1,5-Anhydro-D-fructose: A natural antibiotic that inhibits the growth of gram-positive bacteria and microbial biofilm formation to prevent nosocomial infection

XIAOJIE MENG[1*], KO-ICHI KAWAHARA[1*], HIROAKI MIYANOHARA[2], YASUSHI YOSHIMOTO[3], KAZUHIRO YOSHINAGA[3], SATOSHI NOMA[1], KIYOSHI KIKUCHI[1,9], YOKO MORIMOTO[4], TAKASHI ITO[1], YOKO OYAMA[1], NARIMASA YOSHINAGA[1], BINITA SHRESTHA[1], BINITA CHANDAN[1], KENTARO MERA[5], KO-ICHI TADA[5], NAOKI MIURA[7], YOSHIKO ONO[8], KAZUNORI TAKENOUCHI[1], RYUICHI MAENOSONO[1,2], TOMOKA NAGASATO[1], TERUTO HASHIGUCHI[1] and IKURO MARUYAMA[6]

[1]Department of Laboratory and Vascular Medicine, Cardiovascular and Respiratory Disorders, Advanced Therapeutics; [2]Kagoshima University Medical and Dental Hospital, Kagoshima 890-8544; [3]Nihon Starch Co., Ltd., Kagoshima 891-0122; Departments of [4]Restorative Dentistry and Endodontology, [5]Dermatology, and [6]Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima 890-8520; [7]Laboratory of Veterinary Diagnostic Imaging, Department of Veterinary Medicine, Faculty of Agriculture; [8]Department of Community Health Nursing/Nursing Informatics, School of Health Sciences, Faculty of Medicine, Kagoshima University, Kagoshima 890-0065; [9]Department of Neurosurgery, Yame General Hospital, Fukuoka 834-0034, Japan

Received June 6, 2010; Accepted March 16, 2011

DOI: 10.3892/etm.2011.245

Abstract. Nosocomial infections caused by microbial opportunistic infections or microbial biofilms may occur during hospitalization and increase patient morbidity, mortality and health care costs. Artificial antibiotic agents were initially used to prevent infection; however, the high prevalence of nosocomial infections has resulted in their excessive use, which has led to microbial resistance to these agents. The increase in microbial resistance to antibiotics and the development of antibiotic agents may be the cause of the production of other microbial resistance. Thus, natural compounds that have no adverse side effects would be a preferred treatment modality. Recently, the monosaccharide 1,5-anhydro-D-fructose (1,5-AF), a natural plant compound derived from starch, has been found to have multifunctional properties, including antioxidant, antiplatelet aggregation by thrombin and anti-inflammatory activities. The results of the present study demonstrate that 1,5-AF suppressed the growth of coagulase-negative staphylococci on the hands as well as the growth of *Staphylococcus epidermidis*, which is a cause of opportunistic infections. Furthermore, 1,5-AF suppressed biofilm formation by the methicillin-resistant *Staphylococcus aureus*. In conclusion, 1,5-AF is a natural compound that may be effective in preventing nosocomial infections, without causing adverse side effects.

Correspondence to: Dr Ikuro Maruyama, Department of Systems Biology in Thromboregulation, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8544, Japan
E-mail: rinken@m3.kufm.kagoshima-u.ac.jp

*Contributed equally

Key words: 1,5-anhydro-D-fructose, natural antibiotic, biofilm

Introduction

Nosocomial infections caused by opportunistic infections or microbial biofilms with resistance to antibiotics may occur during hospitalization or treatment at healthcare service units. Recently, the increase in microbial resistance to antibiotics, for example, that of methicillin-resistant *Staphylococcus aureus* (MRSA), has threatened public health on a global scale as it reduces the efficacy of treatments and results in increased patient morbidity, mortality and health care costs (1).

An opportunistic infection is an infection caused by pathogens (bacterial, viral, fungal or protozoan) that do not usually cause disease in a healthy host with a functioning immune system. However, in patients with a compromised immune system, the pathogen has the 'opportunity' to infect through skin injury, a chronic disease, cancer or a drug-induced abnormality. Opportunistic infections are a potential cause of nosocomial infection, and may be resistant to antibiotics. Microbial biofilms, which are polymer-dipped communities of cells responsible for a number of chronic infections, also have extremely high resistance to antibiotics and host defense systems (2,3). However, there are no commercially available specific biofilm inhibitors. Natural compounds

with antibiotic properties, particularly plant-derived ones, are therefore preferred to artificial compounds. However, few of these natural compounds have been identified. Biotechnology companies are currently focused on identifying natural plant compounds with potential use as antimicrobial and antibiofilm drugs (4).

