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***Clostridium difficile* DNA polymerase IIIC: basis for activity of antibacterial compounds**

Andrea Torti^{*}, Andrea Lossani^{*}, Lida Savi^{*}, Federico Focher^{*}, George Edward Wright[†], Neal Curtis Brown[†], and Wei-Chu Xu[†]

^{*}Istituto di Genetica Molecolare, Consiglio Nazionale delle Ricerche, I-27100 Pavia, Italy

[†]GLSynthesis Inc., One Innovation Drive, Worcester, MA 01605 USA

Abstract

Based on the finding that aerobic Gram-positive antibacterials that inhibit DNA polymerase IIIC (pol IIIC) were potent inhibitors of the growth of anaerobic *Clostridium difficile* (CD) strains, we chose to clone and express the gene for pol IIIC from this organism. The properties of the recombinant enzyme are similar to those of related pol IIICs from Gram-positive aerobes, e.g. *B. subtilis*. Inhibitors of the CD enzyme also inhibited *B. subtilis* pol IIIC, and were competitive with respect to the cognate substrate 2'-deoxyguanosine 5'-triphosphate (dGTP). Significantly, several of these inhibitors of the CD pol IIIC had potent activity against the growth of CD clinical isolates in culture.

Keywords

antibacterials; *C. difficile*; competitive inhibitors; DNA polymerase; replication

INTRODUCTION

The Gram-positive anaerobe *Clostridium difficile* (CD) is responsible for the predominant form of bacterial colitis arising during hospitalization. In the United States alone there are nearly one-half million cases of CD-associated disease (CDAD) annually [1,2], and CDAD not only has a high morbidity, it is also a life-threatening disease which has shown increasing mortality rates [3]. Vancomycin and metronidazole are first-line therapy for treatment of CDAD, but recent data have revealed treatment failure and CDAD recurrence after treatment with metronidazole [4], and vancomycin use has been discouraged in hospitals to minimize the risk of vancomycin-resistant enterococci and staphylococci.

DNA polymerase IIIC (pol IIIC) has been shown to be essential for replicative DNA synthesis in aerobic, low G-C Gram-positive bacteria, i.e. those with low guanine-cytosine (G-C) ratio relative to their adenine-thymine (A-T) ratio. Pol IIIC-specific genes of several such Gram-positive bacteria have been cloned and expressed [5–7]. These enzymes share a unique capacity to be inhibited by 6-anilinouracils (AU), 2-phenylguanines (PG) and related compounds as analogs of the specific substrate 2'-deoxyguanosine 5'-triphosphate (dGTP). The compounds bind via a “base-pairing domain” and an enzyme-specific “aryl domain” (see domains depicted for a AU compound below). Through its base-pairing domain, which mimics that of guanine, the molecule base-pairs with an unapposed template cytosine just distal to the DNA primer terminus. Simultaneously, the aryl domain binds an aryl-specific

“receptor” near the enzyme’s dNTP binding site, causing the formation of an inactive ternary complex of inhibitor, DNA and pol III_C [8].

Optimal aryl domain substituents for AU compounds are 3-ethyl-4-methyl and related patterns, and certain substituents on the 3 position of the uracil ring (see, e.g. the structure of 3-HB-EMAU) increase potency against pol III_C and against bacterial growth in culture [9]. The 3-ethyl-4-methyl substituent in 7-substituted N²-phenylguanines [10] (e.g. see structure of 7-HB-EMPG) affords potent and selective inhibition of Gram⁺ pol III_C and bacterial growth. An alternate aryl domain group - 3,4-dichlorobenzyl - gives rise to a N²-benzylguanine series of compounds (see structure of 7-HB-DCBG), which potently inhibit the pol III_Cs [10]. Various 3-substituted 6-anilinouracils [9,10] and guanines [12] have shown good antibacterial activity *in vitro*, but only marginal activity *in vivo* when given systemically. Although protective against systemic *S. aureus* infection in mice when given intraperitoneally, 3-HB-EMAU and its 7-substituted guanine analog 7-HB-EMPG have shown limited potential for development because of poor water solubility and low oral bioavailability. This results, in part, from the hydrophobic and water insoluble properties of the most potent antibacterials. Analogs with basic substituents (diethylamino, morpholinyl) in the 3 or 7 sidechain of uracils or guanines, respectively, form highly water soluble salts, but these also have poor antibacterial activity against aerobes.

