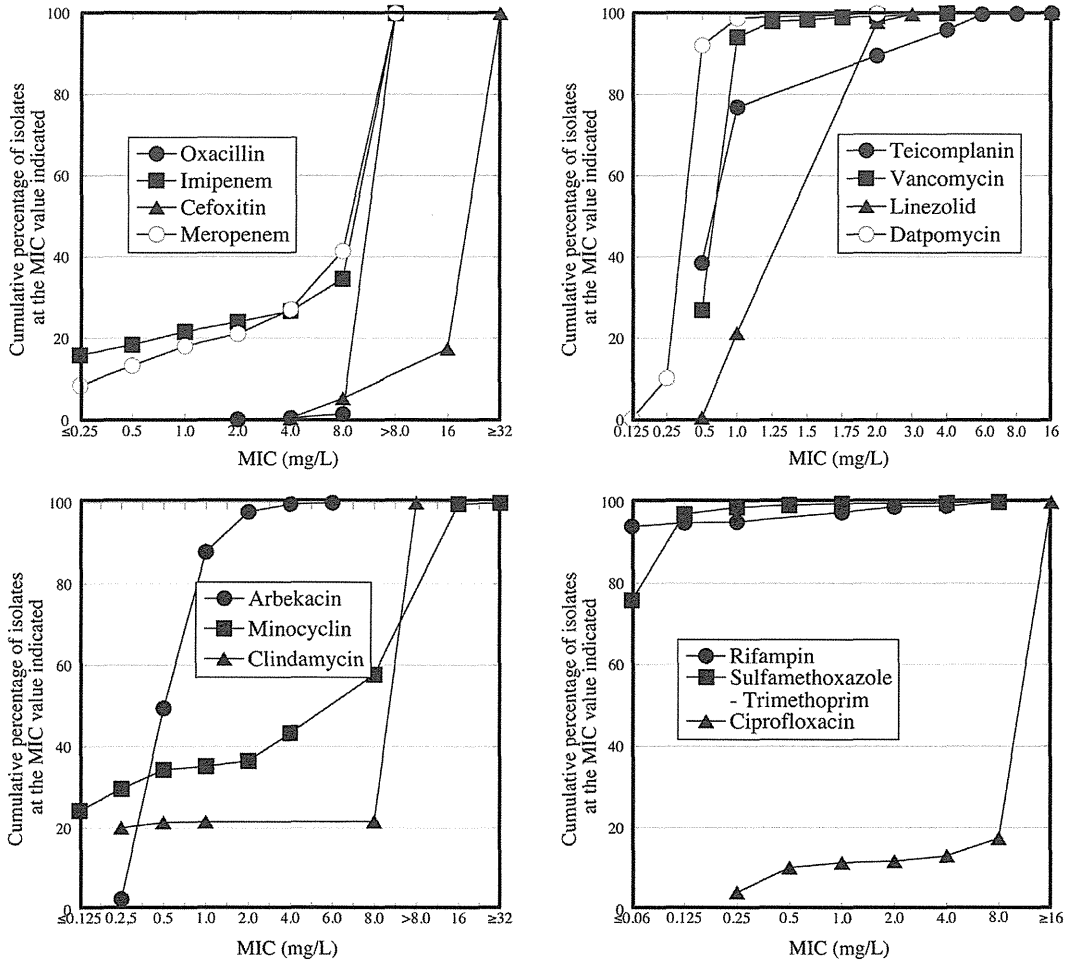

Fig. 1. Cumulative curves of MICs of 14 antibiotics tested for 830 MRSA isolates as determined by the broth microdilution method. Note that VCM, TEIC, LZD, DAP, ST, RFP and ABK showed good antimicrobial activities.

hVISA isolation. On one hand, 5 hospitals had high rates of hVISA isolation such that three, one and one hospitals had hVISA isolates ranging from 12.5% to 15.22%, 23.81% and 47.37%, respectively. The location of latter two hospitals was distant from each other (Table 2). Further analyses revealed that 45 out of 54 hVISA (83.33%) showed BIVR-type vancomycin resistance and, in vise versa, 45 out of 55 BIVR strains were hVISA-type vancomycin resistant (81.82%).

Table 2
Prevelence of VISA, hVISA, BIVR and blaZ-positive strains among all 830 blood-stream MRSA isolates.

Hospital	Region	Sample number	VISA		hVISA		BIVR		hVISA/BIVR phenotype[a]		blaZ positive strains			
											Non-BIVA		*BIVR*	
			Number	%	Number	%	Number	%	Number	%	Number	%	Number	%
A	Kanagawa	108	2	1.85	1	0.93	2	1.85	1	0.93	90	83.33	1	0.92
B	Fukuoka	84	0	0.00	20	23.81	20	23.81	19	22.62	54	64.29	0	0.00
C	Saga	79	0	0.00	1	1.27	1	1.27	1	1.27	77	97.47	0	0.00
D	Aichi	79	1	0.00	0	0.00	0	0.00	0	0.00	79	100.00	0	0.00
E	Kanagawa	24	0	0.00	1	4.17	1	4.17	1	4.17	23	95.83	1	4.17
F	Tokyo	19	0	0.00	9	47.37	5	26.32	5	26.32	10	52.63	0	0.00
G	Hyogo	77	1	1.30	0	0.00	0	0.00	0	0.00	74	96.10	0	0.00
H	Fukuoka	33	1	0.00	1	3.03	0	0.00	0	0.00	30	90.91	0	0.00
I	Tochigi	32	0	0.00	4	12.50	2	6.25	2	6.25	23	71.88	0	0.00
J	Tokyo	46	1	2.17	7	15.22	6	13.04	6	13.04	32	69.57	0	0.00
K	Tokyo	50	0	0.00	7	14.00	9	18.00	7	14.00	42	84.00	0	0.00
L	Chiba	50	0	0.00	0	0.00	0	0.00	0	0.00	48	96.00	0	0.00
M	Hamamatsu	80	2	2.50	2	2.50	7	8.75	2	2.50	64	80.00	0	0.00
N	Nagasaki	69	0	0.00	1	1.45	2	2.90	1	1.45	65	94.20	2	2.90
Total		830	8	0.96	54	6.51	55	6.63	45	5.42	712	94.30	4	7.27

[a] hVISA/BIVR, MRSA strain which has both hVISA and BIVR phenotypes.

We reported earlier that the strain with BIVR phenotype tends to eliminate the *blaZ* gene encoding β-lactamase or inactivate β-lactamase [21]. To see if the same is true in the present isolates, we examined the presence of the *blaZ* gene by PCR. Among 55 BIVR isolates, only 4 were *blaZ* positive that was equivalent to 7.27%, while 94.3% Non-BIVR MRSA were *blaZ* positive (Table 2).

We also identified two LZD non-susceptible isolates from two different hospitals. The nucleotide sequence of all five *rrn* operons in the V domain regions of the 23S rRNA in the two LZD-resistant isolates was analyzed. Both strains carried a G2576T mutation on the 3rd and 5th *rrn* operons (data not shown).

4. Discussion

The bacteremic infections caused by the methicillin-resistant *S. aureus* are problematic due to the high mortality and only limited antibiotics are available for the treatment [24]. In this paper, we carried out nationwide surveillance of antimicrobial susceptibility of bloodborne MRSA isolates to ascertain resistance patterns in order to assist in the enforcement of infection control measures. Our major findings were that over 97% of the isolates were susceptible to teicoplanin, linezolid, sulfamethoxazole/trimethoprim, daptomycin, arbekacin and rifampin, while the majority of the MRSA strains showed resistant to minocycline, meropenem, imipenem, clindamycin, ciprofloxacin, cefoxitin, and oxacillin. On the vancomycin susceptibility, 72 out of 830 isolated (8.67%) showed reduced susceptibility to vancomycin, including 8 strains (0.96%) of VISA, 54 (6.51%) of hVISA, and 55 (6.63%) of BIVR. In addition, the most of the hetero-VISA isolates (83.3%) showed BIVR phenotype.

Vancomycin has been used as the first line for the chemotherapy of MRSA infections. However, problems in the vancomycin therapy of MRSA infections are increasing cases of the treatment failure that raises questions regarding currently employed treatments [25]. Followings are the examples. (i) The vancomycin treatment of high-risk patients with MRSA bacteremic infection often failed even though the MIC of vancomycin was within susceptible ranges. When MICs of vancomycin were <0.5 μg/ml, the treatment failed in 44% of cases, and when that were 1–2 μg/ml, 90% of cases resulted in failure [26,27]. (ii) Infections of MRSA with the vancomycin MICs ≥1.5 μg/ml poorly responded to the vancomycin therapy and high mortality rates were recorded [28]. (iii) Not for all, but some of the apparently vancomycin susceptible MRSA strains contain the population that shows heterogeneous vancomycin-intermediate *S. aureus* (hVISA) [8]. Recently, several lines of evidence documented that the bacteremia by VISA and hVISA were associated with prolonged bacteremia, high rates of complication and poor responses to the vancomycin treatment compared with bacteremia by vancomycin susceptible MRSA [29,30]. Takata et al. reported that bacteremic MRSA strains, which showed the properties of both hVISA and BIVR were associated with a higher probability of mortality, compared with the bacteremic MRSA that shows only one of these properties [11]. Nevertheless, even though the clinical data for the isolates used in the present study were not available for assignment of the isolates with reduced susceptibility to vancomycin into the group of poor response to vancomycin therapy, the relatively high prevalence of the isolates with reduced vancomycin susceptibility in the bloodborne MRSA should not be overlooked.

The Japanese Antimicrobial Surveillance Committee carried out the nationwide antimicrobial susceptibility surveillance of the bacteria from patients with respiratory tract infections (RTIs) for three consecutive years, 2007 through 2009, at the Central Surveillance Laboratory. Among 324 MRSA isolates, none was from blood stream and their overall antibiotic susceptibility patterns against 11 antibiotics were more or less similar to that of the present study throughout (Table 1) [12–14]. Trends of antibiotic susceptibility studied in 2007–2009 are essentially comparable with that in the present study except for the susceptibilities to VCM, TEIC, MINO, LZD and DAP. This study identified a total of 8 VISA isolates (0.96%), whereas not any VISA strain was identified in the previous study (Table 1) [12–14]. The low prevalence of VISA in the non-blood stream MRSA was also reported in our previously study that only 6 VISA isolates (0.25%) had been identified in a retrospective study of the 2446 non-bacteremic MRSA isolates from 1978 through 2005 collected from 17 regionally distant hospitals [31]. Although it is not clear whether there is any inevitable relationship between the incidence of VISA isolate and the sources of specimen, further studies may be required.

Prevalence study of hVISA has been hindered by the lack of a simple and accurate method to identify such strains. Two Etest methods, the macro Etest method and GRD Etest strips, have been developed for the detection of hVISA/VISA. Moreover, their evaluation varied considerably. One group of investigators reported that the macro Etest method had higher specificity (98%) than the GRD E-test [32]. Our studies by a combination of two screening methods and the population analysis showed that overall prevalence of hVISA was 6.51%, among the 830 blood stream MRSA isolates, whereas another group in Japan under the similar conditions reported that 18.5% were hVISA among 162 [11]. The prevalence of the hVISA widely varied from one country to another: Spain, 65%; France, 20%; Germany, 2–14%; Italy, 1%; Brazil, 4%; England, 0–16%; Belgium, 0.4%; Canada, 1.5%; Turkey, 26%; and USA, 0.3–13.9% [32–41]. Although the causes for these gaps are not known at the moment, the possible reasons can be that the precise hVISA is difficult to determine given the ranges of testing methodologies, definitions, and changes in vancomycin susceptibility breakpoints. This may explain the variations in hVISA prevalence detected across hospitals, geographical regions, and patient populations.

Vancomycin remains the reference standard for the treatment of MRSA infections, however, as a result of limited tissue distribution and the emergence of isolates with reduced susceptibility to vancomycin, the need for alternative therapies that target MRSA has become apparent. Although new antibiotics, including linezolid, daptomycin and tigecycline, have been developed, superiority of these agents over vancomycin has not been yet established in the treatment of MRSA. Another problem is frequent emergence of MRSA strains resistant against these new drugs [42,43]. In the present study we identified two linezolid non-susceptible isolates from two different hospitals, both carrying G2576T mutation in domain V of the 23S rRNA gene. We previously showed that the G2576T mutation was prevalent in the linezolid non-susceptible *S. aureus* in Japan [22,44]. Daptomycin became available for clinical use in USA in 2003 and has seen gradually increasing prescription primarily as a second-line agent for severe MRSA infections, but it was not approved in Japan until early 2011. In the present study, we identified 10 isolates with reduced susceptibility to daptomycin with MIC of 2 μg/ml. As all these isolates were isolated from 2008 through 2011, it seems unlike that these strains developed resistance due to the daptomycin administration. There were several reports on the positive correlation between vancomycin- and daptomycin-resistances, and on the development of daptomycin resistance both in vivo and in vitro in *S. aureus* by exposing the strains to vancomycin [45–48]. Since all the daptomycin non-susceptible isolates identified in the present study showed slightly high vancomycin MIC (2 μg/ml, data not shown), the decreased susceptibility of these strains to daptomycin might be, at least partially, due to vancomycin administration during the antibiotic therapy.

Emergence of β-lactam antibiotic induced vancomycin-resistant MRSA (BIVR) was first reported in 2004 [49]. This type of vancomycin resistance is mainly attributable to a rapid depletion of

vancomycin in the medium that was triggered or promoted by β-lactam antibiotics [50]. Therefore, an empiric combination therapy by vancomycin and β-lactam antibiotics for co-infection of MRSA and Gram-negative bacteria might not be an optimal choice of treatment as suggested by our group and others [11,21,49,50]. Recently, clinical impact in BIVR and hVISA has been reported. Among 162 blood-borne MRSA isolates, 30 and 39, equivalent to 18.5% and 24.1%, respectively, were hVISA and BIVR phenotypes, respectively [11]. Furthermore, 60% of hVISA and 46.2% of BIVR were found to show BIVR and hVISA properties, respectively. The study reported that the mortality of the patients who were infected by the hVISA/BIVR two-phenotype MRSA and treated with vancomycin and β-lactam antibiotics trended to be higher compared with the patients infected by either BIVR or hVISA [11]. This study documented that increasing numbers of the vancomycin resistant MRSA such as VISA, hVISA and BIVR were isolated from the MRSA bacteremic specimens. Continued surveillances and evaluation of outcomes for patients infected by MRSA are to be warranted.

