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Title: Novel avenues for *Clostridium difficile* infection drug discovery

Abstract

Introduction: *Clostridium difficile* is the etiologic agent of nosocomial and community-acquired diarrhoea associated with exposure to antibiotics that disrupt the normal colonic flora. As antibacterials currently used for primary *C. difficile* infections favour recurrences, new agents able to neutralize the bacterium without affecting the gut microbiota are badly needed.

Areas covered: The most promising strategies aimed at developing therapies with minimal or no effect on the intestinal flora, such as new narrow-spectrum antibiotics and antimicrobial peptides, bacteriophages and phage lysins, virulence-targeting factors such as riboswitch ligands and quorum sensing-interfering factors, bacteriotherapy based on probiotics and faecal transplant, and toxin-targeting molecules.

Expert opinion: Beyond the development of new antibiotics, virulence-targeting factors or phage cocktails seem promising strategies, which could replace antibiotics avoiding the emergence of resistant strains and the onset of *C. difficile* infection (CDI). Until broad-spectrum antimicrobials will be in use, *C. difficile*-specific lytic phages could help to prevent CDI by eliminating *C. difficile* in patients and in the hospital staff, and for the prevention and treatment of recurrences. Phage therapy is not currently available in Western countries, but, in our opinion, it should have a new chance. Faecal therapy is emerging as a very effective and readily available treatment for recurrences. The shift is from a standardized, drug-based antibacterial therapy toward the forthcoming less expensive and non-patentable procedures of a more personalized medicine. This will imply profound changes affecting both patient-physician interactions and the current profit-oriented approach to the pharmacologic therapy of infections.

Keywords: antibiotics; antimicrobial peptides; *Clostridium difficile*; faecal therapy; phage therapy; probiotics; toxins; virulence factors.

1. Introduction

Clostridium difficile is a Gram-positive anaerobe spore-forming bacillus, which in the late 1970s was identified as the causative agent of antibiotic-associated pseudomembranous colitis [1]. For a thorough retrospective review on *C. difficile* infection (CDI), the reader is referred to [2,3]. The spores of the bacterium are transmitted via the faecal-oral route, and since 2001 CDI is emerging as the leading cause of hospital-acquired diarrhoea in adults, with an incidence and severity increase that makes *C. difficile* one of the most important healthcare-associated pathogens worldwide [4,5]. Furthermore, CDI onset in non-hospitalized patients is a growing problem, as community-acquired cases represent, depending on CDI case definition, 20-50% of all CDI cases identified in the USA, Canada, and Europe [6].

The onset of the disease is associated with the use of antibiotics that disrupt the equilibrium of the intestinal microflora. The antibiotics most frequently implicated prior to 2000 were clindamycin and cephalosporins, especially cefotaxime and ceftazidime. More recently, fluoroquinolones emerged as major inducing agents [7]. Advanced age (over 65), gastrointestinal surgery, permanence in acute or chronic care facilities, and temporary or permanent immunodeficiency of any origin, are significant risk factors.

The increase in CDI frequency and severity is attributed to the spread in Western countries of the hyper-virulent North American Pulsed field type 1, restriction endonuclease analysis type BI, and polymerase chain reaction ribotype 027, known as the NAP1/BI/027 strain. Unlike historic strains, the new one is resistant to newer fluoroquinolones, such as gatifloxacin and moxifloxacin. It is also highly toxigenic *in vitro*, which accounts for the greater severity of symptoms and the high rates of recurrence and mortality [8]. Gene regulation studies suggest that fluoroquinolones could favour *C. difficile* 027 infections not only by disrupting the barrier microbiota, but also by enhancing the expression of *C. difficile* virulence and colonisation factors [9]. Another hypervirulent strain, ribotype 078, was identified in the Netherlands as the predominant strain in pigs and calves.

In humans, it causes a disease with a grade of severity similar to that of the 027 ribotype, but it affects a younger population and is more frequently community-associated [10].

The severity of CDI may range from mild self-limiting diarrhoea to more serious and sometimes fatal conditions such as pseudo-membranous colitis, sepsis syndrome, toxic megacolon, and colonic perforation [11]. The acute disease is initially manifested by nausea, vomiting, diarrhoea with mucus and rarely blood in the stool (5% of cases), abdominal pain, fever, leukocytosis, and dehydration. These symptoms are due to the production of two toxins, referred to as TcdA and TcdB, transcribed from a pathogenicity locus that comprises five genes: *tcdA* and *tcdB* for toxins, and *tcdR*, *tcdE*, *tcdC*, three regulatory genes that encode TcdR, a bacterial transcription initiation factor (σ factor), TcdE, a putative holin, and TcdC, an anti-σ factor, respectively. The expression of *tcdA* and *tcdB* is positively regulated by TcdR and negatively regulated by TcdC [12]. In the NAP-1/027 hypervirulent strains, *tcdC* deletion mutations are associated with an increase by more than a factor of 10 in A and B toxin production [13]. Some strains also produce a third toxin, whose role in CDI pathogenesis is unclear. It is known as binary toxin and is encoded by the *ctdA* and *ctdB* genes, which are located outside the pathogenicity locus. For a thorough and up to date review on the role of A and B toxins in CDI, see [14].

Major therapeutic challenges concerning the chronic form are: i) the treatment of toxin-induced colonic inflammation and of ileus, which prolongs the permanence of the pathogen and prevents the diffusion of orally-administered antimicrobials to the infected areas; ii) the prevention of recurrences, which occur in ~20%-40% of patients, the percentage varying with the infecting strain and the drug used. Since 1983, standard therapy relies on metronidazole (Fig. 1, 1) for the treatment of mild to moderate CDI, and, in the last decade, on vancomycin (Fig. 1, 2) for severe CDI. Unfortunately, these drugs also behave as recurrence-inducing factors, as suggested by the finding that 40% of recurrences are reinfections caused by different strains, and as demonstrated by experiments in hamster infection models [15]. The *C. difficile* resistance to antimicrobials varies widely between countries. Whereas most isolates are susceptible to metronidazole and vancomycin,

decreased sensitivity against metronidazole has been reported in Spain and UK, and sporadic cases of resistance to vancomycin are being reported in Poland, Spain and Scotland [16]. Bacitracin, teicoplanin, and fusidic acid were evaluated for CDI treatment, but they did not show any advantage over metronidazole and vancomycin [17]. In 2011 fidaxomicin (Fig. 1, 3), a new macrocyclic RNA polymerase inhibitor produced by *Actinoplanes deccanensis*, was approved by the US Food and Drug Administration (FDA) for CDI treatment[18]. Fidaxomicin is the last of the four novel antibiotics that entered the market in the last decade (the other three being linezolid, daptomycin and retapamulin). It is administered by the oral route and its efficacy is comparable to that of vancomycin but, thanks to its narrower spectrum of activity, it has a less disruptive effect on the intestinal flora, resulting in greater tolerability and in lower risk of recurrences, especially among non-NAP1-associated infections. The major disadvantage of fidaxomicin is the cost, as the current price of a treatment course exceeds 2500 US dollars [18].

Short-term alternative options for CDI treatment could take advantage of some molecules recently developed for other pathologies. Uncontrolled trials and single-case reports suggest that molecules such as the anti-protozoal agent nitazoxanide, as well as teicoplanin, rifaximin, approved in the US for traveller's diarrhoea, and the broad-spectrum antibiotic tigecycline, none of which approved for CDI, might be useful for the treatment of recurrences or severe complicated cases [19].

This review deals with the most promising strategies aimed at the identification and development of new therapies, and is focused on the following topics: i) new antibiotics; ii) structurally and functionally different agents, including antimicrobial peptides, riboswitch ligands, quorum sensing-interfering factors, bacteriophages, phage lysins, and prebiotics; iii) toxin-targeting molecules; iv) bacteriotherapy, in the form of probiotics or faecal transplant.

2. New antibiotics

The search for new antibiotics to be used against *C. difficile* poses specific problems, linked to the bacterium and its ecosystem: i) the constitutive resistance to a number of in-use antibiotics requires

the identification of new molecular scaffolds to avoid cross-resistance; ii) the new antibiotic should have a very narrow spectrum of activity, so to avoid any further disruption of the protective intestinal microbiota, an event that leaves the patient more susceptible to recurrences; iii) it would be desirable to obtain drugs active against multiple targets, because single target drugs are likely to elicit rapid emergence of resistance; iv) to be approved and have a market, new drugs should at least not be inferior to, and possibly be less expensive than, fidaxomicin, which for now is a pretty good choice. We shall now outline a series of molecules that, at least in part, meet these conditions and are under advanced development.