The prevalence of CDAD provides a strong rationale to discover and develop oral agents as alternatives to vancomycin and metronidazole, the current drugs of choice for the disease. To that end, we have identified purine derivatives with potent inhibition of the growth of CD clinical isolates in culture. We have also cloned and expressed for the first time the CD-specific pol III_C, and show that inhibition of this enzyme by these anti-CD compounds is, as expected, the likely basis for their antibacterial effect. In sum, we have identified a class of purine-based inhibitors of CD pol III_C with considerable promise as antibacterials useful against CDAD.

MATERIAL AND METHODS

Inhibitors

The pyrimidines and purines tested as DNA polymerase inhibitors were prepared as previously described [9–13]. Several of the new inhibitors were prepared as described elsewhere [10].

Antibacterial screening

The test organisms were recent clinical isolates or reference strains acquired from the American Type Culture Collection (ATCC). The growth and test media were those recommended by the Clinical and Laboratory Standards Institute [14] for growth and susceptibility testing of anaerobes. The test organisms were maintained frozen at -80°C . The isolates were sub-cultured on Supplemented Brucella Agar (SBA) plates and incubated for 48 hr at $35-36^{\circ}\text{C}$ in a Bactron II anaerobe chamber. The medium employed for the agar dilution MIC assay of anaerobic bacteria was Brucella Agar supplemented with hemin, vitamin K₁ and 5% lysed sheep blood. Screening was conducted by Micromyx LLC, Kalamazoo, MI.

Drug dilutions and drug-supplemented agar plates were prepared manually. Following inoculation, the drug-supplemented plates were incubated at 35°C for 48 hr in the anaerobic environment (5% hydrogen, 5% carbon dioxide, 90% nitrogen) of the Bactron II. The MIC was read per CLSI guidelines [14].

Cloning of recombinant CD pol IIIC

CD DNA was purchased from the ATCC (ATCC # BAA-1382D-5, strain # 630). The sequence encoding the open reading frame (ORF) CD pol IIIC (gene # 4914021) was PCR-amplified from 100 ng of CD DNA, using the following two primers (5'-phosphorylated): *Sense*: 5'-AATGGAAAGTATTAAGGAATATTTGGATAAG-3', specifically fitted at the 5' end with the recombinase-specific terminus AATG; *Antisense*: 5'-TCCCAACAATGACAATTGATTCTC-3', fitted at the 5' end with the second recombinase-specific terminus TCCC. The PCR-amplified product was inserted into the *E. coli* plasmid pENTRY-Iba10 (IBA-Biotechnology), to generate the recombinant vector, pENTRY-CDpolC, containing the complete CD pol IIIC sequence. Sequencing of the pol IIIC-specific ORF of pENTRY-CDpolC indicated that it was identical to the published sequence [15], except for a single (G→A) mutation at position 2221 which specified substitution of Ile for the expected Val codon.

The ORF of pENTRY-CDpolC was subcloned in pPSG-IBA33 (IBA-Biotechnology) by recombinase-specific recombination, following the procedure suggested by the manufacturer. In this expression vector, the ORF is under T7 promoter control and is expressed as a C-terminal hexahistidine (6-His) tagged fusion protein.

