Bioorganic & Medicinal Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

Review article

Discovery and development of DNA polymerase IIIC inhibitors to treat Gram-positive infections

Wei-Chu Xu[a,*], Michael H. Silverman[b], Xiang Yang Yu[b], George Wright[b], Neal Brown[b]

[a] Department of Chemistry, Worcester State University, 486 Chandler Street, Worcester, MA 01602, USA
[b] Acurx Pharmaceuticals LLC, 22 Camelot Court, White Plains, NY 10603, USA

ARTICLE INFO

Keywords:
ACX-362E
Antibiotic resistance
C. difficile
DNA polymerase
Gram(+) bacteria
Pol IIIC

ABSTRACT

Despite the growing global crisis caused by antimicrobial drug resistance among pathogenic bacteria, the number of new antibiotics, especially new chemical class of antibiotics under development is insufficient to tackle the problem. Our review focuses on an emerging class of antibacterial therapeutic agents that holds a completely novel mechanism of action, namely, inhibition of bacterial DNA polymerase IIIC. The recent entry of this new class into human trials may herald the introduction of novel drugs whose novel molecular target precludes cross-resistance with existing antibiotic classes. This review therefore examines the evolution of DNA pol IIIC inhibitors from the discovery of 6-(*p*-hydroxyphenylazo)uracil (HPUra) in the 1960s to the development of current first-in-class *N*7-substituted guanine drug candidate ACX-362E, now under clinical development for the treatment of *Clostridioides difficile* infection.

1. Introduction

The global threat of antimicrobial resistance (AMR) to existing antibiotics has become a growing concern.[1–4] In the US, Centers for Disease Control and Prevention (CDC) estimated in 2013 that over 2,000,000 people annually suffer infections with bacteria that are resistant to at least one antibiotic, and that more than 23,000 deaths can be attributed to resistant bacterial pathogens. The excess direct healthcare costs from resistant infections may be as high at $20 billion annually, with another $35 billion costs to society in lost productivity.[1] Worldwide, AMR is estimated to cause more than 700,000 deaths annually; unless the trend is reversed, this figure could grow to 10 millions by 2050, with > 4,000,000 deaths per year in both Africa and Asia, and > 300,000 each in Europe, North America, and South America.[2] A global economic model published in 2014 projected that over 35 years, AMR could lead to 300,000,000 premature deaths, loss of growth in gross domestic product of 2–3.5%, and loss of $60–100 trillion worth of economic output.[2]

In 2013, CDC categorized *Clostridioides difficile* (*C. difficile*), vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) as "Serious Threat" to the public health.[1,5] The United States government launched a yearlong AMR Challenge at the United Nations (UN) General Assembly in September 2018, and AMR will continue to be a priority topic at the 2019 UN General Assembly for world leaders.[6] Likewise, World Health Organization (WHO) classified AMR as one of the "Ten threats to global health in 2019"; and listed three carbapenem-resistant pathogens *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacteriaceae* as "Critical Priority"; *vancomycin-resistant Enterococcus faecium*, *Staphylococcus aureus* (methicillin-resistant and vancomycin-resistant) and other four pathogens as "High Priority" in 2017.[7,8] Driven by the epidemiology of emerging resistant infections and continuing unmet medical needs, new antimicrobial drug development is increasingly viewed as a priority.[9]

In the past few years, though, there have been encouraging signs that the antibacterial therapeutic pipeline may be expanding.[10,11] At the same time, gaps remain in the pipeline, and long-term success in staying ahead of emerging resistance is never assured. For the purposes of this review, three gaps are of particular relevance. First, almost all recently developed antibiotics work through well-known and well-exploited bacterial targets. Deak et al.[10] surveyed new antibiotics approved for US marketing between 2010 and 2015 and found only eight approvals; of these, seven drugs had mechanisms of action similar to previously marketed drugs and most were developed for clinical indications for which many adequate therapies already existed.

Second, recent development has focused principally on natural products and their semi-synthetic derivatives, as shown by the classes cited above and the compendiums listed in recent reviews.[9–11] This is especially concerning given the diminishing returns of natural product

* Corresponding author. Tel.: +1 508 929 8294; fax: +1 508 929 8171.
E-mail address: wxu@worcester.edu (W.-C. Xu).

https://doi.org/10.1016/j.bmc.2019.06.017
Received 22 April 2019; Received in revised form 7 June 2019; Accepted 9 June 2019

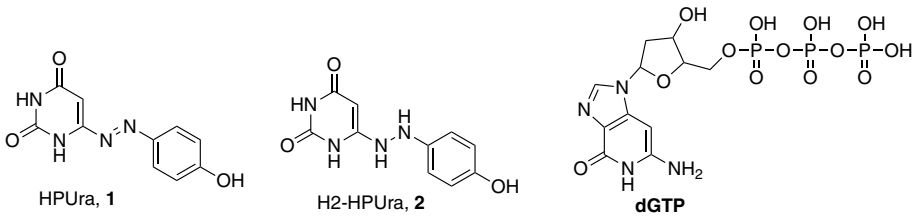


Fig. 1. H2-HPUra (**2**) structurally mimicking dGTP.

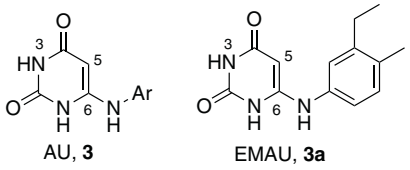


Fig. 2. Anilino-uracils (AUs).

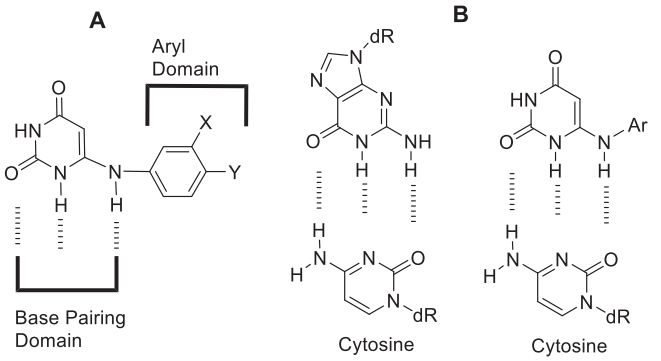


Fig. 3. (A) Relevant domains of the AU molecule. (B) Equivalence of the base-pairing domains of the dGTP (left) and AU (right) molecules.

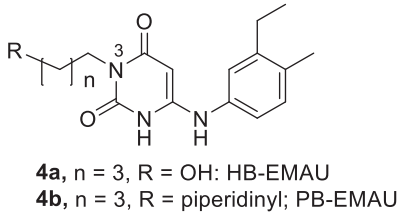


Fig. 4. *N*3-substituted EMAUs.

screening efforts and the need to establish new methods for establishing such screening platforms.[11–13] At the same time, it has been decades since any novel, synthetic, small molecule antibacterial therapeutic agents have been discovered.[11]

Third, the current drug development pipeline is heavily weighted towards anti-Gram-negative (Gram(−)) drugs.[8] Nevertheless, there are still critical and unmet needs in the treatment of infections caused by a variety of Gram-positive (Gram(+)) pathogens, as cautioned in the CDC and WHO lists.[1,8] Gram(+) organisms comprise 7 of the 18 urgent, serious, or concerning drug-resistance threats outlined in the 2013 CDC list; together, these organisms-including *C. difficile*, MRSA and VRE are estimated to cause more than 1.3 million infections and 34,000 deaths in the US annually.[1]

Against the latter background, our review focuses on a novel class of Gram(+) specific, synthetic antibacterial agents, that has emerged from several decades of research and development, with the first clinical candidate molecule, ACX-362E, now in a Phase 1 clinical trial. This class of molecules inhibit a specific target, DNA polymerase IIIC, which has not previously been exploited.

