

In Vitro Cytotoxicity of Native and Rec-Pediocin CP2 Against Cancer Cell Lines: A Comparative Study

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Abstract

Pediocin CP2 is a natural antimicrobial peptide produced by *Pediococcus acidilactici* MTCC 5101. Its recombinant version was created by employing computational protein engineering approach and was expressed as a synthetic fusion protein in recombinant *E. coli* BL21(DE3)-*pedA*. Both native and rec-pediocin were comparatively evaluated for their cytotoxicity against HepG2 (a hepatocarcinoma cell line), HeLa (a cervical adenocarcinoma), MCF7 (a mammary gland adenocarcinoma) and Sp2/O-Ag14 (a spleen lymphoblast) cell lines. Inhibition of cell proliferation was quantitated by MTT assay and induction of apoptosis was studied by genomic DNA fragmentation assay. Results indicated a significantly higher cytotoxicity of rec-pediocin and damage of chromosomal DNA in bacteriocin tested cell lines.

Keywords: Pediocin CP2; Rec-pediocin; Antineoplastic activity; Apoptosis

Introduction

Bacteriocins of lactic acid bacteria have primarily been applied for food preservation but these natural antimicrobial peptides can have applications in wider field of human wellness as therapeutic and prophylactic agents. Their suitability as biopharmaceuticals has been explored *in vitro* as well as *in vivo* through determinations of antimicrobial and cytotoxic effects [1-4]. Results obtained in these experiments suggest that pediocins show a great promise as a class of human therapeutic and prophylactic agents. Pediocins are promising biomolecules for conventional antibiotic treatments for several reasons. In cases where emergence of antibiotic resistance phenotype in important human pathogens is observed, these may provide invaluable tool for fighting against them. The restricted antimicrobial spectrum of class IIa pediocins minimizes their impact on commensal microbiota and tries to maintain gut homeostasis by decreasing colonization of opportunistic pathogens and by eradicating an already established infection. Furthermore, their proteinaceous nature makes them susceptible to degradation and their degradation products are easily metabolized by the body [5,6].

Pediocin CP2 is a plasmid linked class IIa, heat stable, anti-listerial peptide produced by *P. acidilactici* CP2 MTCC5101 [7]. It exerts antibacterial, anti-fungal and spore inhibitory effect against a number of pathogenic and food spoilage organisms [8,9]. As biological and chemical modifications have been suggested to stabilize and increase potency of bacteriocins. Keeping this in view, pediocin CP2 was engineered to enhance its antimicrobial range, production and expression in heterologous *E. coli* BL21(DE3)-*pedA* and highly encouraging results were obtained [10]. In this study, we have tried to establish antiproliferative and cytotoxic effect of native and rec-pediocin CP2 against diverse neoplastic line cells.

Materials and Methods

Procurement and maintenance of cultures

Pediococcus acidilactici MTCC 5101 was revived and maintained in MRS medium containing 0.1% (v/v) Tween-80; pH 6.5 at 37°C. *Listeria monocytogenes* MTCC 657 was procured from MTCC, Chandigarh,

India. It was maintained as broth and agar cultures in Brain Heart Infusion medium at 37°C.

Production and purification of native pediocin CP2

P. acidilactici MTCC 5101 was grown overnight in MRS broth; pH 6.5 at 37°C. Native pediocin CP2 was purified by conventional method of adsorption-desorption [11]. Bacteriocin preparation was then filter sterilized using Millipore 0.45 mm filters [12].

Pediocin activity assay

Bacteriocin activity in native and recombinant cell cultures can also be determined using spot-on-lawn assay [13]. It was carried out by spotting 5 µl CFS or dilutions of pure bacteriocin preparations on MRS bottom agar plates overlaid with 3-4 ml TGE soft agar containing 6 log units of *L. monocytogenes*. Plates were incubated at 37°C for 24 h and inhibition zones were scored. Bacteriocin titre was expressed as reciprocal of the highest dilution showing a definite zone of inhibition or cell lysis in the resultant lawn culture.

