Bioorganic & Medicinal Chemistry Letters 18 (2008) 6161–6164

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Peptides based on CcdB protein as novel inhibitors of bacterial topoisomerases

Eliane Trovatti, Camila A. Cotrim, Saulo S. Garrido, Ronaldo S. Barros, Reinaldo Marchetto *

UNESP – Institute of Chemistry, Department of Biochemistry and Technological Chemistry, Caixa Postal 355, 14800-900 Araraquara, SP, Brazil

ARTICLE INFO

Article history:
Received 6 August 2008
Accepted 2 October 2008
Available online 7 October 2008

Keywords:
Peptides
CcdB toxin
DNA gyrase
Topoisomerase IV

ABSTRACT

The *ccd* toxin–antitoxin system of the F plasmid encodes CcdB, a protein that poisons the essential *Escherichia coli* DNA gyrase, unique type IIA topoisomerase able to introduce negative supercoils into DNA. Based on CcdB structure, a series of linear peptide analogues were obtained by the solid-phase methodology. One of these peptides (*CcdBET2*) displayed inhibition of the supercoiling activity of bacterial DNA gyrase with a concentration required for complete inhibition (IC_{100} = 10 μM) lower than the wild type CcdB. For Topo IV, a second type IIA bacterial topoisomerase, *CcdBET2* was better inhibited the relaxation activity with an IC_{100} of 5 μM (wt CcdB > 10 μM). The replacement of Gly, present in the three C-terminal amino acid residues, by Glu, abolished the capacity to inhibit the gyrase but not the Topo IV activities. These findings demonstrate that the mechanism by which *CcdBET2* inhibits DNA gyrase is different of the mechanism by which inhibits Topo IV. Therefore, *CcdBET2* is a new type II topoisomerase inhibitor with specificity for Topo IV.

© 2008 Elsevier Ltd. All rights reserved.

DNA topoisomerases are enzymes involved in essential processes that control the topological state of DNA in cells.[1,2] Bacterial DNA gyrase and topoisomerase IV (Topo IV) are examples of type II topoisomerases that share significant sequence similarity and have essentially the same catalytic mechanism. They act by forming a transient double-stranded break in DNA, and catalyzing passage of DNA duplex through the break prior to resealing the DNA backbone.[1] DNA gyrase is unique in catalyzing the negative supercoiling of closed circular DNA. In contrast, Topo IV has the ability to relax positive and negative DNA supercoils.[3,4] DNA gyrase and Topo IV are composed of two subunits. The gyrase subunits are named A and B, the corresponding Topo IV subunits are named C and E. For each enzyme, these subunits combine into a heterotetrameric (gyrase, A_2B_2 and Topo IV, C_2E_2) complex to form the functional enzymes. The E-subunit (ParE) and the corresponding gyrase B-subunit (GyrB) contain the site of ATP hydrolysis at the N-terminal domain and a C-terminal domain involved in the interaction with the other subunit (ParC or GyrA) and the DNA substrate. The GyrA and ParC comprise an N-terminal domain (64 kDa) involved in DNA cleavage/relegation and a C-terminal DNA-wrapping domain (33 kDa).

Gyrase and Topo IV have also similarities in their antibiotic sensitivity to topoisomerase inhibitors as coumarins and quinolones.[5,6] The quinolones are synthetic compounds that form an Mg^{+2}-mediated ternary complex with bacterial topoisomerase in the presence of DNA[7,8] and stabilize the transient double-stranded break in DNA, resulting in bacterial cell death.[5,9] Resistance to quinolones usually involves point mutations in defined regions of GyrA and GyrB of DNA gyrase, and the ParC and ParE of Topo IV.[10] The coumarins are natural products of *Streptomyces* species, which inhibit ATPase activity of DNA gyrase and Topo IV by competing with ATP for binding, Mg^{2+} dependent, to the GyrB and ParE subunits of the enzymes, respectively.[11,12] Mutations conferring resistance to the coumarins have been mapped and are known to be located on the N-terminal domain of the GyrB and the ParE, which contain the site of ATP hydrolysis.[13] Other inhibitors as cyclothialidine,[14] simocyclinone D8,[15] albicidin,[16] the plasmid-encoded toxins microcin B17,[17]and CcdB,[18] like quinolones and coumarins inhibit DNA gyrase, however no evidence of the inhibitory activity of these toxins have been found for Topo IV.