CB-183,315 (Fig. 2, 4), by Cubist Pharmaceuticals Inc. (Lexington, MA, USA), is an orally available lipopeptide antibiotic structurally related to daptomycin (Fig. 2, 5), with which it shares the peptide sequence and the ability to alter the membrane potential of target cells [20]. CB-183,315 showed good activity against different *C. difficile* isolates, including those with elevated MICs against metronidazole, moxifloxacin, and vancomycin. The lack of activity of CB-183,315 against *Enterobacteriaceae* and species of the *Bacteroides fragilis* group suggests a low impact, similar to that of fidaxomicin, on the gut normal flora [21]. These properties should result in high cure and low recurrence rates. CB-183,315, which recently completed phase II clinical trials and is currently in phase III, may therefore be considered a suitable candidate for further clinical development [20].

The unrelated compound ramoplanin (Fig. 2, 6), which targets lipid-II, has been in clinical development for many years and in 2009 it was acquired by Nanotherapeutics Inc. (Alachua, FL, USA). Ramoplanin is an oral lipoglycodepsipeptide with good activity against Gram-positive, but not Gram-negative, organisms, and was originally developed for the prevention of bloodstream infections by vancomycin-resistant Enterococci. The drug is not absorbed from the gastrointestinal tract of patients suffering from pseudomembranous colitis, and its *in vitro* activity is comparable to that of vancomycin [22]. In a CDI hamster model both vancomycin and ramoplanin reduced the number of *C. difficile* and led to symptom resolution. In an *in vitro* gut model originally developed by Mcfarlane *et al*. [23], ramoplanin, but not vancomycin, achieved almost complete elimination of

C. difficile spores [22]. In principle, this sporicidal property should diminish the number of relapses caused by the retention of viable spores in the gut, and limit the spreading of infection. Ramoplanin was evaluated in a phase II trial for the treatment of CDI, and was approved by the US Food and Drug Administration for a Special Protocol Assessment non-inferiority trial against vancomycin for phase III [24,25].

Oritavancin (Fig. 3, 7) is an oral semisynthetic lipoglycopeptide originally synthesized by Eli Lilly in an effort to identify vancomycin analogs with better pharmacodynamic characteristics and able to overcome vancomycin resistance. After several changes of ownership, oritavancin was acquired by The Medicines Company (Parsippany, NJ, USA), which is currently developing it in phase III trials as a 1200-mg single dose for the treatment of acute bacterial skin and skin structure infections, including those due to methicillin-resistant *Staphylococcus aureus* [26]. The molecule demonstrated good activity against *C. difficile* in an *in vitro* human gut model [27]. It also has a long half-life (150 to 300 h, compared with a half-life of 6 h for vancomycin) [28] and a low propensity to select resistant strains, probably due to its threefold mechanism of action, which includes transglycosilation and transpeptidation inhibition and cell membrane disruption [26].

Replidyne, Inc. (Louisville, CO. USA) began the development of REP3123 (Fig. 3, 8), a novel diaryldiamine that inhibits the *C. difficile* methionyl-tRNA synthetase and has a narrow spectrum of antibacterial activity, being inactive against many other anaerobes that comprise the normal intestinal flora, such as *Clostridium ramosum*, bifidobacteria, lactobacilli and Gram-negative anaerobes [29,30]. The molecule has bacteriostatic activity, and compared favourably with vancomycin in inhibiting *C. difficile* growth and toxin and spore production *in vitro*. In a CDI hamster model, REP3123 was superior to vancomycin in the overall animal survival. In the words of Ochsner *et al*. [31] REP3123 could be a promising candidate for CDI treatment However, since the completion of preclinical studies in 2009, no clinical trials are reported.

A new prototype drug candidate, MBX-500 (Fig. 3, 9), is currently developed by Microbiotix Inc. and GLSynthesis Inc. (both at Worcester, MA, USA). MBX-500 is a hybrid

molecule formed by an anilinouracil inhibitor of the Gram-positive replication-specific DNA polymerase (pol IIIC), linked to a fluoroquinolone DNA gyrase/DNA topoisomerase IV inhibitor [32]. It was developed to treat antibiotic-resistant Gram-positive aerobic pathogens, but recently it was shown to be active both *in vitro* and *in vivo* against a panel of antibiotic-sensitive and -resistant *C. difficile* isolates, including the NAP1/027 ribotype [33]. The strengths of MBX-500 are: oral administration, local activity with no systemic absorption, narrow antibacterial spectrum, and low probability to select resistant strains, thanks to its ability to target three different bacterial enzymes involved in DNA replication. These characteristics make the molecule an excellent candidate for further development [33].

Novartis (NIBR, Cambridge, MA, USA) is currently developing a molecule named LFF571, obtained by optimization of a macrocyclic natural product, a thiopeptide-based secondary metabolite produced by the rare actinomycete *Planobispora rosea* [34]. The drug, which can be administered by the oral route, is entering a phase II trial to assess the safety and efficacy of multiple daily dosing in patients with moderate *C. difficile* infections [35].

By the end of December 2012 Actelion Pharmaceuticals Ltd. (Allschwil, Switzerland) announced the decision to move forward to phase III clinical development of cadazolid (ACT-179811) in patients suffering from *C. difficile* diarrhoea [36]. Cadazolid is a new chimeric antibiotic with structural elements of the oxazolidinone as well as the quinolone class of antibiotics. It inhibits *C. difficile* protein synthesis leading to strong suppression of toxin production and spore formation. In preclinical studies, cadazolid showed high *in vitro* activity against *C. difficile* clinical isolates coupled with a low impact on bacteria of the normal gut microflora. Other strong points of cadazolid are its low propensity for resistance development and its negligible absorption, resulting in high gut lumen concentrations and low systemic exposure, even in severe CDI cases, when the gut wall is severely damaged and permeability to drugs is potentially increased [37].

3. Antimicrobial peptides (AMPs)

Since the 1990s, AMP development was considered a promising mining field for new drugs against multiresistant pathogens [38]. Antimicrobials of peptidic nature fall into two classes: the gene-encoded, ribosomally synthesized peptides, and the non-ribosomally synthesized peptide antibiotics, typically produced by bacteria and fungi. The latter are assembled by multi-enzyme complexes, contain d-amino acids and other non-proteinogenic amino acids, and often have a cyclic or branched structure [39]. The ribosomally synthesized antimicrobials can be subdivided into two further classes depending on their source: strictly speaking, the term “antimicrobial peptides” refers to peptides of eukaryotic origin, whereas peptides and proteins produced by bacteria are referred to as bacteriocins. Based on their electrical charge, AMPs can be divided into anionic and cationic peptides (AAMPs and CAMPs, respectively). AAMPs, found in vertebrates, invertebrates and plants, are active against bacteria, fungi, viruses, nematodes and insects. Whereas AAMPs have so far received little attention in the literature (for an outline of AAMP characteristics, see the exhaustive review by Harris et al. [40]), CAMPs are included among the most promising candidates for the development of new biomedical alternatives [41]. Prokaryotic bacteriocins, which include the already in use colistin (polymyxin E), daptomycin, and lipopeptides, are attracting renewed interest both as alternatives to conventional drugs and because they are a feature of probiotic bacteria [42,43].

3.1 Lantibiotics (class I bacteriocins)

Lantibiotics are small (19-39 amino acids), heat-stabile, post-translationally modified peptides containing the non-proteinogenic amino acids lanthionine or methyl-lanthionine. They are active against Gram-positive bacteria and some of them, such as nisin and lacticin, are widely used as antibacterial agents by the food and agricultural industry in more than 50 countries [44,45]. Many lantibiotics are extremely potent antibacterial agents with minimum inhibitory concentrations (MIC) in the nanomolar range [46]. Their mode of action involves the binding to lipid II at a site that is

different from the one affected by vancomycin and related glycopeptides, so they are also active against multi-drug resistant strains.

The experimental drug NVB302, which together with mersacidine and cinnamycin belongs to the family of globular type-B lantibiotics, is being developed by Novacta Biosystems Ltd. (Welwyn Garden City, Hertfordshire, UK) with support from a Strategic Award from the Wellcome Trust. NVB302 is a semi-synthetic, chemically modified peptide derived from the parent antibiotic desoxyactagardine B, produced by the actinomycete *Actinoplanes liguriae* [47]. In an *in vitro* human gut model, NVB302 was not inferior to vancomycin in the treatment of CDI, but was associated with faster resolution of the *B. fragilis* group [48]. Recently, NVB302 successfully completed a phase I tolerance clinical trial, and it is scheduled for phase II and III clinical trials in CDI patients [49].

