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An engineered multidomain bactericidal peptide as a model for targeted antibiotics against specific bacteria

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We constructed a peptide consisting of a staphylococcal AgrD1 pheromone fused to the channel-forming domain of colicin Ia and named it pheromonicin. This fusion peptide had bactericidal effects against methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA, respectively), but not against *Staphylococcus epidermidis* or *Streptococcus pneumoniae*. Growth rates, vital staining and colony forming unit (CFU) counts showed that pheromonicin did not merely suppress growth but killed *S. aureus* cells. The specificity of pheromonicin was shown by the absence of bactericidal effects against an accessory gene regulator (*agr*) locus knockout of *S. aureus*, and a dose-dependent inhibition of the bactericidal effects of pheromonicin by competition with corresponding free AgrD pheromone. *In vivo*, all pheromonicin-treated mice survived administration of MRSA that was lethal to controls. No toxicity was detectable in human liver or renal cells in culture, or in livers, kidneys or spleens of pheromonicin-treated mice. The results suggest that these types of chimeric peptides may be of value as antibiotics against specific bacterial infections.

The E_1 family of colicins are channel-forming bacteriocins produced by *Escherichia coli*, which are bactericidal only to *E. coli* and related species[1]. Because they act on the lipid bilayer of cell membranes, colicins could be useful against bacteria that are not their natural targets if they were engineered for insertion into the inner membrane of other bacteria. Colicin Ia[2] was used as a prototype for this targeting study. It kills target cells by forming a voltage-activated channel in the cell membrane, mediated by its 175-residue C-terminal channel-forming domain[3–6]. A 68-residue peptide chain (Lys544–Ile612), extending from α-helix VI to IX of the ten α-helices of the colicin Ia channel-forming domain, is the basic structural element of the ion conductive pore of the colicin Ia channel (refs. 4–6 and X.Q. Qiu *et al. Soc. Neurosci. Abstr.* **27**, 812.24, 2001).

To target the channel-forming domain of colicin Ia to bacterial membranes, we engineered a pheromone fused to colicin Ia. Pheromones are peptides secreted by bacteria into the medium to regulate the protein production of bacteria[7]. A representative model of this regulation is AgrD, an eight-residue peptide encoded by the staphylococcal *agr* locus[8,9]. This pheromone traverses the cell wall and interacts with the AgrC receptor of the cell membrane, initiating a staphylococcal signaling pathway[10,11]. There are four groups of AgrD pheromones among *S. aureus* strains. They are highly specific, and induce a response only in *S. aureus* strains.

RESULTS

Plasmid constructs and peptide purification

The genes encoding three *S. aureus* pheromone peptides—AgrD1 of subgroup 1, YSTCDFIM (GenBank accession no. U85097)[10]; AgrD2 of subgroup 2, GVNACSSLF (GenBank no. AF001782)[10]; and AgrD3 of subgroup 3, YINCDFLL (GenBank no. AF001783)[10]—were introduced into a plasmid to follow colicin Ia channel-forming domains creating the peptides pheromonicin-AgrD1, pheromonicin-AgrD2 and pheromonicin-AgrD3, respectively (**Figs. 1a**,**b**). The structures were designed such that the C-terminal thiolactone loop (Cys4–Met8) of AgrD pheromones remained intact to interact, as it does naturally, with the AgrC membrane receptor of *S. aureus*. **Figure 1c** shows a representative 15% SDS-polyacrylamide gel of pheromonicin-AgrD1 after purification. The band in lane 2 had a migration consistent with a molecular weight slightly larger than that of wild-type colicin, as expected. To determine whether the thiolactone ring had opened to produce the linear form, we looked for evidence of free sulfhydryls, because the only cysteine present in the fusion protein is in the AgrD component. The 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reaction showed no differences in absorption at 412 nm compared to background, indicating no detectable free sulfhydryls in pheromonicins. We did detect free sulfhydryl groups in reversed pheromonicin by this assay, confirming the presence of the linear form of AgrD in this construct. Reversed pheromonicin consists of an AgrD1 pheromone at the