CcdB is the toxin component of the *ccd* toxin–antitoxin (TA) system. TA systems contribute to plasmid stability by a mechanism called post-segregational killing. This mechanism relies on the differential stabilities of the antitoxin and toxin proteins and leads to the killing of daughter bacteria that did not receive a plasmid copy at cell division.[19] TA systems are composed of two genes organized in an operon encoding a toxin and an antitoxin that antagonizes it. The antitoxin, an unstable protein is rapidly degraded, with loss of constitutive expression of the operon as a result of loss of the plasmid, while the toxin, a stable protein, is released to inhibit an essential cellular process. *Ccd* was the first TA system to be discovered and is located on the F plasmid with CcdB being the toxin and CcdA the antitoxin.[20] CcdB is known to act at a different stage of the catalytic cycle of DNA gyrase than the quinolone drugs. Moreover, there is no cross-resistance between quinolones and CcdB[21,22] and there are differences in the ATP requirement for

* Corresponding author. Tel.: +55 16 3301 6670; fax: +55 16 3301 6692.
E-mail address: marcheto@iq.unesp.br (R. Marchetto).

0960-894X/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2008.10.008

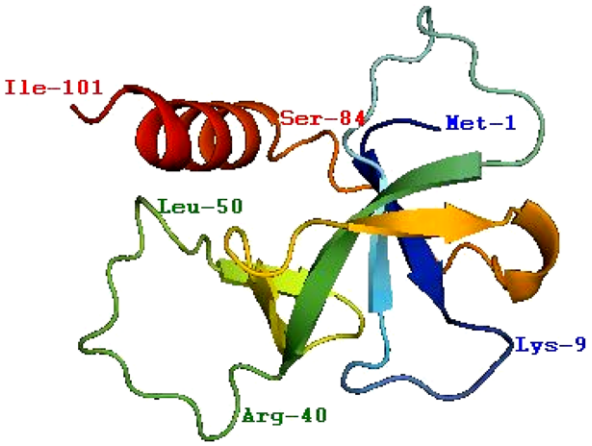

Figure 1. Ribbon diagram of the CcdB. The three segments, C-terminal α-helix, the loop of the wing sheet, and the N-terminal β-sheet, used in the *CcdBET2* design are colored in red, green, and blue, respectively.

cleavage complex stabilization,[23] suggesting that they do not interact at the same site on DNA gyrase.

The structure of CcdB (see Fig. 1) was determined in 1999[24] and the monomer consists of a five-stranded N-terminal β-sheet followed by a C-terminal α-helix. In between two strands of the main β-sheet, a second small three-stranded β-sheet is inserted that sticks out of the molecule as a wing. This wing encompasses loop residues 40–50, which has been hypothesized to the involved CcdA binding.[24] CcdB also forms a dimer with an interface highly hydrophobic formed by a strand (residues 68–72), called S6, several loop segments and the three C-terminal residues of the α-helix. Strand S6 pairs with the same strand of the opposite monomer, in an antiparallel way.[24] Based on CcdB structure we have designed and synthesized a series of linear analogues of CcdB to provide new type II topoisomerase inhibitors. For peptide design we considered the 13 residues C-terminal α-helix, the loop Arg40–Leu50 of the wing sheet, the strand S6, the N-terminal β-sheet (Met1–Leu9) and the residues Gly100 and Ile101 that seem to play a key role in the formation of CcdB–GyrA complex (Fig. 1).