Induction and purification of the recombinant CD pol IIIC

Competent *E. coli* cells (Rosetta-DE3pLysS, Novagen) carrying the isopropyl- β -D-galactoside (IPTG)-inducible expression vector were grown at 37°C in 1.5 L of Luria Broth medium containing chloramphenicol (34 $\mu\text{g/mL}$) and ampicillin (100 $\mu\text{g/mL}$) until OD₆₀₀ reached 0.8. The cultures were chilled on ice and then grown at 18°C in the presence of 50 μM IPTG for 16 hr. The culture was centrifuged at $4,000\times g$ for 20 min, washed once in phosphate buffered saline (PBS) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), and finally centrifuged at $4,000\times g$ for 20 min. The pellet was stored at -80°C until used.

Six grams of the frozen *E. coli* pellet were suspended in 21 mL of 50 mM sodium phosphate buffer, pH 8.0, 20% glycerol, 5 mM β -mercaptoethanol (column buffer) containing 300 mM NaCl, 1 mM PMSF and 1/100 dilution of Sigma protease inhibitor cocktail (product P2714). Lysozyme (1 mg/mL) was added and the mixture was kept on ice for 30 min. The resulting lysate was sonicated (5 $\times$ 10 sec) and ultracentrifuged at $100,000\times g$ for 80 min. The supernatant, which contained the DNA polymerase (pol) activity, was made 3 M in NaCl and quantitatively adsorbed to a 5 mL phenylsepharose column (GE-LifeScience) previously equilibrated in column buffer containing 3 M NaCl. The column was washed with 50 mL of

column buffer containing 2 M NaCl, and the pol activity was eluted with three column volumes of column buffer containing 1% Triton X100. The eluate was adjusted to 300 mM NaCl and 10 mM imidazole, and directly loaded onto a 1 mL Ni-NTA column (His-Trap, GE-LifeScience). The column was washed with 10 mL of column buffer containing 300 mM NaCl and 10 mM imidazole, followed by 10 mL of column buffer containing 300 mM NaCl and 20 mM imidazole. The pol activity was eluted step-wise with column buffer containing 300 mM NaCl and 250 mM imidazole.

The pol-rich fractions of the Ni-NTA eluate were pooled and dialyzed three times against 250 mL each of 50 mM Tris-HCl pH 7.5, containing 1 mM dithiothreitol (DTT), 0.5 mM PMSF and 20% glycerol. The dialyzed enzyme was frozen in small samples at -80°C and was stable in these conditions for several months.

B. subtilis pol IIIC

Transfection and expression of *B. subtilis* (Bs) pol IIIC followed essentially the same procedure as described above, starting from the cDNA [5] (a gift from Dr. Marjorie Barnes).

DNA polymerase (pol) assay

CD and Bs pol IIICs were assayed in 25 μL of a mixture containing 50 mM Bis-Tris-HCl, pH 7.0, 20 mM MgCl_2 , 1 mM DTT, 10% glycerol, 0.4 mg/mL activated calf thymus DNA [16], 25 μM each dATP, dCTP and dGTP, 10 μM [^3H]-TTP (1,500 cpm/pmol), and 0.5 μL CD pol IIIC (5,800 units/mg) or 0.7 μL Bs pol IIIC (4,400 units/mg). (One unit is the amount of enzyme required to incorporate 1 nmol of dNTP in 1 hr at 37°C .) The reaction was incubated for 20 min at 37°C , and then 10 μL of the reaction mixture was spotted on a 96-square glass fiber filter. The filter was rinsed exhaustively with ice-cold 5% trichloroacetic acid containing 5 mM sodium pyrophosphate followed by ice-cold ethanol. The filters were dried and counted as described [17].

CD pol IIIC 3'→5' exonuclease activity assay

Exonuclease substrate preparation: 1 mg of activated calf thymus DNA [16] was added to a reaction mixture containing 100 μM dATP, dCTP, dGTP and 40 μM [^3H]dTTP (750 cpm/pmol), and 50 units of Klenow fragment DNA polymerase I (Promega) in a final volume of 2.5 mL. The mixture was incubated at 37°C for 30 min, stopped by heating at 75°C for 10 min, and then chilled on ice. DNA substrate was precipitated twice with ethanol and ammonium acetate and resuspended in 1 mL of 50 mM Tris-HCl buffer, pH 7.5. The specific activity of the resulting ^3H -labeled DNA was 10^4 cpm/ μg .

