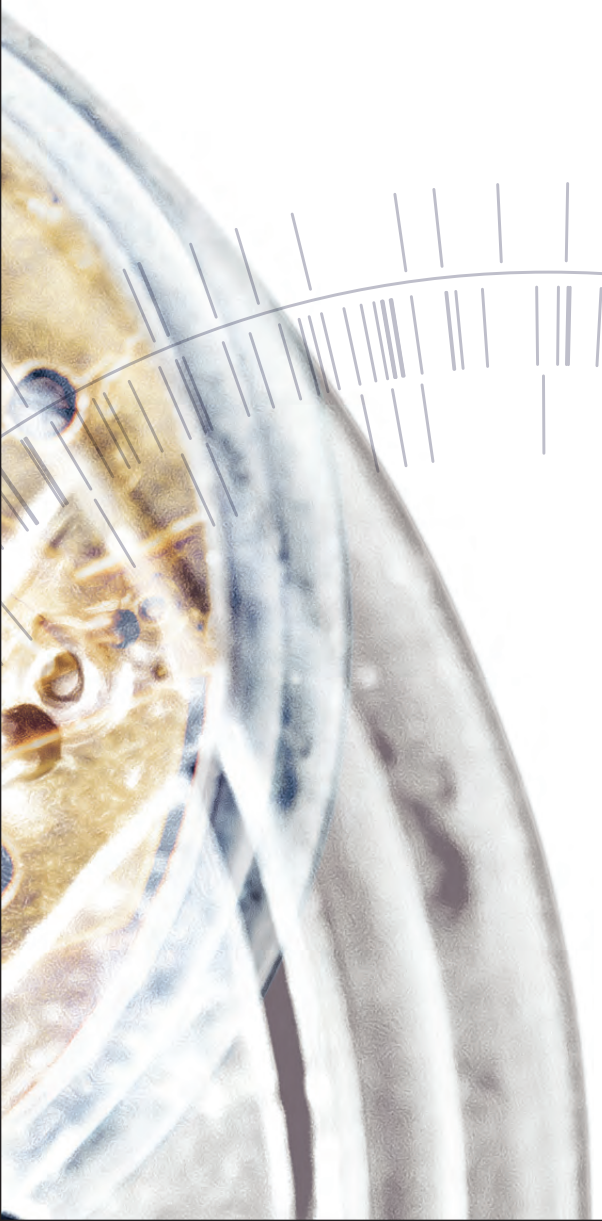
The replication machinery of Clostridium difficile:a potential target for novel antimicrobials

Eijk, H.W. van

Chapter 2

DNA replication proteins as potential targets for antimicrobials in drug–resistant bacterial pathogens

Erika van Eijk [1]
Bert Wittekoek [1]
Ed J. Kuijper [1]
Wiep Klaas Smits [1]



1 Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

With the impending crisis of antimicrobial resistance, there is an urgent need to develop novel antimicrobials to combat difficult infections and multidrug resistant pathogenic microorganisms. DNA replication is essential for cell viability and is therefore an attractive target for antimicrobials. Although several antimicrobials targeting DNA replication proteins have been developed to date, gyrase/topoisomerase inhibitors are the only class widely used in the clinic. Given the numerous essential proteins in the bacterial replisome that may serve as a potential target for inhibitors and the relative paucity of suitable compounds, it is evident that antimicrobials targeting the replisome are underdeveloped so far. In this review, we report on the diversity of antimicrobial compounds targeting DNA replication and highlight some of the challenges in developing new drugs that target this process.

Introduction

The increase in multidrug resistant bacteria has resulted in limited treatment options, and therefore the development of compounds directed against these micro-organisms is of utmost importance. In recent years, the pipeline of new anti-microbials has almost dried up, apart from the approved follow-up compounds (2nd, 3r , 4th generation) which have the same mode of action as their predecessors [1]. The development of antimicrobials derived from existing scaffolds is not without risk, as these compounds may be vulnerable to the same resistance mechanisms. Therefore, exploring new potential targets and/or increasing structural diversity in the next-generation antimicrobials are paramount in minimizing the risk of rapid acquisition of antimicrobial resistance. There are several essential cellular processes that can serve as targets for novel antimicrobials and many of these are exploited by antimicrobials. Of particular interest for this review is DNA replication. Correct replication of DNA by a multi-protein complex, the replisome, and proteins associated with it (**Table 1, Figure 1**) is an essential requirement for cell viability. The 'core' replisome complex consists of helicase, primase, DNA polymerase, sliding clamp, clamp loader and single-stranded DNA binding (SSB) proteins. Stringent coordination of this complex is essential for DNA replication, and inhibition of the function of any of these proteins or their interactions in principle disrupts the process and results in cell death [2]. Other proteins that are crucial for DNA replication include topoisomerase II and DNA ligase.