2. DNA polymerase IIIC and inhibitors

2.1. The Target: DNA polymerase IIIC

Gram(+) and Gram(−) eubacteria contain three major DNA polymerases: pol I, pol II, and pol III, named according to the order of their discovery. The pol III of Gram(+) eubacteria is now formally known as "pol IIIC", is essential for replication of the bacteria chromosome, is a highly conserved enzyme, and is unique to the low G + C class bacteria whose genomes contain < 50% guanine (G) + cytosine (C).[14] The members of this low G + C group include *Streptococcus, Enterococcus, Staphylococcus, Bacillus, Clostridioides, Pneumococcus, Listeria, Mycoplasma,* and *Lactobacillus*. Pol IIIC has little homology with mammalian polymerase, and is not found in either Gram(−) eubacteria or in the high G + C Gram(+) bacteria containing > 50% G + C (i.e., *Actinomyces, Mycobacterium, Streptomyces,* and *Corynebacterium*). These features make pol IIIC an ideal antibacterial drug target for low G + C Gram(+) bacteria.[15,16]

2.2. The evolution of pol IIIC inhibitors

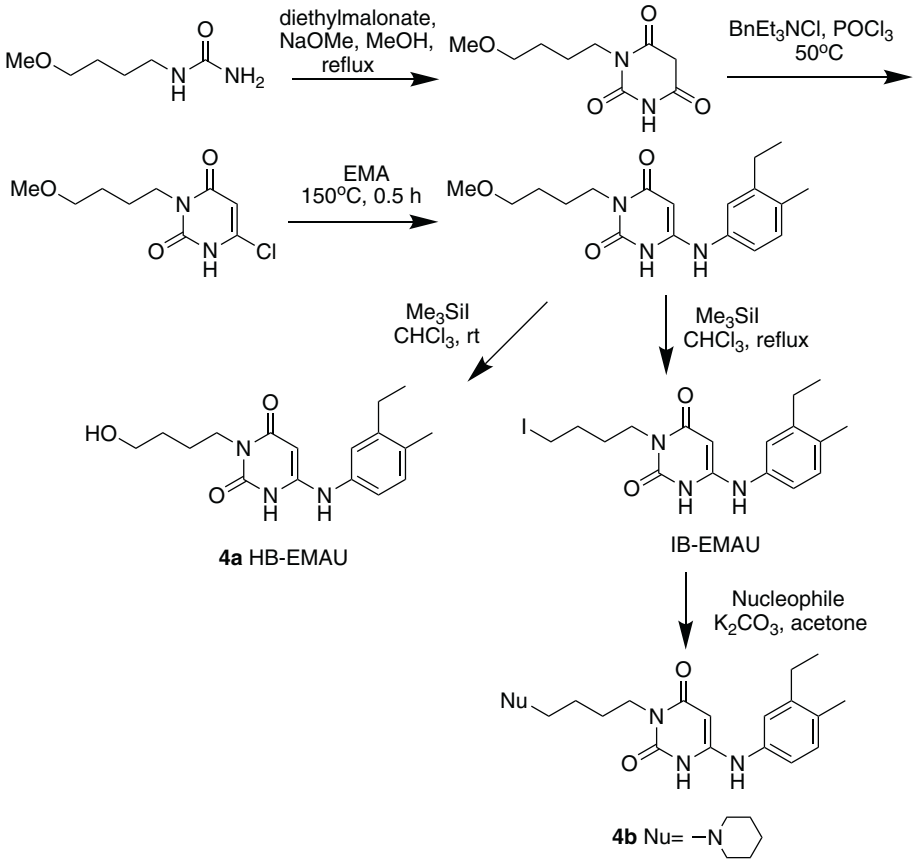
2.2.1. Origin and antibacterial properties of 6-(p-Hydroxyphenylazo)-uracil (HPUra)

The history of these pol IIIC specific inhibitors began in the pharmaceutical division of Imperial Chemical Industries (ICI, now AkzoNobel) in England, where HPUra (**1**), and a series of related 6-arylazopyrimidines were conceived and synthesized. ICI's unpublished research results indicated that these compounds shared strong and highly selective *in vitro* inhibitory activity against a large group of the "low G + C" class of Gram(+) bacteria, with little or no activity vs. cultured mammalian cells, Gram(−) bacteria or, the "high G + C" class of Gram(+) bacteria.

HPUra, although highly effective *in vitro*, had dye-like properties and other properties that precluded its clinical use in humans. As a result, ICI abandoned these arylazopyrimidines despite their potential as novel antimicrobials.[17,18] In 1966 Brown discovered that HPUra worked by selectively blocking Gram(+) bacterial DNA synthesis with no apparent effects on the synthesis of other cellular macromolecules such as RNA and protein.[17]

Table 1
Antibacterial activities (MICs) of selective pol IIIC inhibitors.

Compound	IC_{50} (μM)	MIC (μg/mL)						
	B. subtilis pol IIIC	*B. subtilis*	*S. aureus*	*MRSA*	*E. faecalis*	*E. faecium*	*VRE*	*E. Coli*
4a	0.063	< 1.25	5	5	5	2.5	2.5	> 20
4b	0.028	10	20	40	20	20	10	> 20



Scheme 1. Synthesis of *N*3-substituted uracils.

Table 2
In vitro and *in vivo* activities of EMAIPU (**5**) in rodent model of bacteria septicemia.

IC_{50} (μM)	MIC (μg/mL)			Survival, at dose	
	S. aureus	*S. pneumoniae*	*E. faecalis*	*S. aureus*	*E. faecalis*
0.1	16	8	8	3/6, IV 10 mg/kg	6/6, IV 5 mg/kg

In the late 1970's, Brown and Wright discovered the active form of HPUra, identified pol IIIC as its specific target, and elucidated the molecular mechanism of its action on the target.[19] The native form of HPUra (**1**) does not *per se* inhibit pol IIIC. The pro-drug molecule **1** must first be "activated" by reduction of its azo moiety (i.e., −N═N−) to the hydrazino (i.e., −NH−NH−) form **2** in the intact bacterial cell (Fig. 1).[20] The H-bonding moieties of **2** perfectly mimic those with which the guanine moiety of dGTP, the natural DNA polymerase substrate, binds to template cytosine residues.