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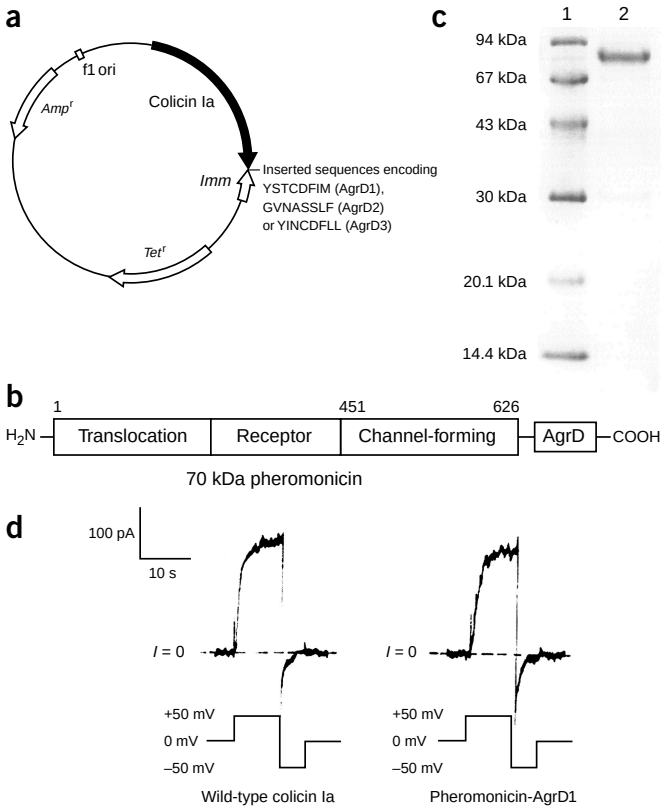

Figure 1 Structure and voltage-dependent channel gating of pheromonicin. (**a**) 8,300-base-pair colicin Ia plasmid used for site-directed mutagenesis of the colicin Ia gene, and subsequent preparation of pheromonicin, consisted of the colicin Ia plasmid with insertion of the genes encoding the *S. aureus* AgrD1, AgrD2 and AgrD3 pheromones, and a random sequence following amino acid position I626 of colicin Ia. *Imm,* immunity protein. (**b**) A domain diagram of the pheromonicin constructs with the AgrD pheromones at the C terminus. (**c**) SDS-polyacrylamide gel of pheromonicin-AgrD1 after purification. Lane 1, molecular weight markers; lane 2, eluted proteins. (**d**) Voltage-dependent gating of wild-type colicin Ia and pheromonicin in artificial lipid bilayers.

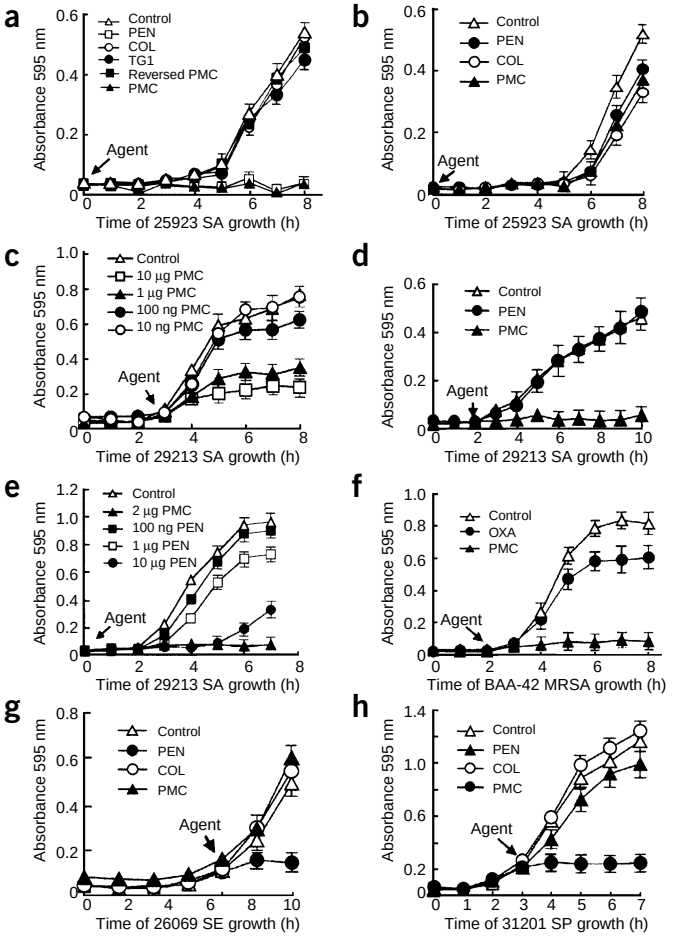

Figure 2 *In vitro* bactericidal activity of pheromonicin against *S. aureus*. (**a**) Penicillin-sensitive *S. aureus* incubated with all additives at 100 ng/ml. (**b**) Additives at 10 ng/ml. (**c**) Growth of penicillin-resistant *S. aureus* cells with additions at 1 µg/ml when A_{595} reached 0.1. (**d**) *S. aureus* cells with additions made before the very early exponential phase. (**e**) Penicillin-resistant *S. aureus* cells with various concentrations of penicillin G or pheromonicin-AgrD1. (**f**) MRSA cells incubated with pheromonicin-AgrD1 or oxacillin at 5 µg/ml. (**g**) *S. epidermidis* after treatment with pheromonicin-AgrD1 or controls (1 µg/ml). (**h**) *S. pneumoniae* exposed to pheromonicin-AgrD1 or controls (1 µg/ml). Additions to medium: control, medium alone; PEN, penicillin G; COL, wild-type colicin Ia; Rev PMC, reversed pheromonicin with N-terminal AgrD1 peptide; TG1, proteins from nontransfected *E. coli* TG1 cells; PMC, pheromonicin-AgrD1.