Despite the potential of replication proteins to serve as a target for antimicrobial compounds, clinical use has primarily been limited to topoisomerase II inhibitors that target DNA gyrase and/or topoisomerase IV (TopoIV). In this review we will discuss inhibitors that target 'core' replisome proteins as well as associated proteins that are crucial for DNA replication. We illustrate three key challenges (antimicrobial resistance, specificity, and exploration of new targets) and potential strategies to meet these challenges using examples of novel DNA replication-targeting antimicrobials active against *Clostridium difficile* and other multidrug resistant pathogens.

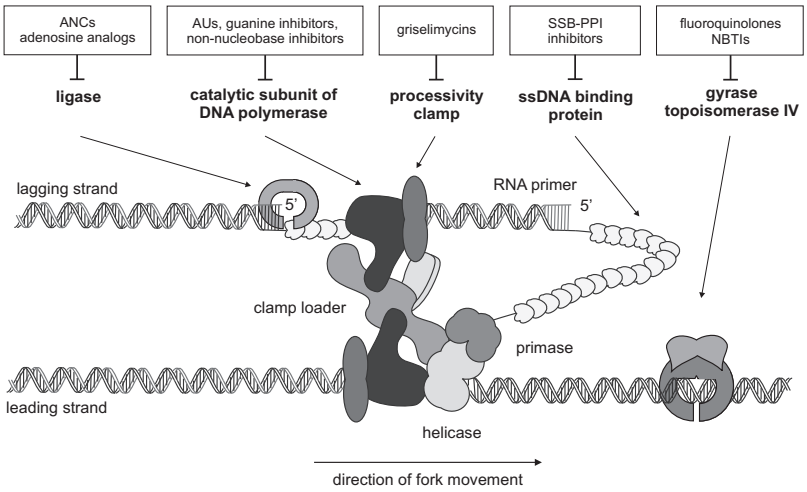

Figure 1. Schematic representation of the variety of targets of antimicrobials in the bacterial replisome.

Indicated is the core of the replisome and the other proteins that have been targeted by antimicrobial compounds. For simplicity, replication initiation proteins and regulators have been omitted from this figure. Important classes of drugs inhibiting specific proteins are boxed. The activity of all proteins is described in the main text. PPI; protein–protein interaction.

Table 1. Diversity of targets in the bacterial DNA replication machinery.

Function	*E. coli*	*B. subtilis*	*C. difficile* [a]	Activity	Inhibitors in (pre) clinical development
Chromosomal replication initiator protein	DnaA	DnaA	CD0001	Initiation of DNA replication at *oriC*	
Replicative helicase	DnaB	DnaC	CD3657 [b]	Unwinding of double stranded DNA at the replication fork	
Replicative helicase loader	DnaC	DnaI	CD3654 [b]	Required for functional loading of the replicative helicase	
Primosome protein	–	DnaB	–	Replication initiation and membrane attachment; enhancing the helicase loading process, origin remodelling	
Primosome protein	–	DnaD	CD3653	Initiation of DNA replication through interactions with other initiation proteins, origin remodelling	
Primase	DnaG	DnaG	CD1454 [b]	Synthesis of primers on the lagging strand	
Primosomal protein N′	PriA	PriA	CD2586		
Sliding clamp	β (DnaN)	DnaN	CD0002		Griselimycins
Clamp loader complex	Multiple proteins, including γ and τ (DnaX)	Multiple proteins, including DnaX	Multiple proteins, including CD0016		
DNA polymerase III α subunit DnaE	DnaE	DnaE	CD3396	Elongation of leading and lagging strand during DNA synthesis (E.coli); initial extension of the RNA primers on the lagging strand (B.subtilis)	Guanine inhibitors
DNA polymerase III α subunit PolC	–	PolC	CD1305	Elongation of both leading and lagging strand during DNA synthesis	AUs, guanine inhibitors, non-nucleobase inhibitors
DNA polymerase I	PolA	PolA	CD1128	Removal of RNA primers and gap filling	
Gyrase	GyrA, GyrB	GyrA, GyrB	CD0005, CD0006	Relaxing the DNA double helix by introducing negative supercoils, catenating and decatenating DNA rings	Fluoroquinolones, NBTIs
Topoisomerase IV	ParE, ParC	ParE, ParC	–		Fluoroquinolones, NBTIs
DNA ligase	LigA	LigA	CD3309	Joining of Okazaki fragments during DNA replication.	ANCs, adenosine analogues
Single-stranded DNA binding protein	SSB	SSB	CD3662, CD3235	Preventing degradation of ssDNA in the replication fork; protein interaction platform	PPI inhibitors

OriC, chromosomal origin of replication.

a Putative; most replication proteins of *C. difficile* are not characterized (apart from PolC [3]).

b These have been recently characterized [4] (Chapters 4 and 5).

