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

Balvir Kumar1*, Praveen P Balgir1, Baljinder Kaur1, Bharti Mittu1 and Ashish Chauhan2

1Department of Biotechnology, Punjabi University, Patiala, Punjab-147002, India
2Department of Chemistry, National Institute of Technology, Jalandhar, Punjab, India

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 Sp2/0-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 reasons. In cases where emergence of antibiotic resistance phenotype in important human pathogens is observed, these may provide invaluable reason for their cytotoxicity against HepG2 (a hepatocarcinoma cell line), HeLa (a cervical adenocarcinoma), MCF7 (a mammary gland adenocarcinoma) and Sp2/0-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.

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 µm 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 5µl CFS or dilutions of pure bacteriocin preparations on MRS bottom agar plates overlaid with 3–4ml TGE soft agar containing 6 log units of L. monocytogenes. Plates were incubated at 37°C for 24h 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 5mM 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.

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.

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which included Ni-NTA affinity chromatography, enterokinase digestion of rec-pediocin and streptactin affinity chromatography [10,11].

**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 X 10^4 and 5 X 10^5 viable cells/ml. After reaching 80% cell viability was calculated using NCL method GTA (2(Nanotechnology Characterization Laboratory, National Cancer Institute, Frederick) as described below; 2 (Nanotechnology Characterization Laboratory, National Cancer Institute, Frederick) as described below;

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 [15]. Viability of the cells exposed to bacteriocin, was measured as a direct proportion of the breakdown of yellow compound tetrazolium bromide to formazan which is used to measure cell proliferation[15]. The cells were treated with different concentrations of rec-pediocin ranging from 1µg/ml and 25µg/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.

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 X 10^5) were cultured for 24h, treated with 1µg/ml rec-pediocin for 48h, and then lysed with 250µl lysis buffer containing 100mM NaCl, 5mM EDTA, 10mM Tris, 0.5% Triton X-100, 0.25% SDS, 0.2% lithium chloride; pH 8.0. 200µg/ml proteinase K was added and digestions were performed at 50°C for 60min. 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 proteolyzing 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 1X TBE buffer at 50V and visualized on a UV transilluminator.

Statistical analysis

Wherever appropriate, data was expressed as mean values ± 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 48h of incubation in a medium containing 1µg/ml and 25µg/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. Sp2/O-Ag14 dropped down to 0% level due to acute toxicity of 25µg/ml of rec-pediocin as compared to cell lines treated with native pediocin, whereas MCF-7, Hep G2 and HeLa models retained only a low level of viability. Total cell viability of Sp2/O-Ag14 (1 X 10^5) were cultured for 24h, treated with 1µg/ml rec-pediocin for 48h, and then lysed with 250µl lysis buffer containing 100mM NaCl, 5mM EDTA, 10mM Tris, 0.5% Triton X-100, 0.25% SDS, 0.2% lithium chloride; pH 8.0. 200µg/ml proteinase K was added and digestions were performed at 50°C for 60min. 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 proteolyzing 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 1X TBE buffer at 50V and visualized on a UV transilluminator.

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<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Growth medium</th>
<th>Atmosphere</th>
<th>Growth temp.</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>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/ml sodium bicarbonate, 100µg/ml penicillin and streptomycin, 10% FBS</td>
<td>95% air ; 5% CO2</td>
<td>37°C</td>
<td>Add fresh medium every 4 to 5 days (depending on cell density) Complete growth medium supplemented with 5% (v/v) DMSO ; liquid nitrogen</td>
</tr>
<tr>
<td>HeLa</td>
<td>Eagle's Minimum Essential Medium containing 100µg/ml penicillin and streptomycin, 10% FBS</td>
<td>95% air ; 5% CO2</td>
<td>37°C</td>
<td>Add fresh medium every 2 to 4 days (depending on cell density) Complete growth medium supplemented with 5% (v/v) DMSO ; liquid nitrogen</td>
</tr>
<tr>
<td>MCF7</td>
<td>Eagle's Minimum Essential Medium containing 0.01 mg/ml bovine insulin; 100µg/ml penicillin and streptomycin, 10% FBS</td>
<td>95% air ; 5% CO2</td>
<td>37°C</td>
<td>Add fresh medium every 2 to 4 days (depending on cell density) Complete growth medium supplemented with 5% (v/v) DMSO ; liquid nitrogen</td>
</tr>
<tr>
<td>Sp2/O-Ag14</td>
<td>Dulbecco's Modified Eagle's Medium containing 100µg/ml penicillin and streptomycin, 10% FBS</td>
<td>95% air ; 5% CO2</td>
<td>37°C</td>
<td>Add fresh medium every 2 to 4 days (depending on cell density) Complete growth medium supplemented with 5% (v/v) DMSO ; liquid nitrogen</td>
</tr>
</tbody>
</table>

