Two diallyl sulphides derived from garlic inhibit meticillin-resistant Staphylococcus aureus infection in diabetic mice

Shih-Ming Tsao,1 Wen-Hu Liu2 and Mei-Chin Yin2

1Department of Infection, Chung Shan Medical University Hospital, Taichung City, Taiwan, Republic of China
2Department of Nutritional Science, Chung Shan Medical University, Taichung City, Taiwan, Republic of China

Correspondence
Mei-Chin Yin
mcyin@csmu.edu.tw

INTRODUCTION
Meticillin-resistant Staphylococcus aureus (MRSA) is a common nosocomial pathogen in Taiwan and other countries (Lowy, 1998; Jang et al., 1999). Healthcare-associated MRSA strains are resistant to many antibiotics (Hiramatsu et al., 1997; Chang et al., 2000), and MRSA infection markedly increases the morbidity and mortality in hospitalized patients. Thus there is a need to develop novel agents with greater inhibitory activity against MRSA.

Our previous animal study revealed that garlic extract and its two diallyl sulphides [diallyl sulphide (DAS) and diallyl disulphide (DADS)] could effectively decrease MRSA viability in blood and organs and reduce the plasma levels of fibronectin and interleukin (IL)-6 in non-diabetic mice (Tsao et al., 2003); however, it remains unknown whether these agents can inhibit MRSA infection and/or alleviate inflammation in diabetic mice.

Diabetes is an immunoinflammatory disease. Several studies have indicated that the cytokine balance is altered in diabetes (Shimada et al., 1996; Muller et al., 2002), which further exacerbates inflammatory, coagulant and oxidative damage in diabetic individuals. Our previous in vivo study indicated that MRSA infection in diabetic mice enhanced inflammation and coagulation by increasing the...
release of C-reactive protein (CRP) and diminishing the release of anti-coagulation factors such as antithrombin III (AT-III) and protein C (Tsao et al., 2006). Furthermore, we observed that MRSA infection in these diabetic mice did not cause high-grade infection and that the MRSA pathogen was markedly present only in kidney (Tsao et al., 2006). Based on the complicated interaction of MRSA infection and diabetes, antimicrobial therapy against MRSA infection in diabetic individuals is more difficult.

The purpose of this study was to examine the inhibitory effects of DAS and DADS on MRSA infection in diabetic mice. The influence of these agents on pathogen distribution and plasma levels of pro-inflammatory cytokines, endothelial injury-associated proteins, and coagulation and anti-coagulation factors, as well as on lipid oxidation levels, was evaluated.

METHODS

Preparation of diallyl sulphides. DAS (purity 97%) and crude DADS (purity 80%) were purchased from Aldrich. DADS was further purified by fractional distillation and its final purity was $\geq 98\%$, as determined by HPLC. In this study, the concentrations of DAS and DADS used were 10 and 1% (w/v), respectively. These concentrations were used because they have been shown to result in a 100% survival rate in non-diabetic mice and increased anti-infection therapy (Tsao et al., 2003). Mineral oil was used as the solvent for DAS and DADS preparation.

Animals. Eight- to nine-week-old male BALB/cA mice (National Laboratory Animal Center, Science Council, Taipei City, Taiwan) were used in this study. Mice were housed under a 12 h light/12 h dark schedule and fed with mouse standard diet no. 1120 and water ad libitum. Use of the mice was reviewed and approved by the Chung Shan Medical University Animal Care Committee. To induce diabetes, mice were treated with streptozotocin [40 mg (kg body weight)$^{-1}$ in 0.1 M citrate buffer, pH 4.5] intraperitoneally for 5 consecutive days. Blood glucose levels were monitored on days 2, 5 and 10 from the tail vein using a one-touch blood glucose meter. Mice with fasting blood glucose levels over 300 mg dl$^{-1}$ were defined as diabetic mice. The diabetic mice were then randomly distributed into different groups. Eight mice were used for each agent at each administration.