Therefore, the first peptide analogue (*CcdBET1*) was built with the residues Ser84 to Ile101 of the CcdB. This amino acids sequence was maintained for the other analogues due its great structural importance and the role that play in the interaction with GyrA. For the second analogue (*CcdBET2*), two fragments of the protein sequence, one including the loop of the wing sheet and the other including an N-terminal β-sheet were added to the primary structure of the *CcdBET1*. A flexible linker was employed to connect these three fragments.[8,25] Taking into account these considerations, the peptide *CcdBET2* was built using the natural fragments 84–101, 40–50, and 1–9, and two residues of ε-amino hexanoic acid (Z) to connect them (Fig. 2). *CcdbET3*, the third CcdB analogue, was synthesized as *CcdBET2* but without the fragment 40–50. So, the amino acids sequence of *CcdBET3* corresponds to the natural fragments 84–101 and 1–9 with a residue of ε-amino hexanoic acid (Z) connecting them. *CcdBET4* analogue was built using the natural fragment 68–101 that includes the C-terminal α-helix, as the other analogues, and the strand S6[24] important for CcdB dimer formation. Finally, the residue Gly100, common for all synthetic peptides, was changed by Glu, an identified mutation that induce the loss of killer activity of the CcdB[26] and, therefore, the ability to form a complex with GyrA. All the peptide analogues, whose primary structures are presented in Figure 2, were synthesized by the solid-phase method, considering the usual polymer salvation parameters.[27]

The ability of the analogues to inhibit the supercoiling reaction of gyrase and relaxation reaction of Topo IV was investigated by titrating CcdB analogues into a fixed concentration of enzyme and DNA. The minimum concentration that produced complete inhibition of supercoiling or relaxation activities was termed the IC_{100}. In standard supercoiling assays at 37 °C with 3.4 nM of gyrase, a relaxed DNA (500 ng) substrate is completely negatively supercoiled in 1 h. *CcdBET2* inhibited this reaction (Fig. 3A) with an IC_{100} value of 10 μM. In addition, *CcdBET2* inhibited the ATP-dependent relaxation reaction of topoisomerase IV, in standard assays (37 °C, 5 nM of enzyme and 400 ng of supercoiled DNA) with an IC_{100} value of 5 μM (Fig. 3B). For both, there is no evidence of cleavage complex formation.

The lack of the loop Arg40–Leu50 yielded *CcdBET3* a peptide that was sixfold weaker than *CcdBET2*, in the supercoiling inhibition. A higher concentration (60 μM) was required for *CcdBET1* (without the loop and the N-terminal β-sheet) inhibit the supercoiling reaction. For the relaxation reaction the loss was lesser, only threefold for both peptide analogues (Table 1). The *CcdBET4*

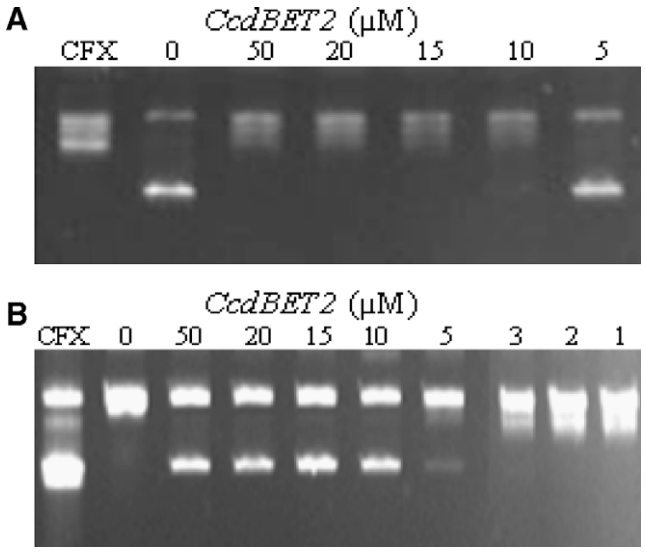

Figure 3. *CcdBET2*-mediated inhibition of DNA supercoiling reactions of DNA gyrase (3.4 nM) from *E. coli* (A) and effect of *CcdBET2* on relaxation of supercoiled DNA by topoisomerase IV (5 nM) from *E. coli* (B). Controls were no drug (lanes 0) and treatment with ciprofloxacin (10 μM), the known topoisomerase inhibitor (lanes CFX).