References

[1] Loffler CA, Macdougall C. Update on prevalence and treatment of methicillin-resistant Staphylococcus aureus infections. Expert Rev Anti Infect Ther 2007;5:961–81.

[2] Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in combined medical-surgical intensive care units in the United States. Infect Control Hosp Epidemiol 2000;21:510–5.

[3] Richards MJ, Edwards JR, Culver DH, Gaynes RP. Nosocomial infections in medical intensive care units in the United States. National nosocomial infections surveillance system. Crit Care Med 1999;27:887–92.

[4] Lode HM. Clinical impact of antibiotic-resistant gram-positive pathogens. Clin Microbiol Infect 2009;15:212–7.

[5] Gemmell CG. Glycopeptide resistance in *Staphylococcus aureus*: is it a real threat? J Infect Chemother 2004;10:69–75.

[6] Ruef C. Epidemiology and clinical impact of glycopeptide resistance in *Staphylococcus aureus*. Infection 2004;32:315–27.

[7] Shoji M, Cui L, Iizuka R, Komoto A, Neoh HM, Watanabe Y, et al. *walk* and *Clpp* mutations confer reduced vancomycin susceptibility in *Staphylococcus aureus*. Antimicrob Agents Chemother 2011;55:3870–81.

[8] Hiramatsu K, Aritaka N, Hanaki H, Kawasaki S, Hosoda Y, Hori S, et al. Dissemination in Japanese hospitals of strains of *Staphylococcus aureus* heterogeneously resistant to vancomycin. Lancet 1997;350:1670–3 [see comments].

[9] Howden BP, Davies JK, Johnson PD, Stinear TP, Grayson ML. Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. Clin Microbiol Rev 2010;23:99–139.

[10] Hanaki H, Yamaguchi Y, Yanagisawa C, Uehara K, Matsui H, Hososaka Y, et al. Investigation of beta-lactam antibiotic-induced vancomycin-resistant MRSA (BVIR). J Infect Chemother 2005;11:104–6.

[11] Takata T, Miyazaki M, Futo M, Hara S, Shiotsuka S, Kamimura H, et al. Presence of both heterogeneous vancomycin-intermediate resistance and beta-lactam antibiotic-induced vancomycin resistance phenotypes is associated with the outcome in methicillin-resistant *Staphylococcus aureus* bloodstream infection. Scand J Infect Dis 2013;45:203–12.

[12] Niki Y, Hanaki H, Matsumoto T, Yagisawa M, Kohno S, Aoki N, et al. Nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese society of chemotherapy in 2007: general view of the pathogens' antibacterial susceptibility. J Infect Chemother 2009;15:156–67.

[13] Niki Y, Hanaki H, Matsumoto T, Yagisawa M, Kohno S, Aoki N, et al. Nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese society of chemotherapy in 2008: general view of the pathogens' antibacterial susceptibility. J Infect Chemother 2011;17:510–23.

[14] Watanabe A, Yanagihara K, Matsumoto T, Kohno S, Aoki N, Oguri T, et al. Nationwide surveillance of bacterial respiratory pathogens conducted by the surveillance committee of Japanese society of chemotherapy, Japanese association for infectious diseases, and Japanese society for clinical microbiology in 2009: general view of the pathogens' antibacterial susceptibility. J Infect Chemother 2012;18:609–20.

[15] Niki Y, Hanaki H, Yagisawa M, Kohno S, Aoki N, Watanabe A, et al. The first nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese society of chemotherapy. Part 1: a general view of antibacterial susceptibility. J Infect Chemother 2008;14:279–90.

[16] Clinical and laboratory standards institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard-eighth edition, vol. 29, no. 2. Wayne, PA: Clinical and Laboratory Standards Institute; 2009M07-a8.

[17] Walsh TR, Bolmstrom A, Qwarnstrom A, Ho P, Wootton M, Howe RA, et al. Evaluation of current methods for detection of staphylococci with reduced susceptibility to glycopeptides. J Clin Microbiol 2001;39:2439–44.

[18] Yamakawa J, Aminaka M, Okuzumi K, Kobayashi H, Katayama Y, Kondo S, et al. Heterogeneously vancomycin-intermediate *Staphylococcus aureus* (hVISA) emerged before the clinical introduction of vancomycin in Japan: a retrospective study. J Infect Chemother 2012;18:406–9.

[19] Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP. A modified population analysis profile (PAP) method to detect heteroresistance to vancomycin in *Staphylococcus aureus* in a UK hospital. J Antimicrob Chemother 2001;47:399–403.

[20] Hososaka Y, Hanaki H, Yanagisawa C, Yamaguchi Y, Matsui H, Nakae T, et al. Nosocomial infection of beta-lactam antibiotic-induced vancomycin-resistant *Staphylococcus aureus* (BIVR). J Infect Chemother 2006;12:181–4.

[21] Hirao Y, Ikeda-Dantsuji Y, Matsui H, Yoshida M, Hori S, Sunakawa K, et al. Low level beta-lactamase production in methicillin-resistant *Staphylococcus aureus* strains with beta-lactam antibiotics-induced vancomycin resistance. BMC Microbiol 2012;12:69.

[22] Ikeda-Dantsuji Y, Hanaki H, Nakae T, Takesue Y, Tomono K, Honda J, et al. Emergence of linezolid-resistant mutants in a susceptible-cell population of methicillin-resistant *Staphylococcus aureus*. Antimicrob Agents Chemother 2011;55:2466–8.

[23] Hososaka Y, Hanaki H, Endo H, Suzuki Y, Nagasawa Z, Otsuka Y, et al. Characterization of oxacillin-susceptible *mecA*-positive *Staphylococcus aureus*: a new type of MRSA. J Infect Chemother 2007;13:79–86.

[24] Pastagia M, Kleinman LC, Lacerda de la Cruz EG, Jenkins SG. Predicting risk for death from MRSA bacteremia. Emerg Infect Dis 2012;18:1072–80.

[25] Chambers HF, Deleo FR. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. Nat Rev Microbiol 2009;7:629–41.

[26] Sakoulas G, Moise-Broder PA, Schentag J, Forrest A, Moellering Jr RC, Eliopoulos GM. Relationship of MIC and bactericidal activity to efficacy of vancomycin for treatment of methicillin-resistant *Staphylococcus aureus* bacteremia. J Clin Microbiol 2004;42:2398–402.

[27] Hidayat LK, Hsu DI, Quist R, Shriner KA, Wong-Beringer A. High-dose vancomycin therapy for methicillin-resistant *Staphylococcus aureus* infections: efficacy and toxicity. Arch Intern Med 2006;166:2138–44.

[28] Lodise TP, Patel N, Lomaestro BM, Rodvold KA, Drusano GL. Relationship between initial vancomycin concentration-time profile and nephrotoxicity among hospitalized patients. Clin Infect Dis Official Publ Infect Dis Soc Am 2009;49:507–14.

[29] Maor Y, Hagin M, Belausov N, Keller N, Ben-David D, Rahav G. Clinical features of heteroresistant vancomycin-intermediate *Staphylococcus aureus* bacteremia versus those of methicillin-resistant S. aureus bacteremia. J Infect Dis 2009;199:619–24.

[30] Neoh HM, Hori S, Komatsu M, Oguri T, Takeuchi F, Cui L, et al. Impact of reduced vancomycin susceptibility on the therapeutic outcome of MRSA bloodstream infections. Ann Clin Microbiol Antimicrob 2007;6:13.

[31] Hanaki H, Hososaka Y, Yanagisawa C, Otsuka Y, Nagasawa Z, Nakae T, et al. Occurrence of vancomycin-intermediate-resistant *Staphylococcus aureus* in Japan. J Infect Chemother 2007;13:118–21.

[32] Adam HJ, Louie L, Watt C, Gravel D, Bryce E, Loeb M, et al. Detection and characterization of heterogeneous vancomycin-intermediate *Staphylococcus aureus* isolates in Canada: results from the Canadian nosocomial infection surveillance program, 1995–2006. Antimicrob Agents Chemother 2010;54:945–9.

[33] Bierbaum G, Fuchs K, Lenz W, Szekat C, Sahl HG. Presence of *Staphylococcus aureus* with reduced susceptibility to vancomycin in Germany. Eur J Clin Microbiol Infect Dis 1999;18:691–6.

[34] Ariza J, Pujol M, Cabo J, Pena C, Fernandez N, Linares J, et al. Vancomycin in surgical infections due to methicillin-resistant *Staphylococcus aureus* with heterogeneous resistance to vancomycin. Lancet 1999;353:1587–8.

[35] Reverdy ME, Jarraud S, Bobin-Dubreux S, Burel E, Girardo P, Lina G, et al. Incidence of *Staphylococcus aureus* with reduced susceptibility to glycopeptides in two french hospitals. Clin Microbiol Infect 2001;7:267–72.

[36] Marchese A, Balistreri G, Tonoli E, Debbia EA, Schito GC. Heterogeneous vancomycin resistance in methicillin-resistant *Staphylococcus aureus* strains isolated in a large Italian hospital. J Clin Microbiol 2000;38:866–9.

[37] Denis O, Nonhoff C, Byl B, Knoop C, Bobin-Dubreux S, Struelens MJ. Emergence of vancomycin-intermediate *Staphylococcus aureus* in a Belgian hospital: microbiological and clinical features. J Antimicrob Chemother 2002;50:383–91.

[38] Geisel R, Schmitz FJ, Thomas L, Berns G, Zetsche O, Ulrich B, et al. Emergence of heterogeneous intermediate vancomycin resistance in *Staphylococcus aureus* isolates in the Dusseldorf area. J Antimicrob Chemother 1999;43:846–8.

[39] Sancak B, Ercis S, Menemenlioglu D, Colakoglu S, Hascelik G. Methicillin-resistant *Staphylococcus aureus* heterogeneously resistant to vancomycin in a Turkish university hospital. J Antimicrob Chemother 2005;56:519–23.

[40] Richter SS, Satola SW, Crispell EK, Heilmann KP, Dohrn CL, Riahi F, et al. Detection of *Staphylococcus aureus* isolates with heterogeneous intermediate-level resistance to vancomycin in the United States. J Clin Microbiol 2011;49:4203–7.

[41] Bae IG, Federspiel JJ, Miro JM, Woods CW, Park L, Rybak MJ, et al. Heterogeneous vancomycin-intermediate susceptibility phenotype in bloodstream methicillin-resistant *Staphylococcus aureus* isolates from an international cohort of patients with infective endocarditis: prevalence, genotype, and clinical significance. J Infect Dis 2009;200:1355–66.

[42] Tsiodras S, Gold HS, Sakoulas G, Eliopoulos GM, Wennersten C, Venkataraman L, et al. Linezolid resistance in a clinical isolate of *Staphylococcus aureus*. Lancet 2001;358:207–8.
[43] Marty FM, Yeh WW, Wennersten CB, Venkataraman L, Albano E, Alyea EP, et al. Emergence of a clinical daptomycin-resistant *Staphylococcus aureus* isolate during treatment of methicillin-resistant staphylococcus aureus bacteremia and osteomyelitis. J Clin Microbiol 2006;44:595–7.
[44] Ikeda-Dantsuji Y, Nakae T, Ariyoshi K, Mizuno H, Moriyama H, Nagura O, et al. Limited detectability of linezolid-resistant *Staphylococcus aureus* by the Etest method and its improvement using enriched media. J Med Microbiol 2012;61:998–1002.
[45] Cui L, Tominaga E, Neoh HM, Hiramatsu K. Correlation between reduced daptomycin susceptibility and vancomycin resistance in vancomycin-intermediate *Staphylococcus aureus*. Antimicrob Agents Chemother 2006;50:1079–82.
[46] Sakoulas G, Alder J, Thauvin-Eliopoulos C, Moellering Jr RC, Eliopoulos GM. Induction of daptomycin heterogeneous susceptibility in *Staphylococcus aureus* by exposure to vancomycin. Antimicrob Agents Chemother 2006;50:1581–5.
[47] Camargo IL, Neoh HM, Cui L, Hiramatsu K. Serial daptomycin selection generates daptomycin-nonsusceptible *Staphylococcus aureus* strains with a heterogeneous vancomycin-intermediate phenotype. Antimicrob Agents Chemother 2008;52:4289–99.
[48] Cui L, Neoh HM, Shoji M, Hiramatsu K. An *rpoB* mutation confers dual heteroresistance to daptomycin and vancomycin in *Staphylococcus aureus*. Antimicrob Agents Chemother 2010;54:5222–33.
[49] Hanaki H, Yamaguchi Y, Nomura S, Haraga I, Nagayama A, Sunakawa K. Method of detecting beta-lactam antibiotic induced vancomycin resistant MRSA (BIVR). Int J Antimicrob Agents 2004;23:1–5.
[50] Yanagisawa C, Hanaki H, Matsui H, Ikeda S, Nakae T, Sunakawa K. Rapid depletion of free vancomycin in medium in the presence of beta-lactam antibiotics and growth restoration in *Staphylococcus aureus* strains with beta-lactam-induced vancomycin resistance. Antimicrob Agents Chemother 2009;53:63–8.

J Infect Chemother 20 (2014) 558–562

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Original article

Distribution of *Candida* species isolated from blood cultures in hospitals in Osaka, Japan

Daiichi Morii*, Masafumi Seki*, José N. Binongo, Ryoichi Ban, Atsuko Kobayashi, Makoto Sata, Shigeki Hashimoto, Junzo Shimizu, Shunji Morita, Kazunori Tomono

Division of Infection Control and Prevention, Osaka University, 2-15 Yamadaoka, Suita, Osaka 565-0871, Japan

ARTICLE INFO

Article history:
Received 28 January 2014
Received in revised form
27 April 2014
Accepted 15 May 2014
Available online 5 July 2014

Keywords:
Candidemia
Distribution
Local epidemiology
Non-*albicans Candida*
Fluconazole susceptibility

ABSTRACT

Background: Candida species are clinically important causes of bloodstream infections because their mortality is very high. Given that some species of Candida are azole-resistant, identifying the distributions of Candida species could facilitate the formulation of an appropriate empirical antifungal therapy. It has been shown that the distribution varies depending on the continent, country, city, and hospital. In this paper, we describe the distributions of species in hospitals in northern Osaka, Japan.

Method: We evaluated blood culture results obtained from six tertiary hospitals in the northern Osaka area between 2004 and 2011. We also obtained comorbidity information from the patients' hospital medical records. Kaplan–Meier curves were drawn to compare the risk of death related to the different species.