N terminus instead of the C terminus of colicin Ia and was expected to have its AgrD component exclusively in linear form owing to AgrD's N-terminal location. Furthermore, after pheromonicins were exposed to Na_2CO_3, which can open the thiolactone ring[12], DTNB reactivity increased to more than 70% of that predicted based on the single cysteine residue of the fusion proteins (data not shown). There was no effect, as expected, on free cysteine or cystine controls under identical conditions, suggesting that thiolactone ring forms are present in the AgrD component of pheromonicins. These results were confirmed by liquid chromatography–mass spectrometry (LC-MS) after tryptic digestion of pheromonicin. Two tryptic fragments of the C terminus were identified: ANKFWGIYSTCDFIM (15 residues) and FWGIYSTCDFIM (12 residues). The calculated doubly charged mass of the linear form of the 15-residue fragment was 898.41 AMU and that of the ring form (linear minus doubly charged H_2O, 17.99/2) was 889.42 AMU. A prominent peak was observed with a mass of 889.54 AMU. For the 12-residue peptide, the calculated doubly charged mass of the linear form was 741.82 AMU and that of the ring form was 732.83 AMU. A peak was observed with a mass of 732.40 AMU. These data confirmed the presence of a thiolactone ring in pheromonicin.

Electrophysiological activity of pheromonicin

Like its precursor, colicin Ia, pheromonicin formed voltage-activated channels on artificial planar lipid bilayer membranes. With the voltage held at +50 mV, the recording showed that pheromonicin-AgrD1-induced current rose rapidly, then reached a quasi-steady state. The current rapidly decayed to zero as soon as the voltage was pulsed to –50 mV (**Fig. 1d**). This behavior is similar to the gating reported for wild-type colicin Ia channel (**Fig. 1d**)[4,13].

Effects of pheromonicin and controls on bacterial cell growth

Protein preparations (100 ng/ml) from nontransfected *E. coli* TG1 cells, wild-type colicin Ia from TG1 cells, or a control—reversed pheromonicin—that lacks the C-terminal thiolactone loop required for activity of AgrD pheromone had no effect on the growth of a

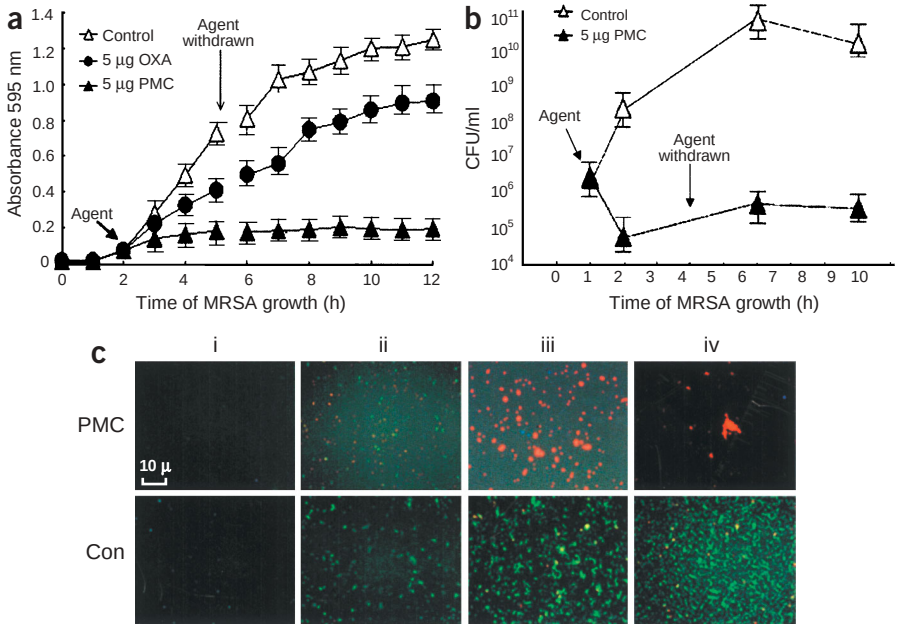

Figure 3 *In vitro* MRSA cell growth after withdrawal of treatments. (**a**) Absorbance assays of cells in the early exponential phase treated with pheromonicin-AgrD1 or oxacillin for 5 h, then replaced by growth medium. (**b**) CFU counts of cells treated with pheromonicin-AgrD1 or medium alone for 5 h followed by replacement with growth medium. (**c**) Cells treated with pheromonicin-AgrD1 or medium alone were stained with propidium iodide and FITC and examined under a fluorescence microscope. Time elapsed after addition of pheromonicin-AgrD1: (i) before addition, (ii) 1 h, (iii) 4 h, and (iv) 5 h . Magnification bar, 10 μm, pheromonicin; Con, control.

penicillin-sensitive strain of *S. aureus* compared to untreated controls (**Fig. 2a**). In contrast, purified pheromonicin-AgrD1 at the same concentration completely inhibited *S. aureus* growth, as did penicillin when added at the beginning of the culture. However, neither pheromonicin nor penicillin inhibited *S. aureus* growth when concentrations were decreased to 10 ng/ml (**Fig. 2b**).

