In Vitro Activity of Cyclic Dipeptides Against Gram-Positive and Gram-Negative Anaerobic Bacteria and Radioprotective Effect on Lung Cells

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Abstract Cyclic dipeptides isolated from *Streptomyces* sp. have been shown to have antimicrobial activity as well as other potentially useful biological activities. The purpose of this study was to compare the *in vitro* activity of two cyclic dipeptides combined against anaerobic bacteria with the activity of other antimicrobial agents. Specifically, the *in vitro* activity of the combination of two cyclic dipeptides was investigated against 140 clinical isolates of anaerobic bacteria by the agar dilution method and was compared with that of erythromycin, cefoxitin, imipenem, clindamycin, and metronidazole. The cyclic dipeptide combination and imipenem were the most active antimicrobial agents tested. In addition, the cyclic dipeptide combination had a radioprotective effect on five normal human lung fibroblast cells, showing survival rates higher (>90%) than either of the two cyclic dipeptides alone (< 80%).

Key words: Combined cyclic dipeptide, anaerobic bacteria, radioprotective effect

(A)

(B)

Fig. 1. Chemical structure of the isolated compound, (A) cyclo(L-phenylalanyl-L-prolyl) and (B) cyclo(L-leucyl- L-prolyl).

Evolution and rapid spread of resistant bacterial strains present a serious hospital-acquired infectious problem and are increasingly responsible for community-acquired infections. Thus, antimicrobial agents effective against resistant organisms are badly needed. Anaerobic bacteria commonly cause serious infections, and increased resistance of anaerobes to several antimicrobial agents has also been problematic in recent years [1, 2, 5]. Thus, evaluation of antimicrobial agents for activity against anaerobic bacteria is imperious. We recently reported efficacies of cyclo(L-leucyl-L-prolyl) and cyclo(L-phenylalanyl-L-prolyl) against VRE (vancomycin-resistant enterococci) and other pathogenic microorganisms, as well as antitumor effect, inhibition of TOPO enzyme, and antimutagenic activity [11–14]. The structure of both compounds is shown in Fig. 1. Furthermore, we found the
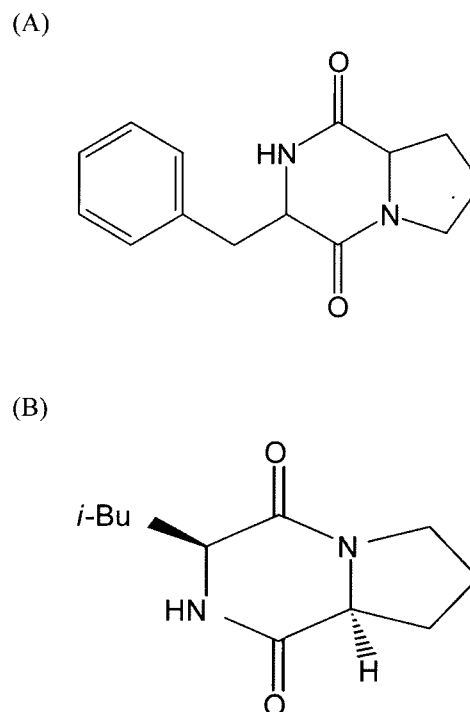
combination of cyclo(leu-pro) plus cyclo(phe-pro) synergically inhibited microorganisms, the TOPO enzyme, tumorigenesis, and mutagenesis [11]. To the best of our knowledge, the combination of two cyclic dipeptides [(cyclo(leu-pro) plus cyclo(phe-pro)] against anaerobic bacteria and its radioprotective effect have never been shown before. We, therefore, examined the *in vitro* effect of combined cyclic dipeptides, and compared the activity with those of several antimicrobial agents, including erythromycin, cefoxitin, imipenem, clindamycin, and metronidazole against several Gram-positive (*Clostridium difficile*, *C. perfringens*, and *Propionibacterium acnes*) and Gram-negative (*Bacteroides*

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Table 1. *In vitro* activity of cyclo(leu-pro) plus cyclo(phe-pro) and other antimicrobial agents against anaerobic bacteria.