Results: Of the 165 cases of candidemia confirmed by blood culture, 66% were male and the mean age was 62 years (range = 0–96). Overall, *Candida albicans* comprised 70 cases (43%), followed by *Candida parapsilosis* with 36 cases (22%), *Candida glabrata* with 25 cases (15%), *Candida tropicalis* with 11 cases (7%), *Candida krusei* with 10 cases (6%), and other Candida species with 13 cases (8%). *C. tropicalis* had higher associated mortality than other species, although it was not statistically significant.

Conclusions: *C. albicans* was the most frequently isolated species, but the proportion of non-albicans Candida species was not negligible. The relatively high frequency of non-albicans Candida species distinguished the Japanese distribution from other areas. This characteristic distribution may have important implications when formulating an empirical antifungal therapy for Japanese clinical practice.

1. Introduction

Candida species are among the most common causes of nosocomial bloodstream infection (BSI) [1]. They are important in clinical settings because the mortality rate associated with candidemia is very high [2,3]. However, conducting an epidemiological study of candidemia is challenging because cultures rarely confirm the infection. Many hospitals in Japan lack suitable facilities for performing reliable mycological assays of *Candida* species. Thus, Japanese physicians use empirical therapy because it is not possible to delay treatment until positive blood culture results are obtained [4]. The incubation time has a statistically significant impact on the in-hospital mortality [5] and delaying empirical treatment for more than 12 h is associated with higher mortality [6].

Numerous surveillance programs have focused on candidemia, which have documented the prevalence of different *Candida* species. In the early 1990s, a population-based surveillance study organized by the Centers for Disease Control and Prevention (CDC) found that *Candida albicans* was the dominant *Candida* species [7], followed by *Candida parapsilosis*. Subsequent surveillance programs have noted an increase in the proportion of non-*albicans Candida* BSI, particularly an increase in *Candida glabrata* [8]. This change in distribution is considered to be important because fungal drug susceptibility testing is not the universal standard of care, so knowledge of the infecting species is used to guide therapy. Both *C. glabrata* and *Candida krusei* are considered to be azole-resistant species [9,10] so any change in the distribution of *Candida* species affects the choice of empirical antifungal therapy. The distribution of *Candida* species varies geographically. In Canada and Europe, for example, the prevalence of *C. albicans* is higher than that in other

* Corresponding authors. Tel.: +81 6 6879 5093; fax: +81 6 6879 5094.
E-mail addresses: morrydaich@alum.emory.edu, morrydaich@hotmail.com (D. Morii), seki@hp-infect.med.osaka-u.ac.jp (M. Seki).

http://dx.doi.org/10.1016/j.jiac.2014.05.009
1341-321X/

regions. The United States (US) has a higher proportion of *C. glabrata* than other regions, while *Candida tropicalis* is disproportionately prevalent in Latin America [11].

In this study, we determined the distributions of *Candida* species in northern Osaka, Japan using data collected during 2004–2011 from tertiary care hospitals.

2. Methods

2.1. Study design

The data used in this study were obtained from tertiary care hospitals in the northern Osaka area of Japan. Blood culture results were evaluated between 2004 and 2011. Six hospitals in the northern Osaka area participated in this study, including one university hospital (1000 beds), one national center for cardiovascular/cerebral diseases (600 beds), and four city hospitals (360–600 beds for each). We received approvals by the ethical review board in Osaka University Hospitals. This study is registered in UMIN (UMIN000008282). A candidemia case was defined as the first isolation of any *Candida* species from blood drawn from a patient at any of the participating hospitals. Medical records were also reviewed to identify the underlying medical conditions, treatments, and potential risk factors.

The detection and species determination of isolates were performed in each hospital laboratory using CHROMagar (Kanto Chemical Co. Inc., Tokyo, Japan), API 20C AUX (SYSMEX bioMérieux Co. Ltd, Kobe, Japan), or a Vitek Yeast Biochemical Card (SYSMEX bioMérieux Co. Ltd, Kobe, Japan). All of the clinical decisions were entrusted to the discretion of the patient's physician. One hospital lacked suitable technology to determine the species type from 2004 to 2009, so only data obtained from this hospital between 2010 and 2011 were included in the analysis.

When identifying underlying conditions and risk factors, we defined "broad-spectrum antibiotics" as fourth generation cephalosporins and carbapenems. Exposure to broad-spectrum antibiotics, anti-MRSA drugs, and prophylactic antifungal agents were defined as the use of these drugs during the 60 days prior to the blood cultures being taken. A central venous line was counted when it was placed in a patient within seven days before the blood culture was taken. Diabetes mellitus, including impaired glucose tolerance, was a clinical diagnosis made by the reviewers of the medical records in each hospital. Hemodialysis was either chronic hemodialysis or other types of active blood purification therapy within the 60 days prior to the blood test.

2.2. Statistical analysis

The relative frequencies were calculated to compare the distributions of different *Candida* species. Kaplan–Meier curves were also drawn to visualize the survival distributions. SAS version 9.3 (SAS Institute Inc., Cary, NC, US) was used for the statistical analyses.

3. Results

A total of 165 cases were detected. Among them, 81 were detected in the university hospital, 3 were in the national center for cardiovascular/cerebral diseases, and 81 were in city hospitals. Table 1 shows the clinical and demographic characteristics of the patients with candidemia. The mean age was 62 years (range = 0–96) and 66% of the patients were male. One person was found to possess two species types. We treated this patient as two cases in the analysis. The department where *Candida* species were isolated most frequently from blood samples was internal medicine with 62 cases. Internal medicine is an aggregate of all types of internal medicine departments, other than cardiology and hematology/oncology. Gastrointestinal surgery was second with 35 cases. In terms of comorbidities, 129 patients (78%) had central venous line placements within seven days prior to their blood samples being tested, 88 patients (53%) were exposed to broad-spectrum antibiotics before *Candida* isolation, and 125 patients (75%) had been hospitalized for more than 18 days at the time of detection.

Table 1
Characteristics of patients with candidemia (n = 165).

Patient characteristics	
Sex (male), *n* (%)	109 (66)
Age (years), mean (SD)	62 (23)
Department, *n* (%)	
Internal medicine	62 (38)
Cardiology	9 (5)
Hematology/oncology	20 (12)
Pediatrics/pediatric surgery	13 (8)
Cardiothoracic surgery	13 (8)
Gastrointestinal surgery	35 (21)
Other surgery	13 (8)
Comorbidity/risk factor, *n* (%)	
Diabetes mellitus	55 (33)
Hemodialysis	22 (13)
Chemotherapy	32 (19)
Immunosuppression	60 (36)
Central venous line	129 (78)
Anti-MRSA drug	20 (12)
Broad-spectrum antibiotics	88 (53)
Fluconazole	19 (12)
Micafungin	44 (27)
Hospital days to isolation, median (25th, 75th percentile)	35 (18, 73)

During the study period, 165 cases of candidemia were confirmed by blood cultures at the participating hospitals. Overall, 42.4% (70 cases) of the infections were due to *C. albicans*, followed by *C. parapsilosis* (21.8%, 36 cases), *C. glabrata* (15.2%, 25 cases), *C. tropicalis* (6.7%, 11 cases), *C. krusei* (6.1%, 10 cases), and other *Candida* spp. (7.9%, 13 cases) (Fig. 1).

The evaluation of the clinical outcomes showed that the 28-day all-cause mortality was 32% for all cases. *C. tropicalis* had a higher in-hospital mortality than other species, although the difference was not statistically significant (Fig. 2). Of the patients who died, 90% died within 31 days of candidemia being diagnosed. With respect to the differences in mortality, the status of comorbidities, rather than the virulence of each species, may explain the higher likelihood of mortality in patients with *C. tropicalis*. In this study, a chi-squared test showed that there was a statistically significant

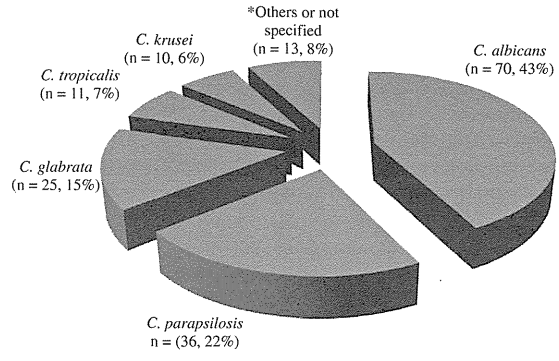

Fig. 1. Overall distribution of *Candida* species (n = 165). * One case of *Candida famata*, one case of *Candida lusitaniae*, and 11 cases of undetermined *Candida* species. *C. albicans* is the most frequently isolated, and *C. parapsilosis* follows.

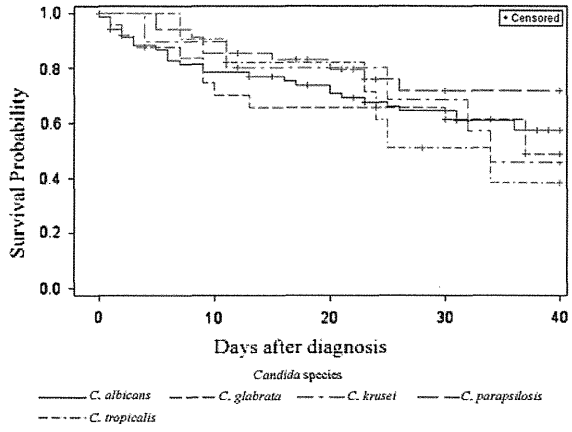

Fig. 2. Survival curve for candidemia patients with each *Candida* species. *C. tropicalis* had a higher in-hospital mortality than other species, although the difference was not statistically significant.

association between diabetes mellitus and *C. tropicalis* (but not with non-*tropicalis Candida*) ($p = 0.027$).

Among all cases, prophylactic fluconazole was given in 19 cases. The crude data shows that the percentages of *C. albicans* and *C. parapsilosis* decreased and the percentages of *C. glabrata* and *C. krusei* increased in the cases where patients were given fluconazole prophylaxis (Table 2). On the other hand, prophylactic micafungin was given in 44 cases. The crude data shows that the percentage of *C. parapsilosis* and *C. krusei* increased in the cases where patients were given micafungin prophylaxis (Table 3).

4. Discussion

The distributions of *Candida* species differ among geographical areas. An international surveillance study, SENTRY study, showed that the prevalence of *C. tropicalis* was higher in Latin America than other regions. The prevalence of *C. parapsilosis* in Latin America was also higher than that in the US [11] (Fig. 3). In the US, *C. glabrata* was significantly more prevalent than other areas. A subanalysis of the SENTRY study showed that there were fairly variable species distributions even within countries and continents. Some species were specific to a particular region. For example, a nationwide sentinel surveillance study conducted in Brazil showed that *Candida pelliculosa* was the fourth most frequently isolated species with a prevalence of 6.2% [12]. This species is not mentioned in The Infectious Diseases Society of America (IDSA) Guidelines 2009 [13], although it was more prevalent than *C. glabrata* (4.9%) and *C. krusei* (1.1%) in Brazil. This suggests that we may need to pay greater attention to the local distribution rather than the continental distribution.

Several population-based or sentinel surveillance studies conducted in the US have shown that 45–58% of candidemia cases

Table 2
Candida species with or without fluconazole prophylaxis.

	Fluconazole prophylaxis +, *n* (%)	Fluconazole prophylaxis −, *n* (%)
C. albicans	6 (32)	64 (44)
C. parapsilosis	3 (16)	33 (23)
C. glabrata	4 (21)	21 (14)
C. tropicalis	2 (11)	9 (6)
C.krusei	4 (21)	6 (4)
Others	0 (0)	13 (9)
Total	19	146

Table 3
Candida species with or without micafungin prophylaxis.

	With micafungin prophylaxis, *n* (%)	Without micafungin prophylaxis, *n* (%)
C. albicans	18 (41)	52 (43)
C. parapsilosis	13 (30)	23 (19)
C. glabrata	7 (16)	18 (15)
C. tropicalis	2 (5)	9 (7)
C. krusei	4 (9)	6 (5)
Others	0 (0)	13 (11)
Total	44	146

were caused by *C. albicans*, 12–24% by *C. glabrata*, and 7–21% by *C. parapsilosis* [7,14–16] (Table 4). However, only one nationwide epidemiological study of *Candida* species is available from Japan [17], which was based on a survey conducted during 1999. According to this study, the prevalence of *C. albicans* was 48%, while that of *C. parapsilosis* was 19%, *C. glabrata* was 16%, *C. tropicalis* was 12%, and *C. krusei* was 3%, among the candidiasis cases reported. Thus, the nationwide trend in the late 1990s appears to have been quite similar to that detected in northern Osaka in the present study. However, local reports from other parts of Japan suggest that there are quite different prevalence rates [18–20] (Fig. 4). One study from a suburban city near Hiroshima (NHO Kure Medical Center) reported an extremely high *C. glabrata* prevalence of 42% during 2002–2008, while the prevalence of *C. albicans* was 29% and that of *C. parapsilosis* was 16% [18]. This difference may be due to contrast of urban area rural area. Urban areas including northern Osaka and Tokyo (Juntendo University and St. Luke's hospital) have relatively low percentage of *C. glabrata*. The distributions of different *Candida* species do not appear to be identical throughout Japan, so it is important to consider local data trends as well as national trends.

The relatively low numbers of *Candida*-positive blood cultures in particular hospitals hinder epidemiological studies of these species. In general, each hospital produces a very low number of positive culture results, which means it is difficult to determine the distributions of species. This is why it may make sense to consolidate data from several hospitals within a district. However, the representativeness of the consolidated distribution may be doubtful if we include more hospitals in the analysis. In addition to spatial considerations, the length of the observation period is also important. The spatial and temporal scales we selected were arbitrary. We collected data from the main tertiary hospitals in northern Osaka for eight consecutive years.

A limitation of our study is that the biological methods used for species identification were not identical. In contrast to several population-based surveys conducted in the US, three different

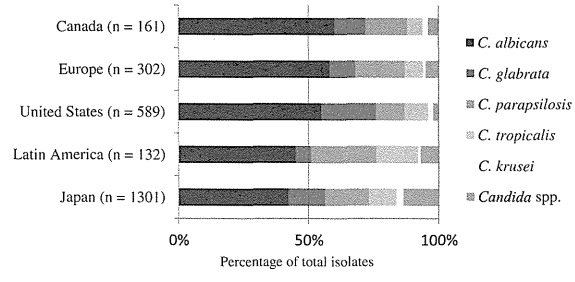

Fig. 3. The global diversity of *Candida* species isolated from bloodstream-infected patients based on the SENTRY program* (1997–2000) and a Japanese study** (2001). *[11], **[17] Japan and Latin America show greater percentage of *non-albicans Candida* than Canada and Europe.