The sugar 1,5-anhydro-D-fructose (1,5-AF) is a recently identified monosaccharide that is formed directly from starch or glycogen through an α-1,4-glucan lyase reaction (EC 4.2.2.13), during which its carbonyl group does not undergo hemiacetal bonding; however, when fully hydrated in an aqueous solution it may play a metabolically active role (5). The compound 1,5-AF has been found in fungi, red algae, *Escherichia coli* and rat liver tissue (6-9). A recent study reported that the 1,5-AF pathway is possibly operative only when the organism is subjected to biotic and abiotic stresses (10). 1,5-AF is likely to act as an antioxidant (11) and a precursor of antibiotics (5). However, the functionality and physiological role of 1,5-AF are largely unknown.

In the present study, the antimicrobial activity of 1,5-AF against a wide range of pathogens, including coagulase-negative staphylococci (CNS), *Staphylococcus epidermidis* and MRSA biofilm formation, was investigated.

Materials and methods

Preparation of 1,5-AF solution. 1,5-AF was provided by Nihon Starch Co., Ltd. (Kagoshima, Japan) as a gift. Fresh 1,5-AF solutions were prepared in sterile H_2O at a concentration of 1 mg/ml.

Determination of the effect of 1,5-AF on coagulase-negative staphylococci. Hands contaminated with coagulase-negative staphylococci were treated as follows: 75% ethanol was spread on one hand and 1% 1,5-AF + 75% ethanol on the other. After 1 min, each hand was placed onto an agar plate. The specimens were incubated at 37˚C for 24 h.

Determination of the effect of 1,5-AF on S. epidermidis. All the keys on computer keyboards at the clinical laboratory of Kagoshima University Hospital were swabbed with sterile cotton swabs moistened with saline. The specimens were incubated at 37˚C for 24 h, then the strain of *S. epidermidis* was identified. *S. epidermidis* from the swab was washed in 1 ml of saline solution, and 0.05 ml of the resulting suspension was spread on either 5% sheep blood agar, 5% sheep blood agar combined with 75% ethanol, or 5% sheep blood agar combined with 1% 1,5-AF + 75% ethanol. The specimens were incubated at 37˚C for 16 h.

Determination of the effect of 1,5-AF on MRSA biofilm. A microtiter plate assay (12,13) was employed to determine the effect of 1,5-AF on biofilm formation. MRSA was obtained from the clinical laboratory of Kagoshima University Hospital and was cultured on sheep blood agar plates for 18 h at 37˚C. Briefly, overnight cultures of MRSA strains were inoculated in tryptic soy broth (TSB; BD Microbiology Systems, Sparks, MD, USA) with 0.25% glucose for 18 h at 37˚C. A 0.5 McFarland standard was used to create inoculum densities of $1.5x10^8$ CFU/ml in PBS using the direct suspension method (14) for the biofilm assay. The biofilm assay was performed in sterile 96-well flat-bottom polystyrene microtiter plates. A volume of 5 μl of the bacterial suspensions containing $1.5x10^8$ CFU/ml was added to the test wells, which already contained 200 μl TSB with 0.25% glucose. The wells were subsequently treated with 2 or 4 μl of 1,5-AF (1 mg/ml), resulting in a final concentration of 10 or 20 μg/ml, respectively. To allow bacteria to form a biofilm, the microplates were incubated for 18 h at 37˚C without shaking. The cells were then decanted, and the wells were washed gently four times with tap water. The cells that remained in the wells were stained with 0.1% crystal violet for 5 min. The wells were then washed four times with tap water. The stained cells were resolved by the addition of 200 μl of 95% ethanol. Absorbance was measured at 570 nm using an ELISA plate reader (ImmunoMini NJ-2300, Japan). Negative controls (bacteria + TSB), vehicle controls (bacteria + TSB + H_2O) and media controls (TSB) were also included. 1,5-AF activity was defined as the ratio of the absorbance of the biofilm remaining after 1,5-AF treatment in comparison to the negative control (15). All experiments were performed in quintuplicate.

Statistical analysis. Results are expressed as the means ± SE. Differences between the means were evaluated using an unpaired two-sided Student's t-test. A value of $P<0.05$ was considered significant.