Another interesting lantibiotic is mutacin 1140, a 22-amino acid peptide produced by *Streptococcus mutans* [50]. It is active against a wide range of Gram-positive bacteria, including *C. difficile*, with a mechanism that involves lipid II binding and sequestration [51]. A synthetic version, named mutacin 1140-S, developed by Oragenics Inc. (Tampa, FL, USA), is concluding preclinical testing. The molecule possesses a set of good pharmaceutical properties, such as chemical stability, negligible toxicity, and *in vivo* efficacy. On the basis of these findings, to which may be added the lack of resistant and genetically stable spontaneous mutants, mutacin 1140-S is scheduled for further development and phase II and III trials [52].

3.2 Two component bacteriocins (class IIb bacteriocins)

Bacteriocin production is considered an important trait of probiotic organisms, and the efficacy of bacteriocin-producing strains of *Lactobacillus salivarius* in the reduction of intestinal infection by *Listeria monocytogenes* was unequivocally demonstrated *in vivo* [53]. A two-component lantibiotic called lacticin 3147, produced by a strain of *Lactococcus lactis* originally isolated from an Irish kefir grain [54], is active against *C. difficile* at low concentrations and physiological pH [55].

However, it was subsequently observed that this bacteriocin has a massive impact, comparable to that of metronidazole and vancomycin, on the normal gut microbiota [56].

In an effort to isolate narrow-spectrum bacteriocins effective against *C. difficile* but with a low impact on gut microbiota, Rea *et al*. screened more than 30,000 bacterial isolates from faecal samples [57]. The screening resulted in the identification of thuricin CD, a new two-component bacteriocin produced by *Bacillus thuringiensis*, which is a spore-forming Gram-positive organism used in agriculture to control insects harmful to crops. The two peptides that make up the molecule of thuricin CD (Trn-α and Trn-β) are active at concentrations of 5 μM and 0.5 μM, respectively. However, the activity is greatly enhanced when both peptides are present, reducing the MIC_{50} to nanomolar values with an optimal 1:2 ratio of Trn-α:Trn-β. Following a series of *in vitro* tests against a broad range of Gram-positive and Gram-negative bacteria, thuricin CD showed a spectrum of activity mainly restricted to a set of spore-forming Gram-positive bacteria including clinically significant and hypervirulent *C. difficile* isolates, with a negligible impact on the intestinal flora [56]. In an *ex vivo* model of the distal colon, thuricin CD compared very favourably with metronidazole, suggesting that it could be used in CDI therapy, provided that its biological activity is protected from proteolytic enzymes by encapsulation technologies to ensure the delivery of biologically active peptides to the colon [58]. The posttranslational modifications found in thuricin CD are unusual, and had not previously been associated with two-peptide bacteriocins. Trn-α and Trn-β both possess three intrapeptide sulphur to α-carbon bridges, an unusual trait resulting from post-translational modifications performed by the TrnC and TrnD enzymes. These enzymes belong to the family of radical S'-adenosylmethionine (SAM) proteins, that catalyze unusual reactions involved in the biosynthesis and degradation pathways of DNA precursors, vitamins, cofactors and antibiotics [59]. Radical SAM protein-encoding genes are rare in bacteriocin-associated clusters but an *in silico* screen for novel thuricin CD-like gene clusters using the TrnC and TrnD radical SAM proteins as driver sequences allowed the identification of fifteen novel thuricin CD-like gene

clusters [60]. This instance shows the possibilities offered by genomic mining of known operons to open new horizons in the field of antimicrobial discovery.

3.3 Host defence peptides (HDPs)

HDPs are components of the host innate defence against pathogens. In mammals, they also participate in the modulation of the specific immune response. Among HDPs, the cationic, cysteine-rich defensins constitute one of the most thoroughly investigated families, which includes many structurally related peptides found in vertebrates, fungi, plants and insects [60]. In humans, defensins contribute to maintain a stable commensal microbiota in the intestinal tract, preventing bacterial overgrowth. It is hypothesized that reduced defensin concentrations compromise host defence and predispose to inflammatory bowel disease [61]. Many mammalian HDPs modulate cytokine production and lower the local inflammatory response by sequestering bacterial lipopolysaccharides and lipoteichoic acids, so preventing their interaction with the Toll-like receptors [62].

Human α-defensins can affect CDI severity by inhibiting *C. difficile* toxin B activity. The human α-defensins HNP-1, HNP-3, and HD-5 prevent the cytotoxic effects of toxin B in the intestinal epithelial cells and in a large array of other cell types [63]. It has been estimated that HD-5, a defensin produced by Paneth cells, might be present in the lumen of small intestine at concentrations of 50-250 μg/mL, which would be sufficient to block the action of toxin B. Whether the low virulence of *C difficile* in the small intestine is related to the occurrence of toxin-inactivating peptides remains to be clarified [63]. However, for now the development of human defensins for therapeutic use has been hindered by production difficulties, cellular toxicity, and concerns about the possible dysregulation of the gut cytokine milieu [64,65].

A cDNA encoding coprisin, a defensin-like peptide, was identified in a bacteria-immunized dung beetle, *Copris tripartitus*, by using differential dot blot hybridization [66]. The core structure of invertebrate defensins is composed of an α-helical domain linked to a two-stranded antiparallel β-sheet with three or four disulphide bonds forming the so-called cysteine-stabilized α-helix β-sheet

motif. Defensin bacterial killing is mediated by membrane disorganization, which takes seconds to minutes, and/or by the binding to intracellular targets, which takes more time (3-5 hours). Neither of the two mechanisms is receptor-based, consistent with the finding that D-peptides are generally as active as L-peptides [67]. The analysis of the natural coprisin peptide, consisting of 43 amino acids, showed that the antibacterial activity resides in the α-helical domain of the molecule. Since D-enantiomeric peptides are known to be extremely resistant to proteases, both D- and L-enantiomeric analogues based on this domain were synthesized [68]. *In vitro* antibacterial assays demonstrated that both enantiomers were almost as active as vancomycin against *C. difficile*, but, unlike vancomycin, they did not inhibit *Lactobacillus* and *Bifidobacterium* species. The activity of coprisin consists in a selective alteration of the plasma membrane of *C. difficile* but not of *Lactobacillus* and *Bifidobacterium* cells, resulting in a significant inhibition of *C. difficile* growth *in vitro*.

These results, together with the observation that in a mouse CDI model the oral administration of a coprisin analogue improved survival rates and diminished inflammatory responses and weight loss, suggest that coprisin analogues could be useful against *C. difficile*. The observation that the coprisin analogue did not prevent inflammation caused by the injection of purified toxin A into the ileal lumen suggests that the coprisin anti-inflammatory activity observed *in vivo* is associated with *C. difficile* growth inhibition rather than with toxin inhibition [68].

It is worth noting that a genetic locus responsible for resistance to AMPs was identified in *C. difficile* by selecting the CD1352 mutant, which is resistant to nisin [69]. The mutation involves the *cprK* gene, which encodes a histidine kinase regulating a nearby putative ABC transporter operon named *cprABC*. These genes share similar sequences with the lantibiotic immunity systems found in lantibiotic-producing bacteria, suggesting that resistance to CAMPs is accomplished through the export of the peptides by the ABC transporter. The finding is quite unusual, for at least two reasons: i) the mutant strain is resistant to nisin and gallidermin and, to a lesser extent, to polymyxin B, whereas typically lantibiotic immunity mechanisms are highly specific for individual

peptides; ii) the *C. difficile cprABC* and *cprK* genes are not associated with a lantibiotic synthesis gene cluster. A homology search for the *cprABC* operon revealed homologs of these genes in all of the other *C. difficile* isolates sequenced to date, suggesting that this operon encodes a universal mechanism of CAMP resistance for the species. Transcriptional analysis of the ABC transporter genes revealed that this operon was upregulated in the presence of nisin in wild-type cells and was more highly expressed in the CD1352 strain. Results obtained with a *cprABC*-disrupted mutant suggest that other yet unidentified CAMP resistance mechanisms must be present in *C. difficile* [69]. As a whole, these data offer new insights into the complex net of interactions between *C. difficile* and the other bacteria comprising the gut microbiota, namely lantibiotic-producing strains, and open unexpected fields of investigation in terms of new targets for therapeutic interventions.