Table 4
Surveillance in the United States.

Reference	Year	City/region	% of total					
			C. albicans	*C. parapsilosis*	*C. glabrata*	*C. tropicalis*	*C. krusei*	Other species
Kao [7]	1992–1993	Atlanta, San Francisco	52	21	12	10	4	–
Hajjeh [14]	1998–2000	Connecticut, Baltimore	45	13	24	12	–	–
Diekema [15]	1998–2001	Iowa	58	7	20	11	2	2

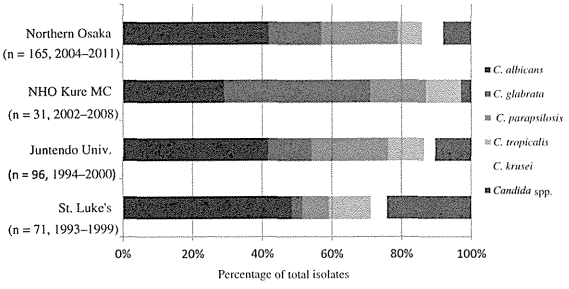

Fig. 4. The domestic diversity of *Candida* species isolated from bloodstream-infected patients in Japanese hospitals. *[18], **[19], ***[20] NHO Kure MC shows greater percentage of *C. glabrata* than other hospitals.

species identification systems were used, which depended on the hospital. The development of a centralized system in Japan would facilitate the standardization of species identification. Sentinel or population-based surveillance via a central laboratory will also be necessary to address concerns about the internal validity of using different biological testing methods.

There are several possible explanations for the different distributions among regions. Prior exposure to antifungal drugs is one possible explanation because patients treated recently with an azole drug have an increased risk of infection with fluconazole-resistant *Candida* [21]. Furthermore, Ben-Ami et al. reported a statistically significant association between fluconazole-resistant species and certain types of antibiotics, such as metronidazole, carbapenem, trimethoprim-sulfamethoxazole, clindamycin, and colistin [22]. A possible selective effect of age is another possible explanation because very young children and neonates are more likely to be infected by *C. parapsilosis* [23]. Several studies have shown that *C. glabrata* infections are more common in older cohorts than younger ones [24,25]. More importantly, differences in general antifungal practices may have caused the different distributions among regions. The Infectious Diseases Society of America's guidelines recommend fluconazole for many forms of candidiasis, including adult nonneutropenic candidemia [13], whereas the European Society of Clinical Microbiology and Infectious Diseases' guidelines grade azoles lower than echinocandins for the treatment of invasive candidiasis and candidemia [4]. In the most recent Japanese guidelines, fluconazole is recommended only for mild candidiasis, otherwise echinocandins are recommended [26]. These different practices may explain the regional differences in the distributions of *Candida* species. Further research is required to determine whether these factors can explain the regional differences in the distributions of *Candida* species.

Funding

None.

References

[1] Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 2004;39:309–17.
[2] Pagano L, Antinori A, Ammassari A, Mele L, Nosari A, Melillo L, et al. Retrospective study of candidemia in patients with hematological malignancies. Clinical features, risk factors and outcome of 76 episodes. Eur J Haematol 1999;63:77–85.
[3] Viscoli C, Girmenia C, Marinus A, Collette L, Martino P, Vandercam B, et al. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). Clin Infect Dis 1999;28:1071–9.
[4] Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, et al. ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect 2012;18:19–37.
[5] Taur Y, Cohen N, Dubnow S, Paskovaty A, Seo SK. Effect of antifungal therapy timing on mortality in cancer patients with candidemia. Antimicrob Agents Chemother 2010;54:184–90.
[6] Morrell M, Fraser VJ, Kollef MH. Delaying the empiric treatment of Candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. Antimicrob Agents Chemother 2005;49:3640–5.
[7] Kao AS, Brandt ME, Pruitt WR, Conn LA, Perkins BA, Stephens DS, et al. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. Clin Infect Dis 1999;29:1164–70.
[8] Pfaller MA, Diekema DJ. Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility. J Clin Microbiol 2002;40:3551–7.
[9] Drago M, Scaltrito MM, Morace G. In vitro activity of voriconazole and other antifungal agents against clinical isolates of *Candida glabrata* and *Candida krusei*. Eur J Clin Microbiol Infect Dis 2004;23:619–24.
[10] Spellberg BJ, Filler SG, Edwards Jr JE. Current treatment strategies for disseminated candidiasis. Clin Infect Dis 2006;42:244–51.
[11] Pfaller MA, Diekema DJ, Jones RN, Sader HS, Fluit AC, Hollis RJ, et al. International surveillance of bloodstream infections due to Candida species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole, and voriconazole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. J Clin Microbiol 2001;39:3254–9.
[12] Colombo AL, Nucci M, Park BJ, Nouér SA, Arthington-Skaggs B, da Matta DA, et al. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. J Clin Microbiol 2006;44:2816–23.
[13] Pappas PG, Kauffman CA, Andes D, Benjamin Jr DK, Calandra TF, Edwards Jr JE, et al. Clinical practice guidelines for the management of candidiasis: 2009 update by the infectious diseases society of America. Clin Infect Dis 2009;48:503–35.
[14] Hajjeh RA, Sofair AN, Harrison LH, Lyon GM, Arthington-Skaggs BA, Mirza SA, et al. Incidence of bloodstream infections due to Candida species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. J Clin Microbiol 2004;42:1519–27.
[15] Diekema DJ, Messer SA, Brueggemann AB, Coffman SL, Doern GV, Herwaldt LA, et al. Epidemiology of candidemia: 3-year results from the emerging infections and the epidemiology of Iowa organisms study. J Clin Microbiol 2002;40:1298–302.
[16] Pfaller MA, Diekema DJ, Jones RN, Messer SA, Hollis RJ. Trends in antifungal susceptibility of Candida spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000. J Clin Microbiol 2002;40:852–6.
[17] Uehara Y, Makmura K, Kamei K, Suzuki K, Kikuchi K, Niimi M. The current situation of severe fungal infection in Japan – a questionnaire based report. Jpn J Antibiot 2001;54:448–72 [in Japanese].
[18] Shimonaka A, Morii D, Tadera K, Shimohana J, Michinaka T. Single center distribution of Candida species. IRYO 2011;65:167–71 [in Japanese].
[19] Yamamoto Y, Mori T, Oguri T. Retrospective analysis of cases with fungemia detected in bloodstream and catheter cultures at Juntendo University Hospital. Juntendo Igaku 2001;28:155–60 [in Japanese].
[20] Kazama I, Furukawa K. A study of candidemia during the six years period from 1993 to 1999 in St. Luke's International Hospital. Kansenshogaku Zasshi 2003;77:158–66 [in Japanese].
[21] Garnacho-Montero J, Díaz-Martín A, García-Cabrera E, Ruiz Pérez de Pipaón M, Hernández-Caballero C, Aznar-Martín J, et al. Risk factors for fluconazole-resistant candidemia. Antimicrob Agents Chemother 2010;54:3149–54.
[22] Ben-Ami R, Olshtain-Pops K, Krieger M, Oren I, Bishara J, Dan M, et al. Antibiotic exposure as a risk factor for fluconazole-resistant Candida bloodstream infection. Antimicrob Agents Chemother 2012;56:2518–23.

[23] Dotis J, Prasad PA, Zaoutis T, Roilides E. Epidemiology, risk factors and outcome of *Candida parapsilosis* bloodstream infection in children. Pediatr Infect Dis J 2012;31:557–60.
[24] Malani A, Hmoud J, Chiu L, Carver PL, Bielaczyc A, Kauffman CA. *Candida glabrata* fungemia: experience in a tertiary care center. Clin Infect Dis 2005;41:975–81.
[25] Malani AN, Psarros G, Malani PN, Kauffman CA. Is age a risk factor for *Candida glabrata* colonisation? Mycoses 2011;54:531–7.
[26] Niki Y, Mikamo H. Japanese guideline for invasive candidiasis. The Japanese Society for Medical Mycology; 2012. http://www.jsmm.org/pulic_comment2-1.pdf.

PLOS ONE

Novel Anti-Microbial Peptide SR-0379 Accelerates Wound Healing via the PI3 Kinase/Akt/mTOR Pathway

Hideki Tomioka[1,2], Hironori Nakagami[3]*, Akiko Tenma[2], Yoshimi Saito[2], Toshihiro Kaga[2], Toshihide Kanamori[2], Nao Tamura[2], Kazunori Tomono[5], Yasufumi Kaneda[4], Ryuichi Morishita[1]*

1 Department of Clinical Gene Therapy, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka, Japan, **2** AnGesMG, Inc., 7-7-15 Saito Bio-Incubator, Ibaraki, Osaka, Japan, **3** Division of Vascular Medicine and Epigenetics, Osaka University United Graduate School of Child Development, 2-1 Yamada-oka, Suita, Osaka, Japan, **4** Division of Gene Therapy Science, Osaka University Graduate School of Medicine, School of Child Development, 2-1 Yamada-oka, Suita, Osaka, Japan, **5** Division of Infection Control and Prevention, Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka, Japan

Abstract

We developed a novel cationic antimicrobial peptide, AG30/5C, which demonstrates angiogenic properties similar to those of LL-37 or PR39. However, improvement of its stability and cost efficacy are required for clinical application. Therefore, we examined the metabolites of AG30/5C, which provided the further optimized compound, SR-0379. SR-0379 enhanced the proliferation of human dermal fibroblast cells (NHDFs) via the PI3 kinase-Akt-mTOR pathway through integrin-mediated interactions. Furthermore SR-0379 promoted the tube formation of human umbilical vein endothelial cells (HUVECs) in co-culture with NHDFs. This compound also displays antimicrobial activities against a number of bacteria, including drug-resistant microbes and fungi. We evaluated the effect of SR-0379 in two different would-healing models in rats, the full-thickness defects under a diabetic condition and an acutely infected wound with full-thickness defects and inoculation with *Staphylococcus aureus*. Treatment with SR-0379 significantly accelerated wound healing when compared to fibroblast growth factor 2 (FGF2). The beneficial effects of SR-0379 on wound healing can be explained by enhanced angiogenesis, granulation tissue formation, proliferation of endothelial cells and fibroblasts and antimicrobial activity. These results indicate that SR-0379 may have the potential for drug development in wound repair, even under especially critical colonization conditions.

Citation: Tomioka H, Nakagami H, Tenma A, Saito Y, Kaga T, et al. (2014) Novel Anti-Microbial Peptide SR-0379 Accelerates Wound Healing via the PI3 Kinase/Akt/mTOR Pathway. PLoS ONE 9(3): e92597. doi:10.1371/journal.pone.0092597

Editor: Ganesh Chandra Jagetia, Mizoram University, India

Received October 5, 2013; **Accepted** February 24, 2014; **Published** March 27, 2014

Funding: This study was supported by a Health Labour Sciences Research Grant in Japan, and partially funded by AnGes MG. No additional funding was received for this study. Hideki Tomioka, Akiko Tenma, Yoshimi Saito, Toshihiro Kaga, Toshihide Kanamori and Nao Tamura are employees of AnGes MG. The funder provided support in the form of salaries for authors, but did not have any additional role in the study design, data collection andanalysis, decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the "Author Contributions" section.

Competing Interests: Ryuichi Morishita is an Editorial Board Member of PLOS ONE. Ryuichi Morishita is a founder of AnGes MG, and a stockholder. AnGes MG has the following patent licenses of AG30/5C (PCT/JP2008/052020: Novel polypeptide and antibacterialagent comprising the same as active ingredient) and SR-0379 (PCT/JP2010/058838: Polypeptides and antibacterial or antiseptic use of same). Hideki Tomioka, AkikoTenma, Yoshimi Saito, Toshihiro Kaga, Toshihide Kanamori and Nao Tamura are employees of AnGes MG. Department of clinical gene therapy is financially supported by AnGes MG, Novartis, Shionogi, Boeringher, and Rohto. Division of VascularMedicine and Epigenetics is financially supported by Bayel. This study is partially supported by the fund of AnGes MG. There are no further patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: nakagami@gts.med.osaka-u.ac.jp (HN); morishit@cgt.med.osaka-u.ac.jp (RM)

Introduction

Antimicrobial peptides are produced by multicellular organisms as a defense mechanism against competing pathogenic microbes [1]. Currently, more than 1,200 antimicrobial peptides have been discovered in animals and plants [2]. In addition to their antimicrobial functions, some peptides, such as LL-37, are known to have other functions. For example, LL-37 is chemotactic for monocytes, T cells, neutrophils and mast cells and also stimulates mast cell histamine release and angiogenesis [3]. These observations have led to the development of a therapeutic concept using antimicrobial peptides as multifunctional effector molecules to prevent infection directly and to promote wound healing in various ulcers. Indeed, many researchers have tried to develop antimicrobial peptides for topical use and systemic application [4] [5]. Before these peptides can be used in clinical applications, several improvements must be made, including 1) increased stability, 2) reduced cytotoxicity and 3) improved antimicrobial activity [6]. To improve the stability of antimicrobial peptides, converting amino acids from L to D conformations has been attempted [7]. Antimicrobial peptides are usually positively charged, between 12 and 100 amino acids in length and form amphipathic structures. The production of shorter analogs may also resolve the cost issue for clinical applications.

We previously developed an antimicrobial peptide named AG30 (angiogenic peptide 30) that contained 30 amino acids and possessed both angiogenic and antibacterial functions [8] [9]. For clinical application in the treatment of drug-resistant ulcers, we have modified AG30 to enhance its angiogenic activity, broaden its antibacterial function, enhance its stability and reduce its cost. In this study, we have demonstrated the production of a stable, shorter peptide, SR-0379, that contains twenty amino acids, including one lysine residue that has been converted to D-lysine. This peptide exhibited antimicrobial activities against a

number of bacteria, including drug-resistant bacteria, and induced the proliferation, tube formation, migration and contraction of human dermal fibroblast cells.