Clinically used antimicrobials targeting DNA replication: topoisomerase II inhibitors

The two bacterial topoisomerase II enzymes – DNA gyrase and TopoIV – modify the topology of DNA during replication [5]. Gyrase and TopoIV are tetramers composed of two GyrA and GyrB subunits (encoded by *gyrA* and *gyrB*) or two ParC and ParE subunits (encoded by *parC* and *parE*), respectively [6,7]. Despite structural similarities [5], the two topoisomerase II enzymes perform distinct functions in prokaryotes. Gyrase is able to introduce negative supercoiling into DNA, thereby relaxing the DNA helix, while TopoIV is specialized in DNA decatenation and unknotting of DNA [8-10]. Some bacteria only encode gyrase in their genome; it is possible that gyrase in these bacteria can efficiently decatenate DNA without TopoIV, as was shown with *Mycobacterium smegmatis* [11].

Existing topoisomerase II inhibitors can roughly be divided into (i) ATPase inhibitors and (ii) topoisomerase II poisons that interfere with the catalytic DNA cleavage/joining process [12]. The first group is represented by the aminocoumarin class, the second group by the fluoroquinolones (FQs).

Aminocoumarins, such as the naturally occurring novobiocin, are cyclic peptides that compete with ATP to bind GyrB [6,13]. Though novobiocin has little structural similarity with ATP, the binding sites of the drug partially overlaps with the binding sites of ATP in GyrB [14]. Due to this overlap, mutations that confer aminocoumarin resistance are likely to affect enzymatic activity of the topoisomerase [12]. Novobiocin was licensed for treatment of infections by staphylococci and other susceptible organisms, but the clinical use of aminocoumarins is very limited due to poor pharmacological properties (e.g. poor solubility, poor absorption). Though improvement of pharmacological properties may yield clinical candidates, none have entered trials yet [15].

Fluoroquinolones (FQs) are the most successful class of antimicrobials targeting DNA replication and one of the most widely used antimicrobials on the market [2]. The FQ mode of action is to stabilize cleaved DNA-topoisomerase II complexes, thereby increasing the number of double-stranded DNA breaks in the bacterial cell [16,17]. Rapid cell death induced by FQs is likely the consequence of

chromosome fragmentation, while inhibition of DNA replication results in reduced cell growth instead of cell death[16]. Most FQs are able to inhibit both gyrase and TopoIV with different efficiencies, with actual target preference depending on the specific compound and the bacterial species against which it is used[16,18-20].

A major concern is the rise of FQ-resistant pathogens. Fluoroquinolones are commonly used to treat infections by Enterobacteriaceae, non-fermenting Gram-negative bacilli (in particular *Pseudomonas aeruginosa* and *Acinetobacter baumannii*) and *Mycobacterium tuberculosis* [21,22], but resistance can also occur when FQs are used to treat infections with a different pathogen. For instance, increased use of FQs and simultaneous development of fluoroquinolone resistance in clinical isolates of *C. difficile* resulted in the emergence of the epidemic PCR ribotype 027 as evidenced from whole-genome sequence data [23,24], even though FQs are not the drug of choice to treat *C. difficile* infections. FQ resistance is mainly acquired through mutations in the so-called quinolone resistance-determining regions (QRDRs) of the gyrase and/or topoIV genes [25]. In most resistant pathogens the mutations are located in the *gyrA* and/or *parC*, and rarely in *gyrB* or *parE*. In most Gram-positive bacteria, TopoIV is the primary target for fluoroquinolones and resistance mutations arise first in *parC*. In contrast, mutations in most Gram-negative bacteria occur first in *gyrA* [26]. Single-step mutations can lead to resistance and the *C. difficile* example illustrates how such a single mutation can fuel an epidemic with detrimental clinical outcome. FQ resistance can also be conferred by non-specific efflux systems that can export quinolones and other antimicrobial agents or by plasmids harbouring a quinolone resistance determinant [21].

Antimicrobials targeting DNA replication under development

There are many different compounds that have been identified as DNA replication inhibitors with potential to be used as an antimicrobial. These have been comprehensively reviewed elsewhere [2,27]. Here, we discuss several classes of DNA replication antimicrobials to highlight the diversity of replication proteins that can be exploited as targets and indicate some of the new developments.

Novel bacterial topoisomerase inhibitors (NBTIs)

Increasing FQ resistance has spurred the development of novel topoisomerase inhibitors that are active against FQ-resistant gyrase or TopoIV. Besides modifying existing FQ scaffolds [28], novel non-quinolone topoisomerase II inhibitors, which are collectively called novel bacterial topoisomerase inhibitors (NBTIs), have been developed [15]. Antimicrobials of this class target the catalytic core of topoisomerase, as do FQs, but NBTIs have a different mode of action. For example, the NBTI GKS299423 (GlaxoSmithKline) was shown to stabilize a pre-cleavage complex, in contrast to the cleavage-complex with double-stranded DNA breaks targeted by FQs [29]. Examples of other NBTI compounds are a series of pyrazole derivatives and related tetrahydroindazoles [30], NXL101 [31], NBTI 5463 [32], gyramides (N-benzyl-3-sulfonamidopyrrolidines) [33], and ACT-387042 and ACT-292706 [34]. None of these compounds have entered clinical trials yet.

