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PEPTIDE-CONJUGATES DERIVED FROM BACTERIAL ParE TOXIN: SYNTHESIS AND BIOLOGICAL STUDIES

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INTRODUCTION

Antimicrobial resistance represents the major public health problem of the 21st century. New therapeutic agents, with innovative modes of action, are urgently needed given the seriousness of the problem. Peptides derived from bacterial toxins could be considered as a way to avoid this global health threat. ParELC3, a peptide derived from bacterial toxin ParE was reported to be a good inhibitor of bacterial topoisomerases *in vitro* and therefore a potential antibacterial agent [1]. However, a permeability limitation prevents its use as a new therapeutic agent. For circumvent this permeability limitation and promote a dual inhibitory targeting, we decided to design and synthesize molecular structures resulting from the conjugation of a ParELC3 analog with quinolone and coumarin groups to permeate the bacterial membrane and act synergistically in the inhibition of different topoisomerases. The peptide ParELC3M and respective conjugates CJHOAc, CJCOQ, CJCFX and CJNCFX (Figure 1b) were chemically synthesized by Solid Phase methodology, purified by RP-HPLC and characterized by mass spectrometry. (ESI-MS). The obtained peptide-conjugates were evaluated for their ability to inhibit topoisomerases activity and prevent the bacterial growth. In standard supercoiling and relaxation assays, the peptide ParELC3M and its conjugates did not inhibit the *E. coli* and *S. pneumoniae* DNA gyrase activity. On the other hand, all conjugates were able to inhibit completely the *M. tuberculosis* DNA gyrase activity and relaxation reaction of *E. coli* and *S. pneumoniae* topo IV with expressive IC_{100} values. In addition, CJCFX and CJNCFX conjugates inhibited *E. coli* topo IA with IC_{100} smaller than 6.25 μmol.L^{-1}. For yeast topoisomerase II, the inhibition was even more effective. The conjugates were effective against *E. coli* and *S. aureus* with MIC values of 25 μmol.L^{-1}. The results also showed low cytotoxicity for both peptide and peptide-conjugates with a cell viability greater than 75% even under the highest tested concentration. The results showed that the insertion of coumarin and fluoroquinolones groups to a peptide chain increase its capacity to permeate the bacterial plasma membrane and reach the cytoplasm, where can exert its activity.

METHODOLOGY

Synthesis, purification and characterization

The peptide ParELC3M and its conjugates (Figure 1) were synthesized by solid-phase methodology, using Fmoc/tBu chemistry with a RinK amide MBHA resin (0.55 mmol.g^{-1}) and DIC/HOBt activation. After deprotection and cleavage with TFA/water/EDT/TIS (94.5:2.5:2.5:0.5) for 2h at RT, the peptides were precipitated, solubilized and dried under vacuum. Analysis and purification was performed by RP-HPLC and the identity of the peptide and peptide-conjugates was confirmed by ESI-MS.

Antimicrobial activity assays

The antimicrobial activity on standard strains of *Staphylococcus aureus* (ATCC 14458) and *Escherichia coli* (ATCC 43895) and *Candida albicans* (ATCC 90028) was evaluated by broth microdilution assay performed in 96-well microplates, as described in the Clinical and Laboratory Standards Institute, reference guide M7-A10 and M27-A3 [2,3] with modifications as previously described [4]. The plates were submitted to spectrophotometric analysis, using a Biotek Instruments EPOCH microplate reader, at a wavelength of 595 nm. This assay was performed in triplicate.

Cytotoxicity assays

The cytotoxicity was investigated by MTT assay in MRC-5 cells (ATCC CCL-171). Briefly, 100 μL of a cell suspension in DMEM-C (DMEM medium supplemented with 10% (v/v) FBS, 40 μg.mL^{-1} gentamicin and 1 μL.mL^{-1} streptomycin) at 5 x 10^4 cells/mL density were added to each well of a sterile 96-wells plate and incubated for 24 h at 37°C in 5% CO_2 for cell adhesion. The cells were washed with PBS buffer, pH 7.4. Aliquots of 100 μL of peptide and conjugates solutions serially diluted in DMEM-C (200 to 25 μmol.L^{-1}) were added to each well, followed by incubation for 72 h at 37°C. The solution was removed and the cells were washed with PBS buffer. Subsequently, 180 μL of DMEM:PBS (1:4) and 20 μL of MTT (1.0 mg.mL^{-1}) solutions were added, followed by incubation for 4 h at 37ºC in 5% CO_2. The supernatant was discarded and 100 μL of isopropyl alcohol:DMSO (1:1) was added and the absorbance read at 540 nm. DMEM-C and cells in DMEM-C were the negative and positive controls, respectively. Cell viability was obtained by the ratio between the optical density (OD) of the treated cells and that of the negative control. This assay was performed in triplicate (Figure 2).