Treatment with SR-0379 significantly accelerated wound healing in a skin ulcer model. In the intracellular mechanism of wound healing, we focused on the PI3K/Akt/mTOR pathway. It has been reported that Akt/mTOR activation, by the ablation of Pten and Tsc1, dramatically increased epithelial cell proliferation, migration, and cutaneous wound healing, while pharmacological inhibition of mTOR with rapamycin delays wound closure [10]. Indeed, SR-0379 would activate the Akt/mTOR pathway which lead to accelerate wound healing. Further application of SR-0379 might provide a new therapeutic option to treat various ulcers, such as diabetic ulcers and severe burns.

Results

Design of SR-0379

For use in clinical applications, we first modified AG30 to improve its stability and reduce its cost. To perform the lead-to-drug-candidate optimization, various approaches to improve stability were evaluated. The metabolic stability of AG30/5C was evaluated by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Figure. S1 in File S1). The major metabolites were 20 amino acids (aa), 18 aa and 17 aa, which overlap the N terminus AG30/5C and 12 aa in the middle of AG30/5C (Fig. 1A). The metabolites of AG30/5C were cleaved by endopeptidases and exopeptidase at multiple sites, with most cleavage sites present in the C-terminus. Those metabolites were synthesized (Fig. 1B), and their proliferation in HUVECs, tube formation in co-culture of HUVECs and NHDFs and antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were evaluated. Treatment with the 20-aa peptide (SR-0007) yielded significant increases in proliferation of HUVECs (Fig. 1C), whereas other metabolites did not have significant effects (data not shown). The stimulatory effects of SR-0007 were equivalent to the effects of AG30/5C (Fig. 1C). Furthermore, the treatment with SR-0007 (10 μg/ml) induced tube form in co-culture of HUVECs and NHDFs with the same level of AG30/5C (Fig. 1D). SR-0007 exhibited similar antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* as AG30/5C (Table 1). These results revealed that SR-0007, which consists of 20 amino acids, is a potent candidate metabolite like AG30. However, SR-0007 was rapidly degraded by rat and human sera at 37°C (Figure 1E). Therefore, we further modified SR-0007 to improve its stability. The lysine in SR-0007 was replaced with D-form lysine, and the resulting compound was named SR-0379. As shown in Fig. 1E, the stability of SR-0379 was significantly improved, suggesting that SR-0379 might be resistant to the actions of known natural peptidases [11].

Antibacterial activity of SR-0379 in drug-resistant/sensitive strains

We examined the antibacterial effects of AG30/5C, SR-0007 and SR-0379 against *E. coli*, *P. aeruginosa* and *S. aureus* (Table 1). Importantly, SR-0379 exhibited more potent antibacterial activity against *E. coli compared* to the original SR-0007 peptide, whereas the minimal inhibitory concentration (MIC) of SR-0379 against *P. aeruginosa* was equivalent to that of SR-0007. A number of strains were then evaluated for their sensitivities to SR-0379 (Table 2). SR-0379 demonstrated potent antibacterial activity against gram-positive and gram-negative aerobes and anaerobes. Additionally, SR-0379 exhibited antibacterial effects against fungi such as *Candida krusei*. Notably, the antimicrobial spectrum of SR-0379 is broader than the spectra for antibiotics such as chloramphenicol or amphotericin B (Table 2) failed to exhibit an inhibitory activity against fungi or bacteria, respectively. Furthermore, we tested the antibacterial effects of SR-0379 on antibiotic-resistant/sensitive strains for use in clinical applications. Unexpectedly, SR-0379 exhibited similar inhibitory effects on various antibiotic-resistant strains, such as methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *Acinetobacter baumannii*, compared to the sensitive strains (Table 3). As antimicrobial peptide functional mechanisms are related to the disruption of the bacterial membrane [12], SR-0379 may exhibit potent antibacterial effects against other antibiotic-resistant strains.

Cellular functions of SR-0379 and its molecular mechanisms

We investigated the effects of SR-0379 on the proliferation, migration and contraction capacity of neonatal normal human dermal fibroblasts (NHDFs) and examined the angiogenic activity of HUVECs in co-culture with NHDFs. In the proliferation assay, the treatment with SR-0379 (1, 3 and 10 μg/ml) resulted in significant increase in the proliferation of fibroblasts in a dose-dependent manner (Fig. 2A). Whereas Normal Human Epidermal Keratinocytes (NHEKs) were treated with SR-0379 (1, 3 and 10 μg/ml), SR-0379 did not affect cell proliferation (Figure S2 in File S1). Similarly, in the tube formation assay, the treatment with SR-0379 (0.5, 2.5 and 10 μg/ml) significantly induced tube formation of HUVECs in co-culture with NHDFs as angiogenic activity (Fig. 2B). In the migration assay, the treatment with SR-0379 (1 and 10 μg/ml) also resulted in significant increases in migration activity (Fig. 2C). In the fibroblast-collagen-matrix contraction assay, the treatment with SR-0379 (1, 3, 10 and 30 μg/ml) significantly induced contraction as a measure of wound healing activity (Fig. 2D). When compared to the activities of FGF2 (0.3 μg/ml), the contraction activity of SR-0379 (30 μg/ml) was more potent, while the other activities were equivalent or less potent in SR-0379. Interestingly, the treatment with SR-0379 significantly increased the mRNA expression of interleukin-8 (IL-8) which was attenuated with pretreatment of Wortmannin (PI3kinase inhibitor, 100 nM) (Figure S3A in File S1). Similarly, the treatment with SR-0379 increased IL-8 protein expression in a dose dependent manner Figure S3B in File S1).

What are the molecular mechanisms of these actions of SR-0379? The phosphorylation effects of SR-0379 on FAK at Tyr397 and Tyr925 and on Akt at Ser473 were examined in NHDFs. As shown in Fig. 3A, treatment with SR-0379 (10 μg/ml) increased the phosphorylation of FAK Tyr397 and Akt Ser473 but not of FAK Tyr925 after 30 minutes, similar to the well-known antimicrobial peptide LL-37. The treatment with SR-0379 at doses of 0.3 to 10 μg/ml also significantly increased the phosphorylation of FAK Tyr397 and Akt Ser473 (Fig. 3B). The involvement of integrins was also tested in NHDFs. Pretreatment with RGD peptide (30, 100 and 300 μM), a small-molecule integrin antagonist, inhibited the activation of FAK and Akt induced by SR-0379 (Fig. 3C). Wortmannin (100 nM) and Rapamycin (1 nM) also inhibited the activation of Akt induced by SR-0379 (Fig. 3D, 3E). The treatment with SR-0379 (10 μg/ml) resulted in a significant increase in cell proliferation of fibroblast, whereas Akt knockdown using siRNA attenuated SR-0379-induced cell proliferation (Figure 3F and Figure S4A in File S1). Similarly, an inhibitor of Akt by Akt inhibitor IV (1 μM) also attenuated SR-0379-induced cell proliferation (Figure S4B in File S1). These results demonstrated the importance of Akt pathway in the effect of SR-0379.

A

Parent compound(AG30/5C)	MLKLIFLHRL KRMRKRLKRK LRFWHRKRYK
Major metabolites	MLKLIFLHRL KRMRKRLKRK (SR-0007)
	MLKLIFLHRL KRMRKRLK
	MLKLIFLHRL KRMRKRL
	LHRL KRMRKRLK

B

AG30/5C	MLKLIFLHRL KRMRKRLKRK LRLWHRKRYK	(Net charge= 17)
SR-0007	MLKLIFLHRL KRMRKRLKRK	(Net charge= 11)
SR-0379	MLKLIFLHRL KRMRKRL DLys RK	(Net charge= 11)

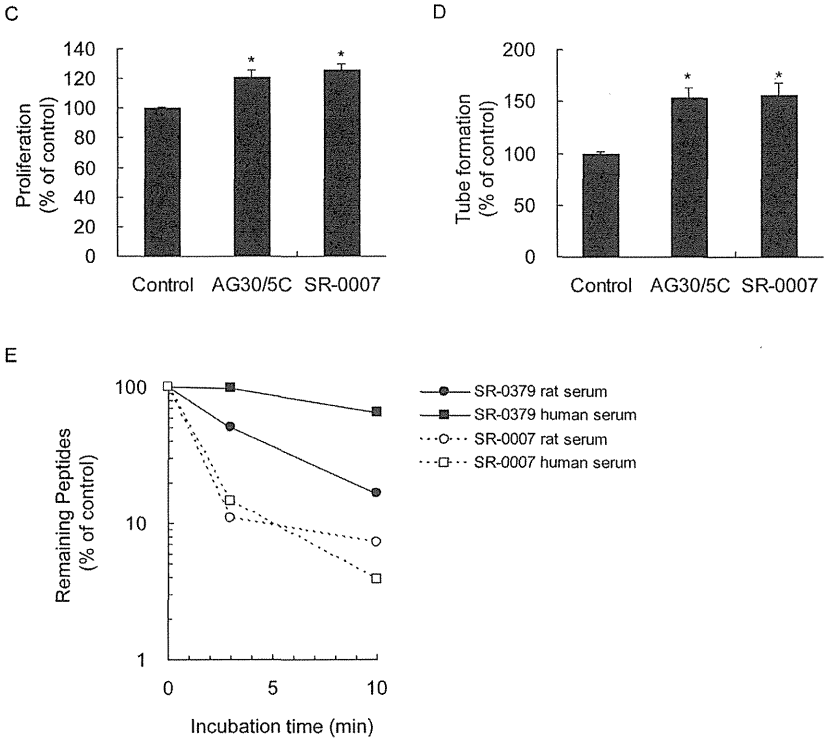

Figure 1. Lead optimization from the angiogenic peptide AG30/5C. A) Major metabolites of AG30/5C determined by MALDI-TOF MS. The parent compound (AG30/5C) was incubated with rat serum *in vitro* 60 minutes. The metabolites were identified by comparison with the pre-incubation peptide. B) Sequences and net charges of AG30/5C and AG30/5C-derived peptides (SR-0007 and SR-0379). The lysine (K) of SR-007 was replaced with D-lysine in SR-0379. C) Effect of AG30/5C (10 μg/ml) and SR-0007 (10 μg/ml) on HUVECs proliferation. N = 3 per group. *P<0.05 vs. control. D) Effect of AG30/5C (10 μg/ml) and SR-0007 (10 μg/ml) on tube formation. The formation of capillary-like structures was observed in co-cultures of HUVECs and NHDFs. N = 5-12 per group. *P<0.05 vs. control. E) Stability of SR-0007 and SR-0379 in rat and human sera. SR-0007 and SR-0379 were quantified before or after incubation *in vitro* with rat and human sera for either 3 or 10 minutes. N = 2.
doi:10.1371/journal.pone.0092597.g001

Acceleration of wound healing by SR-0379 *in vivo*

To evaluate the potential use of SR-0379 in clinical practice, a full-thickness wound model with a skin flap was employed in a streptozotocin-induced diabetic rat model. On day 2, the wound area was quickly and significantly reduced in the SR-0379 (0.2 mg/ml) group but not in the saline and FGF2 groups (Fig. 4A, 4B). As shown in Fig. 4A, the color of the wound surface on day 6 was red (bloody) in the SR-0379 treatment group. In contrast, the color of the wound surface was still dark with necrotic skin visible in both the saline and FGF2 groups. Reduced wound area in the SR-0379 group was sustained on days 6 and 13 (Fig. 4B). The skin wound was completely healed at day 19 with SR-0379 treatment, whereas the wound did not heal until day 24 with saline treatment (Fig. 4C).

To determine the utility of SR-0379 in a clinical situation, we finally evaluated whether the compound could accelerate wound healing in an acute infection wound model, as the presence of infection largely diminishes the wound-healing process. After creating full-thickness defects, *S. aureus* was inoculated. Treatment with SR-0379 (1 mg/ml) significantly reduced the unhealed wound size on days 8 and 15 compared to the saline and FGF2 groups (Fig. 5A, 5B). The effects of SR-0379 on wound healing were more potent than the effects of FGF2, which is currently a standard therapy (Fig. 4B, 5B). To determine the effect of SR-0379 on wound healing, granulation tissue formation was analyzed by subcutaneously implanting a paper disc (Fig. 5C). Granulation tissue weight was significantly increased by treatment with SR-0379 (100 μg/disc). To further evaluate the effect of SR-0379 on

Table 1. MICs of several compounds against *E. coli*, *P. aeruginosa* and *S. aureus*.

Compound	*E. coli* (ATCC25922)	*P. aeruginosa* (ATCC27853)	*S. aureus* (ATCC29213)
	MIC (μg/ml)		
AG30/5C	32	16/32	16/32
SR-0007	64	16/32	16/32
SR-0379	8	16/32	16
Tobramycin	0.25–1	0.25–1	0.12–1
Meropenem	0.008–0.06	0.25–1	0.03–0.12
Oxacillin	-	-	0.12–0.5

The scores indicate the MICs (mg/ml) for *E. coli*, *P. aeruginosa and S. aureus*. MICs represent the individual data from two independent experiments.
doi:10.1371/journal.pone.0092597.t001

wound healing, collagen production and proliferation were measured using the incised wound rat model. The tensile strength after SR-0379 treatment (5 μg) was significantly increased compared to saline treatment. Treatment with SR-0379 induced granulation tissue formation and collagen production, which may accelerate wound healing.

Collagen gel assay is a conventional method to investigate cell behavior that more closely resembles cell behavior *in vivo*. As shown in Figure 2D, the diameter of the collagen gel containing went down by treatment of SR-0379. The promotion of the wound healing with SR-0379 was supported by the enhanced contraction *in vitro*.

Discussion

Recently, wet dressing has been strongly recommended to accelerate the process of wound healing because epithelial cells and dermal fibroblasts proliferate well under wet conditions [13]. However, wounds under wet conditions present an ideal environment for bacterial growth due to the presence of moisture and warmth. Wound infection has become one of the major risk factors in the delay of wound healing [14], although physicians take many precautions to prevent wound infection. Wound colonization is defined as the presence of multiplying microorganisms on the surface of a wound but with no associated clinical signs and symptoms of infection [15]. Recently, the term critical colonization has gained acceptance and is defined as the borderline of colonization and infection. As wound colonization is most frequently polymicrobial and involves numerous microorganisms that are potentially pathogenic, the diagnosis of clinical colonization is difficult for general physicians in a clinical setting. Recombinant growth factor proteins, such as FGF2 and PDGF, have been shown to stimulate ulcer-healing processes in clinical applications [16,17]. However, the wound might fail to be healed by these growth factors in the event of an infection, as these growth factors have no antibacterial properties. Therefore, a wound-healing drug with antibacterial properties would be ideal to avoid the risk of infection during wound care.