2.2.2. Uracil derivatives

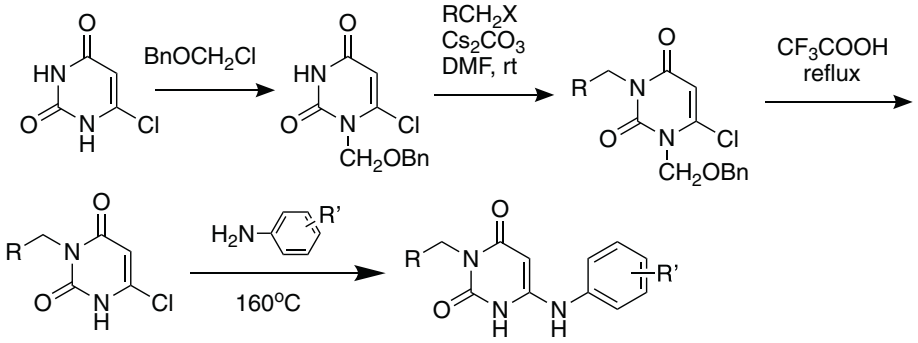
6-(Benzylamino)uracils and 6-anilinouracils (6-AUs, **3**) were also potent inhibitors of *Bacillus subtilis* pol IIIC, and they worked by a mechanism identical with that of **2**.[19–22] Examination of the effects of substituents on the phenyl ring of 6-(benzylamino)uracils and 6-AUs (Fig. 2) led to the discovery that part of the active site of pol III interacted with substituents in the phenyl ring.[21,22] The inhibitory potency was improved by inclusion of small alkyl groups or halogens in the *meta* and *para* positions of the phenyl ring, such as 3-ethyl-4-methyl-aniline (EMA). Qualitative structure-activity relationship (QSAR) study of the varied activities of 6-AUs concluded that the phenyl and pyrimidine rings were mutually perpendicular.[23] The potent AUs included 3′-ethyl-4′-methylanilino-uracil (EMAU, **3a**) with IC_{50} of 1.0 μM on *B. subtilis* DNA pol III.[24]

Structures and mechanisms of action showed that the AUs have two distinct pharmacophores essential for their inhibitory action (Fig. 3A).[25] One is the base-pairing domain comprised of three uracil-specific components: the ring 1-NH, the 2-keto and the 6-NH components. The other is the enzyme-specific domain contributed by an appropriately substituted aryl group at *N*6. The AUs derive their capacity to inhibit pol IIIC by mimicking the guanine component of the dGTP (Fig. 3B).[25]

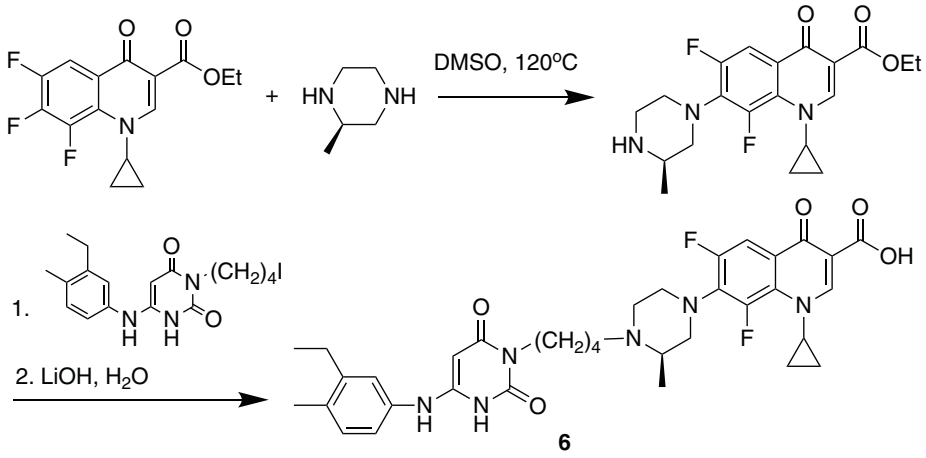
The prototypic AUs have either weak antimicrobial activities or unacceptably low aqueous solubility.[26] Substitution of the uracil's 5-H did not show any improvement in the inhibitory activity.[24] Substitution in the *N*3 position produced more potent and relatively more soluble inhibitors. *N*3-Substitution of the EMAU with hydroxyalkyl [R═HO$(CH_2)_n$−] improved MICs in the range from 0.5 to 15 μg/ml against a broad spectrum of low G + C Gram(+) bacteria, including MRSA and VRE.[25] Hydroxybutyl (HB-EMAU, **4a**), and piperidinylbutyl (PB-EMAU, **4b**) derivatives (Fig. 4) showed very potent *in vitro* antibacterial activities. Compound **4a** protected mice from *S. aureus* infection when administered at 10 mg/kg by IP. However, treatment by the intravenous (IV) or subcutaneous route was hampered by the low water solubility of the compound.[25,27,28]

Though **4b** and its derivatives showed significantly improved pol IIIC inhibitory activities (IC_{50} as low as 0.028 μM), the MICs could not be improved beyond a comparatively modest level of 2 μg/ml (Table 1).[28] This showed pol IIIC inhibitory activities (IC_{50}) do not always linearly correlate with the MICs, because factors other than target sensitivity can affect the antibacterial potency: for example, catabolism of the agent, poor penetration of the bacteria cell, and specific physicochemical parameters are of importance as well.

Zhi et al.[28] synthesized *N*3-substituted-uracils from *N*-(4-



Scheme 2. Improved Synthesis of *N*3-substituted uracils.



Scheme 3. Synthesis of AU-FQ hybrid compound.

Table 3
In vitro pol IIIC inhibitory and antibacterial activities (MICs) of **6** (MBX-500).

IC_{50} (μM)	MIC (μg/mL)						
B. Subtilis pol IIIC	*B. subtilis*	*S. aureus*	*MRSA*	*E. faecalis*	*E. faecium*	*VRE*	*E. Coli*
0.007	0.156	0.313	1.25	1.25	2.5	1.25	5

methoxybutyl)-urea as described in the following scheme. (Scheme 1). However, some of these steps required harsh reagents which were not compatible with some functional groups, and some steps required column chromatography to purify the reaction products.

Kuhl and Svenstrup et al.[29–31] described similar *N*3-substituted EMAU analogues with potent *in vivo* efficacy in murine models of bacterial septicemia. EMAIPU (**5**) inhibited the pol IIIC in the nanomolar range and displayed MICs against *Staphylococci, Streptococci*, and *Enterococci* at about 4 μg/ml. It showed no severe cytotoxicity in *in vitro* assay. The ED_{50} of **5** was 10 mg/kg after IV application in a *staphylococcal* sepsis model in mice (Table 2), but the pharmacokinetics of **5** in mice showed less favorable plasma clearances values.[30]

These *N*3-substituted uracil derivatives like EMAIPU were synthesized through an improved synthesis from 6-chlorouracil.[31] (Scheme 2).