Topoisomerase inhibition assays

The ability of peptides inhibits the topoisomerase supercoiling and relaxation reactions were investigated by gel electrophoresis assays [1].

The standard supercoiling reaction (30 μL) contained relaxed pBR322 DNA (500 ng), *E. coli, S. pneumoniae or M. tuberculosis* DNA gyrase (3.4 nmol L^{-1}) and indicated amounts of samples were carried out in the assay buffer and incubated at 37°C for 1 hour. The reaction was stopped by adding 15 μL of STEB and 60 μL of chloroform: isoamyl alcohol (24:1 v/v). The mixture was centrifuged and the supernatant was analyzed on a 1% agarose gel in TBE buffer. The samples were electrophoresed at 50V for 2 h. The gel was stained with ethidium bromide solution (1 mg mL^{-1}) and analyzed by an Alpha Imager EP System of Alpha Innotech. The ImageJ software was used to process the gel images and quantify the bands (Figure 3).

The *E. coli* or *S. pneumoniae* topoisomerase IV (topo IV) relaxation reactions were carried out in the assay containing supercoiled pBR322 DNA (400 ng), topo IV (5.0 nmol L^{-1}) and indicated amounts of samples, at 37°C for 1 hour. The mixture treatment and the gel analysis were made as described for the gyrase inhibition assay (Figure 4).

The reaction catalyzed by *E.coli* topoisomerase IA (topo IA) was carried out in 30 μL of assay containing supercoiled pBR322 DNA (0.5 μg), topo IA (1U) and indicated amounts of samples, at 37°C for 20 min The gel was analyzed as described for the gyrase and Topo IV inhibition assay (Figure 5A).

For yeast topo II inhibition assay, 30 μL of a standard reaction contained supercoiled pBR322 DNA (0.5 μg), topo II (1U), ATP solution (1 μL) and indicated amounts of each sample, in the assay buffer, was prepared. The mixture was incubated at 37ºC for 30 min. The mixture treatment and the gel analysis were made as above (Figure 5B).