Table 2. *In vitro* activities of SR-0379 against Gram-positive and Gram-negative bacteria and fungi.

		Gram staining	Strain	MIC (μg/ml)	
				SR-0379	Chloramphenicol
Bacteria	Aerobes	G(+)Bacilli	*Micrococcus luteus*	2	1
			Bacillus subtilis	2	4
		G(-)Bacilli	*Salmonella* Enteritidis	8	4
			Salmonella Typhimurium	8	4
			Acinetobacter baumannii	8	16/32
	Anaerobes	G(-)Bacilli	*Propionibacterium acnes*	16/32	NT
			Bacteroides fragilis	32	1
			Fusobacterium nucleatum	16/32	0.25
Fungi			Strain	SR-0379	Amphotericin B
			Penicillium glabrum	8/16	0.125
			Fusarium solani	8	1
			Alternaria alternata	32	1
			Trichophyton mentagrophytes	32	0.125
			Trichophyton rubrum	64	0.125
			Candida krusei	32	2

The scores indicate the MICs (mg/ml) for gram-positive and gram-negative bacteria and fungi. MICs represent the individual data from two independent experiments. NT: Not tested.
doi:10.1371/journal.pone.0092597.t002

Table 3. *In vitro* activities of SR-0379 against seven strains of drug-resistant bacteria.

Bacteria	Drug resistance	MIC (μg/ml)
Pseudomonas aeruginosa	Aminoglycoside-resistant	16
	Carbapenem-resistant	16–64
	Fluoroquinolone-resistant	16/64
Staphylococcus aureus	Methicillin-sensitive	32
	Methicillin-resistant (1)	32
	Methicillin-resistant (2)	32
Acinetobacter baumannii	Multidrug-resistant	16

MICs represent the individual data from two independent experiments.
doi:10.1371/journal.pone.0092597.t003

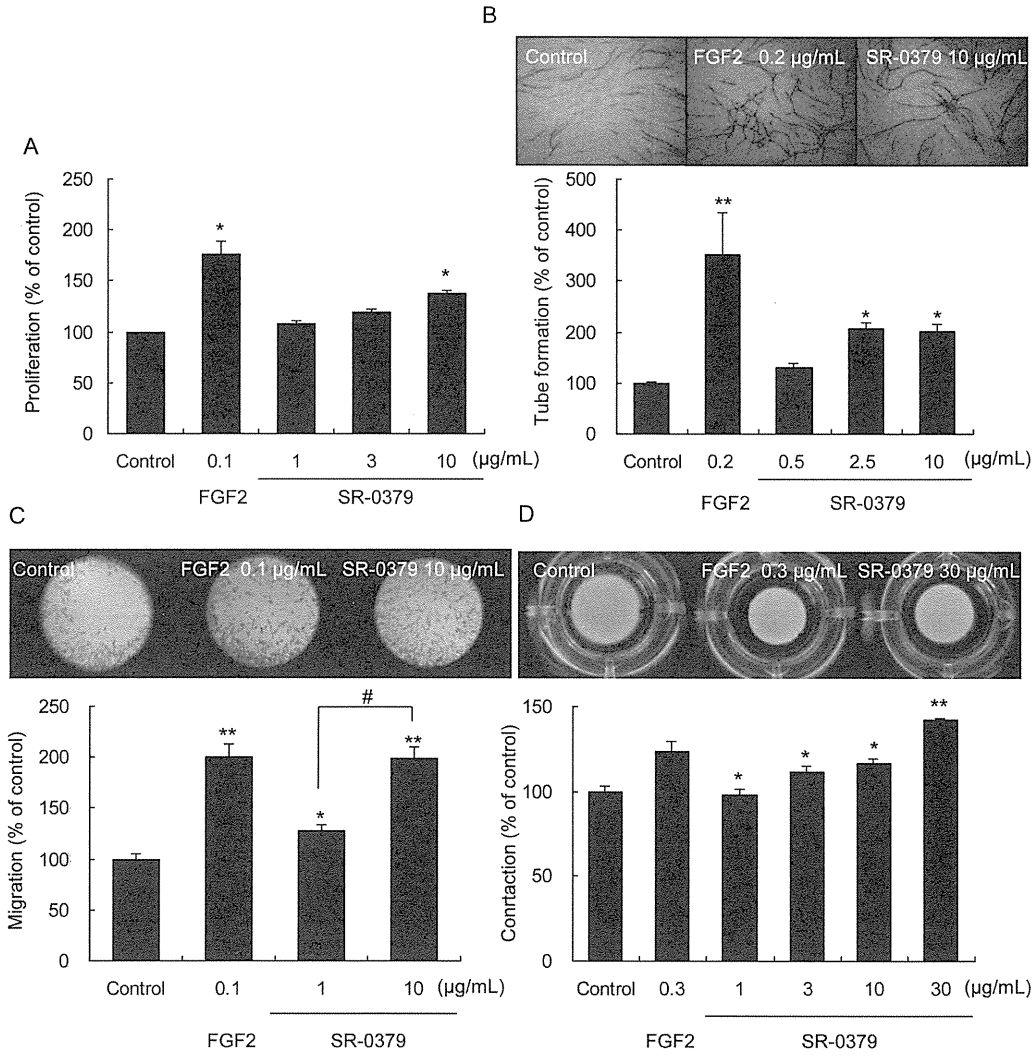

Figure 2. Cellular function of SR-0379. A) Effect of SR-0379 on NHDFs proliferation. NHDFs were treated with SR-0379 (1, 3 and 10 μg/ml) or FGF2 (0.1 μg/ml). N = 4 per group. *P<0.05 vs. control. B) The upper panel shows representative pictures of tube formation in a co-culture of HUVECs and NHDFs (Control, FGF2: 0.2 μg/ml) and SR-0379 (10 μg/ml). The lower panel shows the effects of SR-0379 on tube formation in a co-culture of HUVECs and NHDFs. N = 5 per group. *P<0.05, **P<0.01 vs. control. C) The upper panel shows representative pictures of the migration induced by FGF2 (0.1 μg/ml) and SR-0379 (10 μg/ml). The lower panel shows the effects of FGF2 (0.1 μg/ml) and SR-0379 (1 and 10 μg/ml) on migration. N = 4 per group. *P<0.05, **P<0.01 vs. control, #P<0.01 vs. SR-0379 (1 μg/ml). D) The upper panel shows representative pictures of the fibroblast-collagen-matrix contraction assay with FGF2 (0.3 μg/ml) and SR-0379 (1, 3, 10 and 30 μg/ml). The lower panel shows the effects of FGF2 (0.3 μg/ml) and SR-0379 (1, 3, 10 and 30 μg/ml) on fibroblast-collagen matrix contraction. N = 3 per group. *P<0.05, **P<0.01 vs. control.
doi:10.1371/journal.pone.0092597.g002

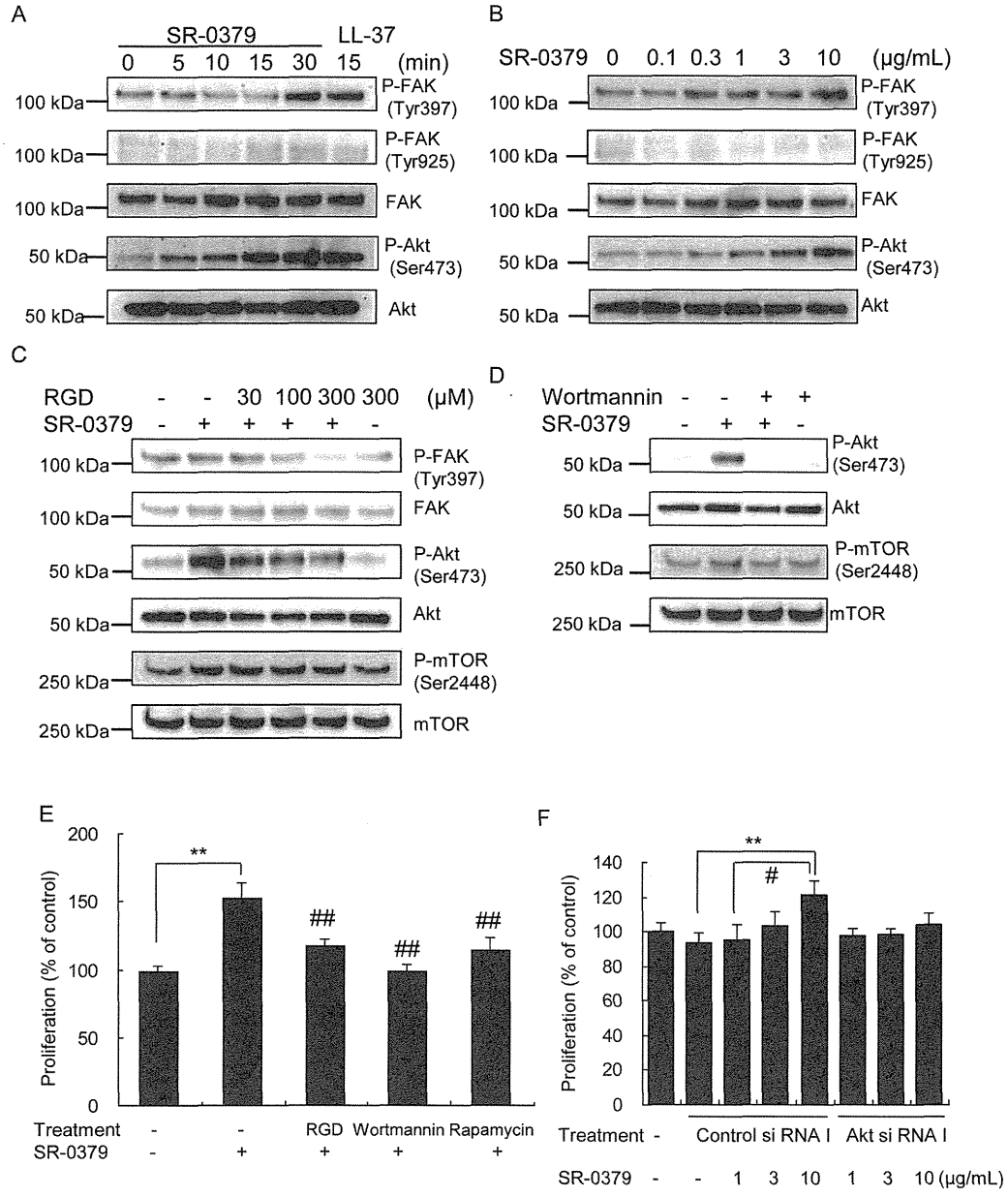

Figure 3. Activation of the PI3 kinase/AKT/mTOR pathway by SR-0379 in NHDFs. A) Effects of SR-0379 on phosphorylated FAK (Tyr397 and Tyr925) and phosphorylated Akt (Ser473) as determined by Western blot. The cells were treated with SR-0379 (10 μg/ml) for 0, 5, 15 and 30 minutes or LL-37 (10 μg/ml) for 15 minutes. B) Effects of SR-0379 on phosphorylated FAK (Tyr397 and Tyr925) and phosphorylated Akt (Ser473) as determined by Western blot. The cells were treated with SR-0379 (0.1, 0.3, 1, 3 and 10 μg/ml) for 30 minutes. C, D) Effects of RGD peptide and wortmannin on the SR-0379-induced phosphorylation of FAK (Tyr397 and Tyr925) and Akt (Ser473) as determined by Western blot. The cells were preincubated with RGD peptide (30, 100 and 300 μM, an inhibitor of integrin-ligand interactions) (C) or wortmannin (100 nM) (D) for 30 minutes and were then treated with SR-0379 (10 μg/ml) for 30 minutes. E) Effects of RGD, wortmannin and rapamycin on the NHDFs proliferation stimulated by SR-0379. The cells were preincubated with RGD (1000 μM), wortmannin (100 nM) or rapamycin (1 nM) for 2 hours and then were treated with SR-0379 (10 μg/ml). N = 3 per group. **P<0.01 vs. control, ## P<0.01 vs. SR-0379 (10 μg/ml). F) Effects of Akt knockdown using siRNA on the NHDFs proliferation stimulated by SR-0379. The cells were pretreated with Akt si RNA or Control si RNA for 24 hours and then were treated with SR-0379 (1, 3 and 10 μg/ml). N = 4 per group. **P<0.01 vs. control si RNA, # P<0.05 vs. SR-0379 (1 μg/ml) treated with control siRNA.
doi:10.1371/journal.pone.0092597.g003

Based on this idea, we developed AG30, an antibacterial peptide with angiogenic activity. Although our previous study demonstrated that AG30/5C is useful to in the treatment of ulcers [8,9], cost and stability are problems for clinical applications. Therefore, in this study, we modified AG30/5C as a lead compound to achieve 1) more stability upon exposure to serum, 2) lower cost, 3) wider antibacterial activity and 4) rapid wound healing. The initial analysis using the metabolites of AG30/5C revealed that a core sequence of 20 aa (MLKLIFLHRLKRMRKRLKRK; SR-0007) was enough to