^{84}SHRENDIKNAINLMFWGI^{101} (***CcdBET1***)

^{1}MQFKVYTYK^{9}-**Z**-^{40}RLLSDKVSREL^{50}-**Z**-^{84}SHRENDIKNAINLMFWGI^{101} (***CcdBET2***)

^{1}MQFKVYTYK^{9}-**Z**-^{84}SHRENDIKNAINLMFWGI^{101} (***CcdBET3***)

^{68}MASVPVSVIGEEVADLSHRENDIKNAINLMFWGI^{101} (***CcdBET4***)

Figure 2. The primary structures of the synthesized CcdB analogues (Z = ε-amino hexanoic acid).

Table 1
Inhibitory activities of peptide analogues of CcdB on bacterial topoisomerase.

Peptide	IC_{100} (μM)[a]	
	DNA gyrase[b]	Topo IV[b]
CcdBET1	60	15
CcdBET2	10	5
CcdBET3	40	15
CcdBET4	ND	ND
CcdBET2M[c]	ND	5

ND, no detectable inhibitory activity.
[a] Concentration of the inhibitor required for complete inhibition of topoisomerase activity.
[b] From *Escherichia coli*.
[c] *CcdBET2* analogue with the substitution Gly → Glu at the position 100.

analogue, which contains the full amino acid sequence of CcdB devoid of the 67 N-terminal residues, did not inhibit the DNA gyrase and Topo IV activities.

From these results it is possible to speculate about of the requirement of the N-terminal sequence (Met1–Lys9) in a probable structure–activity relationship of the peptides synthesized. The best inhibitors (*CcdBET2* and *CcdBET3*) share this fragment and the analogue that showed the lesser activity (*CcdBET1*), not includes the Met1–Lys9 sequence in its primary structure. Probably the peptides with the fragment Met1–Lys9 contain enough structural information to induce the formation of an inactive peptide–enzyme complex. Additionally, in *CcdBET2*, the Arg40–Leu50 fragment seems to improve the molecular adjustments of the peptide in the complex, resulting in the most promising peptide inhibitor of the bacterial topoisomerases. The lack of inhibitory activity observed for *CcdBET4*, analogue devoid of those fragments, corroborates these findings.

Despite DNA gyrase and Topo IV share significant sequence homology and are targets of the same classes of drugs, the mode of action of CcdB on Topo IV has not been a lot investigated. For DNA gyrase, a model for CcdB action involves it binding in the GyrA dimer cavity when the DNA gate is open forming a single CcdB–GyrA complex.[24,28,29] This model places the three C-terminal residues of CcdB, critical to its toxicity,[24] in close proximity to Arg462 residue of the GyrA. Although it is known that Arg462 and the C terminus of CcdB (Trp99–Ile101) play a crucial role in the formation of the CcdB–GyrA complex,[24] data that relate these residues and the ParE subunit of Topo IV have never been alluded. In this context and to explore the role of the C-terminal residues of the *CcdBET2* in its inhibitory activity, the residue Gly100 was replaced by Glu, a change previously found in the CcdB from mutagenesis scan of the *ccd* gene.[26]

The effect of this change in the ability of *CcdBET2* to inhibit the supercoiling or the relaxation of DNA, was investigated by the same standard assays described above but using a fixed concentration of mutated peptide (20 μM). In the conditions of the assays the capacity of the *CcdBET2* to inhibit the DNA gyrase activity was abolished with the change of the amino acid in the position 100 (Fig. 4A). The change of the charge of the residue at position 100 to −1, may push this residue out of the C-terminal hydrophobic pocket, thereby changing the orientation of the residues that play the key role in the formation of the CcdB–GyrA complex. This behavior is in agreement with the CcdB mutants constructed by site-directed mutagenesis that lost their toxic activity and, therefore, their ability to form a complex with GyrA.[24,26] Unlike DNA gyrase, the change of the amino acid at position 100 did not alter the ability of *CcdBET2* to inhibit Topo IV activity (Fig. 4B). Most likely the C-terminal region of CcdB (Trp99–Ile100) is not involved in the interaction with the ParC subunit of Topo IV a proof that the mechanisms of inhibition of the DNA gyrase and the Topo IV by CcdB are different.