4. Riboswitch and quorum sensing targeting

4.1 Riboswitch-mediated modulation of gene expression

Riboswitches, usually located in the 5′ untranslated regions of mRNAs, are RNA sequences that include two functionally distinct domains: the aptamer, and the expression platform. Following the interaction of the aptamer with a specific ligand, the expression platform undergoes a structural change that affects the expression of the adjacent open reading frame (ORF), usually by transcription attenuation and/or ribosome binding site (RBS) sequestration [70]. Thanks to the complexity of the aptamer, whose secondary and tertiary structure can be compared to that of the proteins, the aptamer-ligand interaction is characterized by a high grade of specificity.

To date, at least 20 distinct classes of riboswitches that recognize small-molecule metabolites, divalent cations, or second messengers are reported [71]. Metabolite-sensing riboswitches control gene expression in most bacteria, plants, and fungi [72]. In many bacteria riboswitches are the receptorial component of a feedback mechanism that regulates the expression of genes involved in metabolite biosynthesis or transport. When a metabolite is present at a sufficiently high concentration, its binding to the aptamer represses the ORF expression [73].

In the past few years, computational virtual screening methods based on information about RNA-ligand interactions yielded new RNA binding scaffolds. Since riboswitches evolved as small molecule receptors and are present almost exclusively in bacteria, they are considered good targets [74]. Indeed, the elucidation of the previously unknown mechanisms of action of some antimicrobials such as roseoflavin, pyrithiamine, L-aminoethylcysteine and DL-4-oxalysine, showed that these molecules are riboswitch-targeting compounds [74]. The potential of riboswitches as drug targets is highlighted by a recent paper from Ster *et al*., who demonstrate the effectiveness of a guanine riboswitch ligand analogue in a model of *S. aureus* experimental mastitis in cows [75].

Riboswitches binding the bacterial second messenger cyclic di-guanosyl-5′-monophosphate (c-di-GMP) (Fig. 4, 10) control the expression of genes involved in bacterial pathways affecting virulence, competence, biofilm formation, and synthesis of flagella. An allosteric ribozyme consisting of a c-di-GMP-sensing riboswitch and a group I self-splicing ribozyme was recently identified in *C. difficile* 630 [72]. The proximity of this allosteric ribozyme to the *CD3246* ORF suggests that the coenzyme-mediated regulation of splicing controls the expression of this gene [70].

The knowledge of virulence factors involved in *C. difficile* adaptation to the intestinal environment, toxin production, and resistance to antimicrobials is still scarce. Available data support the hypothesis that *C. difficile* production of flagella contributes to pathogenicity [76,77]. Observations made by Sudarsan *et al*. [78] suggest that *C. difficile* uses a Cd1 riboswitch to regulate transcription of the flagellar protein operon to control motility in response to c-di-GMP signalling. Moreover, a number of conserved genes encoding proteins involved in the synthesis, degradation, or sensing of c-di-GMP were identified in the *C. difficile* genome. This discovery highlights the importance of c-di-GMP signalling in the lifecycle of this pathogen [79]. To determine the functions of c-di-GMP in *C. difficile*, Purcell *et al*. [76] ectopically expressed an active c-di-GMP synthetase or phosphodiesterase to increase or decrease intracellular c-di-GMP, respectively. Results demonstrate that in *C. difficile* 630 intracellular levels of c-di-GMP are inversely related to motility, and suggest that relatively small changes in c-di-GMP can alter motility. The recent finding that

toxin production is regulated by the flagellar regulon adds complexity to the system and supports the importance of further investigation on this issue [80]. Elevated c-di-GMP levels also induced *C. difficile* clumping *in vitro*, suggesting that *C. difficile* can form biofilms in the host [76]. These data are worthy of further investigation, because nothing is known on *C. difficile* biofilm formation in humans. In a recent paper Ethapa *et al*. [77] report that biofilm formation by *C. difficile* has been obtained *in vitro*. These authors found that the process is complex and multifactorial, and suggest that it might be a crucial mechanism for clostridial persistence in the host. Other authors, who investigated the *in vitro* biofilm formation by *C. difficile*, found that bacterial aggregation increases when a mixed flora is present [81]. The possibility of a biofilm-like growth that could maintain long-term colonization in the bowel is suggested by the finding of large aggregates of *C. difficile* cells, described as exaggerated mats, in experimentally infected mice [82]. Biofilm formation could be linked to resistance to antibiotics and occurrence of relapses. The ability of c-di-GMP to regulate *C. difficile* motility and aggregation makes this molecule a key player in CDI pathogenesis and a good starting point to design riboswitch-targeting drugs. Recently, riboswitches derived from the pathogenic bacteria *Vibrio cholerae* and *C. difficile* were used to characterize c-di-GMP analogues. Early findings support the possibility to design novel compounds that target c-di-GMP riboswitches [83].

4.2 Quorum sensing inhibition

Quorum sensing (QS) is a term used to describe a kind of density-dependent bacterial inter-cellular communication based on the constitutive production and secretion of small chemical pheromones also defined quorum sensors or autoinducers (AI). When the AI concentration reaches a critical detection threshold that depends upon bacterial population density (quorum), the AI interaction with species-specific receptors triggers a signal transduction cascade leading to an alteration in gene expression. Such multicellular coordination is essential for successful colonization/infection of the host by many pathogenic bacteria, because it leads to the expression of virulence factors, or biofilm

formation, or both [84]. In Gram-negative bacteria the prototype AI is N-acyl-homoserine lactone (AHL) (Fig. 5, 11), typically synthesized by enzymes belonging to the LuxI family, whereas in Gram-positive bacteria the AI are often small post-translationally modified peptides. In the late 1990s a novel autoinducer, AI-2, was identified in *Vibrio harveyi* cultures, and its production was successively detected in a number of both Gram-negative and -positive bacteria [85]. The AI-2 results from the activity of the *luxS* gene, whose homologues were identified in about one third of the bacterial genomes so far sequenced, including *C. difficile* [86]. AI-2-containing cell-free supernatants from mid-log phase *C. difficile* upregulated the transcript levels of tcdA, tcdB, and tcdE in early-log phase growth *C. difficile*. However, AI-2 did not significantly increase the production of toxin A protein in early-log *C. difficile*. These results suggest that LuxScd-dependent signalling regulates virulence gene expression at the transcriptional level in *C. difficile* [87]. In their study on *C. difficile* biofilm formation, Ethapa et al. observed that a *luxS* mutant strain, contrary to the biofilm-producer wild type strain, was unable to form even a bacterial monolayer [77]. These results strengthen the hypothesis that *C. difficile* possesses a *luxS*-mediated QS system, which may have a role in biofilm formation.

In 2004 Kaufmann *et al*. [88] discovered that an AI produced by *Pseudomonas aeruginosa*, i.e. the N-(3-oxododecanoyl) homoserine lactone (Fig. 5, 12), and its tetramic acid degradation product, 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (Fig. 5, 13), are potent antibacterial agents active against a set of Gram-positive bacteria in the ng/ml range. Starting from this observation, Ueda *et al*. [89] investigated the activity of N-(3-oxododecanoyl) homoserine lactone and of tetramic acid derivatives against *C. difficile*. Their results highlight a correlation between the antibacterial activity and the length of the acyl side chain of tetramic acid. These molecules can be considered potent (effective at 30-50 μM), fast acting (30 min exposure) compounds, whose action likely involves membrane destabilization. However, since they are inhibited by metal cations, their potential in CDI therapy requires confirmation by experiments performed in *in vivo* CDI models.

5. Phage therapy

5.1 Bacteriophages

In the 1910s, d'Herelle realized the viral nature of bacteriophages and promoted their use in the treatment of bacterial infections [90]. In Russia and Georgia, phage therapy became a common treatment for a wide range of infections, and was never abandoned. Phage cocktail preparations, at present mainly produced by Microgen, are available at pharmacies as over-the-counter products, mostly used for diarrheal diseases and pyogenic infections [91]. In Poland, phage therapy is considered an 'Experimental Treatment', which can be used when other therapeutic options do not exist. In France, made-to-order phage preparations from the Institut Pasteur were used until the beginning of the nineties [92]. In other Western countries, phage therapy was declined in the 1940s and its development was abandoned after World War II, mainly because of the success and availability of antibiotics, but also because of treatment failures due to the poor understanding of phage biology and the lack of adequate quality control procedures.