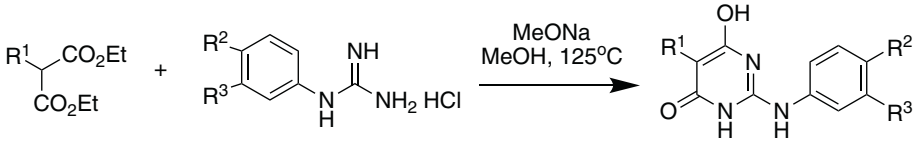
2.2.3. AU hybrids

Zhi et al.[32] in an attempt to improve the antibacterial spectrum and *in vivo* efficacy of the EMAUs, covalently linked the EMAU molecule to a fluoroquinolone (FQ) to create a "AU-FQ" hybrid (Fig. 6). Theoretically, hybridization of two antimicrobial drugs can yield several advantages: (i) The dual targeting can enhance antibacterial potency due to synergistic effects of the two components; (ii) They also may maintain their antibacterial activity against pathogens that are resistant to one of their two components; (iii) This property also should significantly reduce the frequency of developing mutants that simultaneously become resistant to both of the molecule's components.[33]

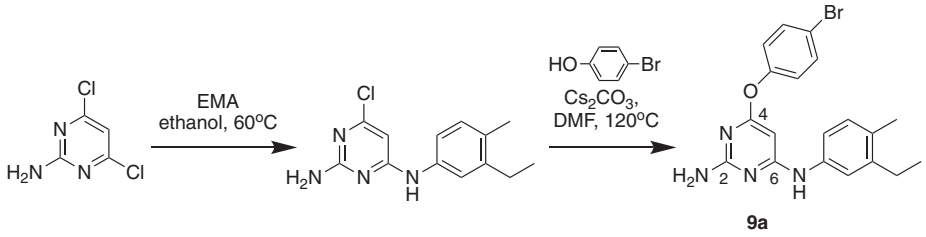
The "AU-FQ" hybrids were prepared as described in Scheme 3.[32] The lead hybrid compound **6** (MBX-500, Fig. 6), was more potent than the parent EMAU compounds as inhibitor of pol IIIC and as antibacterial against Gram(+) bacteria (Table 3).

Compound **6** also inhibited the FQ targets, topoisomerase IV and gyrase, with potencies similar to norfloxacin. It showed no resistance development in a single step passage, whereas the parent components did.[34] Compound **6** had a protective effect against several antibiotic-sensitive and resistant Gram(+) infections in mice.

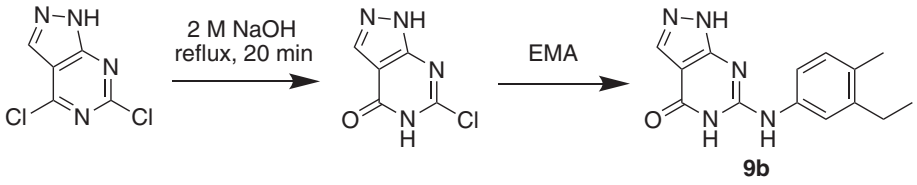
Treatment of *C. difficile*-infected gnotobiotic piglets with MBX-500 at low doses (100 or200 mg/kg twice daily) increased survival. At a higher dose (400 mg/kg twice daily), MBX-500 showed protection of all treated animals, resolution of diarrhea, and clearance of *C. difficile* in 4



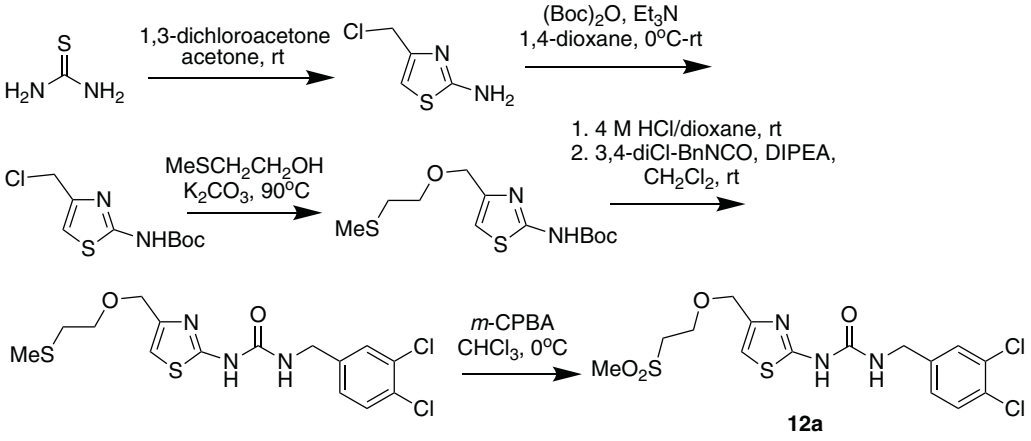
Scheme 4. General synthesis of pyrimidinones.



Scheme 5. Synthesis of anilino-pyrimidine (**9a**).



Scheme 6. Synthesis of anilino-pyrazolo-pyrimidinone (**9b**).



Scheme 7. Synthesis of urea derivatives as pol IIIC inhibitors.

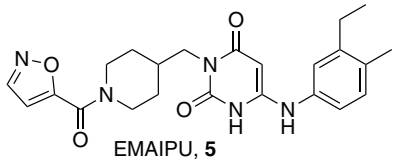


Fig. 5. 6-[(3′-ethyl-4′-methyl-anilino]-3-{[1-(isoxazol-5-ylcarbonyl)piperidin-4-yl]methyl}uracil (EMAIPU, **5**).

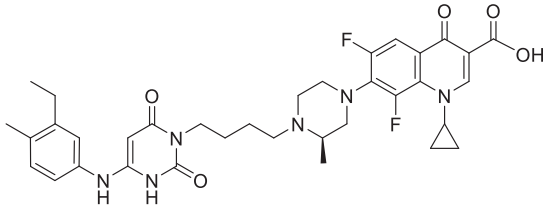


Fig. 6. MBX-500, AU-FQ Hybrid **6.**

of 6 treated animals. These results provide evidence that novel AU-FQ antibiotic, at the highest dose evaluated, has a clinical efficacy in the gnotobiotic piglet model similar to that of vancomycin.[35]

2.2.4. Pyrimidines and pyrimidinones

Wright et al.[24] studied substituents at the 4-position of a series of 2-amino-6-(benzylamino)pyrimidines and concluded that 4-oxo is required to retain the activity. Compound 6-(3′,4′-trimethyleneanilino)-isocytosine (**7**) binds with thymine and is a potent (IC_{50} of 2 μM) pol IIIC inhibitor by competing with dATP (Fig. 7).

Rose et al.[36] prepared (Scheme 4) and 5-substituted-6-hydroxy-2-(anilino)pyrimidinones from aryl guanidine hydrochloride with a substituted malonate.

6-Chloro-pyrimidine (X = Cl, R = Allyl, **8a**), and 2,6-diaminopyrimidinone (X = NH_2, R = H, **8b**) all showed potent antibacterial activities.[36] Compound **8a** showed MIC of 1 μg/mL *in vitro,* but it displayed a strongly negative serum effect. Compound **8b** reduced the serum effect, and selectively inhibit *S. aureus* DNA pol IIIC by competing with dATP with moderate *in vitro* value (MIC ≥ 8 μg/mL). **8c** and **8d** had similar MIC at 16 μg/mL *in vitro,* but **8c** was more water soluble and showed better *in vivo* efficacy, as it protected all 10 animals at 2 × 50 mg/kg in an *in vivo* infection model against *S. aureus*.

Ali et al.[37] prepared 4-(*p*-bromo-phenoxy)-2-amino-6-(anilino)pyrimidine (**9a**, Scheme 5) which displayed an IC_{50} of 10 μM against DNA pol IIIC from S. aureus.