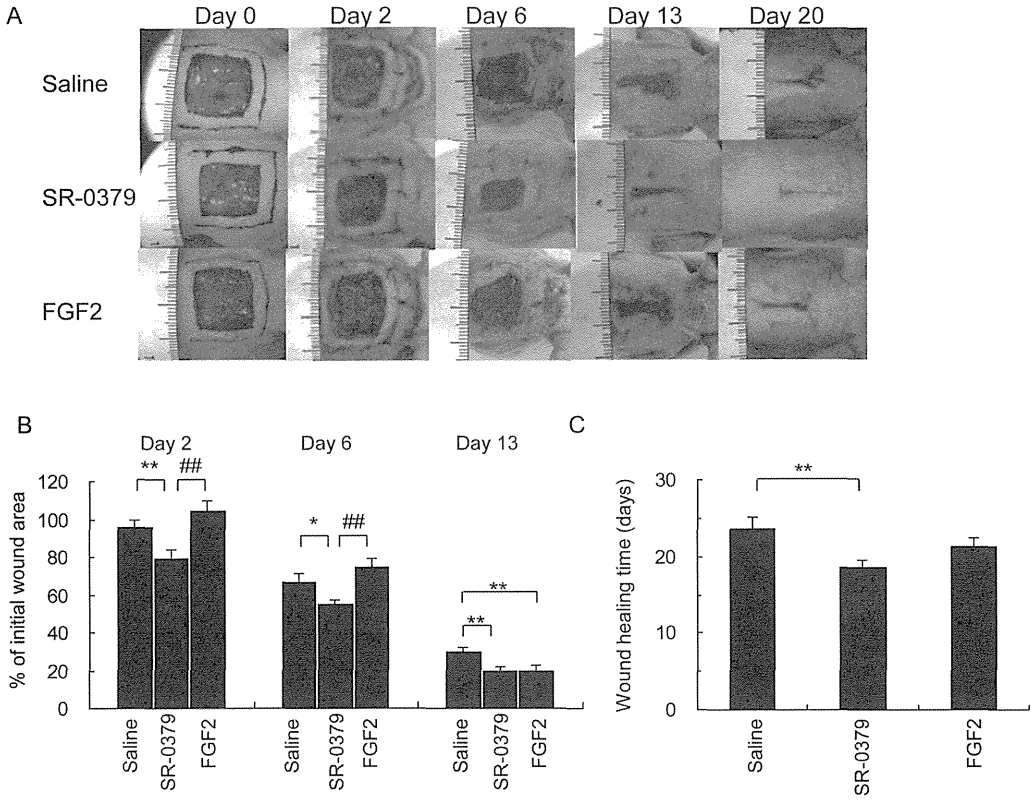

Figure 4. Effects of SR-0379 and FGF2 on full-thickness wound model with flap in diabetic rat model. A) Representative pictures of skin flaps in the streptozotocin-induced diabetic model in the saline (control), SR-0379 (0.2 mg/ml) and FGF2 groups (0.06 mg/ml) on days 0, 6, 13 and 20. B) Quantification of the wound area is represented as a percentage of the initial wound area. N = 6 per group. **P<0.01 vs. control, ##P<0.01 vs. FGF2. C) Days to complete healing by the contraction of full-thickness skin flaps in the streptozotocin-induced diabetic model.
doi:10.1371/journal.pone.0092597.g004

stimulate human umbilical vein endothelial cells (HUVECs) proliferation and tube formation at a level similar to AG30/5C. This smaller peptide led to a reduced cost of peptide synthesis. In the previous reports, the replacement with the D-form amino acid improved the proteolytic resistance of antimicrobial peptides [18]. For example, the D-amino acid variants of host defense peptide chicken cathelicidin-2 showed enhanced stability in human serum, and fully resistant to proteolysis by trypsin and bacterial proteases. The modifications increase the stability and lower cytotoxicity of the peptides without altering their antimicrobial potency. We also confirmed the degradation by the peptide bond cleavages in N-terminus of SR-0379, and the change from L-lysine to D-lysine (SR-0379) increased the resistance to serum. Importantly, SR-0379 displayed broader antibacterial activity than the original AG30 and SR-0007. The bactericidal action of antimicrobial peptides such as pexiganan is thought to result from irreversible membrane-disruptive damage [19] [20] [21]. Especially, from the mechanisms of antibacterial activity, SR-0379 exhibited the same MIC against drug-resistant strains, such as aminoglycoside-, carbapenem- and fluoroquinolone-resistant *P. aeruginosa* and MRSA and the multidrug-resistant *A. baumannii*. SR-0379 might be useful to prevent infection by these drug-resistant bacteria.

In vitro experiments with SR-0379 demonstrated the induction of proliferation, tube formation, migration and contraction. The closure of cutaneous wounds involves three processes: epithelization, connective-tissue deposition and contraction. In particular, contraction is one of the main factors contributing to epidermal wound healing [22]. The fibroblast-collagen matrix contraction model provides a unique way to study mechanisms. Treatment with SR-0379 promoted contraction in this model, which corresponds to wound healing. The stimulatory effect of SR-0379 on the wound healing process was also confirmed by two *in vivo* wound-healing models. Furthermore, SR-0379 was able to induce angiogenesis and granulation tissue formation in the paper disc model and collagen production and proliferation in the incised wound rat model. These results support the potential use of SR-0379 in the wound-healing process. The ulcer model with infection is a unique model that is especially close to a clinical situation. Importantly, SR-0379 treatment resulted in rapid healing without infection compared to FGF2.

Although the multiple functions of antimicrobial peptides are well known, the mechanisms are still unclear. For example, LL-37 is often reported in the analysis of FPR2 (formerly known as FRPL1), the promiscuous Pertussis Toxin (PTX)-sensitive GPCR and the purinergic receptor P2X7 and in the transactivation of epidermal growth factor receptor (EGFR) [3]. The activation of EGFR in epithelial cells, endothelial cells and fibroblasts by LL-37 resulted in activation of the p38 MAPK, ERK1/2 MAPK, NFkB and PI3 kinase pathways. In contrast, although we also examined the contribution of P2X7 receptors to the effect of SR-0379, the specific antagonist of P2X7 (Brilliant Blue G) failed to inhibit the effects of SR-0379 (data not shown). SR-0379 also weakly activated EGFR. Interestingly, SR-0379 strongly activated FAK, while an integrin inhibitor (RGD peptide) blocked the Akt/

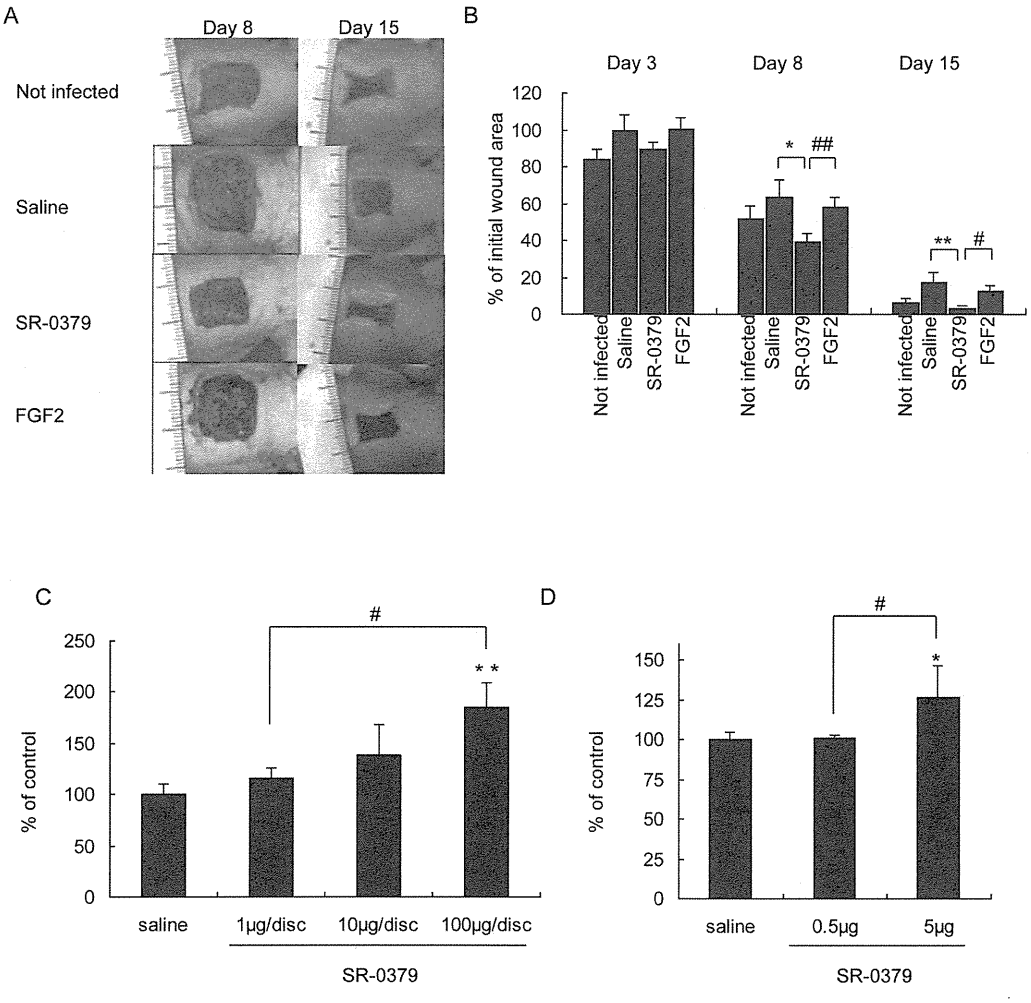

Figure 5. Effects of SR-0379 and FGF2 on the full-thickness skin infected wound model. A) Representative pictures of full-thickness skin flaps in uninfected, saline (control), SR-0379 (1 mg/ml) and FGF2 groups (0.125 mg/ml) on days 8 and 15. B) Quantification of the infected wound area is represented as a percentage of the initial wound area. N = 5 per group. **P<0.01 vs. control, ##P<0.01 vs. FGF2. C) Effects of SR-0379 (1, 10 and 100 μg/disc) on the healing of paper disc implantation in rats. N = 4-5 per group. **P<0.01 vs. control, #P<0.05 vs. 1 μg/disc. D) Effects of SR-0379 (0.5 and 5 μg) on the healing of an experimental open wound in rats. N = 3-4 per group. *P<0.05 vs. control, #P<0.05 vs. 0.5 μg.
doi:10.1371/journal.pone.0092597.g005

mTOR pathway. Downstream of FAK, SR-0379 also activated the PI3 kinase-Akt-mTOR pathway. As mTOR is known to regulate cell growth and survival by integrating nutrient and hormonal signals [10], an inhibitor, rapamycin, attenuated the proliferation induced by SR-0379 in human fibroblasts. The treatment of SR-0379 resulted in increase in cell proliferation of fibroblast, whereas Akt knockdown attenuated the SR-0379-induced cell proliferation These results demonstrate the importance of Akt pathway in the effect of SR-0379.

We have successfully produced SR-0379 as a multifunctional (angiogenic and pro-fibrotic), potent antibacterial peptide with a broad spectrum, including aerobes and anaerobes, Gram-positive and Gram-negative species and drug-resistant and drug-sensitive bacteria and fungi. These properties occur via the activation of PI3 kinase-Akt-mTOR signaling and are useful in the stimulation of wound healing under wet conditions. Further modification of SR-0379 should yield an ideal compound for the treatment of diabetic ulcers, burns and other incurable ulcers. Currently, we plan to test SR-0379 in the treatment of patients with MRSA-positive diabetic and ischemic ulcers.

Materials and Methods

Analysis of the AG30/5C metabolites using MALDI-TOF/MS

Rat sera were collected from rats. AG30/5C was incubated in pooled rat serum at 37°C. Samples were collected before incubation, after 10 minutes of incubation and after 60 minutes of incubation and were precipitated by the addition of an equivalent amount of acetonitrile containing 0.1% trifluoroacetate. The samples were centrifuged, and the supernatants were purified using ZipTip m-C18 (Millipore, MA). Sample solution was mixed with matrix solution (α-cyano 4-hydroxy cinnamic acid). The measurement sample for MALDI (0.4 μL) was applied on a MALDI target plate and dried, and the sequences of the AG30/5C metabolites were confirmed by MALDI-TOF/MS analysis (4700 Proteomics Analyzer, Applied Biosystems, CA).

Serum stability assay

In vitro stability studies were performed by incubating the peptide with rat or human serum. Human sera (Pool of donors, 5 men and 5 women) were commercially purchased from KAC (Kyoto, Japan), which has been permitted only for experiment. We don't use the human biological specimens without the documented informed consent. Rat sera were collected from rats. The peptide (500 μg/ml) was added to serum (300 μL) and incubated at 37°C. A part of samples (90 μL) were taken, and the proteins were precipitated with acetonitrile containing 0.1% trifluoroacetate (200 μL). The precipitate was separated by centrifugation. The supernatants were analyzed by high-performance liquid chromatography (HPLC).

Proliferation, tube formation, cell migration and contraction assays

HUVECs, NHDFs and NHEKs were purchased from Kurabo (Osaka, Japan). The endothelial cells were maintained in HuMedia EB2 and the fibroblasts were maintained in Medium 106S. Both media were supplemented with 1% fetal bovine serum (FBS) as described previously [9]. The epidermal keratinocytes were maintained in HuMedia KB2. Cells were incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 with exchange of medium every 2 days. HUVECs were cultured in 96-well plates at a density of 10,000 cells/well and incubated for 48 hours at 37°C with AG30/5C or FGF2 (recombinant human FGF basic, R&D systems, Inc., Minneapolis, MN). The proliferation of HUVECs, NHDFs and NHEKs was analyzed using a WST-1 assay (Dojindo, Kumamoto, Japan). Tubule formation assay has recently been developed in which endothelial cells are co-cultured with fibroblasts. An angiogenesis assay kit (Kurabo, Osaka Japan) was used according to the manufacturer's instructions. Various concentrations of peptides or FGF2 were added to the medium. After 11 days, the cells were incubated with diluted primary antibody (mouse anti-human CD31, 1:4,000) for 1 hour at 37°C and diluted secondary antibody (goat anti-mouse IgG alkaline phosphatase-conjugated antibody, 1:500) for 1 hour at 37°C; visualization was achieved with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT). The tube-like structures were measured in terms of total tube length with the software (Angiogenesis Image Analyzer, Kurabo, Osaka Japan). Cell migration was evaluated using an Oris cell migration assay kit (Platypus Technologies, LLC., Madison, WI) according to the manufacturer's instructions. Briefly, the assay utilizes cell-seeding stoppers to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2-mm diameter unseeded region, the migration zone, into which the seeded cells migrate. The number of cells that migrated into the detection zone was measured using a plate reader. Cellular collagen gel contraction assays were performed as previously described [23] [24]. A solution of collagen and NHDFs (2×10^6 cells/ml) was added to a 24-well plate at 37°C for 1 hour, and medium supplemented with DMEM containing 10% FBS was then added. The cells were cultured for 24 hours. The culture medium was removed, and DMEM (serum-free) containing SR-0379 or FGF2 was added. The cell-embedded matrix was released from the culture dish surface. At each time point, the lattices were digitally photographed from a fixed distance, and their areas were calculated using image analysis software. In the proliferation assay of fibroblast, RGD peptide, Wotmannin, Akt inhibitor IV and Genistein were obtained from Sigma-Aldrich (St. Louis, MO). Rapamycin was obtained from Funakoshi Co., Ltd.(Tokyo, Japan). Akt siRNA I (#6211) and control siRNA I (#6568) were obtain from Cell Signaling (Boston, MA).