DNA ligase inhibitors

The primary function of bacterial nicotinamide adenine dinucleotide (NAD+)-dependent DNA ligase (LigA) is to join the Okazaki fragments for the completion of lagging-strand DNA replication synthesis [35]. It is an attractive target for the development of novel antimicrobials as the ligase gene is present in all bacterial genomes and was found to be essential in several key pathogens [35,36]. It shares limited similarity to eukaryotic DNA ligase [36,37] and the binding of NAD+ as a substrate for ligase activity is a unique feature of bacterial ligase, limiting the potential for toxicity in humans.

All ligase inhibitors that have been developed to date are competitive inhibitors that bind a hydrophobic pocket near the binding site of the NAD+ substrate [38]. Four predominant classes of LigA inhibitors have been identified and here we will focus on the two most promising classes, the 2-amino-[1,8]-naphthyridine-3-carboxamides (ANCs) and the adenosine analogues [38].

The ANC scaffold was identified in high-throughput screening against *Escherichia coli* LigA (IC_{50} of 25 μM) but displayed better activity against *Staphylococcus aureus* LigA (IC_{50} of 2.5 μM) [39]. The compound was not active against wild-

type *E. coli* cells, however, due to poor permeability and efflux. Optimization of the ANC scaffold yielded compounds with improved activity (MIC 1-8 mg/L) against primarily Gram-positive pathogens (including MRSA and *C. difficile*), and promising results in animal models [39].

Adenosine analogues were identified in high-throughput screening for inhibitors of *Haemophilus influenzae* LigA [40,41]. Biochemical experiments and X-ray crystallography showed that this class of compounds competes with NAD+ and blocks the AMP-binding pocket of the LigA adenylation domain. Adenosine analogues displayed good antimicrobial activities against both Gram-positive and Gram-negative bacteria *in vivo* (MIC 1-8 mg/L) [40]. The initial adenosine analogues had favourable pharmacological properties (mainly good solubility) and one compound showed promise in *S. aureus* thigh infection and *Streptococcus pneumoniae* lung infection animal models [40]. However, the adenosine analogues were rapidly cleared in rats due to metabolism by cytochrome P-450s [41,42]. Despite attempts to optimize the solubility, antimicrobial activity and the clearance of these compounds, there are no reports published on further progress.

Although the hydrophobic pocket near the binding site of the NAD+ substrate enables specific inhibition of the bacterial LigA by competitive inhibitors, it is not directly engaged in interactions with the NAD+ substrate [35]. A spontaneous resistant mutant of *S. aureus* contains a single leucine-to-phenylalanine mutation in the hydrophobic pocket that does not affect the ligase activity of the LigA mutant but leads to a significant loss of target binding by adenosine analogues [35,40]. This relatively high risk of resistance against this class of compounds may have contributed to the fact that no LigA inhibitors have been entered into human trials to date.

DNA polymerase III inhibitors

The bacterial DNA polymerase III α subunit (PolIII) is an essential enzyme for DNA replication as it is responsible for the synthesis of DNA and its potential as antimicrobial target has been noted in recent *in silico* analyses of *E. coli* O157:H7 and *M. tuberculosis* [43,44]. Moreover, the distribution of two different homologues of the α subunit, PolC and DnaE, between Gram-positive and Gram-negative bacteria

presents an opportunity to develop inhibitors specific to either group [45]. The first inhibitor of PolIII, 6-(p-Hydroxyphenylazo)uracil (HPUra) was identified in 1970 [46]. The majority of PolIII inhibitors specifically target PolC of low-G+C Gram-positive bacteria. Indeed, the single DnaE-specific compound reported to date (324C) did not demonstrate any *in vivo* antimicrobial activity against *B. subtilis*, while it was highly active against purified *B. subtilis* DnaE *in vitro* [47] . The reason(s) for this is (are) unclear.

PolIII inhibitors can be categorized into three main classes: (i) the 6-anilinouracils (AU); (ii) the guanine inhibitors; and (iii) the non-nucleobase inhibitors. The AU class of PolIII inhibitors, which includes HPUra, is composed of a uracil-containing base-pairing domain that binds the DNA at cytosine bases and an aryl domain that determines the selectivity and affinity for PolC [48]. AUs competitively inhibit PolC with respect to dGTP through simultaneous binding to the cytosine of the DNA strand and near the active site of PolC, resulting in a ternary inactive complex of AU inhibitor, DNA, and PolC [48,49]. HPUra served as a scaffold for the development of numerous AUs with a broad range of antimicrobial and pharmacological properties. Two promising AUs, 6-(3-ethyl-4-methylanilino) uracil (EMAU) and 6-([3,4-trimethylene]anilino) uracil (TMAU) were highly active against PolC *in vitro*, but required optimization to increase activity against various Gram-positive bacteria, including MRSA [48,50-52]. Improvement of solubility of AUs compromises antimicrobial activity but allowed for the production of compounds that could be delivered intravenously rather than subcutaneously in animal models of infection [48,52-54]. The frequencies of mutations leading to AU resistance ranged from 3.6×10^{-10} to 1.2×10^{-8}, comparable to the frequency of ciprofloxacin resistance [55]. All mutations that conferred AU resistance were located at a specific amino acid in the presumed dNTP/AU-binding site of PolC [55,56]. Unexpectedly, however, the polymerase activity of these mutant PolC enzymes was unchanged in comparison with the wild-type *in vitro*.