The minimal inhibitory concentration at which *S. aureus* cells are inhibited from growing by PMC-AgrD1 according to this assay was about 100 ng/ml.

Timing of the administration of pheromonicin made a difference. When a penicillin-resistant *S. aureus* in very early exponential growth was treated with pheromonicin, a concentration of about 1 μg/ml of pheromonicin-AgrD1 inhibited growth by about 60% (**Fig. 2c**). The very early exponential phase was taken as the period of *S. aureus* cell growth during which the absorbance at 595 nm (A_{595}), reflecting *S. aureus* cell growth, was about 0.1, which occurred during the second to fourth hour after *S. aureus* inoculation. However, when added before the very early exponential phase, 1 μg/ml of pheromonicin-AgrD1 completely inhibited penicillin-resistant *S. aureus* cell growth (**Fig. 2d**). The same concentration of penicillin had no significant inhibitory effect ($P < 0.01$), as expected. From these data, the minimal bactericidal concentration of pheromonicin-AgrD1 needed to inhibit ATCC 25923 and 29213 *S. aureus* cell growth *in vitro* was 0.1–1.0 μg/ml in this absorbance assay.

Even at the highest concentration of penicillin (10 μg/ml), penicillin-resistant *S. aureus* cells still grew from the fifth hour after administration of the antibiotic (**Fig. 2e**). In contrast, only 1 μg/ml of pheromonicin-AgrD1 inhibited growth of these cells by approximately 90% through 7 h of incubation. Considering the difference in molecular weight between pheromonicin (70 kDa) and penicillin (~0.35 kDa), the inhibitory activity of pheromonicin against the penicillin-resistant *S. aureus* strain was approximately 1,000 times greater, on a molar basis, than that of penicillin.

Oxacillin (5 μg/ml) only slightly (20%) inhibited MRSA cell growth when added in the early exponential phase (**Fig. 2f**). In contrast, 5 μg/ml of pheromonicin-AgrD1 inhibited MRSA cell growth by approximately 90% when added at the same time point. Thus, pheromonicin had substantial antibacterial effects against two *S. aureus* strains whose resistance to standard antibiotics present serious clinical therapeutic problems in man.

To determine whether the observed inhibitory activity was generally applicable, the effects of pheromonicin-AgrD1 and penicillin were measured against 136 isolates of MRSA and 207 isolates of MSSA collected from Beijing and Sichuan hospitals in China (**Table 1**). The MIC_{50} of pheromonicin-AgrD1 for MRSA isolates was found to be 8.0 μg/ml, and the MIC_{50} for MSSA isolates was 0.5 μg/ml. However, considering the large difference in molecular weight, to obtain equivalent moles of pheromonicin compared to penicillin, PMC values should be divided by 200. These data showed that PMC-AgrD1 was active against a large number of *S. aureus* isolates and was not limited to a few laboratory strains.

Table 1 Inhibitory activity of pheromonicin-AgrD1 against local *S. aureus*

	S. aureus isolates (no. of samples)			
	MRSA (136)		MSSA (207)	
Agents[a]	MIC_{50} (μg/ml)	MIC_{50} range (μg/ml)	MIC_{50} (μg/ml)	MIC_{50} range (μg/ml)
Pheromonicin-AgrD1	8[b]	<0.0005–64[b]	0.5[b]	0.06–>64[b]
Oxacillin	>128	4–>128	0.125	<0.015–2

[a]As controls, the MIC_{50} (μg/ml) values of pheromonicin, penicillin and oxacillin for MRSA were found to be 0.5, 8 and 8 μg/ml, respectively, and for penicillin-sensitive *S. aureus*, 0.25, 0.125 and <0.06 μg/ml, respectively. [b]Because of the large difference in molecular weight, pheromonicin values should be divided by 200 to obtain the equivalent number of moles of pheromonicin for penicillin. MSSA, methicillin-sensitive *S. aureus;* MRSA, methicillin-resistant *S. aureus;* MIC_{50}, minimum inhibitory concentration, 50%.

Specificity of pheromonicin

Incubation of *S. epidermidis* (**Fig. 2g**) or another unrelated Gram-positive organism, *S. pneumoniae* (**Fig. 2h**), in the presence of pheromonicin-AgrD1 (1 μg/ml) resulted in no significant inhibition of growth of either bacteria ($P < 0.01$). In contrast, growth of both was inhibited when incubated with penicillin (1 μg/ml). These findings confirmed that the growth of the cells were inhibited by penicillin and that the absence of inhibitory effects of pheromonicin was not due to some nonspecific resistance.