Results

Growth inhibition of coagulase-negative staphylococci by 1,5-AF. Hand washing is the most fundamental method for preventing nosocomial infections (16). Alcohol-based hand rubs are the international gold standard method for hand washing (16), but may nonetheless prove ineffective. We therefore examined whether 1,5-AF affects the growth of CNS on CNS-contaminated hands. As shown in Fig. 1, 1,5-AF inhibited the growth of CNS as compared to the control. Colony numbers of CNS from the hand spread with 1,5-AF and 75% ethanol were 99, versus 211 from the control hand spread with 75% ethanol alone.

Growth inhibition of S. epidermidis by 1,5-AF. To further investigate the antimicrobial effect of 1,5-AF, bacterial counts were collected from computer keyboards *S. epidermis* was detected in a specimen from a computer keyboard. (Fig. 2A). We then examined whether 1,5-AF affected the growth of *S. epidermidis*. The strain of *S. epidermidis* was incubated with a 75% ethanol solution with or without the addition of 1% 1,5-AF. In the specimen treated with 1,5-AF (Fig. 2B, right), the growth of *S. epidermidis* was inhibited as compared to the control (Fig. 2B, left).

Inhibition of MRSA biofilm formation by 1,5-AF. Lastly, we examined whether 1,5-AF inhibited the biofilm formation of MRSA by means of a microtiter plate assay. 1,5-AF was found to significantly inhibit the biofilm formation of MRSA (Fig. 3A). However, further investigation revealed that 1,5-AF failed to induce the death of MRSA (Fig. 3B).

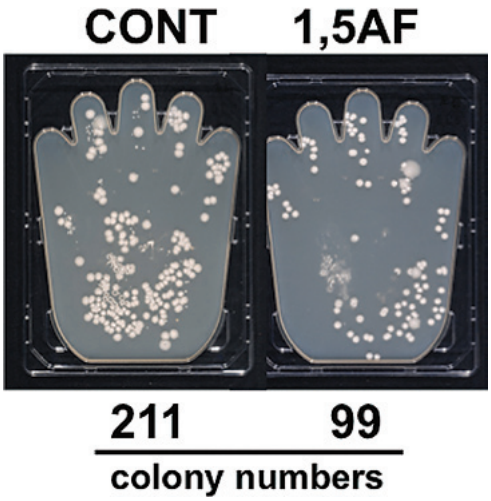

Figure 1. Growth inhibition of coagulase-negative staphylococci (CNS) on hands by 1,5-AF. Each hand was spread with 75% ethanol with or without the addition of 1% of 1,5-AF for 1 min, and then placed on agar plates. The specimens were incubated at 37˚C for 24 h. CONT, control; 1,5AF, 1,5-anhydro-D-fructose.

Figure 2. Growth inhibition of *S. epidermidis* by 1,5-AF. (A) Detection of *S. epidermidis*. All the keys on keyboards at our clinical laboratory were swabbed with sterile cotton swabs, and the specimens were incubated at 37˚C for 24 h. (B) Effect of 1% AF on *S. epidermidis*. *S. epidermidis* suspension from (A) was spread on 5% sheep blood agar, 5% sheep blood agar and 75% ethanol (left), or 5% sheep blood agar and 1% AF + 75% ethanol (right), and incubated for 16 h. CONT, control; 1,5AF, 1,5-anhydro-D-fructose.