The global emergence of resistance to antibiotics, and the low number of drugs based on new molecular scaffolds currently under development by pharmaceutical firms evoke the spectre of a post-antibiotic era [93]. Despite the wide-ranging debate and persistent different opinions, the development of phage therapy, together with antibacterial vaccines, immunostimulants, and antivirulence therapies is by now considered a high priority approach worthy of research and development [94]. The number of papers that support the use of phage therapy reporting controlled trials on animals and humans is significant and rapidly increasing [95-104]. Exhaustive reviews of the clinical experience with phage therapy at the Eliava institute of Tiblisi (Georgia) and at the Hirszfeld Institute of Wroclaw (Poland) are now available in English [105,107].

To get an idea of the pros and cons of phage therapy the reader is referred to the up to date report by Brüssow [91] on the debates held at recent international conferences on the subject, and to [108]. Based on the experience gained in Eastern countries, best results can be achieved by using

customized cocktails of phages selected among those able to grow on bacteria isolated from the patient. Phage therapy centres in Georgia and Poland keep collections of phages, which are constantly updated every six months by selecting mutants that are more active. The quality-controlled procedure for the production of a bacteriophage cocktail to be used in clinical trials has been described in detail [96]. Phage suspensions can be standardized and patented, and can undergo the validation process based on preclinical and clinical trials. Custom phage preparations may also be developed on request for a single patient, but in this case the product cannot be patented or tested before use and it is not compatible with the current licensing rules. In order to make phage therapy research and development more attractive to pharmaceutical firms, and to allow its exploitation at its best, new regulatory frameworks are needed both in Europe and in the U.S [109,110].

Today, in Western countries the field of phage therapy for CDI has yet to be explored. To our knowledge, only two experimental studies were published on the direct action of lytic bacteriophages on *C. difficile*: the first one, that goes back to 1999, reports on experiments performed in a model of hamster infection, whereas in the second one, published in 2010, an *in vitro* batch fermentation model of a *C. difficile* colonised system was used [111,112]. The results of both studies concur to indicate that phages might be an excellent option for CDI treatment and prevention.

Currently, Novolytics Ltd. (Warrington, UK), a company whose mission is to exploit phage technology to overcome resistance to antibiotics, is developing topical formulations of a bacteriophage cocktail against MRSA, and has a commercial collaboration with a leading UK university to design a new phage cocktail targeted at *C. difficile* [113]. In Bangladesh, Nestlé is currently recruiting for a clinical trial to assess safety, tolerability and efficacy of the oral administration of T4 phages in young children with diarrhoea due to enterotoxigenic and/or enteropathogenic *E. coli* infections [35].

In Western countries, the adoption of phage therapy is thwarted by safety concerns, because of the lack of formal and well-controlled large-scale clinical trials. These concerns are mainly

related to three aspects: i) bacterial, pyrogen or toxin contamination of phage preparations; ii) the possible manifestation of the Herxheimer effect due to the rapid and massive phage-induced bacteriolysis; iii) the transfer of bacterial genes encoding virulence factors or antibiotic resistance by generalised transduction [110]. These concerns can be overcome, considering that modern purification and control techniques make the release of toxic preparations highly unlikely, the release of endotoxin can be prevented by appropriate bacteriophage engineering [114], and the emergence of phage-resistant strains with increased virulence is an extremely unlikely event.

On the other hand, phage therapy has properties that in our view make it particularly suitable for CDI treatment and prevention: i) the extremely narrow spectrum of action; ii) the lack of adverse effects on the host and the possibility to be used in patients allergic to classic antibiotics; iii) the fact that a single oral dose may be sufficient; iv) the rapidity of action. Moreover, the lack of interference with antibiotics makes phages good candidates for combination therapies, and the oral administration removes concerns regarding systemic toxic side effects.

To achieve the best results with phage therapy, it is mandatory to isolate the causative agent of the primary infection and to screen it *in vitro* against a library of phages to select the most effective. The isolation of *C. difficile* from infected subjects is more difficult than toxin detection, but the availability of new culture media that allow bacterial detection in 24 hours with high sensitivity is going to facilitate the task [115]. Given the low overall impact on patients, phage therapy might be used not only for the therapy of overt CDI, but also for the prevention of CDI onset and recurrences.

5.2 Endolysins

Bacteriophage endolysins (lysins) are two-domain proteins that perform two basic functions: substrate recognition and enzymatic hydrolysis of bacterial peptidoglycan. The N-terminal domain harbours the enzymatic activity, whilst the cell wall-binding domain located at the C-terminal directs the enzyme to its substrate [116]. Lysins were originally developed to control mucous

membrane infections, on the assumption that they can lyse Gram-positive bacteria from the outside, whereas the outer membrane of Gram-negative bacteria prevents their direct interaction with peptidoglycan [117,118]. Lysins have a short half-life (15-20 min), but their action is so rapid that *in vitro* nanogram quantities kill sensitive Gram-positive bacteria in seconds after contact. *In vivo* experiments, performed on a murine model of nasopharyngeal pneumococcal colonization, showed that five hours after the local treatment with a purified lysin the number of *S. pneumoniae* CFU recovered from infected animals was reduced to almost undetectable levels [119]. Moreover, lysins are *per se* non-toxic and, unexpectedly, not easily inactivated by antibodies [120]. Since the peptidoglycan, which is the lysin target, is not present in eukaryotic cells, it can be expected that lysins will be well tolerated by humans.

The potential of lysins as therapeutic tools against CDI was investigated at the Institute of Food Research (Norwich, UK) by researchers of the Mayer group, who identified and characterized an endolysin, called CD27L, derived from a *C. difficile*-infecting bacteriophage [121]. Subsequently, a structure-activity analysis of CD27L demonstrated that molecular modifications affect the level of activity and/or host range [122]. These results provide a sound basis for further developments, considering that the lysin approach may be more attractive to pharmaceutical firms than the whole-phage approach, in terms of better marketing and patenting opportunities.

6. Probiotics, prebiotics and faecal biotherapy

6.1 Probiotics

Probiotics are defined as “live bacteria having a beneficial effect on the host when consumed in adequate amounts” [123]. They act by modulating the gut microbiota, by maintaining the integrity of the gut barrier, and by modulating the local immune response [124]. These effects are supposed to rely on various mechanisms such as bacteriocin production, competition for available nutrients, and modulation of the gut cytokine production. Probiotic consumption is considered generally safe, and complications rare. A review on probiotic safety evidences that, although cases of bacteremia and

endocarditis as well as cases of fungemia (*S. boulardii*) are described in the literature, there is no overall increase in population risk based on usage data [125]. However, a placebo controlled study on the effect of a multispecies probiotic including six different strains of freeze-dried, viable bacteria (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus salivarius, Lactococcus lactis, Bifidobacterium bifidum,* and *Bifidobacterium lactis*), administered to severe acute pancreatitis patients, showed that mortality in the probiotic-treated group was about twice as high as in the placebo group, with a higher incidence of mesenteric ischemia [126]. Although to date this is the only trial to infer such a relationship, it is enough to suggest that probiotics should be avoided in critically ill patients. A recent study commissioned by the U.S. Agency for Healthcare Research and Quality reiterates the apparent safety of probiotics when used to prevent or treat diseases but acknowledges limited safety reporting of existing studies. Accordingly, a recent position paper issued by the U.S. FDA clarifies that its limited oversight of probiotics as a "food" applies only to the ingestion of these agents by healthy individuals to maintain gastrointestinal health. This document also asserts that the use of probiotics to prevent, treat, or mitigate disease would classify these agents as "drugs" and would require the same stringent approval process as any pharmaceutical product or device [127]. Probiotics are not recommended for CDI in the 2010 SHEA/IDSA treatment guidelines. [128] However, a placebo-controlled clinical trial assessed the efficacy of *Lactobacillus acidophilus + L. casei* capsules in the prophylaxis of both antibiotic-associated diarrhoea and *C. difficile*-associated diarrhoea [129].

