Proceedings of the 36th European Peptide Symposium
Michal Lebl (Editor)
European Peptide Society, 2022 *https://doi.org/10.17952/36EPS.2022.031*

Peptide Dimerization Effect on Bacterial Topoisomerases Activity

Camila Aguiar Rocha, Jonatas Medeiros de Almeida Angelo, Edson Crusca, and Reinaldo Marchetto

UNESP - Institute of Chemistry, Department of Biochemistry and Organic Chemistry, Caixa Postal 355, 14.800-900 - Araraquara, São Paulo, Brazil

Introduction

Bacterial DNA topoisomerases are essential enzymes that play critical roles in many biological processes involving DNA and consequently the cell growth. Type II DNA topoisomerases are attractive targets to antibacterial therapy [1]. The increasing incidence of drug-resistant bacteria emphasizes the urgent need for a new generation of antibacterial drugs. In this context, WRWYCRCK, a peptide conceived as of a positional scanning screen of an L-amino acid synthetic peptide combinatorial library [2], was synthesized, oxidized and investigated regarding the disulfide bonds effect in the inhibition of topoisomerases activity and microorganisms' growth. The dimeric peptides showed best results of inhibition for all tested topoisomerases compared to monomer peptide. The CM3 peptide, the parallel two-disulfide bonds dimer was the most effective. For bacterial growth, the CM1 dimer (BR1020190256524) was the most effective. The dimerization of WRWYCRCK peptide was sufficient to improve its activity on type I topoisomerases and to turn it into a new inhibitor of bacterial type II topoisomerases.

Results and Discussion

One of the main threats to human health are infectious diseases caused by multidrug-resistant microorganisms. In addition, studies show that the number of new antibiotics on the market has decreased significantly [3]. Therefore, it is urgent the development of new antimicrobial drugs, to be more potent and less susceptible to the different mechanisms of drug resistance. A new and important approach for the development of new antimicrobials is through the synthesis of DNA topoisomerases inhibitor peptides [4]. In this context, the octapeptide WRWYCRCK (Lin1) and its linear derivatives were synthesized by solid-phase methodology employing the Fmoc/tBu strategy, considering the usual polymer solvation parameters [5]. Acetamidomethyl and trityl groups were used as cysteine side chain protectors as a way to direct disulfide bonds. Cysteine residue at 5 or 7 position was also replaced by alanine to change the number and position of disulfide bonds in the dimers (Figure 1). The peptides were analyzed and purified by RP-HPLC and the chemical identity was confirmed by mass spectrometry (positive ES-MS): 1240.6, 1311.6, 1208.5 and 1208.5 g/mol (Lin1, Lin2, A^7Lin1 and A^5Lin1, respectively).

$Ac^{1}WRWYCRCK^{8}\text{-}NH_2$ ***Lin1***

$Ac^{1}WRWYCRC(Acm)K^{8}\text{-}NH_2$ ***Lin2***

$Ac^{1}WRWYCR\mathbf{A}K^{8}\text{-}NH_2$ ***A^7Lin1***

$Ac^{1}WRWY\mathbf{A}RCK^{8}\text{-}NH_2$ ***A^5Lin1***

Fig. 1. Primary structure of synthesized linear peptides.

Lin1 and its linear derivatives were then oxidized with air or iodine to obtain their respective dimeric forms (Table 1), which were obtained with good yield (40 - 50%) and high purity (98 to 99%). The peptides' ability to inhibit the supercoiling reaction of DNA gyrase and the relaxation reaction of topoisomerase IV (Topo IV) and Topo IA was investigated by gel electrophoresis. The assays were performed by titrating Lin1 and its dimeric forms into a fixed concentration of topoisomerase and DNA followed by IC_{100} determination (the minimum concentration that produces complete inhibition of supercoiling or relaxation activity). In the standard supercoiling and relaxation assays at 37°C, an

Table 1. Primary structure and molecular mass characterization of dimers.

Peptide	*Primary structure*	*MW (g.mol^{-1})*	*ES+*	*ES m/z (g.mol^{-1})*
CM1	Ac^{1}WRWY**C**RAK^{8}-NH$_2$	1,241.49	+2	621.3
	Ac^{1}WRWY**C**RAK^{8}-NH$_2$		+3	414.5
CM2	Ac^{1}WRWYAR**C**K^{8}-NH$_2$	1,312.56	+2	656.8
	Ac^{1}WRWYAR**C**K^{8}-NH$_2$		+3	438.2
CM3	Ac^{1}WRWY**C**R**C**K^{8}-NH$_2$	1,209.43	+3	605.3
	Ac^{1}WRWY**C**R**C**K^{8}-NH$_2$		+4	403.8

initial screening at 100 µmol/L selected CM1, CM2 and CM3 as the better topoisomerases inhibitors (complete inhibition). Lin1 did not inhibit DNA gyrase and Topo IV activities.