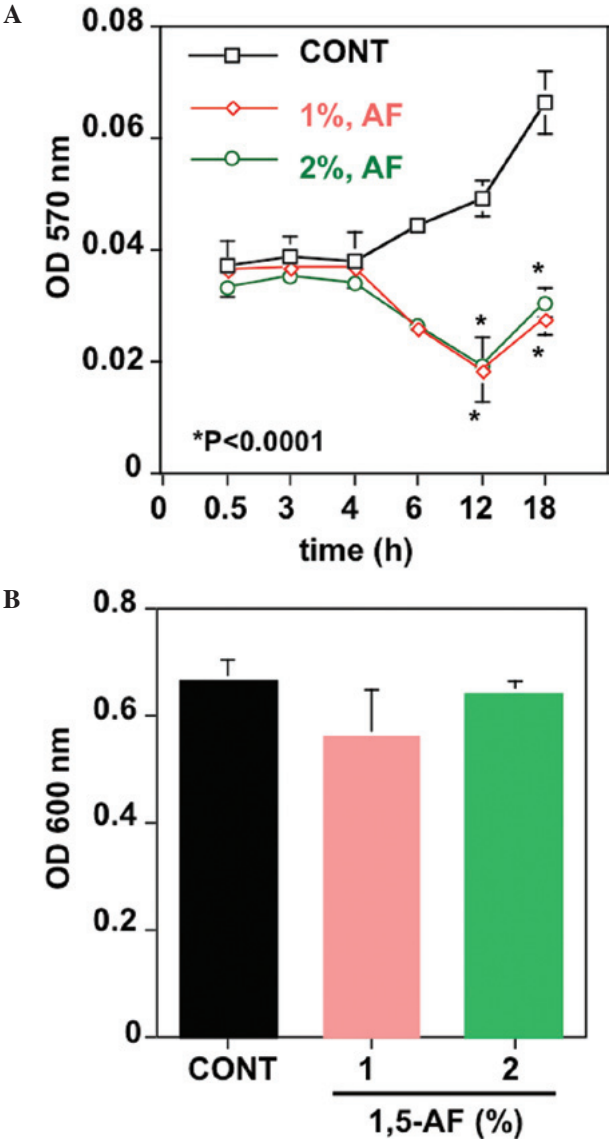

Figure 3. Inhibition of biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) by 1,5-AF. (A) Inhibition of MRSA biofilm by 1,5-AF. Cultured MRSA was incubated for 0.5-18 h in a 96-microplate and then removed by washing with PBS. The plates were stained with 0.1% crystal violet for biofilm production. (B) Measurement of MRSA growth with or without 1,5-AF. Cultured MRSA was incubated for 18 h, then cell growth was measured at 600 nm. CONT, control; 1,5AF, 1,5-anhydro-D-fructose.

Discussion

In the present study, we demonstrated that the monosaccharide 1,5-AF had an antimicrobial effect on the proliferation of CNS and *S. epidermidis*. Furthermore, 1,5-AF significantly suppressed the formation of MRSA biofilm. These results suggest that the antibiotic properties of 1,5-AF may be clinically effective in preventing opportunistic infection and the formation of biofilm, such as MRSA, with microbial resistance to antibiotics.

In healthy subjects, internal tissues, such as the blood, brain and muscle, are normally free of microorganisms. However, surface tissues, such as the skin and mucous membranes, are constantly in contact with environmental organisms and

readily become colonized by various microbial species. The normal flora of humans consists of a few eukaryotic fungi and protists, with bacteria being the most numerous and obvious microbial components. CNS are among the normal flora of the human skin and mucous membrane. CNS that are aerobic and gram-positive cocci are likely to consist mainly of *S. epidermidis*. Opportunistic infections have previously been shown to be caused by *S. epidermidis* (16), and result in nosocomial infections. Thus, inhibition of the increase in opportunistic infections as well as overcoming microbial resistance to antibiotics is essential for preventing nosocomial infections.

It is believed that 1,5-AF may inhibit opportunistic infections and microbial resistance to antibiotics. Recent studies have found that 1,5-AF has multifunctional properties, including acting as antioxidant for scavenging reactive oxygen species induced by phorbol myristate acetate in THP-1 cells, copper-mediated LDL oxidation, antiplatelet aggregation by thrombin and anti-inflammation, and inhibition of the following: translocation of nuclear factor-κB by lipopolysaccharide stimulation, expression of inducible nitric oxide synthesis protein *in vitro*, cytokines, including macrophage chemoattractant protein, interleukin-6, and tumor necrosis factor (10,11,17,18). 1,5-AF contains pre-antimicrobial substances. These are first converted to the intermediate enolone ascopyrone M, which is then converted to the antimicrobial microthecin in fungi belonging to morels, such as *Morchella* (*M*) *costata* and *M. vulgaris,* and in the red algae *Gracilariopsis lemaneiformis* (10). Our results, which are consistent with those of previous studies (10,11,17,18), indicate that treatment with 1,5-AF suppresses the growth of CNS and *S. epidermidis*. Additionally, pre-treatment with 1,5-AF may suppress the growth of MRSA (data not shown). We also found that, 1,5-AF inhibited MRSA biofilm formation, though it did not inhibit MRSA growth. Thus, 1,5-AF has both a suppressive (pre-treatment) and therapeutic (inhibition of biofilm formation) effect. These findings suggest that 1,5-AF has important antibiotic effects that could aid in the prevention of nosocomial infections.