Several bacterial and fungal species were studied or are currently under study to determine their efficacy against CDI either as single probiotic agents or in combination with other agents. These agents include *Saccharomyces boulardii* and several *Lactobacillus, Clostridium, Streptococcus,* and *Bifidobacterium* species. *S. boulardii* and *Lactobacillus rhamnosus* GG are two of the best characterized probiotic organisms for use in CDI. A meta-analysis of the efficacy of *S. boulardii* performed in 2010 points out that this yeast may have a favourable impact on CDI prevention, based on the following evidence: i) it produces a serine protease that directly degrades

C. difficile toxins; ii) it destroys the colonic receptor site for *C. difficile*; iii) it upmodulates the immune response to toxin A and B. Furthermore, the treatment with *S. boulardii* was effective in several experimental models of CDI in hamsters, gnotobiotic mice, rats, and turkeys [130]. However, the usefulness of *S. boulardii* in CDI is still debated. In a review of the available literature published in 2009, Miller concluded that probiotics, *S. boulardii* included, do not have a role in CDI prevention or therapy [132]. However, since 1989, sporadic human cases or small case series reports concur to the view that *S. boulardii* shows promise for CDI prevention, and a recent paper by Johnson *et al*. suggests that the primary prevention of CDI with probiotics may be achievable [131]. In conclusion, most authors state that more clinical trials of sufficient size and with rigorous design are needed to confirm these findings and to develop consistent treatment protocols.

In 2011, Cartman advocated the development of new generation of probiotics based on *Clostridium* species [133]. In fact, since 1985 it is known that intestinal colonization with a nontoxigenic *C. difficile* strain protects hamsters against a challenge with toxigenic *C. difficile* [134]. Similar experiments performed in 2009 in the hamster model showed that colonisation by non-toxigenic *C. difficile* during antibiotic administration is an effective prevention strategy against infection with toxigenic strains [135].

The mechanism by which nontoxigenic strains prevent colonization by toxigenic *C. difficile* strains has not been elucidated, but the use of nontoxigenic *C. difficile* spores to prevent primary or recurrent CDI is an attractive strategy [136].

In a controlled study, the co-administration of spores of *Clostridium butyricum* MIYAIRI, commonly sold in Japan as tablets for balancing the intestinal flora, significantly reduced the incidence of antibiotic-associated diarrhoea in children [137]. Recently, ViroPharma Inc. (Exton, PA, USA) completed a phase I clinical trial showing that the administration of an oral suspension of spores of the nontoxigenic *C. difficile* strain M3 (VP20621) is well tolerated and effectively induces

the colonization of the gastrointestinal tract of subjects pretreated with vancomycin [138]. The preparation is now scheduled for a phase II trial.

6.2 Prebiotics

Since the late 1990s, the word prebiotic has been used to indicate "dietary substances that by inducing specific changes in the composition and/or activity of the gastrointestinal microbiota confer benefits upon host health" [139]. Today, prebiotics are considered potentially useful tools to manipulate the microbiota composition. Non-digestible oligosaccharides like the chicory fructans reach the caecum without undergoing structural changes, but they are not found in the stools, being metabolized by the colonic flora. Their ability to increase the number of bifidobacteria and lower the intraluminal pH interferes with the engraftment of incoming pathogenic germs. In addition to this properly demonstrated effect, other less evident functions of prebiotics affect the bioavailability of minerals and the metabolism of lipids, resulting in potential subtle benefits on a variety of pathological conditions including intestinal infections, cardiovascular disease, type II diabetes, obesity, osteoporosis and cancer [140,141]. A study performed on normal and antibiotic-treated faecal microbiotas *in vitro* showed that the addition of nondigestible oligosaccharides enhanced resistance against *C. difficile* colonization in antibiotic-free, but not in clindamycin-treated cultures [142]. In a randomized study on 72 infants, the administration of fructo-oligosaccharides had no significant impact on intestinal flora and *C. difficile* counts or toxin detection in faeces [143]. These results indicate that the research in this field is still at a very basic level.

6.3 Faecal biotherapy

The rationale of faecal biotherapy, or faecal transplantation (FT), relies on the observation that normal colonic flora controls *C. difficile* overgrowth. The transplant of the entire faecal ecosystem obtained from a healthy donor is highly effective, achieving 73-100% clinical resolution rates in recurrent or refractory CDI 140,141 [144,145]. The transplant can be delivered by nasogastric tube,

colonoscopy or retention enema. The last methodology is the less expensive and the less likely to cause injuries to patients [146]. Given the by now widely recognized effectiveness of FT, the major concerns on its use relate to the possible transfer of pathogens, especially viruses. Indeed, the donor screening is perhaps the most expensive part of the procedure. This problem could be overcome by the collection of multiple donations, which could be stored frozen, from a small number of thoroughly screened donors. The feasibility of this kind of procedure is supported by a recent paper by Jorup-Rönström et al. [147].

The main possible evolution of faecal transplant consists in bacteriotherapy, i. e. a treatment based on defined bacterial cocktails able to restore the physiological microbiota and to displace *C. difficile* from the gut. In 1989, Tvede and RaskMadsen reported that a mixture of ten different facultative aerobic and anaerobic bacteria was able to resolve *C. difficile* infection in a small number of human patients [148]. Perhaps the importance of this finding was not fully appreciated at the time, and there were no further developments. However, recently Lawley *et al*., working with a CDI mouse model, demonstrated the effectiveness of MixB, a mixture of six intestinal bacteria including three previously described species, *Staphylococcus warneri*, *Enterococcus hirae*, *Lactobacillus reuteri*, and three novel species, *Anaerostipes* sp. nov., *Bacteroidetes* sp. nov. and *Enterorhabdus* sp. nov., in the resolution of intestinal disease and contagiousness [149]. The protocol that was used to identify the six active bacterial species is rather complex and can be performed more easily in an animal model, but this work lays the conceptual and practical foundations for the development of bacteriotherapy. This approach overcomes the concerns about the psychological impact on patients, and is more attractive to the pharma industry, because bacterial cocktails could be patented.

7. Toxin inhibition

In principle, virulence factor targeting has the double advantage of avoiding the selection of resistant strains and the disruption of normal microbiota. The pathogenic effect of *C. difficile* is

mediated by toxins A and B, that share 45% sequence similarity and have four conserved regions: an N-terminal enzymatic region, a protease domain, a C-terminal receptor-binding region, and a translocation region. Following endocytosis by the cells of the intestinal mucosa, the toxins undergo a complex process in which the autocatalytic activity of the protease domain releases the enzymatic N-terminal region into the cytosol. This enzyme irreversibly glucosylates the RhoA family of small GTPases inducing cell apoptosis [150].

Various strategies have been explored to achieve intraluminal neutralization of *C. difficile* toxins, mainly by using antibodies or binding agents. For an exhaustive review of this issue, the reader is referred to [136]. Passive immunity with intravenous monoclonal antibodies targeting both toxins seem a promising approach [151 Gerding 2012]. In fact, in a phase II clinical trial the administration of monoclonal antibodies against *C. difficile* toxin A and B by i.v. infusion significantly reduced the rate of recurrences [152]. The active immunity option is also being actively investigated, and phase I trials demonstrated the antigenicity of toxoid-based vaccines [153].

Toxin-binding agents such as cholestyramine and colestipol or tolevamer showed poor efficacy both in *in vitro* models of human gut and in phase III clinical trials [154]. A different approach involves the identification of molecules able to selectively inhibit the enzymatic activities of the two toxins, i.e. the autocatalytic protease and the cytotoxic effector domain [155]. In this field, the identification of inhibitors of the cysteine protease of the B toxin [155] is a promising starting point, which support the validity of the approach and is worthy of further development. A problem that could arise with this category of drugs is that almost all of the protease inhibitors currently approved for anti-HIV therapy cause diarrhoea and hyperlipidemia [156]. Whereas hyperlipidemia may be a small concern, due to the short duration of CDI therapy, drug-induced diarrhoea could exacerbate the symptoms of the patients. Therefore, this problem should be taken into account in the development of protease inhibitors.

8. Expert opinion

The strategies to solve the CDI problem must cope with the multiple challenges posed by the bacterium. Since *C. difficile* is commonly found in wild and domestic animals, its spores are destined to remain in the environment indefinitely, and the eradication of the species is not feasible. Toxoid-based vaccines are being developed, and some of them are in phase I clinical trials [35]. One problem of strategy based on vaccines is to define the pre-emptive or therapeutic use of the vaccine, and the choice of subjects that should be vaccinated, considering that CDI has an incidence of 20-30/100,000 in Western countries. The focused, therapeutic administration of anti-toxin monoclonal antibodies, currently in phase II clinical trials, would probably be less expensive.