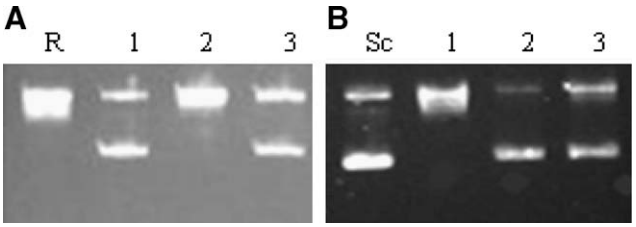

Figure 4. Effect of *CcdBET2* mutation on supercoiling of gyrase (A) and relaxation of Topo IV (B) reactions. The reactions were performed in the presence of *CcdBET2* (lanes 2) and *CcdBET2M* (lanes 3). Controls (lanes 1) were performed in the absence of peptides. SC and R represent supercoiled and relaxed pBR322, respectively.

Although a number of antibacterial agents have been developed, the continuous emergence of bacterial resistance is a problem that needs to be overcome. As bacterial topoisomerases are the most validated targets in antimicrobial therapies, the development of inhibitors that might simultaneously target both DNA gyrase and Topo IV, with equal potency has the potential to reduce the emergence of target-based resistance. Therefore, targeting both enzymes, *CcdBET2* is the newer inhibitor of bacterial topoisomerases that differs in its interaction mode and likely in the mechanism that inhibits DNA gyrase or Topo IV. For inhibition of supercoiling activity, the IC_{100} of *CcdBET2* (10 μM) is very close of obtained for CcdB in conditions of very low rate reaction (gyrase concentration 1.5 nM and temperature of 25 °C).[29] With Topo IV, the IC_{100} for inhibition of relaxation activity (5 μM) is less than CcdB (highest than 10 μM), obtained under conditions not shown.[29]

In summary, this is the first example of a synthetic peptide derived from a bacterial toxin with simultaneous inhibitory activity in DNA gyrase and topoisomerase IV. Interesting is that a molecule as *CcdBET2*, dimensional and structurally different has the same or higher activity than the original toxin structure. Certainly the *CcdBET2* sequence contains enough structural information to induce the formation of an inactive peptide–enzyme complex as the CcdB–GyrA or CcdB–ParC complexes. It is noteworthy that *CcdBET2*, differently of the CcdB, inhibits preferentially the Topo IV relaxation activity, evidence that this peptide has a mechanism that likely differs at the molecular level from CcdB. In fact, the substitution at C-terminal Gly100 rendered an inactive peptide on gyrase activity, consistent with the role of the three C-terminal residues in the interaction with Arg462 of GyrA.[28] This substitution had no effect on Topo IV. Therefore, the interaction of *CcdBET2* with Topo IV is made by a region of the molecule which the three C-terminal residues are not involved.

This study has identified a new class of potent type II topoisomerase inhibitors which might serve as lead for potential antibacterial agents.

Acknowledgments

This work was supported by FAPESP in the form of Grant (03/04492-3) and scholarship (C.A. Cotrim) and by CNPq Ph.D. fellowships (E. Trovatti and S.S. Garrido). R. Marchetto is recipient of research fellowship from CNPq.

References and notes

1. Corbett, K. D.; Berger, J. M. *Annu. Rev. Biophys. Biomol. Struct.* **2004**, *33*, 95.
2. Schoeffler, A. J.; Berger, J. M. *Biochem. Soc. Trans.* **2005**, *33*, 1465.
3. Ullsperger, C.; Cozzarelli, N. R. *J. Biol. Chem.* **1996**, *271*, 31549.
4. Crisona, N. J.; Strick, T. R.; Bensimon, D.; Croquette, V.; Cozzarelli, N. R. *Genes Dev.* **2000**, *14*, 2881.
5. Peng, H.; Marians, K. J. *J. Biol. Chem.* **1993**, *268*, 24481.
6. Khodursky, A. B.; Zechiedrich, E. L.; Cozzarelli, N. R. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 11801.