Exonucleolytic activity was determined in the absence of dNTPs under the same conditions of the DNA polymerase assay. Reaction mixtures (15 μL) were incubated for 10 min at 37°C with 0.4 mg of ^3H -labeled DNA. Results were determined in a liquid scintillation counter as described above.

Inhibitor assays

Prior to their use in DNA polymerase assays, compounds were prepared as 20 mM stock solutions in reagent grade dimethylsulfoxide (DMSO). Control, "solvent" experiments indicated that DMSO at concentrations up to 5% did not interfere with DNA polymerase activity. K_i values for inhibitor candidates were determined by the truncated, dGTP-deficient assay method [18]. Each 25 μL assay contained 50 mM Bis-Tris-HCl pH 7.0, 20 mM MgCl_2 , 1 mM DTT, 10% glycerol, 0.4 mg/mL activated calf thymus DNA (prepared as previously described), 25 μM dATP and dCTP, 10 μM [^3H]-TTP (1500 cpm/pmol) and limiting amounts of enzymes. Reactions were incubated for 20 min at 37°C in the presence of at least 6 different concentrations of each compound as dilutions in DMSO. Control

assays contained the same amount of DMSO. Reactions were stopped and enzyme activity was determined as described above. The K_i of a given inhibitor is defined as the concentration which yielded 50% inhibition of the control activity; the “truncated assay” used gives K_i directly for this multi-substrate enzyme [18].

RESULTS

Characterization of recombinant CD pol III_C

Following cloning and expression of the gene for CD pol III_C, fractions emerging from the Ni column were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and, where applicable, protein was estimated by the method of Bradford [19]. Results are shown in Figure 1. As shown in lanes 8–12, the final Ni-NTA purification step yielded three major proteins: the largest displayed an apparent molecular weight of 165 kilo Daltons (kD), the size expected for the full length, His-6-terminated CD pol III_C. The other two proteins displayed molecular weights of 140 and 70 kD. Western blotting experiments with a His-6-specific monoclonal antibody probe (results not shown) confirmed the presence of the His-6 terminus on the 165 species. The 140 kD species was also His-6 antibody-reactive, while the 70 kD band was not. The latter results suggest that the 140 kD species is a C-terminal-truncated form of the polymerase, possibly a proteolytic product of the full-length 165 kD species or a product of translation from a downstream ATG codon. The lack of antibody reactivity of the 70 kD band is consistent with its identity as the molecular chaperon DnaK, a 70 kD *E. coli* protein which is commonly present as a contaminant in recombinant proteins purified by Ni-NTA adsorption/elution [20].

As expected from its highly conserved primary structure, the recombinant CD pol III_C behaved very much like the pol III_Cs of the aerobic, low G:C Gram-positive organisms, i.e., aerobic bacilli, staphylococci, streptococci, and enterococci. It closely approximated them with respect to size (see Figure 1), and it fully conserved their essential active site region [21]. And, despite the presence of a C-terminal His-6, the CD pol III_C displayed the expected specific activity and preferences for primer:template, pH, divalent cation, salt and specific activity (results not shown) [22]. The K_m s for the individual dNTP substrates (dTTP, 4 μ M; dATP, 7 μ M; dCTP, 12 μ M; dGTP, 1.3 μ M) also were in the range of the corresponding values published for the pol III_Cs of the low G:C aerobic Gram-positives [22].

CD pol III_C showed a potent 3' $\rightarrow$ 5' exonuclease activity, assayed as described in Materials and Methods section, which was not inhibited by the compounds tested in this study (data not shown).