Bacterial strains. Ten clinical MRSA isolates were obtained from infected patients in Chung Shan Medical University Hospital (Taichung City, Taiwan). The bacterial strains used in the present study were identical to those used by Tsao et al. (2006). All isolates were identified using Vitek AMS (bioMérieux) and API 20E (bioMérieux) kits. Antibiotic-resistance profiles using vancomycin, meticillin, cefotaxime and tetracycline were determined by disc diffusion (Sigma). Discs were placed on the surface of Mueller–Hinton agar plates supplemented with 2% NaCl and seeded with MRSA. Inhibition zones were measured after 24 h incubation at 35 °C. Interpretation of resistance was based on the National Committee for Clinical Laboratory Standards criteria (NCCLS, 1999). The 10 MRSA isolates were susceptible to vancomycin and resistant to the other tested antibiotics. All cultures were routinely maintained on Mueller–Hinton agar plates at 25 °C until used. Overnight MRSA cultures in broth were diluted with PBS and adjusted to an OD$_{600}$ of 0.3 (about 10$^{5}$ c.f.u. ml$^{-1}$) (Hanaki et al., 1995). Prior to infection, MRSA cultures were diluted with PBS to 10$^{8}$ c.f.u. ml$^{-1}$ to give 10$^{5}$ c.f.u. per mouse in a volume of 200 μl.

Experimental design. Diabetic mice were infected by injecting 200 μl MRSA/PBS suspension containing 10$^{7}$ c.f.u. via the tail vein. After infection, mice were kept under standard laboratory conditions with free access to food and water. Our previous study showed that MRSA was clearly present only in the kidney at day 4 post-infection (p.i.) in diabetic mice (Tsao et al., 2006). In order to examine the effect of DAS and DADS on MRSA viability, these agents were given at day 4 p.i. in this study. Thus at day 4 p.i., 200 μl DAS or DADS was administrated orally twice with an interval of 12 h. Vancomycin at a concentration of 1% (200 μl of a 10 mg l$^{-1}$ aqueous solution) was given orally as a comparator. Eight hours after each administration, mice were killed with carbon dioxide. The blood, kidney and spleen of each mouse were collected. Blood samples used for bacterial culture, and for cytokine and inflammation analysis, were collected into tubes without anti-coagulant. Plasma was separated from erythrocytes immediately after blood collection. Blood samples used for measuring coagulation and anti-coagulation factors were collected into tubes containing sodium citrate. Kidney (200 mg) or spleen (100 mg) was homogenized in 2 ml PBS (pH 7.2) in a motor-driven Teflon-glass homogenizer (Glas-Col). The filtrate was used for analysis.

Culture. Serial dilutions (100 μl) from kidney filtrate were cultured on Mueller–Hinton agar plates supplemented with 2% NaCl. After incubation for 24 h at 35 °C, colonies were counted and calculated as log$_{10}$ c.f.u. g$^{-1}$. The limit of detection was 100 c.f.u. g$^{-1}$.

Proinflammatory cytokine measurement. Plasma levels of IL-6 and TNF-α were detected by ELISA using Cytoscreen Immunoassay kits (BioSource International). Samples were run in duplicate according to the manufacturer’s instructions. The sensitivity of the assay was a lower limit of 5 pg ml$^{-1}$ for IL-6 and 10 pg ml$^{-1}$ for TNF-α.

Measurement of inflammation and endothelial injury markers. Plasma levels of CRP, fibrinogen and fibronectin were measured as inflammation and endothelial injury markers. CRP levels (μg ml$^{-1}$) were determined with a commercial ELISA kit (Anogen). Fibronectin levels (g l$^{-1}$) were assayed using a commercial kit (Iatron Laboratories) based on the principle of salting out. Fibrinogen (mg ml$^{-1}$) was assayed using rabbit anti-rat fibrinogen antibody and quantified by solid-phase immunoenzymic ELISA (Sakata et al., 2000).

Measurement of coagulation and anti-coagulation factors. Levels of the coagulation factor plasmogen activator inhibitor-1 (PAI-1) and the anti-coagulation factors AT-III and protein C were measured in this study. PAI-1 activity (U ml$^{-1}$) was assayed using a commercial kit (Trinity Biotech). The activity (%) of AT-III and protein C in plasma was determined by chromogenic assays using commercial AT-III and protein C kits (Sigma), according to the manufacturer’s instructions, and was given as the ratio in normal human plasma.

Lipid oxidation determination. The concentration of malondialde-hyde (MDA; nmol ml$^{-1}$) in the filtrate from kidney and spleen was determined by an HPLC method (Jain & Palmer, 1997). Briefly, 0.2 ml kidney or spleen filtrate was mixed with 0.8 ml PBS, and 0.5 ml trichloroacetic acid (30 %) was added. After vortexing and standing in ice for 2 h, samples were centrifuged at 1500 g for 15 min. One millilitre of supernatant was mixed with 0.25 ml thiobarbituric acid (1 %) and the mixture was incubated in a boiling water bath for 15 min. The concentration of the MDA-thiobarbituric acid complex was assayed using HPLC equipped with a reverse-phase Shodex KC-812 column with a UV/Vis detector at 532 nm.