Bacteriostatic or bactericidal effects

When pheromonicin-AgrD1 or oxacillin was added to the medium of MRSA cells in the early exponential phase and withdrawn 5 h later,

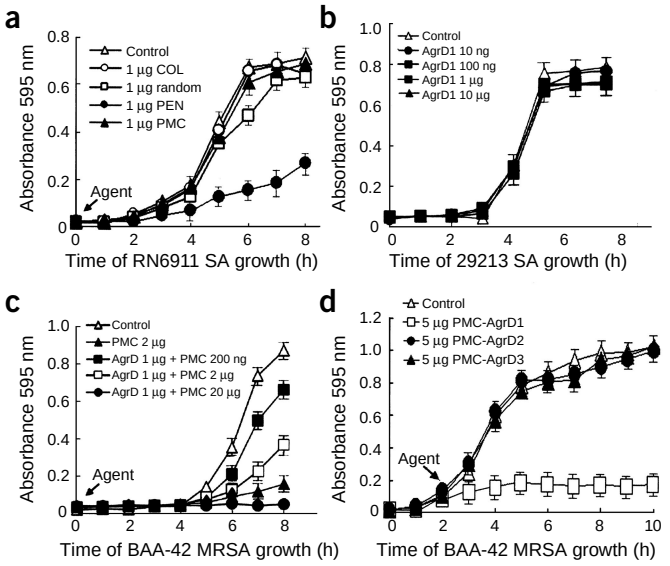

Figure 4 Effects of pheromonicins and controls on cell growth of *S. aureus*. (**a**) An *agr* locus knockout *S. aureus* incubated with wild-type colicin Ia, a random eight-residue peptide linked at the C terminus of colicin Ia, penicillin and pheromonicin-AgrD1, all at 1 μg/ml. (**b**) Penicillin-resistant *S. aureus* cell growth in the presence of increasing concentrations of free AgrD1 pheromone alone. (**c**) Free AgrD1 pheromone, 1 μg/ml, was added to MRSA cells to compete with increasing concentrations of pheromonicin-AgrD1. (**d**) Pheromonicin-AgrD1, pheromonicin-AgrD2, pheromonicin-AgrD3 incubated at 5 μg/ml with MRSA cells. COL, wild-type colicin Ia; Random, random eight-residue peptide linked at C terminus of colicin Ia; PEN, penicillin; PMC, pheromonicin-AgrD1; MRSA, methicillin-resistant *S. aureus*.

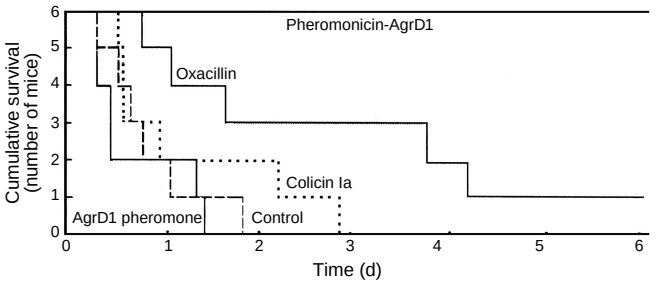

Figure 5 Cumulative survival of mice after *S. aureus* infection. One hour after intraperitoneal injection of *S. aureus,* mice were injected intravenously with free AgrD1, oxacillin, wild-type colicin Ia or pheromonicin-AgrD1, all at 3 mg/kg, in separate groups. The numbers of surviving animals at various time points were determined, and Kaplan-Meier analysis was used to determine the significance of differences between the pheromonicin and control groups.

cells treated with pheromonicin-AgrD1 did not resume growth, whereas cells treated with oxacillin resumed growth with medium change alone (**Fig. 3a**). However, because absorbance does not necessarily reflect actual growth, we also counted colony-forming units (CFUs)[14]. CFU counts confirmed that after withdrawal of pheromonicin-AgrD1, cells did not resume growing, arguing against a transient growth arrest (**Fig. 3b**). The number of MRSA cells stained (red) by propidium iodide, a vital dye, increased after 1 and 4 h of exposure to pheromonicin (5 μg/ml) and then decreased by the fifth hour after exposure as the total number of fluorescein isothiocyanate (FITC)-stained *S. aureus* cells (green) declined (**Fig. 3c**). Propidium staining of controls, with only medium added, did not increase, but the total cell number did increase substantially. The data supported the conclusion that pheromonicin-AgrD1 (5 μg/ml) permanently inhibited growth of MRSA cells, and this effect was associated with an increase in permeability to a vital dye.