SAR study by Ali et al.[38] of 6-anilinopyrazolo[3,4-*d*]pyrimidin-4-one (**9b**, Scheme 6) derivatives showed that 4-oxo and pyrazolo NH must both be present to retain the optimal activity by competing with dATP. Compound **9b** showed IC_{50} of 71 μM and MIC ≥ 9 μg/mL *in vitro* against *B. subtilis, E. faecium, S. aureus, S. pneumonia*. Although **9b** showed *in vivo* efficacy in *S. aureus* infected mice model at 10 mg/kg by the IP, the low water solubility hampered treatment by the IV or subcutaneous route.

2.2.5. Non-nucleobase inhibitors

Butler et al.[39] discovered pyrazole-carboxylic-acid hydrazide

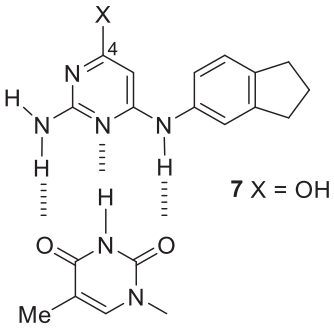


Fig. 7. Compound **7**H-bonds with thymine.

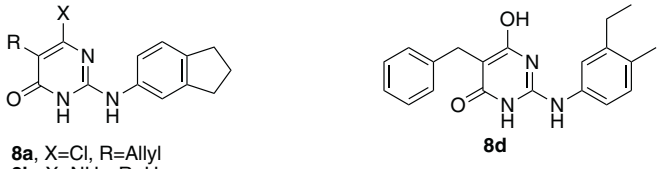


Fig. 8. Pyrimidines and pyrimidinones.

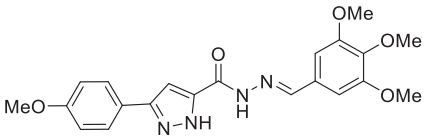


Fig. 9. Pyrazole carboxylic acid hydrazide **10**.

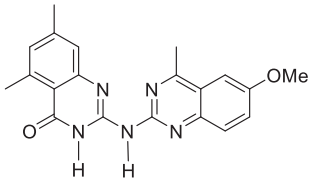


Fig. 10. BisQuinol **11**.

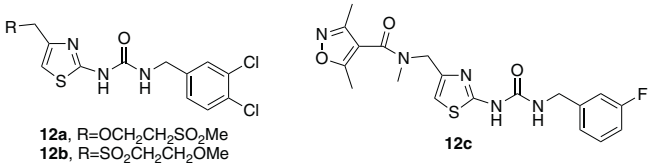


Fig. 11. Heterocyclic urea pol IIIC inhibitors.

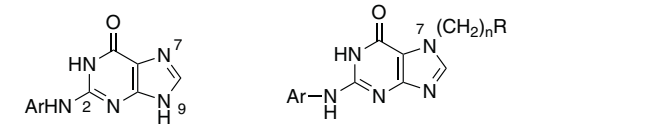


Fig. 12. Substituted guanines.

compounds as potent DNA pol IIIC inhibitors with antibacterial activities from the high throughput screening (HTS). These compounds possessed none of the heterocyclic nitrogenous bases (i.e. guanine, cytosine, adenine, thymine or uracil) in the structure, though they appeared to mimic or compete with one or more dNTPs to inhibit the DNA pol IIIC. The lead compound **10** (Fig. 9) is a very potent pol IIIC inhibitor (IC_{50} = 0.2 μM) with MICs as low as 0.625 μg/mL against *B. subtilis*.[39,40]

Guiles et al.[41] discovered quinazolin-2-ylamino-quinazolin-4-ols (Fig. 10) as a new class of DNA polymerase IIIC inhibitors mimicking dGTP. The lead compound **11** showed potent *in vitro* activity with IC_{50} of 0.3 μM and MIC at 16 μg/mL against *S. aureus* in the presence of 50% human serum. In a simple murine model *in vivo* test, compound **11** protected 50% of the animals from a *S. aureus* IP infection with a single dose of 25 mg/kg.

Substituted heterocyclic urea pol IIIC inhibitors have shown potent *in vitro* antibacterial activities. Substituted urea was prepared through amine and aryl isocynate as depicted in the synthesis of compound **12a** (Scheme 7).[42]

Compound **12a** exhibited MIC_{90}'s ranging from 1 to 8 μg/mL against MRSA, vancomycin-resistant *E. faecium* and macrolide-resistant *S. pyogenes*. The MICs of **12b** were in the range from 0.5 to 4 μg/mL in clinically relevant resistant strains. Compound **12c** exhibited MIC_{90}'s ranging from 1 to 4 μg/mL.[42] These derivatives are currently at the lead optimization stage as oral Gram(+) antibacterial agents.

2.2.6. Guanines

The platform of guanine, the actual base which these pol IIIC specific inhibitors mimic, was studied.[43,44] *N*2-(3′-Ethyl-4′-methyl)-phenyl-guanine (EMPG, **13a**, Fig. 12) and *N*2-(3′,4′-dichloro)-benzyl-guanine (DCBG, **13b**) are potent pol IIIC inhibitors, though weaker than the relative uracil counterpart.[45]

Although many of the *N*3-substituted AUs (Figs. 2–6) pyrimidines (Figs. 7–9), ureas (Fig. 11) and *N*2-substituted-guanine agents (Fig. 12) studied showed strong pol IIIC inhibitory activity and/or antibacterial activity *in vitro*, none of them proved to be a worthy candidate for development as a clinical agent due to the various structural challenges discussed above.

Alkylation of **13a** or **13b** produced a mixture of *N*9 and *N*7 alkylation isomers (Scheme 8).[45] The *N*7 alkylation isomers isolated were found to be the most active pol IIIC inhibitors. Substituents in the *N*7 position of the guanines are nearly isosteric with those in the *N*3 position of the uracil-based inhibitors. *N*7-(4-Hydroxybutyl)-EMPG (7-HBEMPG, **13c**, Fig. 12), *N*7-(4-Hydroxybutyl)-DCBG (7-HBDCBG, **13d**, R═OH) and *N*7-Morpholinobutyl-DCBG (7-MorBDCBG, **13e**) were considerably more active against *B. subtilis* pol IIIC and showed potent antibacterial activities (Table 4). Particularly, *N*7-Morpholinoethyl-DCBG (7-MorEDCBG, **ACX-362E**, also know as **362E**, or **GLS-362E**) showed very potent *in vitro* and *in vivo* activities against broad spectrum of *C. difficile* pathogens, and will be discussed in Section 2.2.8.