Statistical analysis. A total of 10 clinical MRSA isolates were used. Eight mice were used for each agent at each administration. Data were expressed as the means ± SD of 10 experiments (n=10). Data were
analysed by analysis of variance and computed using the SAS General Linear model procedure (SAS, 1990). The difference among means was determined by the least significant difference test with significance defined at $P < 0.05$.

**RESULTS**

The anti-MRSA effect of vancomycin, DAS and DADS in the kidney is shown in Fig. 1. Compared with the untreated groups, these agents given once or twice significantly decreased MRSA viability in the kidney ($P < 0.05$), and the administration of each agent twice showed a greater inhibitory effect than when given once ($P < 0.05$). The influence of these agents on plasma levels of IL-6 and TNF-$\alpha$ is presented in Fig. 2. MRSA infection significantly elevated the plasma levels of IL-6 and TNF-$\alpha$ in diabetic mice ($P < 0.05$). Compared with the untreated groups, vancomycin, DAS or DADS given once did not affect the plasma levels of IL-6 or TNF-$\alpha$ ($P > 0.05$); however, DAS and DADS given twice significantly decreased both IL-6 and TNF-$\alpha$ levels in plasma ($P < 0.05$). Vancomycin given twice did not significantly decrease the plasma level of IL-6 and TNF-$\alpha$ ($P > 0.05$). The influence of these agents on the plasma levels of CRP, fibrinogen and fibronectin is shown in Table 1. MRSA infection caused significant elevation in these biomarkers ($P < 0.05$). Compared with the untreated groups, treatment with DAS and DADS significantly diminished CRP, fibronectin and fibrinogen levels in plasma ($P < 0.05$), and the administration of each agent twice showed a greater suppressive effect than when given once ($P < 0.05$). Vancomycin given twice also significantly decreased the plasma levels of CRP and fibronectin ($P < 0.05$). The influence of these agents on the activity of PAI-1, AT-III and protein C is shown in Table 2. Compared with the untreated group, DAS or DADS treatment did not affect PAI-1 activity ($P > 0.05$), but DAS or DADS given twice significantly increased AT-III activity ($P < 0.05$) and DADS given twice significantly elevated levels of protein C activity ($P < 0.05$). Vancomycin treatment did not significantly affect the activity of PAI-1, AT-III or protein C ($P > 0.05$). The anti-oxidative effect of these agents in the kidney and spleen is shown in Fig. 3. MRSA infection significantly enhanced lipid oxidation in the kidney and spleen, determined by MDA levels ($P < 0.05$), which were significantly decreased by one or two

**Fig. 1.** MRSA levels (log$_{10}$ c.f.u. g$^{-1}$) in kidney from mice with MRSA infection without treatment (untreated) or treated with 1% vancomycin (Van), 10% DAS or 1% DADS. I and II represent one and two administrations of each agent. Data are means ± SD ($n=10$).

**Fig. 2.** IL-6 and TNF-$\alpha$ levels (pg ml$^{-1}$) in plasma from uninfected mice and from mice infected with MRSA without treatment (untreated) or treated with 1% vancomycin (Van), 10% DAS or 1% DADS. I and II represent one and two administrations of each agent. Data are means ± SD ($n=10$).
treatments with DAS or DADS \((P < 0.05)\). Compared with the untreated group, vancomycin given twice also exhibited a suppressive effect on lipid oxidation \((P < 0.05)\).

**DISCUSSION**

Our previous study found that MRSA infection in diabetic mice did not cause marked high-grade infection and that the MRSA pathogen was markedly present only in the kidney at day 4 p.i. (Tsao et al., 2006). The results of our present study showed that oral administration of DAS or DADS at day 4 p.i. could effectively reduce MRSA viability in the kidney from \(10^7\) to \(10^3\) c.f.u. \(g^{-1}\) in diabetic mice. These results support the potential use of DAS or DADS as agents for \textit{in vivo} therapy against MRSA infection. However, further studies in other animals such as rat or rabbit or in humans are necessary to verify the therapeutic effect of these agents in other species.