Microorganism (no. of isolates)	Antimicrobial agent	MIC (mg/l)		
		MIC_{50}	MIC_{90}	Range
Bacteroides fragilis (n=22)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.032	0.125	0.032–0.5
	Cyclo(leu-pro)	0.25	0.5	0.25–2.0
	Cyclo(phe-pro)	0.5	1.0	0.25–4.0
	Erythromycin	1.0	2.0	0.25–8.0
	Cefoxitin	8.0	32	1.0–>64
	Imipenem	0.125	0.5	0.125–1.0
	Clindamycin	0.25	0.5	0.125–2.0
	Metronidazole	0.5	1.0	0.25–4.0
Bacteroides ovatus (n=25)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.25	0.5	0.125–1.0
	Cyclo(leu-pro)	1.0	2.0	0.5–4.0
	Cyclo(phe-pro)	1.0	4.0	1.0–8.0
	Erythromycin	0.5	1.0	0.25–8.0
	Cefoxitin	0.5	4.0	1.0–8.0
	Imipenem	0.125	0.25	0.032–0.5
	Clindamycin	2.0	4.0	0.5–8.0
	Metronidazole	4.0	32	2.0–>64
Clostridium difficile (n=20)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.064	0.125	0.032–0.5
	Cyclo(leu-pro)	0.125	0.25	0.125–0.5
	Cyclo(phe-pro)	0.125	0.5	0.125–1.0
	Erythromycin	1.0	4.0	0.25–8.0
	Cefoxitin	0.5	1.0	0.125–2.0
	Imipenem	0.125	0.25	0.064–0.5
	Clindamycin	0.5	1.0	0.125–4.0
	Metronidazole	0.125	0.5	0.25–1.0
Clostridium perfringenes (n=20)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.5	1.0	0.125–2.0
	Cyclo(leu-pro)	1.0	2.0	0.5–4.0
	Cyclo(phe-pro)	1.0	2.0	1.0–4.0
	Erythromycin	2.0	8.0	0.25–16
	Cefoxitin	1.0	2.0	1.0–32
	Imipenem	0.125	0.5	0.064–1.0
	Clindamycin	0.5	2.0	0.25–4.0
	Metronidazole	2.0	4.0	1.0–16
Propionibacterium acnes (n=35)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.064	0.125	0.032–0.25
	Cyclo(leu-pro)	0.125	1.0	0.125–2.0
	Cyclo(phe-pro)	0.125	1.0	0.125–2.0
	Erythromycin	1.0	2.0	0.5–8.0
	Cefoxitin	4.0	32	2.0–>64
	Imipenem	0.5	1.0	0.25–4.0
	Clindamycin	2.0	4.0	1.0–16
	Metronidazole	1.0	2.0	0.5–8.0
Peptostreptococci (n=18)	Cyclo(leu-pro)+Cyclo(phe-pro)	0.5	2.0	0.125–4.0
	Cyclo(leu-pro)	1.0	4.0	1.0–4.0
	Cyclo(phe-pro)	0.5	8.0	1.0–8.0
	Erythromycin	0.5	2.0	0.25–8.0
	Cefoxitin	0.5	1.0	0.125–4.0
	Imipenem	0.25	1.0	0.25–4.0
	Clindamycin	8.0	32	0.125–4.0
	Metronidazole	4.0	64	2.0–>64

fragilis, *B. ovatus*, and *Peptostreptococci*) anaerobic bacteria. Furthermore, we investigated the protective effect of the drug combination on several normal human lung fibroblast cell lines.

Antimicrobial agents of known activity were obtained from suppliers: erythromycin (Serva, Feinbiochemia GmbH & Co., Heidelberg, Germany), cefoxitin (The Upjohn Co. Chicago, U.S.A.), imipenem (Merk & Co., Inc., West Point,

U.S.A.), clindamycin and metronidazole (Sigma Chemical Co., St. Louis, MO, U.S.A.). The 140 strains of anaerobic bacteria used in this study were clinical isolates obtained between April 2003 and February 2004 from Korea Cancer Center Hospital (KCCH) (Seoul, Korea). All isolates were identified by standard criteria [6, 16], and the species and number of strains tested are shown in Table 1. MICs were determined by the agar dilution method as described earlier by Rhee et al. [14]. In brief, both cyclic dipeptides were dissolved in water with 8% methanol, and pH was adjusted to 4.0 with 2 M NaOH. A pH-adjusted 8% methanol-water solution without dissolved substances was used as a negative control. The concentrations of antimicrobial agents tested ranged from 0.125 to 128 mg/l. Colony suspensions equivalent to a 0.5 McFarland standard were prepared and inoculated onto antibiotic-containing medium using a Cathra Systems replicating device (MCT Medical, Inc., St. Paul, MN, U.S.A.) to yield a final inoculum of 10^4 CFU/spot. The plates were incubated in ambient air at 35°C for 24 h. The MIC was defined as the lowest antibiotic concentration that completely inhibited growth.