Production and purification of recombinant pediocin CP2

Recombinant pediocin was expressed using T7 driven pET32(b)-*pedA* in *E. coli* BL21(DE3)-*pedA* by adding IPTG in the 4h old culture broth. Over expressed Rec-pediocin in the form of inclusion bodies (IBs) was extracted from the cell lysates by urea lysis and it was renatured using refolding buffer containing 5 mM concentration of imidazole and β-mercaptoethanol. Since rec-pediocin protein bears two affinity purification tags, thus it was purified from the crude cell extract by employing tandem affinity purification (TAP) approach which included Ni-NTA affinity chromatography, enterokinase digestion of rec-pediocin and streptactin affinity chromatography [10,11].

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Cell lines used for testing *in vitro* cytotoxicity of rec-pediocin

HepG2, a hepatocarcinoma cell line was procured from NCCS, Pune, India. HeLa ATCC CCL2 (a cervical adenocarcinoma cell line of *Homo sapiens*), MCF7 ATCC-HTB-22 (a mammary gland adenocarcinoma cell line of *Homo sapiens*) and Sp2/O-Ag14 ATCC-CRL-1581 (a spleen lymphoblast cell line of *Mus musculus*) were gifted by Dr. Sanjog Jain, Niper, Mohali. Cell proliferation was carried out as per standard method [14]. Cell lines were seeded in the recommended growth medium supplemented with 10% fetal bovine serum (FBS) and 100µg/ml penicillin and streptomycin and cultured in a humidified 5% CO₂ incubator at 37°C (Table 1). Cell densities were maintained between 5 × 10⁴ and 5 × 10⁵ viable cells/ml. After reaching 80% confluence, spent medium was discarded. Cell layers were rinsed with 0.25% (w/v) trypsin and 0.53mM EDTA solution to remove all traces of serum which may contain trypsin inhibitor. 2.0 to 3.0 ml Trypsin-EDTA solution was added to each flask and cells were observed under the inverted microscope and observed for dispersion. Dispersion was facilitated by keeping the flasks at 37°C. 6.0 to 8.0 ml of fresh growth medium was added; cells were aspirated by gently pipetting and transferred to new culture vessels. Cell lines were exposed to two different concentrations of rec-pediocin ranging from 1 µg/ml and 25 µg/ml for 48 hours at 37°C. For exposure time over 24 hours, the

tissues were fed with the fresh medium. Cell counts were taken using haemocytometer. After the required exposure time, MTT assay was carried out to determine overall cell viability.

MTT cell viability assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was carried out according to the protocol given by Shashi et al. [15]. Viability of the cells exposed to bacteriocin, was measured as a direct proportion of the breakdown of yellow compound tetrazolium to dark blue water insoluble formazon. Only the metabolically active cells can show this reaction which can be solubilized with 100µl DMSO and then quantified. The liquid in the plate wells was combined with the liquid from the tissue. Mixture is then assayed spectrophotometrically at 570 nm using 96 wells plate reader to determine level of tetrazolium degradation. % cell viability was calculated using NCL method GTA 2 (Nanotechnology Characterization Laboratory, National Cancer Institute, Frederick) as described below;

$$\% \text{ cell viability} = \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Cellfree sample blank}} \times 100}{\text{OD}_{\text{Medium control}}}$$

Cell lines	Growth medium	Atmosphere	Growth temp.	Growth condition	Storage
Hep G2	1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium containing 2.5mM L-glutamine, 15mM HEPES, 0.5mM sodium pyruvate, 1200mg/l sodium bicarbonate, 100µg/ml penicillin and streptomycin, 10% FBS	95% air; 5% CO ₂	37°C	Add fresh medium every 4 to 5 days (depending on cell density)	Complete growth medium supplemented with 5% (v/v) DMSO; liquid nitrogen
HeLa	Eagle's Minimum Essential Medium containing 100µg/ml penicillin and streptomycin, 10% FBS	95% air; 5% CO ₂	37°C	Add fresh medium every 2 to 4 days (depending on cell density)	Complete growth medium supplemented with 5% (v/v) DMSO; liquid nitrogen
MCF7	Eagle's Minimum Essential Medium containing 0.01 mg/ml bovine insulin; 100µg/ml penicillin and streptomycin, 10% FBS	95% air; 5% CO ₂	37°C	Add fresh medium every 2 to 4 days (depending on cell density)	Complete growth medium supplemented with 5% (v/v) DMSO; liquid nitrogen
Sp2/O-Ag14	Dulbecco's Modified Eagle's Medium containing 100µg/ml penicillin and streptomycin, 10% FBS	95% air; 5% CO ₂	37°C	Add fresh medium every 2 to 4 days (depending on cell density)	Complete growth medium supplemented with 5% (v/v) DMSO; liquid nitrogen

Table 1: Growth parameters of cell lines used to assay *in vitro* cytotoxicity of native and rec-pediocin.