Pheromone's role in the specificity of pheromonicin effects

Exposure of an *agr* locus knockout strain of *S. aureus* to pheromonicin-AgrD1 did not inhibit growth of these cells (**Fig. 4a**). Similarly, neither a construct in which a random peptide of the same length as AgrD1 linked to the C terminus of colicin nor control colicin alone had any inhibitory effects (**Fig. 4a**). Incubation of penicillin-resistant *S. aureus* with free AgrD1 alone in increasing concentrations did not inhibit growth (**Fig. 4b**). Pheromonicin-AgrD1 inhibition of MRSA cell growth was blocked up to 90% in a concentration-dependent manner by the addition of free AgrD1 pheromone (**Fig. 4c**). Although pheromonicin-AgrD1 strongly inhibited MRSA cell growth, pheromonicin-AgrD2 and pheromonicin-AgrD3 had no significant effects ($P < 0.01$; **Fig. 4d**). All these data indicated that the specificity of pheromonicin was dependent on the AgrD1 peptide–AgrC receptor interaction. Both AgrD and colicin components were necessary, but neither component alone was sufficient.

In vivo activity of pheromonicin

To assess the effects of pheromonicin *in vivo*, mice were injected with BAA-42 MRSA cells intraperitoneally, followed by intravenous injections of pheromonicin-AgrD1 or controls. All mice in the AgrD1 alone, colicin alone and untreated groups died in less than 3 d (**Fig. 5**). Seventy-six percent of oxacillin-treated animals died within 5 d. However, 100% of the mice treated with intravenous pheromonicin-AgrD1 (3 mg/kg) survived the 6-d experimental period.

Assessment of toxicity of pheromonicin to mammalian cells

There were no obvious differences in cell counts (**Fig. 6a**,**b**) or lactate dehydrogenase (LDH) levels (**Fig. 6c**,**d**) in cultured human Chang liver cells and human embryonal kidney cells incubated with pheromonicin-AgrD1 as compared to untreated controls. There was no microscopic evidence of necrosis or inflammation in the livers, kidneys or spleens of mice that received several doses of pheromonicin-AgrD1 (3 mg/kg) (**Fig. 6e**). These data further attest to the specificity of pheromonicin for bacterial cells, and suggest that pheromonicins may be tolerated by mammalian systems without evident toxicity.

DISCUSSION

To our knowledge, this is the first report of the use of an unrelated bactericidal pheromone to replace the ligand domain of a channel-forming bacterial toxin for directing bactericidal properties of the toxin toward a selected target.

The *agr* locus is conserved throughout *Staphylococci*[10,11], but each bacterial species produces pheromones with target specificity. Recently, it has been found that all *S. aureus* subgroups are sensitive to the *S. epidermidis* pheromone, but only the subgroup 4 pheromone of *S. aureus* inhibited the *S. epidermidis* Agr response[15]. The *S. aureus* Agr pheromone used in the present study was a subgroup 1 pheromone, which was predicted to result in a chimeric bacteriocin with unique specificity to act on *S. aureus*, but not on other staphylococcal species or other bacteria. The absence of effects of pheromonicin on *S. epidermidis* and *S. pneumoniae* or inhibition by purified pheromone was consistent with this specificity. It is noteworthy that the effects of the

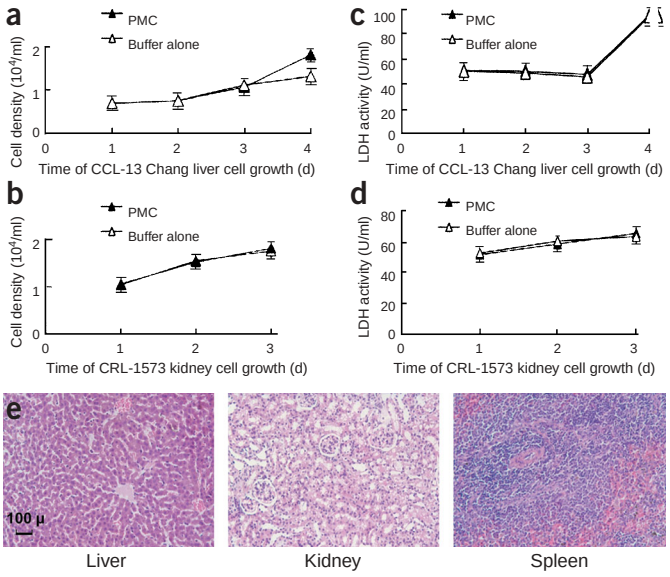

Figure 6 Toxicity of pheromonicin in human cells. (**a**,**b**) Cells were incubated in medium with pheromonicin-AgrD1 (200 µg/ml) or without, and at various time points, stained with trypan blue, and counted by light microscopy. (**c**,**d**) LDH levels in the medium were measured. Chang CCL-13 liver cells, **a** and **c**. CRL-1573 renal cells, **b** and **d**. (**e**) The liver, kidney and spleens of mice treated with PMC for 5 weeks were stained with hematoxylin and eosin. Magnification bar, 100 µm, PMC, pheromonicin.