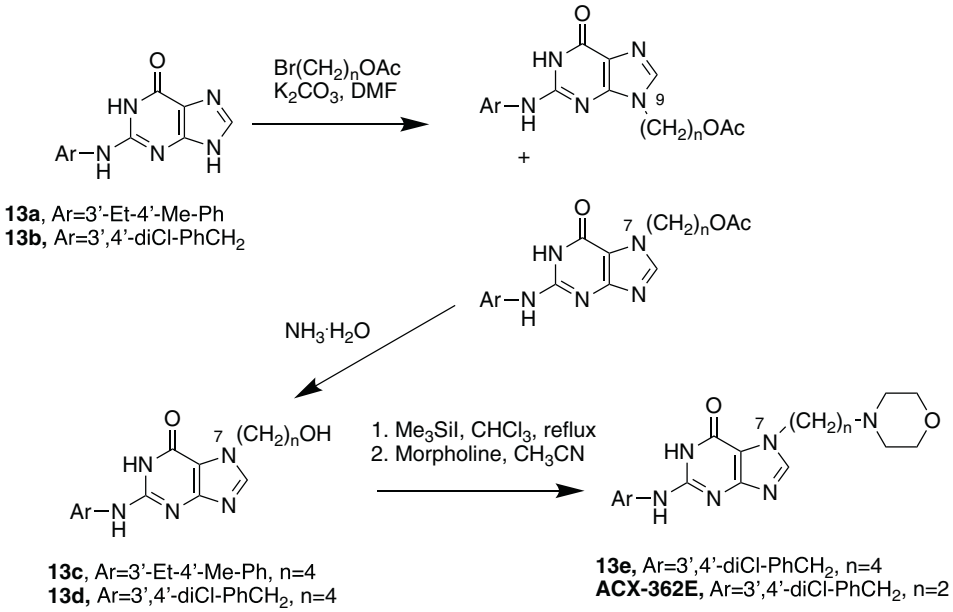
Column chromatography was required to separate the *N*7-alkylated product from the *N*9-isomer produced in the above alkylation of DCBG or EMPG (Scheme 8), which prohibited the production of the final product. A more efficient and greener process has been developed to prepare **ACX-362E** in large scale production without using column chromatography (Xu & Wright, unpublished result).

2.2.7. Purine isosterics

Xu et al.[46] prepared and studied purine isosteric heterocyclic compounds 3-Deazaguanine (**14a**), 8-azaguanine (**14b**) and 8-methylguanine (**14c**) analogues (Scheme 9). 3-Deaza isosteres of DCBGs (**14a**) had MICs similar to those of the DCBGs, while modification at 8-position (**14b** or **14c**) resulted in 2- to 10-fold loss of the activity compared with the 8-H counterparts. Other purine isosterics could be synthesized for SAR study to search for more "druggable" compounds.

2.2.8. *ACX-362E against C. difficile*

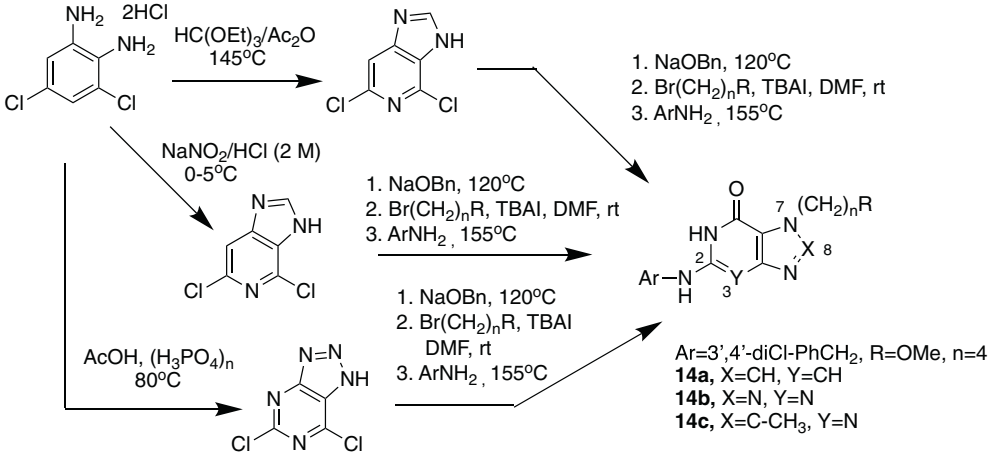
Clostridioides is a spore-forming, toxigenic, "low G + C" Gram(+) anaerobic bacterium that causes antibiotic-associated colitis. It colonizes the human intestinal tract after the normal gut flora have been disrupted associated with the use of broad-spectrum antibiotics. *C. difficile* infection (CDI) is one of the most common health care-



Scheme 8. Synthesis of *N*7-DCBGs.

Table 4
In vitro pol IIIC inhibitory and antibacterial activities (MICs) of guanines.

Compound	IC_{50} (μM)	MIC (μg/mL)						
	B. Subtilis pol IIIC	*B. subtilis*	*S. aureus*	*MRSA*	*E. faecalis*	*E. faecium*	*VRE*	*E. Coli*
13c	0.47	15	30	30	20	20	10	> 80
13d	0.19	15	30	30	10	7.5	3.75	> 80
13e	0.051	10	20	40	10	10	5	> 80



Scheme 9. Synthesis of purine isoterics as pol IIIC inhibitors.

Table 5
In vitro antibacterial activity of 7-morpholinylalkyl-DCBGs and comparators against 23 *C. difficile* strains.

Compound	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)
13e	4	16
ACX-362E	2	4
Vancomycin	1	4
Metronidazole	1	4

associated infections and a significant cause of morbidity and mortality, especially among hospitalized elderly patients. *C. difficile* was estimated to cause almost half a million infections in the United States in 2011, and 29,000 died within 30 days of the initial diagnosis.[47] Development of novel antibacterial agents against *C. difficile* has drawn intensive attention recently.[48]

*N*7-substituted-*N*2-DCBGs selectively inhibit *C. difficile* pol IIIC, an unexploited target in *C. difficile* drug development.[49] One promising DCBG derivative, *N*7-Morphilino-ethyl-*N*2-DCBG (**ACX-362E**, 7-Mor-EDCBG) has recently been selected for development as the first anti-

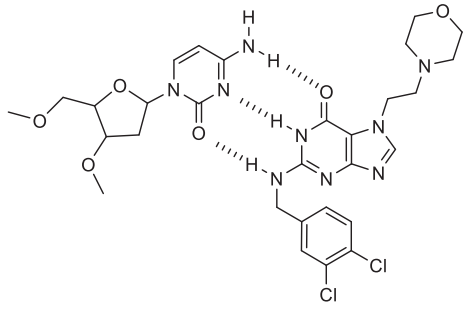


Fig. 13. ACX-362E form H-bonding with cytosine residue.

clostridial agent. The *in vitro* antibacterial activities of **ACX-362E** were as potent as **13e** against several low G + C Gram(+) strains screened (unpublished data), and it displayed MIC_{50} and MIC_{90} values (2 and 4 μg/mL) close to vancomycin and metronidazole against 23 different *C. difficile* strains, including vancomycin and metronidazole-resistant strains (Table 5). Its specificity for the novel pol IIIC target endows **ACX-362E** with strong potential for bypassing the resistance that is emerging in *C. difficile* during the prolonged application of the other agents in current use.[49]

Torti et al.[50] explored the basis for potent inhibition of *C. difficile* in culture by **ACX-362E**. They cloned and expressed the target pol IIIC and confirmed that it is the primary target in *C. difficile*. **ACX-362E** inhibited both *C. difficile* pol IIIC and *B. subtilis* pol IIIC by the same mechanism (Fig. 13).[50,51] It strongly suggests that it may be possible to develop a single "broad spectrum" variant of **ACX-362E** that is active against multiple low G + C Gram(+) pathogens, including pathogens which are resistant to the existing, "conventional" antibiotics — for example, MRSA, VRE and emergent strains that are resistant to linezolid or daptomycin.