Bacteriocin	Concentration (µg/ml)	O.D of sample	O.D of cell free sample blank	% cell viability
MCF-7				
Rec-pediocin	1	0.309 ± 0.055	0.289 ± 0.012	6.135 ± 0.071
	25	0.363 ± 0.084	0.356 ± 0.029	2.147 ± 0.118
Native pediocin CP2	1	0.489 ± 0.011	0.306 ± 0.016	56.135 ± 0.092
	25	0.350 ± 0.019	0.315 ± 0.019	10.736 ± 0.097
HeLa				
Rec-pediocin	1	0.439 ± 0.015	0.300 ± 0.015	42.638 ± 0.102
	25	0.341 ± 0.018	0.304 ± 0.013	11.349 ± 0.119
Native pediocin CP2	1	0.479 ± 0.017	0.289 ± 0.019	58.282 ± 0.034
	25	0.457 ± 0.011	0.409 ± 0.011	14.723 ± 0.128
Sp2/O-Ag14				
Rec-pediocin	1	0.454 ± 0.012	0.289 ± 0.012	50.613 ± 0.24
	25	0.353 ± 0.015	0.356 ± 0.015	0.0 ± 0.0
Native pediocin CP2	1	0.546 ± 0.012	0.306 ± 0.017	73.619 ± 0.165
	25	0.402 ± 0.018	0.315 ± 0.015	26.687 ± 0.106
Hep G2				
Rec-pediocin	1	0.361 ± 0.012	0.289 ± 0.012	22.085 ± 0.241
	25	0.374 ± 0.015	0.356 ± 0.014	5.521 ± 0.018
Native pediocin CP2	1	0.449 ± 0.016	0.306 ± 0.029	43.865 ± 0.231
	25	0.319 ± 0.019	0.315 ± 0.018	1.26 ± 0.169

Table 2: Cytotoxic effect of native and rec-pediocin on cancerous cells.

DNA fragmentation assay

Fragmentation of the genomic DNA was studied in the most sensitive cell line according to Muller et al. [16] procedure with slight modifications. Sp2/O-Ag14 (1×10^5) were cultured for 24 h, treated with 1mg/ml rec-pediocin for 48 h, and then lysed with 250 μ l lysis buffer containing 100 mM NaCl, 5 mM EDTA, 10 mM Tris, 0.5% Triton X-100, 0.25% SDS, 0.2% lithium chloride; pH 8.0. 200 μ g/ml proteinase K was added and digestions were performed at 50°C for 60 min. Lithium chloride was also added to assist cell lysis and as an inhibitor of nucleases. After incubation, suspension was centrifuged at 13,000 rpm for 3 mins, aqueous phase was transferred to fresh tube containing deproteinizing mixture of phenol, chloroform and isoamyl alcohol (25:24:1) and again centrifuged at 3,000 rpm for 3 min. DNA was precipitated from the aqueous phase with 3 volumes of chilled ethanol containing 0.3 M sodium acetate at 4°C. Samples were subjected to electrophoresis in 1% w/v agarose gel using $1 \times$ TBE buffer at 50V and visualized on a UV transilluminator.