Inhibition by substrate analogs

Various synthetic pyrimidines and purines, i.e. 6-aminouracils and 2-substituted-guanines, previously shown to inhibit the pol III_Cs of aerobic, low G-C Gram-positive bacteria as competitors of dGTP, were tested for inhibition of the CD pol III_C. Structures and acronym descriptions are shown below. The results of Table 1 show that the CD pol III_C is sensitive to the same compounds as the classical Gram-positive pol III_C, e.g. that from *B. subtilis*. However, it can be seen that CD pol III_C is 5–10-fold less sensitive to some compounds compared with the Bs enzyme. This result is consistent with the lower sensitivity of some pol III_Cs to inhibitors, e.g. that from *S. aureus* [6]. However, the *relative* potencies of certain compounds, e.g. EMAU vs. CIMAU, are essentially identical with those published for the pol III_Cs of Bs and other aerobic, Gram-positive bacteria.

Among the “DCBG” purine analogs, the 7 isomers retained significant inhibition, whereas the 9 isomers, exemplified by the 9-methyl derivative 362D (Table 1), were weak inhibitors (data not shown). This distinction between potent 7 and weak 9 isomers in purine-based derivatives is a consistent finding for reported examples [10,12].

Mechanism of inhibition

Because we have found potent anti-CD activity of certain “DCBG” compounds (see below), we chose a lead analog in this class for mechanistic studies. The structurally unique active site of pol IIIC has been well established as the target of the DCBGs in low G:C Gram-positive *aerobes*. The high primary structure similarity of the *anaerobic* CD-specific pol IIIC and the pol IIICs of *aerobes* strongly suggested that pol IIIC was also the target of these compounds in CD cultures. The results summarized below in Table 2 and Figure 2 strongly support that suggestion.

As shown in Table 1, the “5-(N-morpholinyl)pentyl” compound 7-MorPn-DCBG (359E) was a potent inhibitor of the CD pol IIIC, displaying a K_i value of less than 1 μ M. Significantly, the action of 359E on the CD pol IIIC was specifically competitive with dGTP; as shown by the Lineweaver-Burke plots of Figure 2, variation of [dGTP] (panel A) revealed competitive kinetics, while variation of [dTTP] (panel B) was clearly noncompetitive. In sum, these results indicate that 7-MorPn-DCBG (359E), as expected, inhibits CD pol IIIC as a dGTP analog.

Anti-anaerobe activity

Representative pol IIIC inhibitors were tested for antibacterial activity against various anaerobic bacteria in culture, including numerous strains of *C. difficile*. As summarized in Table 2, all pol IIIC inhibitors had some activity against *Lactobacillus* and *Clostridium* spp., although they lacked activity, as expected, against the Gram-negative anaerobe *Bacterioides fragilis*. DCBG derivatives appeared to be the most potent antibacterials against *C. difficile* strains. Compounds based on “EMAU” (6-anilinouracils) and “EMPG” (N²-phenylguanines) were less active, although these compounds have been found to be potent inhibitors of the growth of Gram-positive aerobes [11,12]. In particular, the 7-isomers of DCBGs were more active than 9-isomers (data not shown), and the morpholinyl derivatives, e.g. 7-MorE- (362E) and 7-MorPn-DCBGs (359E), were more active than other substituted derivatives (Table 2).

DISCUSSION

The goal of this work was to explore the basis for potent inhibition of *Clostridium difficile* in culture by 7-substituted DCBGs and to discover a novel DCBG analog with the potential for use in treating human CD-associated diarrhea (CDAD). The cloning and expression of

the likely target pol III_C and demonstration that it is strongly inhibited by classical substrate analogs that inhibit aerobic pol III_Cs indicate that pol III_C is, by analogy, the primary target in *Clostridium difficile*. Specifically, 7-[(N-morpholinylalkyl)DCBGs appear to have many of the essential characteristics of a novel antimicrobial useful for treating CDAD. First, the compounds are strong inhibitors of CD growth *in vitro* with relatively low activity for relevant representatives of the normal colonic flora (Table 2). Second, they show the expected potency for inhibition of CD pol III_C and a mechanism of pol III_C inhibition fully consistent with the DCBG inhibitor class (Table 1, Figure 2). Third, and most importantly, they are effective orally, and safe at high doses, in therapy of a relevant hamster model of CDAD (to be published).