The guanine inhibitors of bacterial DNA polymerase III have a purine moiety as the base-pairing domain instead of the uracil ring of the AUs. They act via the same active site -directed competitive inhibition as AUs, but are active against both PolC and DnaE [49,57,58]. Two lines of guanines inhibitors have been developed: the *N*2-(3,4-dichlorobenzyl) guanines (DCBGs) and the *N*2-(3-ethyl-4-methylphenyl)guanines (EMPGs). *N*7-substituted DCBGs and EMPGs displayed potent

in vitro antimicrobial activities against several Gram-positive pathogens but showed limited efficacy in animal models [49,58]. Similar to the AUs, the DCBGs have poor solubility in water and no attempts have been made to produce soluble analogues so far.

The non-nucleobase class of DNA polymerase III inhibitors includes anilinopyrimidinediones (APs) and quinazolin-2-ylamino-quinazolin-4-ols (BisQuinols). The APs are structural isomers of AUs and are also competitive inhibitors of dGTP [59]. They show minimal cytotoxicity and moderate antimicrobial activities (MIC ranging from 8 to 16 mg/L) [59]. In contrast to AUs and APs, BisQuinols have been suggested to compete with the DNA template, rather than nucleotides [60]. Though BisQuinol analogues were able to inhibit Gram- positive pathogens, they were unselective for mammalian Polδ, which raises concerns about cytotoxicity.

There is no information on resistance development against the guanine inhibitors or non-nucleobase inhibitors, but considering the similarities in mode of action to the AUs, caution is warranted.

Inhibitors of other replication related proteins

The sliding clamp, or A subunit of the replication machinery, is a polymerase processivity factor [61,62]. The protein can be targeted by novel griselimycins [63]. Griselimycin is a natural product of *Streptomyces griseus* with specific activity against the Corynebacterineae suborder, including *Mycobacterium* species [64]. The development of this class of compounds as anti-tuberculosis drugs was initially abandoned as rifampicin became available for treatment, but revisited in light of its activity against drug-resistant isolates [65]. Poor pharmacokinetic properties of griselimycin were addressed by the total synthesis of derivatives [63]. In particular, cyclohexylgriselimycin was highly active against *M. tuberculosis in vitro* and in a mouse model of infection, comparable to isoniazid. Evidence for the mechanism of action came from observations that the griselimycin biosynthetic operon contains a sliding clamp homologue capable of conferring resistance to a susceptible *Streptomyces* strain, the selective amplification of a *dnaN* containing chromosomal fragment in mycobacterial strains with evolved resistance (i.e. resistant strains harbouring multiple copies of the *dnaN* gene), and the crystal structure of griselimycin in complex with DnaN [63].

Single-stranded DNA at the replication fork is stabilized and protected by single-stranded binding (SSB) proteins [66]. These proteins are also an integral part of nucleoprotein complexes involved recombination and repair [67,68]. Although present in all domains of life, amino acid sequence, subunit composition and oligomeric state of these proteins differ substantially between organisms [67]. The essential role of SSB protein-protein interactions (SSB-PPI) and low sequence similarity between eukaryotic and prokaryotic SSBs allows the potential development of SBB-PPI inhibitors that could serve as novel antimicrobials [68]. Indeed, small molecules that interfere with the interaction between SSB and one or several binding partners have been identified by a high- throughput fluorescence polarization assay [68,69]. Inhibition by these compounds is based on mimicking the SSB protein C-terminus, which acts as a platform for interaction with other proteins, or more targeted inhibition of the SSB protein/Exonuclease I (binding partner) interface [68].

Challenges in developing novel antimicrobials targeting replication

We consider three main challenges in the development of novel antimicrobial compounds. First, novel antimicrobials should overcome resistance to known drugs and minimize development of resistance against the new drug. Second, they should preferably be specific to the microorganism/pathogen of interest to prevent dysbiosis of the host microbiome. The third challenge is to move away from modification and optimization of existing scaffolds that inhibit established cellular targets and explore novel targets and mechanisms of action. In the following section, we illustrate these challenges with a focus on compounds inhibiting the multidrug resistant organism *C. difficile*.