pheromonicin-AgrD1, although substantial even against antibiotic-resistant strains, were not uniform across all strains. A possible explanation is that the strains belonged to different Agr groups. A major proportion of *S. aureus* strains belong to the AgrD1 group, and only 5% of the MRSA strains belong to the AgrD2 and AgrD3 groups (ref. 11). Our results indicated that pheromonicins with AgrD2 or AgrD3 components did not affect the cell growth of either an MRSA strain or a penicillin-sensitive *S. aureus* strain, suggesting that neither strain belonged to the AgrD2 or AgrD3 groups. The observed difference in activity of the pheromonicin-AgrD1 against the strains does not appear to be due to differences in Agr grouping. Other differences in *S. aureus* interactions with pheromonicin, possibly with the colicin component, might be responsible.

Although the pheromone component of pheromonicin would be expected to stimulate the Agr signal pathway of *S. aureus* cells of the same Agr group[10], the observed substantial growth inhibition of *S. aureus* cells by the pheromonicin indicated that the toxic effect of the colicin Ia component of the fusion molecule was predominant. The absence of any effect of the pheromonicin on *agr* locus knockout *S. aureus* cells confirmed that interaction of the AgrD component with AgrC receptor was required, and that activity of colicin Ia either alone, or linked to a random peptide of the same length as AgrD1, did not reproduce the observed antibacterial effects.

Chemical and mass spectral data confirmed that a thiolactone ring is present in pheromonicin. The mechanism by which this ring formation occurs has not been previously described or determined, but is under investigation.

Broad-spectrum antibiotics are useful when the specific organism causing an infection is unknown. However, their use may result in selection and proliferation of resistant bystander organisms, creating pathogens from previously harmless organisms, and can lead to complications such as antibiotic-associated diarrhea, colitis or fungal overgrowth. Thus, when a single offending organism has been identified, there may be advantages to the use of a narrow-spectrum antibiotic. Currently, there are few examples of such agents. However, because pheromonicins could theoretically be tailored to specific bacteria by the introduction of pheromone fragments specific to particular organisms, the development of a wide variety of narrow-spectrum antibiotics could be possible in the future.

METHODS

Mutagenesis of pheromonicin peptide chains and protein purification. The YSTCDFIM amino acid sequence of AgrD1 pheromone was constructed to follow position I626 of colicin Ia by double-stranded oligonucleotide mutagenesis (QuickChange kit, Stratagene) using a Promega pSELECT-1 plasmid containing the colicin Ia gene (P. Gosh, University of California, San Francisco, CA, USA) to form pheromonicin-AgrD1. The oligonucleotide used, containing the desired YSTCDFIM mutation, was 5′-GCGAATAAGTTCTGGGGTATT-**TATTCCACCTGTGATTTTATAATG**TAAATAAAATATAAGACAGGC-3′ (**Fig. 1a**,**b**). Harvested plasmid was transfected into TG1 *E. coli* cells to produce pheromonicin. TG1 cells harboring pheromonicin plasmids were grown in FB medium containing 50 µg/ml ampicillin (to select for the plasmid) and were resuspended in 60–80 ml borate buffer (50 mM borate buffer, pH 9.0, with 2 mM EDTA and 2 mM dithiothreitol) containing 0.5 mM phenylmethylsulfonyl fluoride. The cells were sonicated and debris removed by centrifugation for 90 min at 75,000*g*. Nucleic acids were removed by addition of 1/5 volume streptomycin sulfate. Dialyzed extracts were applied to a CM-Sepharose column (2.5 × 12 cm; Pharmacia Biotech). Proteins were recovered by stepwise elution with 0.1, 0.2 and 0.3 M NaCl in borate buffer and collected in 0.5-ml fractions[16,17]. The total protein concentration of eluate was about 5 mg/ml. As determined from 15% SDS-polyacrylamide gel assays, pheromonicin eluted by 0.2 M NaCl comprised about 90% of total eluted protein (**Fig. 1c**). Fusion proteins pheromonicin-AgrD2 (GVNACSSLF) (*agrD2*), pheromonicin-AgrD3 (YINCDFLL) (*agrD3*), a random control sequence (SMTTVGGG) and reversed pheromonicin (*agrD1* gene introduced at N terminus of colicin Ia) were prepared similarly.

To determine whether the AgrD components of the pheromonicins contained inactive linear forms rather than the thiolactone rings that are required for interaction with AgrC receptor, we measured free sulfhydryl groups[18]. Samples in PBS were mixed with DTNB and their absorbance measured at 412 nm, using fresh free L-cysteine in dilutions to produce a standard curve. Thiolactone cleavage reduction was accomplished by treatment with Na_2CO_3, pH 9.0 for 2 min[19], followed by the DTNB assays described above. Free cysteine and cystine were used as controls. To obtain direct evidence for or against the presence of a thiolactone ring, pheromonicin was digested by trypsin at 37 °C overnight and then run through a C18 high-performance liquid chromatography column, and measured by Mariner electrospray ionization–time of flight mass spectrometer[20] (Laboratory for Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, Bronx, NY, USA).

