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In Vivo Activities of Recombinant Divercin V41 and Its Structural Variants against *Listeria monocytogenes*▽

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Recombinant divercin RV41 (DvnRV41) and its structural variants were used in this study to assess their antilisterial activities in vivo in mice challenged intravenously with *Listeria monocytogenes* EGDe. Treatment with DvnRV41 before and after infection permitted a conclusion as to the capacities of this peptide to retain activity and reduce growth of *L. monocytogenes* EGDe. Moreover, the use of structural variants for the first time in vivo and the reductions of their activities confirmed the importance of certain amino acids in antilisterial activity.

Listeriosis is a bacterial infection occurring in animals and humans after ingestion of as few as 100 bacteria per gram of food (17). Treatment of listeriosis requires 14 days and a high dose of amoxicillin in combination with an aminoglycoside (5), although treatment options using alternate forms of antibiotics exist (14). With prompt detection, treatment of "human" listeriosis with antibiotics is efficient (13), but the need to prevent the rise of resistant strains stands as a concern requiring attention. The use of novel but safe molecules, such as bacteriocins, is of major interest and could be undertaken in this therapy. A growing number of medical applications of bacteriocins have been reported in the literature (1, 3, 6, 7, 8, 15, 16), highlighting the potential biotechnological applications of bacteriocins.

The present study is focused on recombinant divercin RV41 (DvnRV41), a class IIa bacteriocin produced from a synthetic gene (10). DvnRV41 possesses an N-terminal region that is extended in comparison to the wild-type version (10, 12). Assays for demonstrating the effectiveness of DvnRV41 and its structural variants (W19F, Q21S, A22G, and P40V) (12) in an animal model were conducted. To the best of our knowledge, this is the first report showing the use of structural variants of class IIa bacteriocins in vivo. DvnRV41 and its structural variants were purified to homogeneity as recently reported (12), while their antagonism was assessed by the agar diffusion method (9, 11) and by MIC determination (2) for activity against *Listeria monocytogenes* EGDe (4).

MICs were determined using two serial dilutions of DvnRV41 or its variants in Elliker broth placed in wells of a microtiter plate (Nunc). Each well was inoculated with 50 μl of the indicator strain at a concentration of 10^5 CFU/ml. The microtiter plate cultures were incubated at 30°C for 18 h; optical density at 600 nm was measured at regular intervals (1 h) by using an UltraMicroplate reader (Bio-Tek Instruments). MICs were calculated using the highest dilution showing complete inhibition of the strain tested. MIC determination was repeated independently at least three times. As evidenced by the agar diffusion test and determination of MICs, DvnRV41 was a more active peptide than the structural variants (12), which exhibited differences in their antilisterial activities (Table 1). The antilisterial activities of these peptides in mice were assessed as follows. Six- to 8-week-old female BALB/c mice (Charles River Laboratories, l'Arbresle, France) were randomly divided into groups of two animals, and each group received 2 μg of purified peptide (DvnR41 or the W19F, Q21S, A22G, or P40V variant) intravenously (i.v.) in the tail. Mice were surveyed for 72 h postinjection in order to observe development of signs of localized inflammation, vomiting, diarrhea, respiratory distress, depression, and listlessness. After this period, none of these adverse effects was registered.

L. monocytogenes EGDe was grown aerobically overnight at 37°C under shaking in tryptone soya broth (TSB) medium. One milliliter of listerial culture was adjusted in saline buffer (0.9% [wt/vol] NaCl) to an inoculum size of approximately 1.5×10^3 CFU/50 μl. The inoculum was injected intravenously into the mice, which had been randomly divided into groups of five animals (Table 2). Afterwards, the animals were observed for 72 h for the presence of any adverse effect before being euthanized by cervical dislocation. Spleens were recovered, homogenized, diluted, and plated onto solid Palcam medium. Plates were incubated for 16 h at 37°C, and then colonies were counted. Mouse groups 1 to 5 received 2 μg of either DvnRV41 or one of the structural variants (W19F, Q21S, A22G, or P40V) 30 min after listerial infection (postchallenge), while groups 6 to 10 received equal amounts of the aforementioned peptides 15 min before listerial infection (prechallenge). Consequently, all peptides used in this study were shown to be active against *L. monocytogenes* EGDe under both pre- and postchallenge conditions. The highest level of antagonism was obtained with DvnRV41. The Q21S and A22G structural variants were also very potent regarding the reduction of CFU counts (3.15 and 3.11 $\log_{10}$ CFU versus the control levels) and the conversion of the spleen cultures (2 out of 5 for both peptides). These structural variants displayed potent

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TABLE 1. MICs obtained for *L. monocytogenes* EGDe[a]

Peptide	Amino acid modification	*L. monocytogenes* EGDe MIC (μg/ml)
DvnRV41	None	[0.03–0.15]
Variant 1	W19F	[15.77–31.54]
Variant 2	Q21S	[10.98–21.96]
Variant 3	A22G	≤8.63
Variant 8	P40V	[1.93–3.86]

[a] MIC determination was performed as recently reported (2, 12) and required at least three independent experiments.

activities in vivo despite the differences in MICs observed in vitro (12).

The times of injection prechallenge and postchallenge were significant only for DvnRV41, where in vivo antilisterial activity was diminished following administration before infection. Under this circumstance, it is hypothesized that the amount of DvnRV41 present at the time of infection is likely "affected" by a metabolic pathway that is not clearly understood; meanwhile, the peptide and total volume present remained intact following administration after infection. Remarkably, DvnRV41 showed a higher level of antilisterial activity in vivo than the Q21S, A22G, P40V, and W19F structural variants. DvnRV41 caused a 5.3-$\log_{10}$ reduction in viable cell count under postchallenge conditions and a 3.1-$\log_{10}$ reduction in viable cell count under prechallenge conditions. The lowest level of reduction in total bacteria was 1.8 $\log_{10}$ CFU, observed for the W19F and A22G structural variants following administration before and after infection, respectively.

In addition to the concept of structure/function relationships, this study shows that class IIa bacteriocins are able to retain antilisterial activity in vivo, which argues positively for the use of such peptides to complement current antibiotics in the treatment of listeriosis. Furthermore, these peptides are free of side effects, are easily formulated, and have confirmed antilisterial activity. Since this is a preliminary study, it does not have the power to show statistical significance.

TABLE 2. Planning of the trial and reduction in *L. monocytogenes* EGDe colonizing the spleens of challenged mice following administration of peptides[a]

Group	Peptide	No. (%) of mice with negative cultures (n = 5)	Log_{10} no. of CFU/spleen[b]
1	DvnRV41	4 (80)	0.97 ± 2.17
2	W19F	1 (20)	4.41 ± 2.46
3	Q21S	2 (40)	3.10 ± 2.93
4	A22G	2 (40)	3.14 ± 2.93
5	P40V	1 (20)	3.23 ± 1.88
6	DvnRV41	2 (40)	3.13 ± 2.86
7	W19F	1 (20)	3.92 ± 2.32
8	Q21S	2 (40)	2.83 ± 2.75
9	A22G	1 (20)	4.43 ± 2.50
10	P40V	1 (20)	4.25 ± 2.38
11	Untreated control	0	6.25 ± 0.19

[a] For groups 1 to 10, five mice received 2 μg of peptide (at 30 min postchallenge for groups 1 to 5 and 15 min prechallenge for groups 6 to 10). Mice in all groups were infected with an *L. monocytogenes* EGDe challenge dose of 1.5×10^3 CFU.

[b] The CFU value is the mean of gathered data for 5 mice, and the average and standard deviation are shown.

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