NHDFs were plated at a density of 5000 cells per well in 96-well culture plates in the corresponding culture media without antibiotics one day prior to transfection. Lipofectamine RNAi-MAX was purchased from Invitrogen. The lipofectamin (2 μL) was gently added to 100 μL medium and the mixture was incubated for 20 minutes at room temperature. The Akt or Control siRNA was added to the mixture and was incubated for 5 minutes. Transfection complexes were added to each well. NHDFs were incubated for 24 hours at 37°C in a CO_2 incubator and then SR-0379 (10 μg/ml) was added. NHDFs proliferation was analyzed using a WST-1 assay.

Measurement of MICs against Bacteria and Fungi

Antimicrobial activity of the peptides was evaluated against *Escherichia coli* JCM 5491, *Pseudomonas aeruginosa* JCM 6119, *Staphylococcus aureus* JCM2874, *Salmonella* Typhimurium JCM1652, *Acinetobacter baumannii* JCM6841, *Bacteroides fragilis* JCM11019, *Fusobacterium nucleatum* JCM11025, *Penicillium glabrum* JCM22534, *Fusarium solani* JCM11383, *Alternaria alternata* JCM5800 (RIKEN, A research institution for basic and applied science in Japan), *Micrococcus luteus* NBRC13867, *Bacillus subtilis* NBRC3134, *Propionibacterium acnes* NBRC107605, *Trichophyton mentagrophytes* NBRC6124, *Trichophyton rubrum* NBRC9185, *Candida krusei* NBRC1395 (National Institute of Technology and Evaluation, Tokyo, Japan), *Salmonella* Enteritidis IID604 (The Institute of Medical Science, The University of Tokyo, Tokyo, Japan). Additionally, the clinical isolates (Drug-sensitive/resistant *Pseudomonas aeruginosa* and *Staphylococcus aureus*, Osaka University Hospital) and multidrug-resistant *Acinetobacter baumannii* (ATCC BAA-1605) were used. The MICs (expressed as μg/ml) of AG30/5C, SR-0007 and SR-0379 were determined by the broth microdilution method as previously described [8,9]. Serial two-fold dilutions of peptide were added to 0.1 ml of medium containing each type of bacteria and fungi at concentrations of 0.4×10^4 – 5×10^4 CFU/ml. The plates were incubated at 37°C with vigorous shaking for 24 or 48 hours. The MICs were determined as the lowest concentrations of peptide that inhibited visible bacterial growth.

Western blot analysis

Protein extracts (15 μg) were resolved by 10% SDS-PAGE and were then transferred to nitrocellulose membrane. Western blotting was performed. Phospho FAK (Tyr397), Akt, phospho Akt (Ser 473), mTOR, phospho mTOR (Ser2448) and α-Tubulin antibodies were obtained from Cell Signaling (Boston, MA). FAK antibody was obtained from Millipore (Billerica, MA). Phospho FAK (Tyr925) antibody was obtained from Abcam (Cambridge, MA).

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis and ELISA

Expression of the human IL-8 mRNA was measured using real-time reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from the tissue samples using ISOGEN reagent (NIPPON GENE, Toyama, Japan). Complementary DNA (cDNA) was synthesized using the Thermo Script RT-PCR System (Invitrogen, Carlsbad, CA). Relative gene-copy numbers for IL-8 mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by real-time RT-PCR using TaqMan Gene Expression Assays (IL-8: Hs00174103_m1; GAPDH: 4352934). Absolute gene-copy numbers were normalized to GAPDH using a standard curve.

The cell free culture supernatants were harvested after treatment of SR-0379 (1, 3 and 10 μg/ml) at 24, 48 and 72 hours.

The amount of IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Effect of SR-0379 on wound healing in a streptozotocin-induced diabetic model

This experimental protocol was approved by the committee for ethics in animal studies of AnGes MG. Male HWY/Slc rats (7 weeks) were given a single intravenous injection of 65 mg/kg streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO), and whole-blood glucose was monitored 24 hours later. This strain is hair less in adult and suitable for wound healing model. The glucose level criterion for diabetes was set at 300 mg/dl. STZ-induced diabetic rats were anesthetized. The square flap (1.73 cm × 1.73 cm) was made in the back of rats. In the center of the flap, the square wound (1.41 cm × 1.41 cm) with full-thickness defect was made (area per wound; 2 cm^2). In the flap model, skin was cut in three directions of square wound to partially block the blood flow to wound. SR-0379 (0.2 mg/ml, 50 μl), FGF2 (0.06 mg/ml, 50 μl) or saline (control) was administered to each wound (each time point from day 0 to 28). Dressings (Perme-roll, Nitto Denko, Japan) were applied to the wounds. We took a picture of wound with scale every time and calculated the size of scanned image using software (http://hp.vector.co.jp/authors/VA004392/Download.htm#lenara).

Effect of SR-0379 on wound healing in a cyclophosphamide-induced immunodeficient infection model

Male HWY/Slc rats (7 weeks) were given a single intravenous injection of 100 mg/kg cyclophosphamide (CPA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were anesthetized for the preparation of a full-thickness skin flap 24 hours later. CPA-treated rats with white blood cell counts lower than 5,000 were used. The bacteria (*S. aureus*, 1×10^5 CFU/ml) was applied to each wound on days 0, 1, 2 and 3. SR-0379 (1 mg/ml, 50 μl), FGF2 (125 μg/ml, 50 μl) and saline (control, 50 μl) were administered to the wound at time points on days 0 to 27. Dressings (Perme-roll, Nitto Denko, Japan) were applied to the wounds. Healing size was evaluated by photographing the wound area at a close and fixed distance. The remaining unhealed wound size was measured from the image.

Evaluation of granulation tissue formation in a paper disc implantation model

Granulation tissue formation was determined as described previously [25]. A paper disc containing saline or SR-0379 (1, 10 and 100 μg) was implanted into the subcutaneous tissue on the backs of 9-week-old Crl:CD(SD) rats under anesthesia. Four or five rats were used for each experimental condition. The paper disc was removed on day 8 and the granulation tissue around the paper disc was weighed after the removal of absorbed fluids with paper wipe.

Evaluation of collagen production and proliferation in the incised wound rat model

The dermises of Crl:CD(SD) rats (7 weeks) were incised under anesthesia. In the back of rats, we cut the skin (30 mm) and sutured 3 points (Nylon thread, Natsume Seisakusho Co., Ltd., Tokyo, Japan). SR-0379 (0.5 and 5 μg per day) was topically administered in sutured wound one a day for 5 days, and during the period the suture was removed at day 3. At day 6, extracted skin was fixed in one side and pulled in another side. The tension was monitored until the opening of sutured wound. In this evaluation, the increase in tension reflects the strength of sutured wound.

Statistical analysis

All values are expressed as the means + SEM. Analysis of variance and a subsequent Fisher's Least Significant Difference test were used to determine the significance of differences in multiple comparisons.

Supporting Information

File S1 Supporting figures S1–S4. Figure S1, MALDI-TOF MS analysis. A) Major metabolites of AG30/5C determined by MALDI-TOF MS. Parent compound (AG30/5C) was incubated with rat serum *in vitro* for 10 minutes and 60 minutes. The metabolites were identified by the comparison with that from pre-incubation. Figure S2, Effect of SR-0379 on cell proliferation. Normal Human Epidermal Keratinocytes (NHEKs) were treated with SR-0379 (1, 3 and 10 μg/ml). The results were shown as percent increase compared with control (no treatment). N = 3 per group. *P<0.05 vs. control. Figure S3, Effect of Akt pathway on SR-0379-induced cell proliferation. A) Knockdown of Akt expression by siRNA was confirmed with western blot analysis anti-Akt antibody and anti-α-tubulin antibody. The sample was extracted from NHDFs with no treatment (NT), non-target siRNA (C: control) and Akt siRNA. B) Effects of Akt inhibitor on NHDFs proliferation stimulated by SR-0379. The cells were preincubated with Akt inhibitor IV (1 μM) for 1 hour and then were treated with SR-0379 (1, 3 and 10 μg/ml). N = 3 per group. *P<0.05 vs. control, **P<0.01 vs. control, ## P<0.01 vs. SR-0379 (1 μg/ml), †† P<0.01 vs. SR-0379 (3 μg/ml), ‡‡ P<0.01 vs. SR-0379 (10 μg/ml). Figure S4, Up-regulation of interleukin-8 (IL-8) induced by treatment of SR-0379. A) IL-8 mRNA expression was quantified by real time PCR and shown as a relative expression compared with that of GAPDH mRNA. NHDFs were treated with SR-0379 (10 μg/ml) for 24 hours. Effects of Wortmannin (PI3kinase inhibitor, 100 nM) and Genistein (Tyrosine-specific protein kinase inhibitor, 100 nM) on SR-0379-induced IL-8 mRNA expression. N = 3 per group. *P<0.05 vs. control, **P<0.01 vs. control, ## P<0.01 vs. SR-0379 (no inhibitor). B) IL-8 levels in culture supernatants form NHDF was measured by ELISA at 24, 48 and 72 hours after treatment. NHDFs were treated with SR-0379 (1, 3 and 10 μg/ml) for 72 hours. N = 2.
(PDF)

Acknowledgments

We thank Ms. Ryoko Sata and Mr. Shintaro Komaba for their technical assistance.

Author Contributions

Conceived and designed the experiments: HN KT YK RM. Performed the experiments: HT HN AT YS T. Kanamori T. Kaga NT. Analyzed the data: HT AT T. Kanamori. Contributed reagents/materials/analysis tools: HT AT T. Kaga. Wrote the paper: HT HN.

References

1. Ganz T, Selsted ME, Szklarek D, Harwig SS, Daher K, et al. (1985) Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 76: 1427–1435.
2. Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res. England. pp. D933–937.
3. Vandamme D, Landuyt B, Luyten W, Schoofs L (2012) A comprehensive summary of LL-37, the factotum human cathelicidin peptide. Cell Immunol 280: 22–35.
4. Gordon YJ, Romanowski EG, McDermott AM (2005) A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. Curr Eye Res. England. pp. 505–515.
5. Hancock RE, Sahl HG (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. United States. pp. 1551–1557.
6. Fox MA, Thwaite JE, Ulaeto DO, Atkins TP, Atkins HS (2012) Design and characterization of novel hybrid antimicrobial peptides based on cecropin A, LL-37 and magainin II. Peptides. United States: Crown 2012. Published by Elsevier Inc. pp. 197–205.
7. Won A, Khan M, Gustin S, Akpawu A, Seebun D, et al. (2011) Investigating the effects of L- to D-amino acid substitution and deamidation on the activity and membrane interactions of antimicrobial peptide anoplin. Biochim Biophys Acta. Netherlands: 2010 Elsevier B.V. pp. 1592–1600.
8. Nishikawa T, Nakagami H, Maeda A, Morishita R, Miyazaki N, et al. (2009) Development of a novel antimicrobial peptide, AG-30, with angiogenic properties. J Cell Mol Med. England. pp. 535–546.
9. Nakagami H, Nishikawa T, Tamura N, Maeda A, Hibino H, et al. (2012) Modification of a novel angiogenic peptide, AG30, for the development of novel therapeutic agents. J Cell Mol Med 16: 1629–1639.
10. Squarize CH, Castilho RM, Bugge TH, Gutkind JS (2010) Accelerated wound healing by mTOR activation in genetically defined mouse models. PLoS One 5: e10643.
11. Ge Y, MacDonald DL, Holroyd KJ, Thornsberry C, Wexler H, et al. (1999) In vitro antibacterial properties of pexiganan, an analog of magainin. Antimicrob Agents Chemother 43: 782–788.
12. Gottler LM, Ramamoorthy A (2009) Structure, membrane orientation, mechanism, and function of pexiganan–a highly potent antimicrobial peptide designed from magainin. Biochim Biophys Acta. Netherlands. pp. 1680–1686.
13. Dyson M, Young S, Pendle CL, Webster DF, Lang SM (1988) Comparison of the effects of moist and dry conditions on dermal repair. J Invest Dermatol 91: 434–439.
14. Woo KY, Coutts PM, Sibbald RG (2012) A randomized controlled trial to evaluate an antimicrobial dressing with silver alginate powder for the management of chronic wounds exhibiting signs of critical colonization. Adv Skin Wound Care. United States. pp. 503–508.
15. Edwards R, Harding KG (2004) Bacteria and wound healing. Curr Opin Infect Dis. United States. pp. 91–96.
16. Akita S, Akino K, Imaizumi T, Tanaka K, Anraku K, et al. (2006) The quality of pediatric burn scars is improved by early administration of basic fibroblast growth factor. J Burn Care Res. United States. pp. 333–338.
17. Wieman TJ, Smiell JM, Su Y (1998) Efficacy and safety of a topical gel formulation of recombinant human platelet-derived growth factor-BB (becaplermin) in patients with chronic neuropathic diabetic ulcers. A phase III randomized placebo-controlled double-blind study. Diabetes Care 21: 822–827.
18. Molhoek EM, van Dijk A, Veldhuizen EJ, Haagsman HP, Bikker FJ (2011) Improved proteolytic stability of chicken cathelicidin-2 derived peptides by D-amino acid substitutions and cyclization. Peptides 32: 875–880.
19. Boman HG (1995) Peptide antibiotics and their role in innate immunity. Annu Rev Immunol 13: 61–92.
20. Hancock RE (1997) Peptide antibiotics. Lancet 349: 418–422.
21. Jacob L, Zasloff M (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. Ciba Found Symp 186: 197-216; discussion 216–123.
22. Kao HK, Chen B, Murphy GF, Li Q, Orgill DP, et al. (2011) Peripheral blood fibrocytes: enhancement of wound healing by cell proliferation, re-epithelialization, contraction, and angiogenesis. Ann Surg 254: 1066–1074.
23. Grinnell F (2000) Fibroblast-collagen-matrix contraction: growth-factor signalling and mechanical loading. Trends Cell Biol. England. pp. 362–365.
24. Carlson MA, Longaker MT (2004) The fibroblast-populated collagen matrix as a model of wound healing: a review of the evidence. Wound Repair Regen. United States. pp. 134–147.
25. Okumura M, Okuda T, Okamoto T, Nakamura T, Yajima M (1996) Enhanced angiogenesis and granulation tissue formation by basic fibroblast growth factor in healing-impaired animals. Arzneimittelforschung 46: 1021–1026.