7. Sissi, C.; Perdonà, E.; Domenici, E.; Feriani, A.; Howells, A. J.; Maxwell, A.; Palumbo, M. *J. Biol. Chem.* **2001**, *311*, 195.
8. Marchetto, R.; Nicolás, E.; Castilho, N.; Bacardit, J.; Navia, M.; Vila, J.; Giralt, E. *J. Peptide Sci.* **2001**, *7*, 27.
9. Hiasa, H.; Yousef, D. O.; Marians, K. J. *J. Biol. Chem.* **1996**, *271*, 226424.
10. Morris, J. E.; Pan, X. S.; Fisher, L. M. *Antimicrob. Agents Chemother.* **2002**, *46*, 582.
11. Hardy, C. D.; Cozzarelli, N. R. *Antimicrob. Agents Chemother.* **2003**, *47*, 941.
12. Garrido, S. S.; Scatigno, A. C.; Trovatti, E.; Carvalho, D. C.; Marchetto, R. *J. Peptide Res.* **2005**, *65*, 502.
13. Fujimoro-Nakamura, M.; Ito, H.; Oyamada, Y.; Nishino, T.; Yamagishi, J.-I. *Antimicrob. Agents Chemother.* **2005**, *49*, 3810.
14. Goetschi, E.; Angehrn, P.; Gmuender, H.; Hebeisen, P.; Link, H.; Masciadri, R.; Nielsen, J. *Pharmacol. Ther.* **1993**, *60*, 367.
15. Flatman, R. H.; Howells, A. J.; Heide, L.; Fiedler, H.-P.; Maxwell, A. *Antimicrob. Agents Chemother.* **2005**, *49*, 1093.
16. Hashimi, S. M.; Wall, M. K.; Smith, A. B.; Maxwell, A.; Birch, R. G. *Antimicrob. Agents Chemother.* **2007**, *51*, 181.
17. Heddle, J. G.; Blance, S. J.; Zamble, D. B.; Hollfelder, F.; Miller, D. A.; Wentzell, L. M.; Walsh, C. T.; Maxwell, A. *J. Mol. Biol.* **2001**, *307*, 1223.
18. Couturier, M.; Bahassi, E. M.; Van Melderen, L. *Trends Microbiol.* **1998**, *6*, 269.
19. Hayes, F. *Science* **2003**, *301*, 1496.
20. Ogura, T.; Hiraga, S. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 4784.
21. Bernard, P.; Couturier, M. *J. Mol. Biol.* **1992**, *226*, 735.
22. Critchlow, S. E.; O'Dea, M. H.; Howells, A. J.; Couturier, M.; Gellert, M.; Maxwell, A. *J. Mol. Biol.* **1997**, *273*, 826.
23. Bernard, P.; Kézdy, K. E.; Van Melderen, L.; Steyaert, J.; Wyns, L.; Pato, M. L.; Higgins, M. P.; Couturier, M. *J. Mol. Biol.* **1993**, *234*, 534.
24. Loris, R.; Dao-Thi, M.-H.; Bahassi, E. M.; Van Melderen, L.; Poortmans, F.; Liddington, R.; Couturier, M.; Wyns, L. *J. Mol. Biol.* **1999**, *285*, 1667.
25. Scatigno, A. C.; Garrido, S. S.; Marchetto, R. *J. Peptide Sci.* **2004**, *10*, 566.
26. Bahassi, E. M.; Salmon, M. A.; Van Melderen, L.; Bernard, P.; Couturier, M. *Mol. Microbiol.* **1995**, *15*, 1031.
27. Marchetto, R.; Cilli, E. M.; Jubilut, G. N.; Schreier, S.; Nakaie, C. R. *J. Org. Chem.* **2005**, *12*, 4561.
28. Dao-Thi, M.-H.; Van Melderen, L.; De Genst, E.; Afif, H.; Buts, L.; Wyns, L.; Loris, R. *J. Mol. Biol.* **2005**, *348*, 1091.
29. Smith, A. B.; Maxwell, A. *Nucleic Acids Res.* **2006**, *34*, 4667.