**Table 1:** Growth parameters of cell lines used to assay in vitro cytotoxicity of native and rec-pediocin.
Treatment of various forms of cancer. Therefore, suggest a great therapeutic potential of rec-pediocin for hepatocarcinoma. Sp2/O-Ag14 indicated only a low level of viability of mammary gland carcinoma is followed by Hep G2 of line studied. Further the results confirmed that in terms of sensitivity both native as well as rec-pediocin treated cell lines and MCF-7, a mammary gland adenocarcinoma cell line being the most sensitive 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 1µg/ml rec-pediocin, while it showed 100% viability loss when treated with 25µg/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-deprivation stops cell division in G1/S and leads to apoptosis 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

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Concentration (µg/ml)</th>
<th>O.D of sample</th>
<th>O.D of cell free sample blank</th>
<th>% cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec-pediocin</td>
<td>1</td>
<td>0.309±0.055</td>
<td>0.289±0.012</td>
<td>6.135±0.071</td>
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<td></td>
<td>25</td>
<td>0.363±0.084</td>
<td>0.356±0.029</td>
<td>2.147±0.118</td>
</tr>
<tr>
<td>Native pediocin CP2</td>
<td>1</td>
<td>0.489±0.011</td>
<td>0.306±0.016</td>
<td>56.135±0.092</td>
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<td></td>
<td>25</td>
<td>0.350±0.019</td>
<td>0.315±0.019</td>
<td>10.736±0.097</td>
</tr>
<tr>
<td>HeLa</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec-pediocin</td>
<td>1</td>
<td>0.439±0.015</td>
<td>0.300±0.015</td>
<td>42.638±0.102</td>
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<tr>
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<td>0.341±0.018</td>
<td>0.304±0.013</td>
<td>11.349±0.119</td>
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<tr>
<td>Native pediocin CP2</td>
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<td>0.289±0.019</td>
<td>58.282±0.034</td>
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<td>0.457±0.011</td>
<td>0.409±0.011</td>
<td>14.723±0.128</td>
</tr>
<tr>
<td>Sp2/O-Ag14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec-pediocin</td>
<td>1</td>
<td>0.454±0.012</td>
<td>0.289±0.012</td>
<td>50.613±0.24</td>
</tr>
<tr>
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<td>0.353±0.015</td>
<td>0.356±0.015</td>
<td>0.0±0.0</td>
</tr>
<tr>
<td>Native pediocin CP2</td>
<td>1</td>
<td>0.546±0.012</td>
<td>0.306±0.017</td>
<td>73.619±0.165</td>
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<td></td>
<td>25</td>
<td>0.402±0.018</td>
<td>0.315±0.015</td>
<td>26.687±0.106</td>
</tr>
<tr>
<td>HepG2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rec-pediocin</td>
<td>1</td>
<td>0.361±0.012</td>
<td>0.289±0.012</td>
<td>22.085±0.241</td>
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<td>0.374±0.015</td>
<td>0.366±0.014</td>
<td>5.521±0.018</td>
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<tr>
<td>Native pediocin CP2</td>
<td>1</td>
<td>0.449±0.016</td>
<td>0.306±0.029</td>
<td>43.865±0.231</td>
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<td>0.319±0.019</td>
<td>0.315±0.018</td>
<td>1.269±0.169</td>
</tr>
</tbody>
</table>

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

Figure 1: Cell viability loss in pediocin treated cancerous cell lines.

Figure 2: Analysis of genomic DNA on 1% agarose gel. Lane 1, fragmented genomic DNA of bacteriocin treated cells; Lane 2, 0.05 to 10Kb DNA ruler (Novagen).
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.

References

1. Kheadr E, Bernoussi, N, Lacroix C, Fliss I (2004) Comparison of the sensitivity of various cancerous cell lines tested in the study. The cytotoxic effect on cancerous cells from human origin was also reported earlier.