Six normal human lung fibroblast cell lines were obtained from the KCCH and maintained in alpha-MEM containing 15% fetal bovine serum (Summit Biotechnology, Ft. Collins, CO, U.S.A.) and antibiotics. The seed volume was 5×10^3 CFU/ml, and exponentially growing cells were used for most experiments. Both drugs alone [cyclo(phe-leu) or cyclo(phe-pro)] and the drug combination [cyclo(phe-leu) plus cyclo(phe-pro)] were administered to the cells at 1 μg/ml. Control cells were not pretreated with any drug. After treatment with drugs for 24 h, irradiations were carried out using a ^{137}Cs irradiator at a dose rate of 20 Gy/min. Colorimetric assays were performed by modification of a previously described assay method [7, 9, 10, 17]. Briefly, assays were carried out in 96-well plates. After 24 h of incubation, the cells were fixed in 50% cold TCA for 1 h at 4°C in the dark. The media and TCA were removed, and the plates were rinsed five times with water and then air-dried. The cells were stained by addition of 50 μl of 0.4% sulforhodamine B (SRB) (Sigma, St. Louis, MO, U.S.A.)

in 1% acetic acid for 10 min. The stain was removed and the cells were washed five times with 1% acetic acid and subsequently air-dried. One-hundred μl of 10 mM unbuffered Tris was added to each well to dissolve the dye. The plates were shaken for 5 min until the dye was uniformly distributed and then read on an Emax Precision Plate Reader (Molecular Devices, Sunnyvale, CA, U.S.A.) at 490 nm. Media were used as a blank for these assays. The antimicrobial activities of cyclo(leu-pro) combined with cyclo(phe-pro) and 5 antimicrobial agents against selected anaerobic bacterial strains are shown in Table 1. The drug combination and imipenem were highly active against *B. fragilis*, *B. ovatus*, *C. difficile*, *C. perfringens*, and *Propionibacterium acnes*, with $MIC_{90}s$ (90% of isolates were inhibited) ranging from 0.032 to 0.25 mg/l. MICs were high (8 mg/l) for only 3 of the 22 *B. fragilis* strains tested. Furthermore, the drug combination was extremely active against all the anaerobic bacteria, with $MIC_{50}s$ of ≤0.25 mg/l, except *Peptostreptococci*. Overall, the drug combination was more active than either of the two cyclic dipeptides alone, as well as cefoxitin, imipenem, clindamycin, and metronidazole. The drug combination was least effective against *Peptostreptococci* with MIC_{50} of 0.5 and MIC_{90} of 2.0 mg/l. Furthermore, MICs of clindamycin and metronidazole were 8.0 and 4.0 mg/l ($MIC_{50}s$) and 32 and 64 mg/l ($MIC_{90}s$), respectively. Although imipenem was active against all Gram-negative and Gram-positive anaerobic bacteria, 70% of *Peptostreptococci* and 10% of *Clostridium perfringens* were resistant to this agent. It should be noted that clindamycin was active against 90% of *B. fragilis*, whereas all other strains were resistant to this agent. Erythromycin was overall active against 90% of strains, and only 20% of *C. difficile* were resistant to this agent. In contrast, metronidazole was most active only against *C. difficile* with MIC_{50} of 0.125 mg/l and MIC_{90} of 0.5 mg/l. However, other strains were resistant to this agent. The survival of exponentially growing normal human lung fibroblasts (K2288, K3114, K4008, K4108, K4111, and K5001) after prereatment with cyclo(leu-pro), cyclo(phe-pro), cyclo(leu-pro) plus cyclo(phe-pro), or not treated with drugs control was

Table 2. Radioprotective effect of cyclo(leu-pro), cyclo(phe-pro), or cyclo(leu-pro) plus cyclo(phe-pro) on various normal human lung fibroblast cells.