ACX-362E has many desirable anti-*C. difficile* properties. This agent is poorly absorbed from the GI tract and essentially nontoxic when given orally.[49] In a hamster model of *C. difficile* colitis, oral **ACX-362E** was as effective as oral vancomycin, the current agent of choice for treating severe forms of the human disease. Treatment for 7 days with **ACX-362E** at 50 mg/kg twice daily reduced the recurrence rate to 40% and delayed death when the disease recurred, and when the same treatment regimen was continued for a total of 14 days, there was no recurrence observed during the remainder of the 34-day observation period.[49] Given these promising results, **ACX-362E**, as noted above, has now entered human trials, becoming the first pol IIIC-specific agent to enter clinical investigation.[52]

3. Conclusion

Contemporary small-molecule, synthetic antibacterial drugs have taken decades to emerge from laboratory discovery to clinical introduction and wide-scale human therapeutic use.[1,10,11,53–55] The newest introduction of this type, linezolid (2000) is considered a recent advance, yet the origins of the oxazolidinone class date to 1955 – with 40 years elapsing before a viable clinical candidate emerged from this class.[53,54] Likewise, the fluoroquinolone class, which became well established in the late 1980s, has its roots in nalidixic acid, discovered three decades earlier.[53,55] Years of successive investigations led from empiric observations of the *in vitro* antibacterial activity of HPUra through the identification of pol IIIC as the target enzyme, increasingly sophisticated SAR analyses, and the development of more highly potent and selective molecular classes. This process has led to the DCBG class, and to **ACX-362E**, the first pol IIIC inhibitor known to have entered clinical trials.[52] Data regarding the eventual clinical utility await the conduct of randomized and controlled efficacy trials in patient populations.

Given the novelty of the pol IIIC target, newer generations of inhibitors could become potentially important agents for the treatment of other serious bacterial infections. Since **ACX-362E**, its derivatives, and other structurally related pol IIIC inhibitors act through a heretofore clinically unexploited mechanism, there is a good reason to believe they will not share cross-resistance with any available class of antibiotics. Furthermore, as fully synthetic molecules unrelated to any known natural products and devoid of any history of use in the food supply, pol IIIC inhibitors may eventually be extremely useful agents to treat such high-priority pathogenic bacteria as MRSA, VRE, and multidrug-resistant *Streptococcus pneumoniae*, as well as Gram(+) bacteria showing resistance to other commonly used drugs like linezolid and daptomycin.

ACX-362E and related molecules arose from years of traditional medicinal chemistry and an approach requiring the use of laborious, trial-and-error, SAR. While this course was successful in creating at least one clinical candidate molecule, the DCBG series still provides challenges with respect to "druggable" features such as water solubility and bioavailability. Nevertheless, the tools of molecular modeling and structure-based drug design may well provide new and powerful ways to deal with these obstacles and produce agents with improved inhibitory potency and features that favor its oral and parenteral use.

Declaration of Competing Interest

None.

Acknowledgements

The authors would like to acknowledge the review and support of Robert J DeLuccia of Acurx Pharmaceuticals LLC.

Financial support by Dr. Aisiku Interdisciplinary STEM Research Team Initiative grant to W Xu through Worcester State University is gratefully acknowledged.

References

1. CDC Antibiotic Resistance. Antibiotic Resistance Threats in the United States. https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf/; 2013 Accessed 28 March 2019.
2. O'Neill J, Antimicrobial Resistance: Tracking a Crisis for the Health and Wealth of Nations. Review on Antimicrobial Resistance. https://amr-review.org/Publications.html/; 2014 Accessed 28 March 2019.
3. FDA Guidance Document. Antibacterial Therapies for Patients with an Unmet Medical Need for the Treatment of Serious Bacterial Diseases. https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM359184.pdf/; 2017 Accessed 28 March 2019.
4. Laxminarayan R, Duse A, Wattal C, et al. Antibiotic resistance—the need for global solutions. *Lancet Infect Dis.* 2013;13:1057–1098.
5. Biggest Threats and Data. https://www.cdc.gov/drugresistance/biggest_threats.html/; 2019 Accessed 28 March 2019.
6. United Nations (UN) General Assembly. https://www.cdc.gov/drugresistance/intl-activities/un-general-assembly.html/; 2019 Accessed 28 March 2019.
7. Ten Threats to Global Health in 2019. https://www.who.int/emergencies/ten-threats-to-global-health-in-2019/; 2019 Accessed 18 April 2019.
8. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. https://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf/; 2017 Accessed 28 March 2019.
9. WHO Antibacterial Agents. Antibacterial Agents in Clinical Development: An analysis of the antibacterial clinical development pipeline, including tuberculosis. 1-48. https://www.who.int/medicines/news/2017/IAU_AntibacterialAgentsClinicalDevelopment_webfinal_2017_09_19.pdf./; 2017 Accessed 28 March 2019.
10. Deak D, Outterson K, Powers J, Kesselheim A. Progress in the fight against multidrug-resistant bacteria? A review of U.S. food and drug administration–approved antibiotics, 2010–2015. *Ann Int Med.* 2016;165:363–372.
11. Theuretzbacher U. Antibiotic innovation for future public health needs. *Clin Microbiol Infect.* 2017;23:713–717.
12. Czaplewski L, Bax R, Clokie M, et al. Alternatives to antibiotics—a pipeline portfolio review. *Lancet Infect Dis.* 2016;16:239–251.
13. Lewis K. Platforms for antibiotic discovery. *Nat Rev Drug Discov.* 2013;12:371–387.
14. Kornberg A, Baker T. *DNA Replication.* Sausalito, CA: University Science Books; 2005.
15. Wright G, Brown N. DNA polymerase IIIC: a new target for antibiotic development. *Curr Opin in Anti-Infect Invest Drugs.* 1999;1:45–48.
16. Butler M, Wright G. *A Method to Assay Inhibitors of DNA Polymerase IIIC Activity. New Antibiotic Targets-2008, Chapter 3.* Human Press; 2008.