Subsequently the IC_{100} for the selected peptides were determined (Figure 2) and showed that CM3 was better, inhibiting 37% of the supercoiling reaction of gyrase and relaxation reaction of Topo IV with IC_{100} values of 25 and 10 µmol/L, respectively (Table 2). CM1 and CM2 showed only partial inhibition at 50 and 75 µmol/L, respectively. The dimers were more efficient in inhibiting DNA gyrase and Topo IV compared to the monomer, which did not inhibit the activity of this enzyme, evidencing the importance of dimerization in the inhibitory activity of Lin1. The two disulfide bonds C^5-$C^{5'}$ and C^7-$C^{7'}$ of CM3 were shown to be important for peptide-enzyme interactions, especially to Topo IV. Topoisomerases inhibitors, as CM3 peptide, which act simultaneously in both DNA gyrase and Topo IV, may greatly contribute to reduce the emergence of target-based resistance.

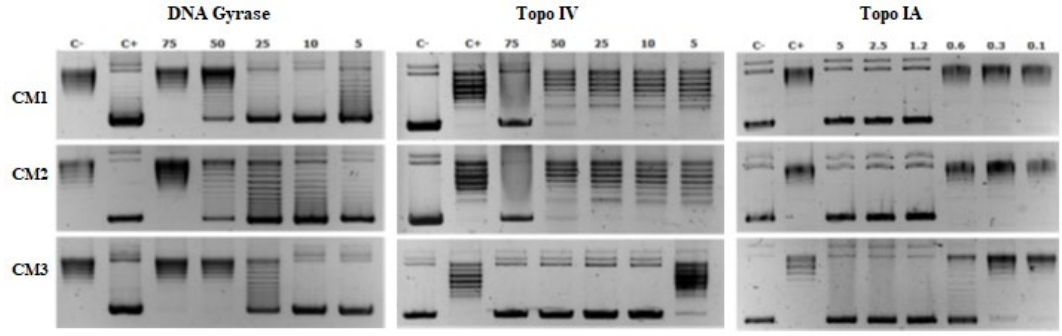

Fig. 2. IC_{100} determination of dimeric peptides CM1, CM2 and CM3 for DNA supercoiling of DNA gyrase and DNA relaxation of TopoIV and Topo IA reactions. Line C-: relaxed plasmid pBR322 for DNA Gyrase and supercoiling DNA for TopoIV and TopoIA. Line C+: relaxed pBR322+DNA gyrase; supercoiling DNA+TopoIV; supercoiling DNA+TopoIA, in the absence of peptide. The indicated values are the peptide in µmol/L. The enzymes are from E. coli.

As bacterial topoisomerase IA is essential for cellular viability of a number of important bacterial pathogens, this enzyme also represents a valid target for novel antibiotics aimed at overcoming antimicrobial resistance [6]. Again, CM3 was the best inhibitor (Table 2). Completely inhibited the Topo IA relaxation reaction with an IC_{100} of 0.6 µmol/L. CM1 and CM2, which differ only in disulfide bond position, inhibited the enzyme identically (1.2 µmol/L) but less efficiently than CM3, which has

two disulfide bonds. Lin1 peptide, which does not contain any disulfide bonds, was the least potent, as observed for type II topoisomerases. The enzymatic assays showed that the presence of two disulfide bonds seems to favor the inhibitory activity of the octapeptide Lin1, regardless of the topoisomerase type. The CM3 dimer, which has two specific and targeted disulfide bonds, was more effective in inhibiting type I and type II topoisomerases when compared to those with only one (CM1 and CM2) or no disulfide bond (Lin1). Probably, the greater structural rigidity of dimers containing two disulfide bonds should favor the inhibition of different topoisomerases involving the formation of a more stable peptide-enzyme complex with lower rotational mobility.

Table 2. Inhibitory activities of monomeric and dimeric peptides on bacterial topoisomerases.