Treatment group	Cells					
	K2288[a]	K3114	K4008	K4108	K4111	K5001
Radiation 20 Gy[c]	50[b]	48	52	54	60	34
Cyclo(phe-pro)[d]	73	68	70	84	65	92
Cyclo(leu-pro)[e]	69	82	76	75	70	74
Cyclo(leu-pro)+Cyclo(phe-pro)[f]	89	92	96	90	95	92

Data are means from five separate experiments.
[a]Normal human lung fibroblast cells isolated from various patients; [b]Survival rate; [c]Control; [d]Treatment with cyclo(pro-phe) (1 μg/ml) before irradiation 20 Gy; [e]Treatment with cyclo(leu-pro) (1 μg/ml) before irradiation (20 Gy); [f]Treatment with cyclo(phe-pro)+cyclo(leu-pro) (1 μg/ml) before irradiation (20 Gy).

examined by SRB proliferation assay (Table 2). Interestingly, the drug combination enhanced the survival over that observed after treatment with each drug alone, resulting in survival rates of 89% to 96% in five different cell lines. Remarkably, the drug combination raised the survival of the K4008 cell line to 96%. Cyclo(pro-phe) protected two cell lines, K4108 and K5001, with resulting survival rates of 84% and 92%, respectively. In contrast, cell line survival rates were less than 76%, except for K3114 (82%), following treatment with cyclo(pro-leu). The survival rate of the control was less than 60%. Antibiotic combinations are used for a variety of reasons, one of which is to achieve antibiotic synergy, resulting in more effective inhibition or killing of organisms resistant to therapeutic doses of single antibiotics [8]. Combination of cyclic dipeptides exhibited synergy against VRE (vancomycin-resistant enterococci), various pathogenic bacteria, and yeasts [12]. Nevertheless, to the best of our knowledge, the effectiveness cyclic dipeptide combinations against anaerobic bacteria has never before been evaluated. Anaerobic infections usually require combination therapy or excision of the infected tissue together with antimicrobial therapy [15]. In the present study, we found that a combination of dipeptides was effective against anaerobic Gram-negative and Gram-positive bacteria. As expected, the drug combination was significantly more active against various anaerobic bacteria than other antimicrobial agents such as erythromycin, cefoxitin, imipenem, clindamycin, and metronidazole. In addition, the MIC of 0.064 mg/l of the drug combination against *C. difficile* was less than the MIC 4.0 mg/l of ALP20 (a new penem antibiotic, Astra Clinical Research Center, Sodertalje, Sweden) against *C. difficile*, using the same MIC test by Carl *et al.* [1]. Our results indicate that, of the approved antimicrobials tested, imipenem had the greatest activity against Gram-negative bacteria (*B. fragilis* and *B. ovatus*) with MICs ranging from 0.032 to 1.0 mg /l. Intriguingly, the MICs of the drug combination ranged from 0.032 to 0.5 mg/l with all strains. Furthermore, the dipeptide combination exerted a significant radioprotective effect: In 6 different normal human lung fibroblast cells, a low concentration of the dipeptide combination (1 μg/ml) increased the survival rate up to 89 to 96%, whereas the survival rates of cells treated with either cyclo(pro-phe) or cyclo(pro-leu) alone were less than 84%. Interestingly, Graz *et al.* [3, 4] reported that cyclic dipeptides selectively inhibited the growth of a carcinoma and allowed the normal cell population to recover. The effects of these cyclic dipeptides appear to be related to energy metabolism, histone acetylation, phosphorylation, and induction of lineage-specific gene expression. The results in the present study suggest that cyclic dipeptides are highly selective and may provide a radically new direction in cancer therapy. In summary, we are the first to demonstrate that the combination of the two antimicrobial cyclic dipeptides,

cyclo(leu-pro) and cyclo(phe-pro), exhibits strong activity against anaerobic bacteria. In addition, the drug combination had a radioprotective effect on normal human cells. Therefore, the drug combination appears to be a potent inhibitor of anaerobic bacteria *in vitro*. Additional investigation of these and other cyclic dipeptides is merited, especially with regard to bioavailability, toxicity, and stability, because such combinations may be beneficial treatment modality for bacterial infection as well as cancer.

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