CDI onset could be prevented by the development and proper use of narrow-spectrum antibiotics, which should substitute the broad-spectrum drugs currently in use for common infections. However, huge advances in this field are unlikely, mainly because of low marketing opportunities. Specific anti-*C. difficile* narrow-spectrum antibiotics based on new molecular scaffolds and non inferior to fidaxomicin are being developed. In the next future, they should be able to lower the rate of recurrences and to overcome the problem of resistance,which by now is sporadically occurring against vancomycin and metronidazole.

More ambitious strategies aim to replace antibacterials with virulence-targeting factors. The research on molecules that target riboswitches and QS is still at a very basic level, whereas the field of AMPs is rapidly developing and bacteriocin research could profitably merge with probiotic optimization studies. Probiotics can be added as adjuvants to antibiotic therapy, but after more than ten years of use their utility is still debated.

In our view, phage therapy constitutes a tool that is worthy of a second chance in Western countries. It has the advantage of short term availability and does not require huge investments, thanks to the wide experience gained in France and in the East Europe countries. The isolation of lytic phages targeting *C. difficile* should not be a problem, considering that recently virulent phages against *C. perfringens* were easily isolated and characterized [157]. The association of phage therapy with antibiotics gives synergic results, but the ultimate goal should be to use antibiotics as the last

option, instead of the first, with positive outcomes on the resistance problem. For phage therapy to work at its best, the ideal clinical setting would include the possibility to rapidly identify and match the infecting bacterium to large phage bank databases. In order to achieve this result, the interdisciplinary cooperation of bioinformaticists, health care professionals, and phage researchers is needed [158]. The adoption of this approach in Western countries would require the modification of some regulatory guidelines in Western Europe and in the U.S., and, equally important, a change in the way of thinking of physicians and health policy decision-makers. This should involve a shift from the standard-oriented therapeutic approach towards a more personalized medicine in which specific products (i.e. phages or phage cocktails) are specially prepared for the treatment of an individual patient. Whereas the medical and scientific community is becoming aware of the problem of antibiotic resistance, in Western countries the knowledge of the possibilities of phage therapy is rather poor.

The situation, which in some respects resembles the difficulties that hindered the introduction of the first vaccines, is appropriately illustrated by a recent event: in 2011, Germany experienced the largest epidemic ever recorded due to Shiga toxin-producing *E. coli*. The genome sequences of the pathogen revealed a food-borne clonal outbreak due to the enteroaggregative *E. coli* O104:H4 strain. In these infections, the administration of antibiotics is counter-indicated because they can activate toxin expression. In addition, the epidemic strain was highly resistant to antibiotics, which left few options beyond supportive therapy. A total of 3,842 cases were reported, with 18 deaths due to *E. coli* gastroenteritis and 35 deaths due to haemolytic uremic syndrome [159]. Phages and phage cocktails targeting the O104:H4 strain were available at the Nestlé Research Center (NRC, Lausanne, Switzerland), at the Pasteur Institute in Paris and at the Eliava Institute (Tiblisi, Georgia). Neither the medical community nor the public health authorities inquired about or asked for these phages during the outbreak, that lasted about two months, and the offer of phages by NRC was not considered by the German public health sector [91]. We can attribute this kind of

reaction to the poor knowledge about phage therapy in the West, and assume that in Russia, in Poland or in France the emergency would have been handled with a different approach.

Severe refractory CDI and repeated recurrences could benefit from toxin neutralization, a possibility that needs further investigation. The best and already available option for the treatment of these often severe and otherwise hopeless conditions is FT. Despite the difficulties of psychological order, for now FT remains the most successful treatment for recurrences. It is technically less challenging and less risky than blood transfusion, and there are no major obstacles to its adoption.

Finally, we must say that both phage therapy and FT are therapeutic procedures inherently not suitable for patenting: as such, they do not attract investments for development and refining, and their implementation requires the commitment of publicly-funded institutions, national reference centres and hospital laboratories.

Article highlights

- Infectivologists are facing a world-wide increase of antibiotic-associated severe *C. difficile* enteric infections, whose management is currently far from satisfactory.
- New antibiotic-like molecules or antimicrobial peptides like thuricin CD, which has a very narrow spectrum of action and can selectively target *C. difficile* without disrupting the normal gut microbiota, are under development.
- More ambitious and long-term alternative strategies involve the development of non-antimicrobial molecules targeting the expression of virulence factors by acting on riboswitches or quorum sensing.
- Phage therapy could be an excellent alternative to antibiotics for both CDI prevention and treatment. It deserves a second chance in Western countries, where it is little known.

- Probiotic co-administration with antimicrobials may limit the incidence of CDI. However, their usefulness is still debated and they are not validated for the treatment of established CDI. The development of non-toxigenic *C. difficile* strains looks promising.
- Faecal therapy is emerging as the most successful treatment for severe refractory CDI and relapses. Standardized mixtures of faecal bacteria able to reconstitute the normal intestinal microbiota are under development.

Conflict of interest

The authors declare no conflict of interest.

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Papers of special note are highlighted as either of interest (●) or of considerable interest (●●) to readers.

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Figure legends

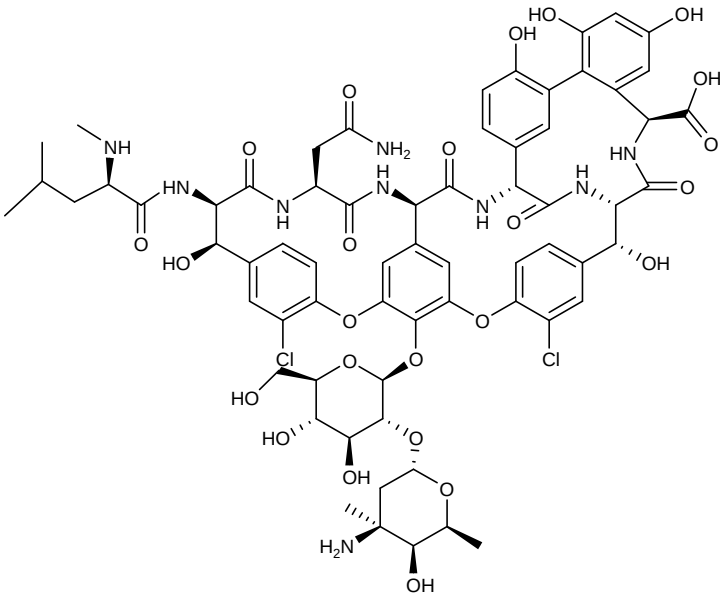
Fig. 1. Chemical structures of drugs approved for CDI.

Fig. 2. Chemical structures of antibacterial molecules active against *C. difficile* currently under development.

Fig. 3. Chemical structures of antibacterial molecules active against *C. difficile* currently under development.

Fig. 4. Chemical structure of the riboswitch ligand cyclic di-guanosyl-5′-monophosphate.

Fig. 5. Chemical structures of QS inhibitors.



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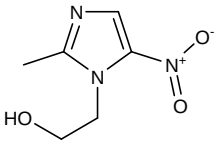

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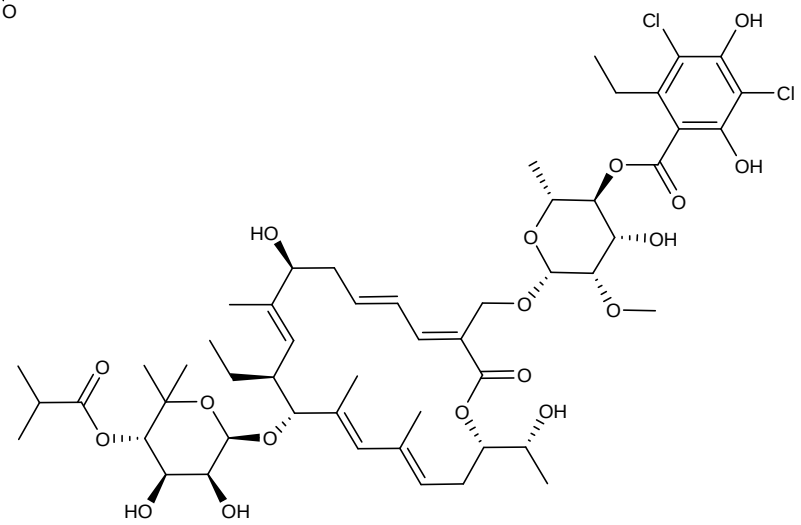
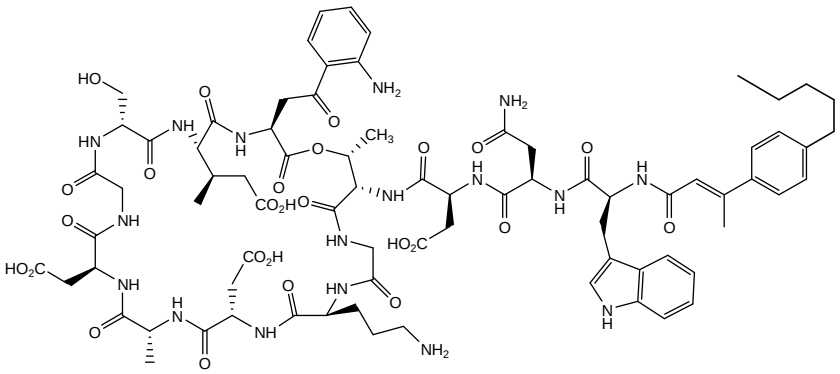


fidaxomicin (3)