Our previous study indicated that MRSA infection in non-diabetic mice caused elevation of IL-6 up to \(104\) pg ml\(^{-1}\) at 16 h p.i. and that this pro-inflammatory cytokine could effectively be reduced to a level of 40–50 pg ml\(^{-1}\) by one administration of DAS or DADS (Tsao et al., 2003). The results from the present study showed that MRSA infection in diabetic mice resulted in the production of IL-6 and TNF-\(\alpha\) up to about 290–300 pg ml\(^{-1}\) at day 4 p.i. and that two administrations of DAS or DADS were required in order to reduce both IL-6 and TNF-\(\alpha\) levels down to 180–200 pg ml\(^{-1}\) in these MRSA-infected diabetic mice. These results support the suggestion that these agents are able to decrease immune complex formation and alleviate infection-induced inflammation reactions; however, diabetic mice apparently require higher doses of DAS or DADS or a shorter administration interval. It should be noted that, without the influence of MRSA infection, diabetic mice had higher IL-6 levels than non-diabetic mice (83 compared with \(32\) pg ml\(^{-1}\)) (Tsao et al., 2006). Thus the higher pro-inflammatory cytokine level or imbalanced cytokine profile that occurs under diabetic conditions might affect the dosage used or the administration interval of the antibiotic agent such as DAS or DADS. As these agents were able markedly to suppress IL-6 and TNF-\(\alpha\) production, these agents might also be beneficial for other inflammation-associated diseases.

It has been indicated that the pro-inflammatory cytokines IL-6 and TNF-\(\alpha\) are central mediators for the regulation of several biomarkers such as CRP (McCarty, 1999; Mohamed-Ali et al., 2001; Tomita et al., 2004), which subsequently modulates the progression of inflammation and endothelial dysfunction. In the present study, the interaction of MRSA infection and diabetes definitely enhanced the elevation of IL-6 and TNF-\(\alpha\); thus the observed upregulation of CRP, fibronectin and fibrinogen could be explained. Fibronectin is an extracellular matrix protein, responsible for the adherence and internalization of pathogens into host cells (Cossart, 1997). Our previous study found that DAS and DADS could effectively suppress MRSA-induced fibronectin production in MRSA-infected.

**Table 1.** Plasma levels of CRP, fibronectin and fibrinogen in uninfected mice and in mice infected with MRSA without treatment (untreated) or treated with 1 % vancomycin (Van), 10 % DAS or 1 % DADS (I and II represent one and two administrations of each agent)

Data are means \(\pm\) SD \((n=10)\). *Means in a column without a common letter are significantly different \((P < 0.05)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP (µg ml(^{-1}))</th>
<th>Fibronectin (mg ml(^{-1}))</th>
<th>Fibrinogen (g l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>32.6 (\pm) 1.4(^a)</td>
<td>3.97 (\pm) 0.17(^a)</td>
<td>4.02 (\pm) 0.21(^a)</td>
</tr>
<tr>
<td>Untreated</td>
<td>53.4 (\pm) 2.0(^a)</td>
<td>5.47 (\pm) 0.38(^d)</td>
<td>6.13 (\pm) 0.34(^e)</td>
</tr>
<tr>
<td>Van-I</td>
<td>54.0 (\pm) 2.1(^e)</td>
<td>5.50 (\pm) 0.46(^d)</td>
<td>6.10 (\pm) 0.37(^e)</td>
</tr>
<tr>
<td>Van-II</td>
<td>50.1 (\pm) 2.2(^e)</td>
<td>5.15 (\pm) 0.37(^c)</td>
<td>6.06 (\pm) 0.28(^e)</td>
</tr>
<tr>
<td>DAS-I</td>
<td>47.3 (\pm) 1.1(^c)</td>
<td>5.12 (\pm) 0.35(^a)</td>
<td>5.79 (\pm) 0.18(^a)</td>
</tr>
<tr>
<td>DAS-II</td>
<td>41.7 (\pm) 1.3(^c)</td>
<td>4.68 (\pm) 0.27(^b)</td>
<td>5.36 (\pm) 0.30(^b)</td>
</tr>
<tr>
<td>DADS-I</td>
<td>44.2 (\pm) 1.5(^c)</td>
<td>4.93 (\pm) 0.13(^c)</td>
<td>5.81 (\pm) 0.23(^c)</td>
</tr>
<tr>
<td>DADS-II</td>
<td>38.1 (\pm) 1.0(^b)</td>
<td>4.21 (\pm) 0.21(^c)</td>
<td>5.09 (\pm) 0.37(^b)</td>
</tr>
</tbody>
</table>

**Table 2.** Activity of PAI-1 \((U ml\(^{-1}\))\), AT-III\(\%\) and protein C \(\%\) in uninfected mice and in mice infected with MRSA without treatment (untreated) or treated with 1 % vancomycin (Van), 10 % DAS or 1 % DADS (I and II represent one and two administrations of each agent)