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Fig. 1. SDS-PAGE analysis of CD pol IIIC purification. Each sample was loaded in a volume of 8 μ l. Lane (1), clarified supernatant. (2), phenylsepharose flow through. (3), phenylsepharose wash. (4), phenylsepharose eluate/Ni-NTA load. (5), Ni-NTA flowthrough. (6), Ni-NTA wash 1. (7), Ni-NTA wash 2. (8)–(12), serial dilutions of the pooled Ni-NTA peak fractions: (8), 6.4 μ g protein. (9), 3.2 μ g. (10), 1.6 μ g. (11), 0.64 μ g. (12), 0.32 μ g. (M) Full-Range Rainbow molecular weight markers (GE Healthcare).

Fig. 2. Lineweaver-Burke plots of the effects of dGTP (panel A) and dTTP (panel B) on inhibition of CD pol IIIIC by 359E. The concentrations of inhibitor are the same in both assays: ▲= 10 μM ; △= 5 μM ; ○= 2.5 μM ; ●= 0 μM .

Table 1

Inhibitors of CD and Bs pol IIICs.

Cpd	Acronym	K_i values, μM^*		
		CD pol IIIC	Bs pol IIIC	
			this work	published
59D	EMAU	1.5	0.28	0.19
24A	CIMAU	6.2	0.64	0.62
179E	HB-EMAU	0.37	0.13	0.06
194B	MorB-EMAU	0.41	0.053	0.05
300A	7-HPn-EMPG	2.9	0.74	0.28
325E	7-EOE-DCBG	1.22	0.55	0.33
332E	7-ValOPn-DCBG	1.57	0.48	0.25
362C	7-MeDCBG	0.89	0.32	-
362D	9-MeDCBG	ca. 100	nt	-
362E	7-MorE-DCBG	0.325	0.34	-
363A	7-MorPr-DCBG	0.325	0.16	-
258D	7-MorB-DCBG	0.13	0.14	-
359E	7-MorPnDCBG	0.125	0.27	-

* assayed at 4% DMSO and – dGTP (truncated reaction) as described.

nt, not tested

Table 2

Activity (MIC, µg/ml) of pol IIIC inhibitors and antibiotics against anaerobes.

		pol IIIC inhibitors (see structures)								antibiotics	
Organism *	Strain No.	179E HB EMAU	315C 7-MPn EMPG	332E 7-ValPn DCBG	325E 7-EOE DCBG	362C 7-Me DCBG	362E 7-MorE DCBG	359E 7-MorPn DCBG	Vanco	Metron	
<i>L. casei</i>	1722 †	32	32	>32	nt	32	16	16	>16	>16	
<i>L. acidophilus</i>	0681	32	16	8	nt	8	4	2	2	>16	
<i>Bi. brevi</i>	3967 †	>32	>32	32	nt	>32	>32	>32	2	>16	
<i>E. lentum</i>	1274 †	>32	>32	32	nt	>32	>32	>32	2	0.5	
<i>C. difficile</i>	3581	8	8	4	2	2	2	2	1	1	
“	3584	4	8	2	2	1	1	2	0.5	0.5	
“	3588	1	4	1	1	1	0.5	0.5	2	0.5	
“	4381 †	4	8	2	2	2	1	2	1	0.5	
<i>Ba. fragilis</i>	0123 †	>32	>32	>32	>32	>32	>32	>32	>16	1	

^{*} L., Lactobacillus; Bi, Bifidobacterium; E, Eubacterium; C, Clostridium; Ba, Bacillus.

[†] ATCC strains