RESULTS

Synthesis and Structures

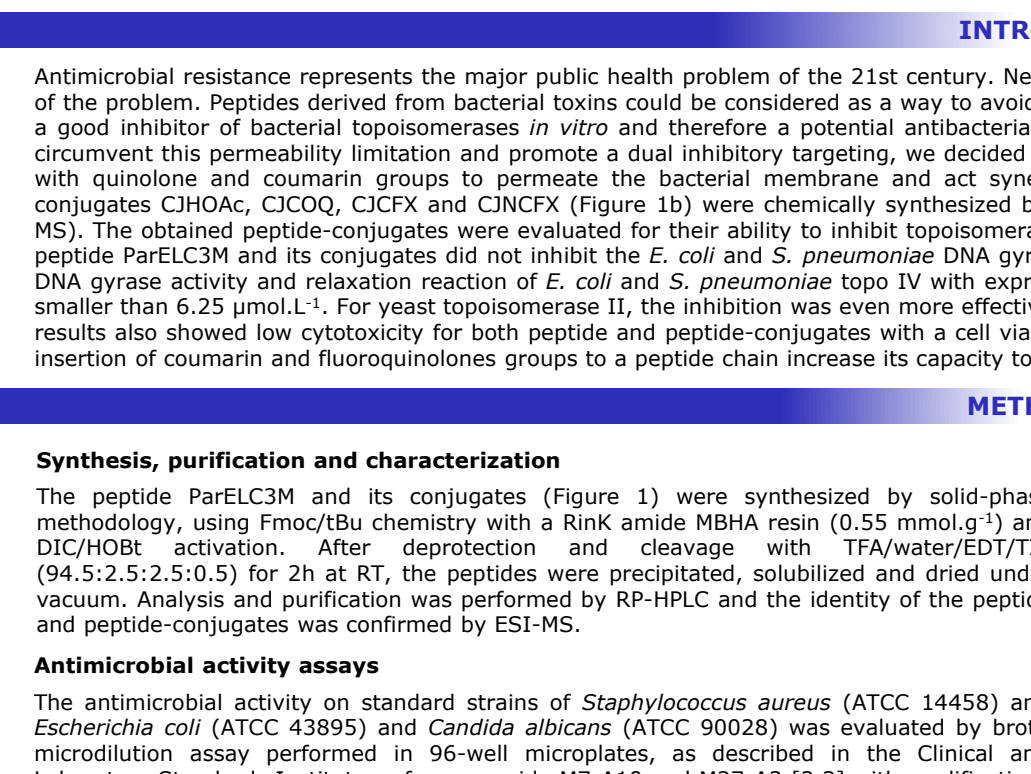

Figure 1. ParELC3M, CFX, CICPOQ and HOCAc structures (A); Synthesis scheme of peptide-conjugates CJCFX, CJCOQ, CJHOAc (B) and CJNCFX (C)

Characterization

Sample	Calculated MW (g.mol^{-1})	ES+	ES m/z (g.mol^{-1})	Obtained MW (g.mol^{-1})
ParELC3M	2,467.99	+3 +4	824.00 618.49	2,469.18
CJCCFX	2,739.30	+3 +4	914.71 686.09	2,740.74
CJCOQ	2,689.61	+3 +4	898.35 674.02	2,692.05
CJHOAc	2,628.13	+3 +4	877.67 658.27	2,629,54
CJNCFX	2,910.77	+3 +4	971.46 728.86	2,911.41

Antimicrobial Assays

Sample	Minimal inhibitory concentration (MIC)					
	E. coli		*S. aureus*		*C. albicans*	
	μmol.L^{-1}	%	μmol.L^{-1}	%	μmol.L^{-1}	%
ParELC3M	100	ND	100	ND	100	ND
CJHOAc	100	ND	100	ND	100	ND
CJCOQ	100	ND	100	ND	100	ND
CJCFX	100	96	50	96	100	ND
CJNCFX	100	95	25	98	100	ND

Cytotoxicity Assays

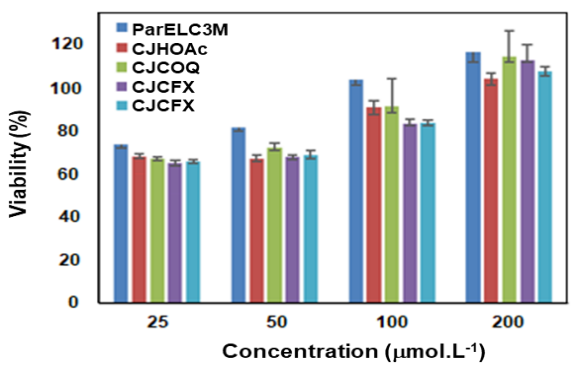

Figure 2. Cell viability assay for MRC-5 cells for ParELC3M peptide and its conjugates. Incubation time =72 h. Standard deviation are indicates by vertical bars.

Gel electrophoresis assays

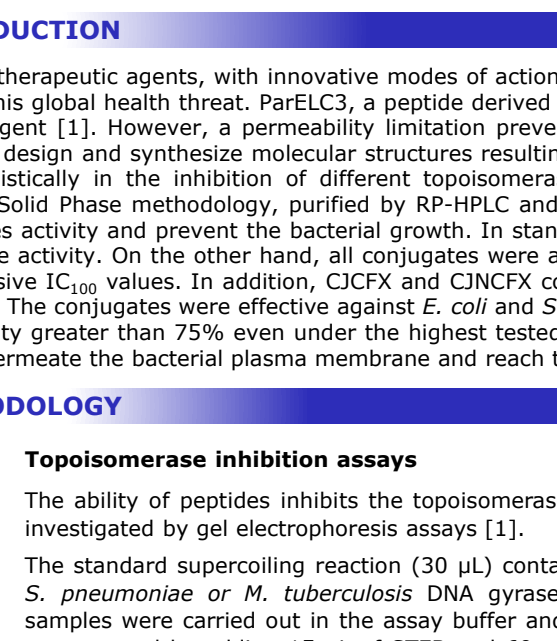

Figure 3. Initial screening at 100 μmol.L^{-1} of ParELC3M, CJHOAc, CJCOQ, CJCFX and CJNCFX for the DNA supercoiling reaction catalyzed by **DNA Gyrase** from (A) *E. coli,* (B) *S. pneumoniae* and (C) *M. tuberculosis. (*OC=nicked open circular; R=relaxed topoisomers; SC= supercoiled topoisomers