17. Brown N, Handshumacher R. Inhibition of the synthesis of DNA in bacteria by 6-(p-hydroxyphenylazo)-2,4-dihydroxypyrimidine. I. Metabolic studies in *Enterococcus faecalis*. *J Biol Chem.* 1966;241:3083–3089.
18. Brown N. 6-(*p*-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in phage-infected *Bacillus subtilis*. *Proc Natl Acad Sci USA.* 1970;67:1454–1461.
19. Brown N, Wright G. *The molecular basis for the inhibitory action of 6-(arylazo)pyrimidines on replicative DNA synthesis of gram-positive bacteria. Drug Action at the Molecular Level*. Baltimore: Univ. Park Press; 1977.
20. Clements J, D'Ambrosio J, Brown N. Inhibition of *Bacillus subtilis* DNA polymerase III by phenylhydrazinopyrimidines: demonstration of a drug-induced DNA: enzyme complex. *J Biol Chem.* 1975;250:522–526.
21. Brown N, Gambino J, Wright G. Inhibitors of *Bacillus subtilis* DNA polymerase III. 6-(arylalkylamino)uracils and 6-anilinouracils. *J Med Chem.* 1977;20:1186–1189.
22. Wright G, Brown N. Inhibitors of *Bacillus subtilis* DNA polymerase III. 6-Anilinouracils and 6-(alkylamino)uracils. *J Med Chem.* 1980;23:34–38.
23. Wright G, Gambino J. Quantitative structure-activity relationships of 6-anilinouracils as inhibitors of *Bacillus subtilis* DNA polymerase III. *J Med Chem.* 1984;27:181–185.
24. Trantolo D, Wright G, Brown N. Inhibitors of *Bacillus subtilis* DNA polymerase III. Influence of modifications in the pyrimidine ring of anilino- and (benzylamino) pyrimidines. *J Med Chem.* 1986;29:676–681.
25. Tarantino P, Zhi C, Wright G, Brown N. Inhibitors of DNA polymerase III as novel antimicrobial agents against gram-positive eubacteria. *Antimicrob Agents Chemother.* 1999;43:1982–1987.
26. Wright G, Brown N. Deoxyribonucleotide analogs as inhibitors and substrates of DNA polymerases. *Pharmacol Ther.* 1990;47:447–497.
27. Tarantino P, Zhi C, Gambino J, Wright G, Brown N. 6-Anilinouracil-based inhibitors of *Bacillus subtilis* DNA polymerase III: antipolymerase and antimicrobial structure-activity relationships based on substitution at uracil N3. *J Med Chem.* 1999;42:2035–2040.
28. Zhi C, Long Z, Gambino J, et al. Synthesis of substituted 6-anilinouracils and their inhibition of DNA polymerase IIIC and gram-positive bacterial growth. *J Med Chem.* 2003;46:2731–2739.
29. Kuhl A, Svenstrup N, Ladel C, et al. *Antimicrob Agents Chemother.* 2005;49:987–995.
30. Svenstrup N, Ehlert K, Ladel C, Kuhl A, Häbich D. New DNA polymerase IIIC inhibitors: 3-subtituted anilinouracils with potent antibacterial activity in vitro and in vivo. *ChemMedChem.* 2008;3:1604–1615.
31. Svenstrup N, Kuhl A, Ehlert K, Häbich D. Improved synthesis of antibacterial 3-substituted 6-anilinouracils. *Bioorg Med Chem Lett.* 2008;18:3215–3218.
32. Zhi C, Long Z, Manikowski A, et al. *J Med Chem.* 2006;49:1455–1465.
33. Klahn P, Brönstrup M. Bifunctional antimicrobial conjugates and hybrid antimicrobials. *Nat Prod Rep.* 2017;34:832–885.
34. Butler M, Shinabarger D, Citron D, et al. MBX-500, a hybrid antibiotic with *in vitro* and *in vivo* efficacy against toxigenic *Clostridium difficile*. *Antimicrob Agents Chemother.* 2012;56:4786–4792.
35. Steele J, Zhang Q, Beamer G, Butler M, Bowlin T, Tzipori S. MBX-500 is effective for treatment of *Clostridium difficile* infection in gnotobiotic piglets. *Antimicrob Agents Chemother.* 2013;57:4039–4041.
36. Rose Y, Ciblat S, Reddy R, et al. Novel non-nucleobase inhibitors of *Staphylococcus aureus* DNA polymerase IIIC. *Bioorg Med Chem Lett.* 2006;16:891–896.
37. Ali A, Aster S, Graham D, et al. Design and synthesis of novel antibacterial agents with inhibitory activity against DNA polymerase III. *Bioorg Med Chem Lett.* 2001;11:2185–2188.
38. Ali A, Taylor G, Ellsworth K, et al. Novel pyrazolo[3,4-d]pyrimidine-based inhibitors of Staphlococcus aureus DNA polymerase III: design, synthesis, and biological evaluation. *J Med Chem.* 2003;46:1824–1830.
39. Butler M, Cardinale S, Barnes M, et al. Novel inhibitors of bacterial DNA polymerase IIIC. *Interscience Conference on Antimicrobial Agents and Chemotherapy.* 2005; 2005:F-1875..
40. Butler M, Antibacterial pyrazole carboxylic acid hydrazides. 2004; WO2004094370A2.
41. Guiles J, Sun X, Critchley I, et al. Quinazolin-2-ylamino-quinazolin-4-ols as novel non-nucleoside inhibitors of bacterial DNA polymerase III. *Bioorg Med Chem Lett.* 2009;19:800–802.
42. Guiles J, Jarvis T, Strong S, Sun X, Antibacterial Heterocyclic Ureas. 2014; US 8716320B2.
43. Wright G, Brown M, Baril E, Brown N. Design and characterization of N2-arylaminopurines which selectively inhibit replicative DNA synthesis and replication-specific DNA polymerases: guanine derivatives active on mammalian DNA polymerase alpha and bacterial DNA polymerase III. *Nucl Acids Res.* 1982;10:4431–4440.
44. Butler M, Dudycz L, Khan N, Wright G, Brown N. Development of novel inhibitor probes of DNA polymerase lll based on dGTP analogs of the HPUra type: base, nucleoside and nucleotide derivatives of N2-(3,4-dichlorobenzyl)guanine. *Nucl Acids Res.* 1990;18:7381–7387.
45. Wright G, Brown N, Xu W, et al. Active site directed inhibitors of replication-specific bacterial DNA polymerases. *Bioorg Med Chem Lett.* 2005;15:729–732.
46. Xu W, Wright G, Brown N, et al. 7-Alkyl-N(2)-substituted-3-deazaguanines. Synthesis, DNA polymerase III inhibition and antibacterial activity. *Bioorg Med Chem Lett.* 2011;21:4197–4202.
47. Lessa F, Mu Y, Bamberg W, et al. Burden of *Clostridium difficile* infection in the United States. *N Engl J Med.* 2015;372:825–834.
48. Tran M, Kullar R, Goldstein E. Investigational drug therapies currently in early stage clinical development for the treatment of *clostridioides (clostridium) difficile* infection. *Expert Opin Investig Drugs.* 2019. https://doi.org/10.1080/13543784.2019.1581763.
49. Dvoskin S, Xu W, Brown N, Yanachkov I, Yanachkova M, Wright G. A novel agent effective against *Clostridium difficile* infection. *Antimicrob Agents Chemother.* 2012;56:1624–1626.
50. Torti A, Lossani A, Savi L, et al. *Clostridium Difficile* DNA polymerase IIIC: basis for activity of antibacterial compounds. *Curr Enzym Inhib.* 2011;7:147–153.
51. Eijk E, Boekhoud I, Kuijper E, Bos-Sanders I, Wright G, Smits W. Genome location dictates the transcriptional response to PolC inhibition in *Clostridium difficile*. *Antimicrob Agents Chemother.* 2019;63:e01363–e1418. https://doi.org/10.1128/AAC.01363-18.
52. Acurx Announces First-In-Man Clinical Trial Data of ACX-362E for CDI. https://www.acurxpharma.com/news-media/press-releases/; 2019 Accessed 22 March, 2019.
53. Durand G, Raoult D, Dubourg G. Antibiotic discovery: history, methods and perspectives. *Int J Antimicrob Agents.* 2019;53:371–382. https://doi.org/10.1016/j.ijantimicag.2018.11.010.
54. Zurenko G, Yagi B, Schaadt R, et al. *In vitro* activities of U-100592 and U-100766, novel oxazolidinone antibacterial agents. *Antimicrob Agents Chemother.* 1996;40:839–845.
55. Wolfson J, Hooper D. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity *in vitro*. *Antimicrob Agents Chemother.* 1985;28:581–586.