Challenge: antimicrobial resistance

The use of any antimicrobial agent exerts a selective pressure on susceptible bacterial populations, thereby creating an environment where the development of antimicrobial resistance is selected for. It can be assumed that resistance to any antimicrobial is unavoidable. Though resistance is reported to nearly all of the

discussed replication antimicrobials, the likelihood of developing resistance varies greatly between classes. For example, mutations that confer resistance to LigA inhibitors do not affect the activity of the enzyme, while most mutations leading to aminocoumarin resistance impair gyrase functioning. Both antimicrobials target the active site of their target enzymes, but the binding sites of LigA inhibitors are located in a region that is not directly involved in substrate (NAD+) binding of LigA. Antimicrobials targeting DNA replication proteins that bind their targets at the active site, such as the aminocoumarins, have a relative low risk for resistance, since mutations that affect drug-protein binding tend to lead to a non-functional protein.

LigA inhibitors, PolC inhibitors and most aminocoumarins target one specific protein and therefore, a single mutation is frequently sufficient to cause resistance. FQs are able to target both gyrase and TopoIV. As a result, a high level of FQ resistance usually requires the presence of mutations in both gyrase and TopoIV [70], which reduces the risk of resistance development. However, variations in the potency of FQs against gyrase and TopoIV still enable the emergence of resistant bacteria [16] and for organisms that only have gyrase encoded in their genome, such as *C. difficile*, a single mutation is still sufficient to cause resistance. Indeed, a single mutation in *gyrA* (Thr82Ile) was most frequently found in FQ-resistant clinical *C. difficile* isolates [71].

Two promising strategies that might reduce development of resistance (and cross-resistance) are (i) to target multiple proteins in the bacterial cell, using so-called hybrid antimicrobials, or (ii) to use multiple binding sites in a single target. Multi-targeting reduces the chance of resistance development and results in compounds that remain active against mutants resistant to either one of the parent compounds. Though polypharmacological modelling and rational design of multi-targeting drugs is a major challenge [72,73], interesting progress has been made in recent years.

In FQ hybrid antimicrobials, a FQ moiety is covalently linked to another pharmacophore, with a distinct cellular target [74]. Various FQ hybrid classes have been developed, but of particular interest here are the 6-anilinouracil-fluoroquinolone (AU-FQ) hybrids, since both moieties target DNA replication. The AU-FQ hybrid class was created by linking various FQs to the *N*3 of the PolIIIC inhibitor HB-EMAU, with different linkers to modulate antimicrobial activity and pharmacological properties [75]. AU-FQ hybrids are highly active against Gram-positive bacteria, and some compounds had moderate activity against the Gram-negative *E. coli*. MBX-500,

one of the best AU-FQ hybrids, displayed 3-fold stronger inhibition of *B. subtilis* PolC than the AU moiety alone, while it showed 5- to 10-fold less potent inhibition of *B. subtilis* TopoIV and gyrase in comparison to the FQ component [76]. Despite these differences in target inhibition, MBX-500 had strong antimicrobial activities against *Bacillus* species (MIC of 0.156 mg/L), *S. aureus* strains (MIC ranging from 0.625 to 5 mg/L) and various other Gram-positive bacteria. As expected, MBX-500 retained high antimicrobial activity against strains that were resistant to the FQ component. Resistance to MBX-500 was only found in an AU- and FQ-resistant *S. aureus* that carried mutations in both targets, thereby providing evidence that MBX-500 truly acts via a dual-targeting mechanism. Indeed, the spontaneous mutation frequency against MBX-500 was low ($<5.6x10^{-10}$ at 4 x MIC); no resistant *S. aureus* strains could be isolated after a single passage, whereas *S. aureus* did develop resistance against the individual AU and FQ components [76]. The antimicrobial properties of MBX-500 were also investigated in *C. difficile* [77], where fluoroquinolone resistance is widespread among clinical strains [71]. The compound was active against a panel of 30 *C. difficile* isolates (MIC range 1-4 mg/L), which included several multi-resistant strains and isolates from the epidemic PCR ribotype 027. Thus, dual target antimicrobials are promising in the fight against resistant pathogens, and the low mutation frequencies (e.g. no spontaneous resistance) indicate a possible reduced risk of resistance development with comparison to single-target compounds.

Another approach to avoid cross-resistance and reduce the risk of resistance is to identify multiple (novel) binding sites or multiple modes of actions in a single established target, as exemplified by the novel topoisomerase II inhibitor kibdelomycin [78]. Biochemical studies in *S. aureus* showed that kibdelomycin is a potent ATPase inhibitor of both topoisomerase II enzymes, although it is >80-fold more active against the ATPase subunit of gyrase than TopoIV. The co-crystal structures of kibdelomycin bound to the N-terminal domains of *S. aureus* GyrB and ParE revealed a novel mode of 'dual' ATPase inhibition by blocking ATP binding and destabilizing GyrB/ParE subunit dimerization [79]. Kibdelomycin displays strong antimicrobial activities against predominantly Gram-positive bacteria, including MRSA and *C. difficile* [80,81]. The MIC_{90} for *C. difficile* was 0.5 mg/L, similar to the novel therapeutic fidaxomicin, but more potent than metronidazole and vancomycin [80]. Importantly, kibdelomycin was equally active against FQ-resistant and -susceptible strains and showed favourable pharmacokinetics in mice and promise in a hamster model of *C. difficile* infection [80]. Kibdelomycin also retained antimicrobial activity against