The present findings suggest that 1,5-AF may serve as a novel natural antibiotic compound with properties including the suppression of gram-positive bacteria growth and MRSA biofilm formation. This novel function of 1,5-AF may be useful in the treatment of infectious diseases.

Acknowledgements

We thank Nobue Uto and Tomomi Morizono for excellent technical assistance. This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (ID: 21390483, K.-I. K.).

References

1. Coast J, Smith RD and Millar MR: Superbugs: should antimicrobial resistance be included as a cost in economic evaluation? Health Econ 5: 217-226, 1996.
2. Lewis K: Riddle of biofilm resistance. Antimicrob Agents Chemother 45: 999-1007, 2001.
3. Fux CA, Stoodley P, Hall-Stoodley L and Costerton JW: Bacterial biofilms: a diagnostic and therapeutic challenge. Expert Rev Anti Infect Ther 1: 667-683, 2003.
4. Schachter B: Slimy business – the biotechnology of biofilms. Nat Biotechnol 21: 361-365, 2003.
5. Yu S, Bojsen K, Svensson B and Marcussen J: α-l,4-Glucan lyases producing 1,5-anhydro-D-fructose from starch and glycogen have sequence similarity to α-glucosidases. Biochim Biophys Acta 1433: 1-15, 1999.
6. Baute M-A, Baute R and Deffieux G: Fungal enzymic activity degrading 1,4-a-D-glucans to 1,5,-D-anhydrofructose. Phytochemistry 27: 3401-3403, 1988.
7. Broberg A, Kenne L and Pedersen M: Analysis of 1,5-anhydro-D-fructose, microthecin, and 4-deoxy-glycero-2,3-diulose in algae using gas chromatography-mass spectrometry in selected ion monitoring mode. Anal Biochem 268: 35-42, 1999.
8. Shiga Y, Kametani S, Kadokura T and Akanuma H: 1,5-Anhydroglucitol promotes glycogenolysis in *Escherichia coli*. J Biochem 125: 166-172, 1999.
9. Kametani S, Shiga Y and Akanuma H: Hepatic production of 1,5-anhydrofructose and 1,5-anhydroglucitol in rat by the third glycogenolytic pathway. Eur J Biochem 242: 832-838, 1996.
10. Yu S and Fiskesund R: The anhydrofructose pathway and its possible role in stress response and signaling. Biochim Biophys Acta 1760: 1314-1322, 2006.
11. Yamaji K, Sarker KP, Maruyama I and Hizukuri S: Antioxidant effects of 1,5-anhydro-D-fructose, a new natural sugar, in vitro. Planta Med 68: 16-19, 2002.
12. Christensen GD, Simpson WA, Younger JJ, Baddour LM, Barrett FF, Melton DM and Beachey EH: Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitiative model for the adhesion of staphylococci to medical devices. J Clin Microbiol 22: 996-1006, 1985.
13. Mango K, Nishi J, Wakimoto N, *et al*: Biofilm formation by and accessory gene regulator typing of methicillin-resistant *Staphylococcus aureus* strains recovered from patients with nosocomial infections. Infect Control Hosp Epidemiol 27: 188-190, 2006.
14. Isenberg HD: Clinical Microbiology Procedures Handbook. ASM Press, Washington, DC, pp1.5.1-1.5.18, 2004.
15. Djordjevic D, Wiedmann M and McLandsborough LA: Microtiter plate assay for assessment of Listeria monocytogenes biofilm formation. Appl Environ Microbiol 68: 2950-2958, 2002.
16. Darmstadt GL, Nawshad Uddin Ahmed AS, Saha SK, *et al*: Infection control practices reduce nosocomial infections and mortality in preterm infants in Bangladesh. J Perinatol 25: 331-335, 2005.
17. Meng X, Kawahara K, Nawa Y, *et al*: 1,5-Anhydro-D-fructose attenuates lipopolysaccharide-induced cytokine release via suppression of NF-kappaB p65 phosphorylation. Biochem Biophys Res Commun 380: 343-348, 2009.
18. Meng X, Kawahara K, Matsushita K, *et al*: Attenuation of LPS-induced iNOS expression by 1,5-anhydro-D-fructose. Biochem Biophys Res Commun 387: 42-46, 2009.