Electrophysiological and optical absorbance assay. Channel-forming experiments were done on planar lipid bilayer membranes formed at 20–22 °C from asolectin (lecithin type IIS; Sigma) as previous described[4,13].

***In vitro* minimal inhibition and minimal bactericidal assays.** *S. aureus* penicillin-sensitive (American Type Culture Collection (ATCC) 25923), *S. aureus* penicillin-resistant (ATCC 29213) (Peking Union Medical College, Beijing, China), *S. aureus* methicillin-resistant (ATCC BAA-42), RN6911 *agr* locus knockout (R. Novick, New York University, New York, NY, USA) in which the AgrC receptor domain is inactivated, *S. epidermidis* (China Medical Culture Collection (CMCC) 26069) and *S. pneumoniae* (CMCC 31201) were grown in 100 ml Klett flasks containing 10 ml of BM medium (1% tryptone, 0.5% yeast, 0.5% glucose, 1% NaCl, 0.1% K_2HPO_4) for *Staphylococci*, and BM medium with 2.7 g MH culture/100 ml for *Streptococci*. Cells were inoculated to an initial cell density of 2.5×10^5 CFU/ml and shaken at 37 °C. Free AgrD was synthesized by Shenyang Biotechnic (Shanghai, China).

Known concentrations of pheromonicin were prepared by diluting purified preparations. The same range of protein concentrations (10 ng/ml–10 µg/ml)

was used for controls: total medium protein from nontransfected *E. coli* TG1 cells, wild-type colicin Ia from TG1 cells transfected with wild-type colicin Ia plasmid, reversed pheromonicin from TG1 cells transfected with a gene encoding AgrD pheromone introduced at the N terminus of the colicin Ia gene, a random fusion protein with the SMTTVGGG peptide introduced at the C terminus of colicin Ia, penicillin and oxacillin. Results were expressed as means ± s.e.m. in turbidometric absorbance units measured at 595 nm. In some experiments, CFUs were counted as described[20] to confirm actual cell growth.

To determine whether the observed inhibitory activity was generally applicable, effects were measured against 136 MRSA isolates and 207 MSSA isolates collected from hospitals in Beijing and Sichuan, China, and identified by antimicrobial assay (Viktar) and API assay (BioMerieux); *S. aureus* penicillin-sensitive and penicillin-resistant and MRSA cells were used as controls. *S. aureus* cells (10^5 CFU/ml) were inoculated on the surface of MH medium (CMCC) with 2% agar and twofold diluted agents by Denley 400 multipoint inoculator. Cells were incubated 18–24 h at 37 °C and inhibition zones were measured to determine MIC_{50} in accordance with the National Committee for Clinical Laboratory Standards 2002.

Viability of treated cells. MRSA cells were incubated with pheromonicin, oxacillin or growth medium alone for 5 h. Then, the medium was replaced with growth medium without antibiotic and samples were assayed by absorbance, by CFU counting, or by vital dye exclusion with propidium iodide and FITC[21] with manual counting under a fluorescence microscope (Leica DMR) at 1,000× with B/G/R UV/blue/green filter and excitation wavelengths of 420, 495 and 570 nm.

Toxicity studies on mammalian cells. CCL-13 Chang liver and CRL-1573 human embryonal kidney cell lines (ATCC) were incubated as described previously[22]. At regular time points, cells were stained with trypan blue and counted microscopically. Biochemical evidence for mammalian cell toxicity was sought by using a Roche LDH International Federation of Clinical Chemistry colorimetric assay with a Hitachi 7170A analyzer at 340 nm to measure the LDH activity in the medium[23]. To assess potential toxicity *in vivo*, 10 Kunming (KM) mice, Animal Center, Chinese Academy of Science (Kunming, China), half male and half female, weighing 25–30 g, were injected with 3 mg/kg pheromonicin intravenously once a week for the first week, then intramuscularly once a week thereafter. Mice were killed and their organs sectioned, stained with hematoxylin and eosin and examined microscopically 5 weeks after treatment.

***In vivo* bactericidal activity.** KM mice, half male and half female, weighing 25–30 g were injected intraperitoneally with 0.5 ml of MRSA ATCC BAA-42, 5.5×10^5 CFU/ ml. One hour after *S. aureus* injection, mice were injected via tail vein with 0.9% saline alone ($n = 6$), or with free AgrD1 ($n = 6$), wild-type colicin Ia ($n = 6$), oxacillin ($n = 6$) or pheromonicin-AgrD1 ($n = 6$), all at 3 mg/kg. The number of surviving animals at various time points was determined. Three *in vivo* assays were done. Kaplan-Meier analysis was used to determine the significance of differences between the pheromonicin and control groups.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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