Data are means \(\pm\) SD \((n=10)\). *Means in a column without a common letter are significantly different \((P < 0.05)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PAI-1 ((U ml(^{-1})))</th>
<th>AT-III (%)</th>
<th>Protein C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>8.3 (\pm) 1.4(^a)</td>
<td>96 (\pm) 4(^e)</td>
<td>81 (\pm) 5(^c)</td>
</tr>
<tr>
<td>Untreated</td>
<td>18.2 (\pm) 2.3(^b)</td>
<td>53 (\pm) 6(^e)</td>
<td>49 (\pm) 6(^c)</td>
</tr>
<tr>
<td>Van-I</td>
<td>17.5 (\pm) 1.8(^b)</td>
<td>54 (\pm) 4(^e)</td>
<td>50 (\pm) 4(^a)</td>
</tr>
<tr>
<td>Van-II</td>
<td>18.3 (\pm) 2.0(^b)</td>
<td>55 (\pm) 5(^a)</td>
<td>48 (\pm) 5(^a)</td>
</tr>
<tr>
<td>DAS-I</td>
<td>17.6 (\pm) 2.1(^b)</td>
<td>57 (\pm) 3(^a)</td>
<td>47 (\pm) 6(^a)</td>
</tr>
<tr>
<td>DAS-II</td>
<td>16.9 (\pm) 1.7(^b)</td>
<td>63 (\pm) 3(^b)</td>
<td>55 (\pm) 6(^a)</td>
</tr>
<tr>
<td>DADS-I</td>
<td>17.2 (\pm) 2.3(^b)</td>
<td>59 (\pm) 4(^b)</td>
<td>54 (\pm) 3(^a)</td>
</tr>
<tr>
<td>DADS-II</td>
<td>16.4 (\pm) 2.2(^b)</td>
<td>70 (\pm) 5(^b)</td>
<td>64 (\pm) 5(^b)</td>
</tr>
</tbody>
</table>
non-diabetic mice (Tsao et al., 2003), which could reduce the amount of fibronectin available for MRSA and consequently decrease the adherence and/or internalization of MRSA into host cells. The present study also found that DAS and DADS could effectively decrease the release of two other inflammation and endothelial injury biomarkers, CRP and fibrinogen, which consequently might alleviate MRSA-induced inflammatory damage and endothelial dysfunction. It was interesting to find that DAS or DADS given once did not affect IL-6 and TNF-α levels, but significantly decreased fibronectin, CRP and fibrinogen levels. Thus the down-regulation effect of DAS or DADS on these three inflammatory-associated biomarkers could not be attributed only to the suppression of IL-6 and TNF-α by these agents.

Our previous study found that the interaction of diabetes and MRSA infection markedly upregulated PAI-1 activity and downregulated AT-III and protein C activities (Tsao et al., 2006), which meant that anti-coagulation and fibrinolysis systems were impaired and that the development of coagulation and thrombosis was favoured. The present study also found that DAS or DADS given twice could elevate the activity of AT-III, an anti-coagulant factor. Thus these agents might be able to alleviate haemostatic disorders in MRSA-infected diabetic mice by enhancing anti-coagulation action, as AT-III can inhibit the activity of a number of proteases in the coagulation cascade (Asakawa et al., 2000).

MRSA-induced oxidative stress and the anti-oxidative effect of DAS or DADS in non-diabetic mice were observed by us in a previous study (Tsao et al., 2003). Based on their enzymic and non-enzymic anti-oxidative capabilities (Wu et al., 2001; Yin et al., 2002), we suggested that these agents could directly improve oxidative stress and indirectly enhance anti-infective therapy. DAS and DADS are compounds naturally formed in foods of the species Allium, such as garlic, Chinese leek and onion. In the present study, a single oral administration of 10% DAS or 1% DADS provided 21.5 μg DAS or 3.6 μg DADS, which was safe and effective for MRSA infection therapy in diabetic mice with a body weight of 20–22 g. Therefore, these two agents at these concentrations could be considered to be novel antibiotics.

In conclusion, DAS and DADS effectively inhibited MRSA viability, suppressed MRSA infection-induced elevation of IL-6, TNF-α, fibronectin, CRP and fibrinogen, elevated AT-III activity and decreased MRSA-induced oxidative damage in diabetic mice. These data suggest that these agents could provide anti-bacterial, anti-inflammatory, anti-coagulant and anti-oxidative functions against MRSA infection in diabetic individuals.

REFERENCES