Figure 1



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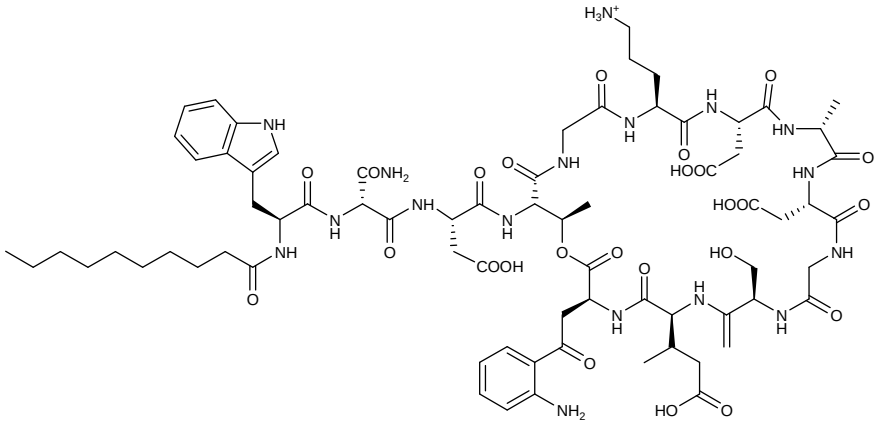

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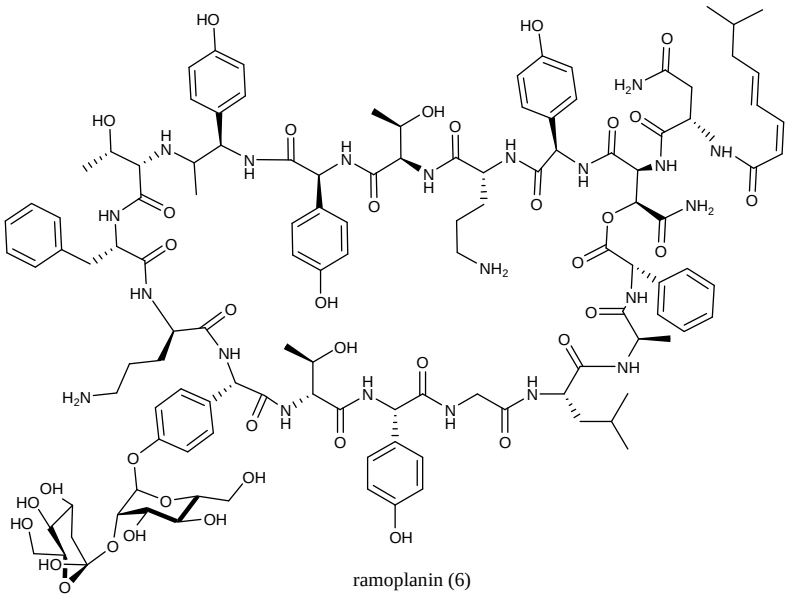
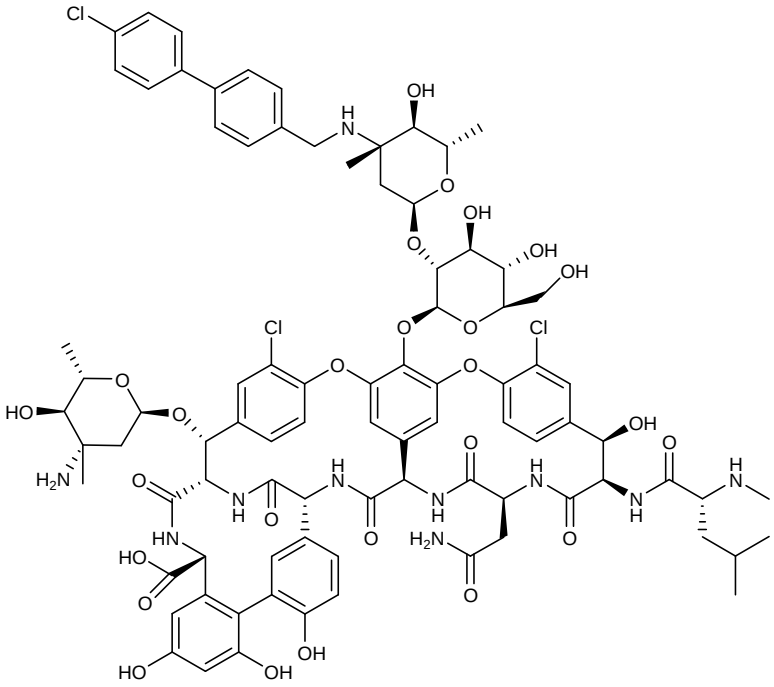


ramoplanin (6)

Figure 2



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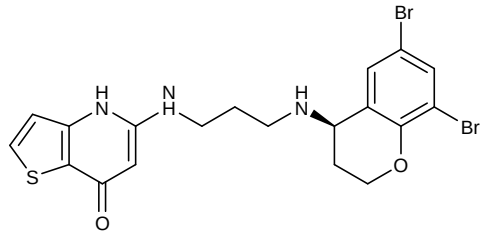

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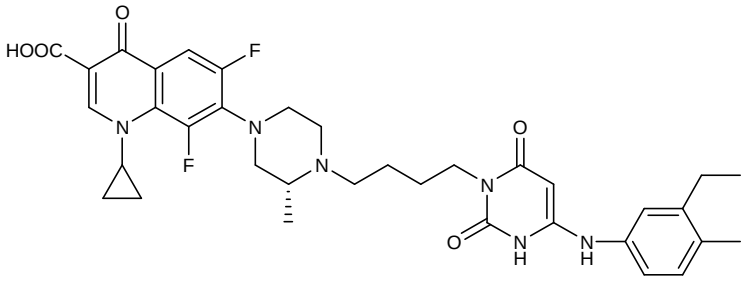

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Figure 3

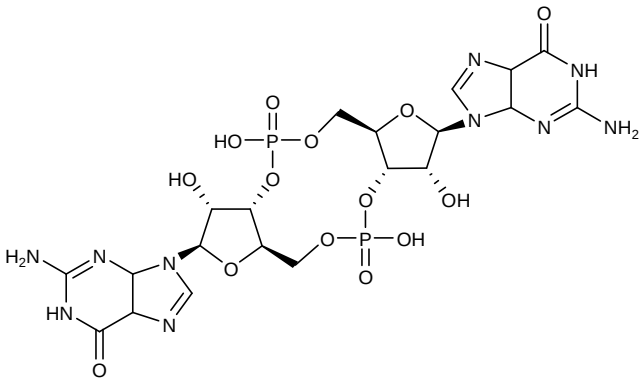

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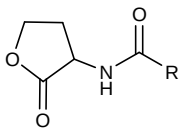
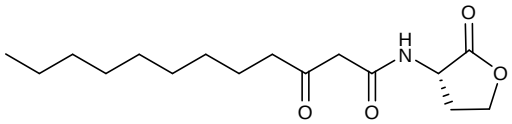
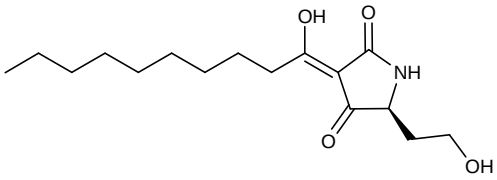
Figure 4



AHL (11)



3-oxo-AHL (12)



3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione (13)

Figure 5