S. aureus strains resistant to novobiocin and coumermycin A1, two known ATP inhibitors of topoisomerase II, and showed a low frequency of resistance development ($<5.4x10^{-10}$) [78]. The MIC of kibdelomycin against a coumermycin-resistant *S. aureus* strain carrying three mutations in GyrB was modestly increased from 1 to 4 mg/L relative to the susceptible wild-type strain. This was attributed to one of the three mutations (Ile175Thr) in GyrB, which also interfered with kibdelomycin binding. Although the reduced susceptibility of the coumermycin A1 resistant strain warrants some caution, the data suggests that complete resistance caused by a single point mutation is not likely to occur due to the dual binding mode of this compound.

Challenge: specificity of novel antimicrobials

The normal gut microbiota is a diverse community of microbes that lives in a complex ecological system with its host [82]. With the increasing knowledge on interactions and dynamics of the microbiota with its host, it becomes evident that the human microbiome is of great importance for the prevention and treatment of infectious diseases. It can act as a protective barrier that prevents colonization of the gut by non-commensal pathogens and opportunistic pathogens that are already present. This so-called colonization resistance [83] is greatly influenced by the diversity of the gut microbiota. Many broad-spectrum antimicrobials used in the clinic are known to affect the composition of the gut microbiota [84,85]. Colonization of the gut by pathogenic *C. difficile* is believed to require the disruption of the gut microbiota [86,87], and patients with recurrent *C. difficile* infection (CDI) have a reduced diversity of their microbiota compared with healthy controls and patients with initial CDI [88]. There is a strong association between the use of broad-spectrum antimicrobials and increased risk of (recurrent) disease [89,90]. Patients treated with the narrow-spectrum antimicrobial fidaxomicin had lower *C. difficile* recurrence rates in comparison with broad-spectrum vancomycin [91], which was attributed to the minimal impact of fidaxomicin on the gut microbiota, especially on bacteria of the *Bacteroides* cluster, clostridial cluster XIVa and bifidobacteria [91,92]. The example of *C. difficile* underscores that it is desirable for novel antimicrobials to limit the impact on the human microbiota as a whole.

Two strategies to generate or identify compounds with a narrow spectrum are (i) to test derivatives of an existing inhibitor for increased specificity and (ii) to screen for compounds with a selective inhibition of a specific pathogen.

The first strategy has been used to identify and develop PolC inhibitors with increased species-specificity. Many PolC inhibitors have a broad Gram-positive spectrum and are therefore likely to significantly affect the composition of the microbiota. However, the isolation and purification of the *C. difficile* PolC as well as polymerases from various other organisms enabled the identification of a series of novel 7-substituted DCBG inhibitors that showed improved potency and specificity against *C. difficile* [3]. One of these compounds, 362E, showed potent antimicrobial activity against *C. difficile* strains, similar to the first-line therapeutics metronidazole and vancomycin (MIC_{90} of 4 mg/L) [3,93]. Limited *in vitro* tests indicated that 362E is inactive against certain other Gram-positive anaerobes [3], but the impact of 362E on the gut microbiota has not been reported to date. 362E protected hamsters from death due to *C. difficile* similar to vancomycin and, though recurrent CDI was observed in both treatment groups when animals were treated for 3 days, prolonged treatment with 362E resulted in a reduced recurrence rate [93]. Further experimental evidence is needed to confirm the selectivity of this compound for *C. difficile* during *in vivo* treatment.

A second approach is to screen compounds *de novo* for specific activity against certain pathogens and couple this to an assay to determine the desired mode of action. SMT19969 (ridinilazole) is a bis(4-pyridyl)bibenzimidazole that has been specifically developed as an novel front-line antimicrobial for CDI treatment (MIC 0.06 – 0.5 mg/L) [94,95] with a low chance of resistance development [96]. Although the mode of action awaits validation, compounds of the bisbenzimidazole class were shown to bind the minor groove of the DNA duplex and are thought to inhibit DNA replication through inhibition of DNA helicases and/or topoisomerases [97]. SMT19969 showed high selectivity for *C. difficile* relative to other intestinal isolates during *in vitro* susceptibility testing. In a Phase I clinical trial, treatment with SMT19969 resulted in minimal changes in the composition of the faecal microbiota of healthy human subjects, except for a significant reduction in total clostridial count [98] and this was confirmed in an *in vitro* gut model of CDI [99]. SMT19969 was superior to vancomycin and, in a subset of strains, to fidaxomicin in a hamster model of recurrent CDI [95,98]. A clinical Phase II trial is currently ongoing where the efficacy of SMT19969 treatment will be compared with vancomycin treatment in CDI patients.