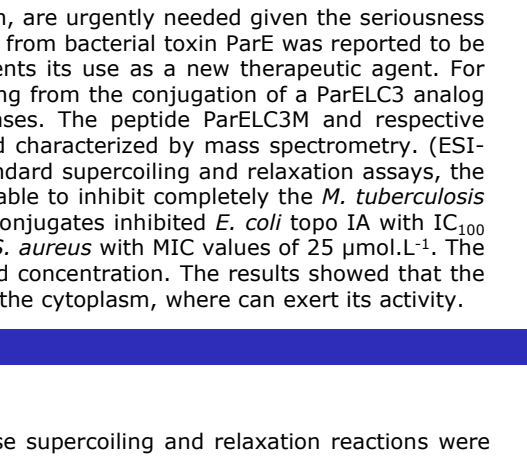

Figure 4. Initial screening at 100 μmol.L^{-1} of ParELC3M, CJHOAc, CJCOQ, CJCFX and CJNCFX for the DNA supercoiling reaction catalyzed by **Topo IV** from (A) *E. coli* and (B) *S. pneumoniae*. OC= nicked, open circular; R=relaxed topoisomers; SC= supercoiled topoisomers

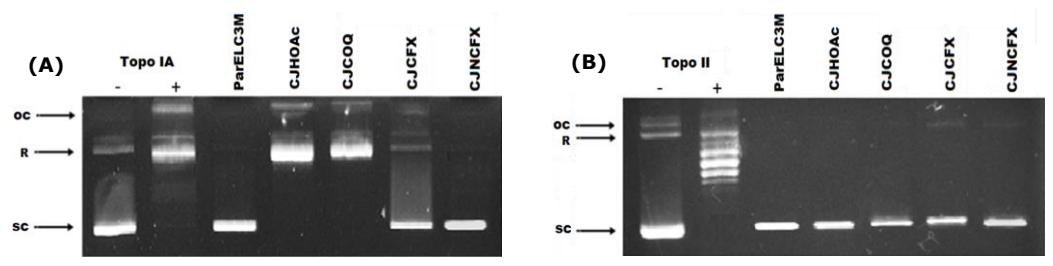

Figure 5. Initial screening at 100 μmol.L^{-1} of ParELC3M, CJHOAc, CJCOQ, CJCFX and CJNCFX for the DNA relaxation reaction catalyzed by **Topo IA** from *E. coli* **(A)** and DNA supercoiling reaction catalyzed by Topo II from Yeast **(B)**. OC= nicked, open circular; R=relaxed topoisomers; SC= supercoiled topoisomers

Sample	IC_{100} (μmol.L^{-1})						
	DNA Gyrase			Topo IV		Topo IA	Topo II
	E. coli	*S. pneumoniae*	*M. tuberculosis*	*E. coli*	*S. pneumoniae*	*E. coli*	*yeast*
ParELC3M	75	ND	50	25	50	100	12.5
CJHOAc	ND	ND	50	50	50	ND	3.125
CJCOQ	ND	ND	50	50	50	ND	6.25
CJCFX	75	ND	12.5	25	50	6.25	3.125
CJNCFX	75	ND	25	25	50	6.25	3.125

IC_{100} = Concentration of the peptide required for complete inhibition of topoisomerase activity;
ND = No detectable inhibitory activity.

CONCLUSIONS

- The primary target of the ParELC3M conjugate with CFX is Topo IV enzyme unlike free CFX that is DNA gyrase.
- In Gram-negative bacteria, the main targets of peptide conjugates are Topo IV and Topo IA enzymes while for Gram-positives the target is DNA gyrase.
- Peptide-CFX conjugates showed bactericidal activity, with CJNCFX as the most active. This fact may be related to the C3/C4 ketoacid region of the quinolone, which is free in this conjugate.
- Peptide-CFX conjugates inhibited the yeast Topo II activity with very expressive IC_{100} values, but were unable to inhibit the growth of *C. albicans*, basically due the inability to permeate the fungal cell membrane.
- The results showed that the insertion of coumarin and fluoroquinolones groups to ParELC3M increase its capacity to permeate the bacterial (but not the fungal) plasma membrane and reach the cytoplasm, where exert its activity.

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