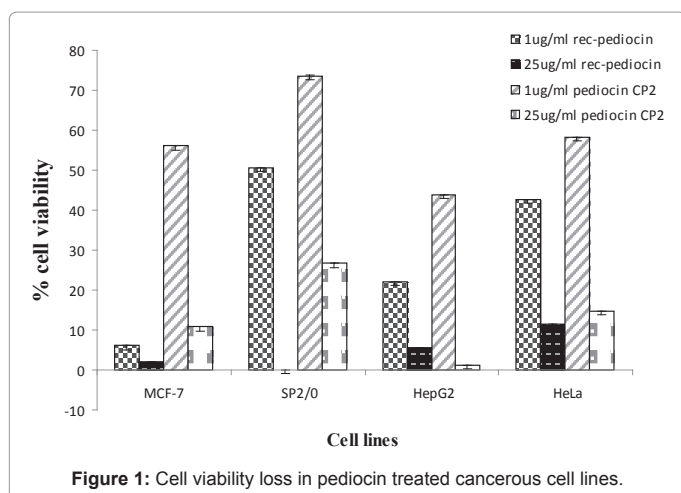
Statistical analysis

Wherever appropriate, data was expressed as mean values \pm standard deviations. A probability value of p-value <0.05 was used as the criterion for statistical significance.

Results

Anti-cancerous activity of rec-pediocin

Cytotoxicity of native and rec-pediocin was comparatively investigated on several cancerous cell lines with different morphologies and physiology. Table 2 and figure 1 show cell viability after 48 h of incubation in a medium containing 1 μ g/ml and 25 μ g/ml of native and rec-pediocin. Sp2/O-Ag14 presented a highest sensitivity to rec-pediocin CP2, whereas MCF-7, Hep G2 and HeLa cell lines were sensitive at different degrees to the toxic effect of native and rec-pediocin. After 48 hours of exposure to rec-pediocin, epithelial tissue models retained only a low level of viability. Total cell viability of Sp2/O-Ag14 dropped down to 0% level due to acute toxicity of 25 μ g/ml of rec-pediocin as compared to cell lines treated with native pediocin, where they have retained 26.687% viability. The % viability of MCF-7 cell lines exposed to 25 μ g/ml of rec-pediocin and native pediocin CP2 was 2.147% and 10.736% respectively, whereas that of HepG2 cell lines was 5.521% and 1.226% respectively. HeLa cells exhibited less degree of sensitivity towards rec-pediocin when compared to other cancerous



cell lines of spleen lymphoblast, hepatocarcinoma and mammary gland adenocarcinoma.

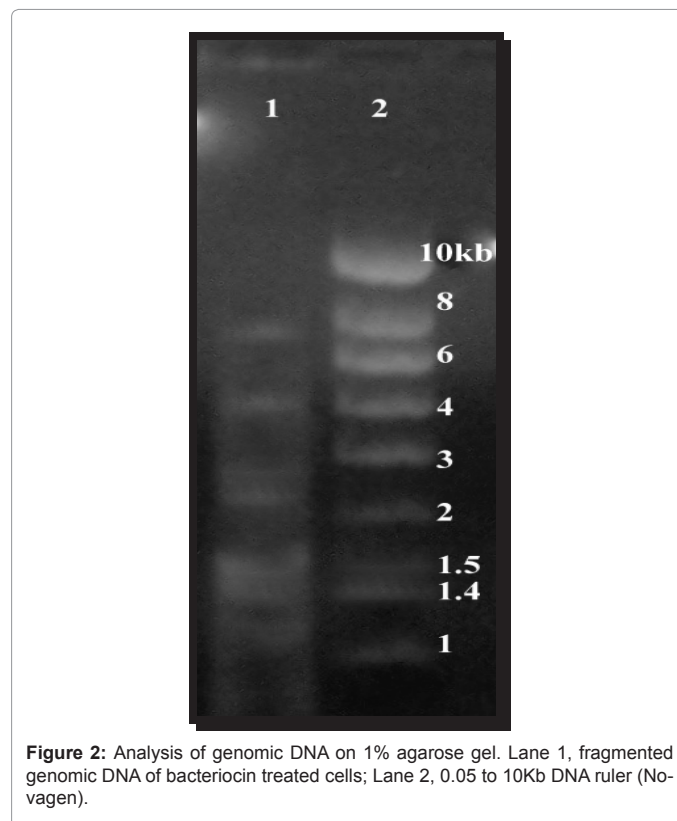
Results indicated a concentration dependent viability loss in both native as well as rec-pediocin treated cell lines and MCF-7, a mammary gland adenocarcinoma cell line being the most sensitive cell line studied. Further the results confirmed that in terms of sensitivity MCF-7 of mammary gland carcinoma is followed by Hep G2 of hepatocarcinoma. Sp2/O-Ag14 indicated only a low level of viability loss (approx. 49%) upon treatment with 1 μ g/ml rec-pediocin, while it showed 100% viability loss when treated with 25 μ g/ml rec-pediocin. Results obtained in the study were significant at p-value <0.05 and therefore, suggest a great therapeutic potential of rec-pediocin for treatment of various forms of cancer.