Peptide	***IC_{100} (µmol/L)***		
	DNA gyrase	***Topo IV***	***Topo IA***
Lin1	ND	ND	>10[d*]
CM1	>50[a*]	75	1.2
CM2	>50[b*]	75	1.2
CM3	>25[c*]	10	0.6

*IC_{100} = Concentration of the peptide required for complete inhibition of topoisomerase activity; ND = No detectable inhibitory activity; *estimated by AlphaView software (AlphaImager®EP); [a]83% inhibition of E. coli gyrase at 50 µmol/L; [b]70% inhibition of E. coli gyrase at 50 µmol/L; [c]37% inhibition of E. coli gyrase at 25 µmol/L; [d]42% inhibition of E. coli topo IA at 10 µmol/L*

The antimicrobial activity of peptides was evaluated by analysis the growth of *E. coli* (ATCC 43895), *S. aureus* (ATCC 14458) and *C. albicans* (ATCC 90028), in a microdilution test [7]. The MIC values were considered as the lowest concentration of peptide able to inhibit at least 90% of the strains growth, within 24 hours for the bacteria and 48 hours for the fungus. It was observed that the oxidation of one or two cysteine residues of Lin1 affects differently the growth of the tested microorganisms. CM1 showed the best growth inhibition results of all groups of microorganisms. It was observed that the oxidation of one or two cysteine residues of Lin1 affects differently the growth of the tested microorganisms. Differently of topoisomerases inhibition studies, CM1, CM2 and CM3 showed very similar activities. CM1 was the most effective in inhibiting the growth of the three microorganisms tested (Table 3). Apparently, the C^5-$C^{5'}$ disulfide bond allows CM1 to have greater rotational mobility, compared to CM2 and CM3, probably due the greater molecular symmetry provided by this bond. This characteristic, added to the composition of the intracellular medium and concentration conditions, different from those used in the enzymatic inhibition assays, reflects in a better adjustment of the CM1

Table 3. Antimicrobial activity of monomeric and dimeric peptides.

Peptide	***MIC (µmol/L)***		
	S. aureus	***E.coli***	***C. albicans***
Lin1	5	0.6	1.2
CM1	2.5	0.1	0.2
CM2	2.5	0.3	0.6
CM3	10	0.3	1.2

MIC = Minimal inhibitory concentration

to its binding site with the target enzyme and, consequently, better results in growth inhibition assays. The intracellular environment effect is quite evident when considering the Lin1 activities. Lin1 was the peptide with the lowest inhibitory activity for all topoisomerases, but showed great antimicrobial activity, especially for Gram-negative bacteria and fungi. Unlike the medium used for the enzymatic inhibition assays, the intracellular medium can provide conditions for the natural oxidation of Lin1, favoring their dimerization and consequently improving their inhibitory capacity on topoisomerases and their antimicrobial activity. Furthermore, the microorganism's membrane permeability must also be considered. Molecules with greater rotational freedom such as CM1 can be transported more easily across the biological membrane.

The dimerization of WRWYCRCK was sufficient to improve its activity on type I topoisomerases and to turn it into a new inhibitor of bacterial type II topoisomerases. Dimeric peptides from WRWYCRCK demonstrated a good potential to constitute a new generation of antimicrobial agents.

Acknowledgments

We gratefully acknowledge São Paulo Research Foundation (FAPESP) for financial support (2018/19468-6) and National Council for Scientific and Technological Development (CNPq) to the research fellowship to R. Marchetto.

References

1. Delgado, J.L., et al. *Biochem. J.* **475**, 373-398 (2018), https://doi.org/10.1042/BCJ20160583
2. Fujimoto, D.F., et al. *J. Molec. Biol*. **5**, 891-907 (2006), https://doi.org/10.1016/j.peptides.2012.12.025
3. Fernandes, P. Martens, E. *Biochem. Pharmacol*. **133**, 152-163 (2017), https://doi.org/10.1016/j.bcp.2016.09.025
4. Lau, J.L., Dunn, M.K. *Bioorg. Med. Chem*. **26**, 2700-2707 (2018), https://doi.org/10.1016/j.bmc.2017.06.052
5. Marchetto, R., et al. *J. Org. Chem*. **12**, 4561-4568 (2005), https://doi.org/10.1021/jo9611632
6. Yang, R., et al. *Int. J. Mol. Sci.* **20**, 1116 (2019), https://doi.org/10.3390/ijms20051116
7. Almeida, C.V., et al. *BBA Gen. Sub.* **1865**, 129937 (2021), https://doi.org/10.1016/j.bbagen.2021.129937