In another example, as discussed above, kibdelomycin showed potent antimicrobial activities against a large panel *C. difficile* isolates [80]. In contrast to metronidazole, kibdelomycin is mostly inactive against Gram-negative anaerobes, including

the *Bacteroides fragilis* group, similar to the narrow-spectrum drug fidaxomicin [80]. The narrow spectrum of fidaxomicin is believed to contribute to its favourable characteristics in treating recurrent CDI [91], but the effect of kibdelomycin on recurrent disease has not been investigated yet. MBX-500 also had little activity against most Gram-negative anaerobes and was selectively active against *C. difficile* strains among Gram-positive anaerobes [77]. MBX-500 was shown to be efficacious in a gnotobiotic piglet model of acute CDI, with 100 percent survival rate and only mild CDI symptoms [100]. Though the efficacy of MBX-500 was comparable to vancomycin in a hamster model of CDI, it was superior in a murine model of recurrent CDI and associated with improved weight gain in both animal models [77]. The weight gain of infected animals treated with MBX-500 and the results in the murine model suggests that this compound might have a low impact on the gut microbiota.

Narrow-spectrum antimicrobials are useful to prevent or treat opportunistic infections such as CDI that are associated with dysbiosis of the microbiome. Careful assessment of the effects on microbiota should be considered for all antimicrobials developed in the future.

Challenge: identifying novel targets in the bacterial replisome

Despite the success of the FQs, the number of compounds targeting DNA replication in clinical use or development is relatively low compared to inhibitors of other cellular processes such as protein synthesis. Why is this group of antimicrobials underdeveloped, considering that there are many more proteins in the bacterial replisome?

A major issue is the poor characterization of the DNA replication machinery of important pathogens. For instance, the DNA replication proteins in *C. difficile* are merely predicted through homology with proteins in other organisms (**Table 1**) but functional evidence is limited to *C. difficile* PolC so far [3]. It can be expected that the extensive biochemical and structural characterization of replisome proteins of drug-resistant pathogens might lead to the identification of unique features that can be used to design antimicrobials that specifically target this specific species.

Another possible explanation for the underdevelopment of antimicrobials targeting DNA replication is that screens for replication inhibitors have been notori-

ously difficult. Most inhibitors are evaluated through *in vitro* biochemical assays that measure inhibition of either enzymatic activity or DNA replication and these are not always suitable for high-throughput screening [27]. Moreover, compounds identified this way may display potent *in vitro* activity, while they have no activity against bacterial cells *in vivo* due to undesirable pharmacokinetic properties. Besides some of the examples mentioned (aminocoumarins, ANCs and PolIII inhibitors), this is illustrated by two studies on inhibitors of the bacterial primosome [101,102]. Inhibitor peptides that target the DnaB-DnaG helicase-primase complex in *Bacillus stearothermophilus* were able to inhibit purified proteins *in vitro* [101], but were inactive *in vivo*. A highly active triaminotriazine inhibitor of the *Pseudomonas aeruginosa* replicative helicase DnaB did not show antimicrobial activity towards wild-type *P. aeruginosa* or *E. coli*, showed poor activity against *S. aureus* and showed problematic toxicity towards mammalian cells [102]. To circumvent the limitations of biochemical assays, screening could be performed using a whole-cell assay. By limiting the levels of a certain replisome protein or affecting its function in cells [103,104], screens can be enriched for compounds that target DNA replication. A successful application of a whole-cell screen is the identification of vibrepin as an inhibitor of DNA replication in *Vibrio* [104]. *Vibrio* species, including the causative agent of cholera (*Vibrio cholerae*) have a bipartite genome, where replication of the second chromosome is dependent on the replication initiator RctB. In the screen, the growth of an *E. coli* strain harbouring an RctB-dependent plasmid with an antimicrobial resistance determinant was evaluated in the presence and absence of the antimicrobial and inhibition of growth was indicative of an RctB inhibitor. Vibrepin was found to interfere with chromosome II origin opening by RctB *in vitro* and inhibited various *Vibrio* species (MIC 0.4-2.0 mg/L), but also inhibited various other species via an RctB-independent mechanism [104]. This example also illustrates how differences between replication machineries can be exploited to work towards species-specific antimicrobial compounds.

Outlook

Despite the challenges that are discussed with respect to antimicrobials targeting DNA replication, we feel it is important to focus research on the identification of novel therapeutic targets in the bacterial replication machinery, whether they are

individual proteins or protein-protein interactions [27], for several reasons. First, DNA replication is essential for cell viability and inhibiting any essential protein would be detrimental for cell survival. Second, most components of the bacterial replisome are substantially different from their eukaryote counterparts and can therefore be exploited to develop compounds with minimal cytotoxicity. Third, antimicrobials directed at novel targets are likely to avoid existing resistance mechanisms. And finally, some components are well conserved between most bacteria, such as the DNA initiator protein DnaA, while others are more restricted to certain species. Therefore, the bacterial replisome can be used to develop both broad- and narrow-spectrum antimicrobials. Recent insights on the structure of several DNA replication proteins and their inhibitors and the development of new assays that enable high-throughput screening for inhibitors of DNA replication are expected to influence the rate of success of this class of antimicrobials considerably [2].

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