DNA fragmentation in bacteriocin treated cells

Apoptosis in tissues was examined by DNA fragmentation assay. Sp2/O-Ag14 cells were treated with rec-pediocin at 1mg/ml for 48 h. Rec-pediocin was reported to increase fragmentation of genomic DNA in cancerous cells as indicated by ladder formation in 1% agarose gel (Figure 2). These results provided an evidence that rec-pediocin induces cell-cycle arrest and apoptosis in cells.

Discussion and Conclusions

Antineoplastic properties of bacteriocins such as colicin [17], microcin [18], pediocin [19] and pyocin [20] have been inadequately revealed towards diverse neoplastic line cells. Microcin B17 was shown to inhibit DNA gyrase in the bacterial cells which leads to accumulation of double-stranded DNA breaks [21]. In this respect, bacteriocins show similarity to that of commonly used antineoplastic drugs such as quinolones. Interestingly, microcin B17 (bearing oxazole and thiazole groups) shares structural homology similar to bleomycin, a peptide used



in the treatment of cancers especially of Hodgkin disease and germinal cancers for more than 30 years [22]. Pyocins F and S produced by *Pseudomonas aeruginosa* show structure homology with bacteriophage tails [23]. Antineoplastic activity of pyocin has been established against mouse hepatocarcinoma and lymphoblastic leukemia using HepG2 and Im9 cell lines, whereas human fetal foreskin fibroblast was unaffected [24]. Its uptake is possibly mediated by iron-related receptors in bacterial cells [25] and transferrin receptors in mammalian cells [26]. This mechanism is reinforced by the fact that iron deprivation stops cell division in G1/S and leads to apoptosis in some neoplastic cell lines [27]. However, detailed *in vivo* investigation is required on potential use of pediocins as therapeutic agents or prophylactic compounds.

Carl Vogt [28] described the principle of apoptosis which shows it as a programmed death of cells, which may occur even in multicellular organisms. Various biochemical changes such as cell membrane damage, cell shrinkage, nuclear fragmentation, chromatin condensation and genetic DNA fragmentation take place during apoptosis. DNA fragmentation takes place at the end of apoptosis, which includes activation of calcium and magnesium dependent nucleases that degrade genomic DNA of susceptible cells. Currently used anticancer drugs have been shown to induce apoptosis in susceptible cells [29]. Nuclear DNA of cells that have entered in the phase of apoptosis shows a characteristic ladder pattern of oligonucleosomal fragments, which is regarded as the hallmark of apoptosis [14]. A series of studies have provided convincing evidence suggesting that the antimicrobial peptides or bacteriocins produced by lactic acid bacteria inhibit growth of cancer cells [30]. Inhibition of cell proliferation by colicins [17], microcin [18], pediocin [19] and pyocin [20] has been established in breast carcinoma, breast adenocarcinoma, osteosarcoma, leiomyosarcoma, fibrosarcoma, T cell lymphoma, cervix carcinoma, Burkitt lymphoma, pulmonary carcinoma, colon adenocarcinoma, lymphoblastic leukemia, and hepatocarcinoma.

The results presented here indicate cytotoxic effect of rec-pediocin on various cancerous cell lines tested in the study. The cytotoxic effect on cancerous cells from human origin was also reported earlier [31]. The uniqueness of the bacteriocins lies in their interaction with the cell surface without penetrating the target cells, yet affecting cell division and DNA synthesis [32]. Bacteriocins are highly specific in their membrane interaction which is related to the unique receptors found in different bacterial species or types [33]. Preliminary experiments with rec-pediocin have shown its cytotoxicity against cancerous cell lines and which is attributed through the induction of programmed cell death or apoptosis. In future, this information could be integrated and exploited to fully explore the suitability of rec-pediocin as *in vivo* therapeutics.

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