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Prophylaxis and Treatment of Generalyzed Infection Induced by Methicillin-Resistant Staphylococcus Aureus (MRSA) in Vivo with Hypothalamic Proline Rich Peptides Galarmin and D-15 Galarmin

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Dr. Gregory T. Papanikos
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Abstract

Epidemiological data indicate that Staphylococcus aureus and particularly methicillinresistant strains of S.aureus (MRSA) are responsible for the majority of complicated cases of Staphylococcus infections and are increasingly implicated as a cause of nosocomial and community associated infections worldwide. Proline-rich peptides (Galarmin and analogues) are new brain cytokines isolated from neurosecretory granules of hypothalamus by Prof. A. Galoyan and coworkers with a broad-spectrum of biological activities including antibacterial, antitumor, and immunomodulatory properties. This allowed us to conclude that Galarmin and its analogues can be efficient against generalyzed infection induced by MRSA on mice model in vivo.

Received data indicate that Galarmin and its analogue d-15 Galarmin are strong remedies for the prophylaxis and treatment of MRSA infection in vivo. Galarmin at the concentration of 1 μg/mice express its highest protective effect for the prophylaxis (administration 24h before infection) and treatment (1h post-infection) by increasing the survival of experimental animals up to 100% over the control (non-treated) group. For the parallel administration more efficient are higher concentrations of Galarmin: 5 and 10 μg, which increase the survival of animals by 50-60%. In that case the absence of bacterial growth from the blood of treated animals was observed. The most efficient protective concentration of d-15 Galarmin is 16 μg administrated 8h before the infection which increase the survival of infected animals by 80%.

Keywords:

Introduction

Despite the availability of effective antimicrobial agents, *Staphylococcus aureus* continues to cause life-threatening infections, including septic shock [1-3]. Recent increase of methicillin-resistant *Staphylococcus aureus* (MRSA) strains at large hospitals as well as community settings (community associated) started to pose great difficulty in selecting antimicrobial agents for the management of the infections they cause [4-6]. It may also be called multidrug-resistant *S. aureus*, oxacillin-resistant *S. aureus* (ORSA). Healthcare-associated MRSA strains are resistant to the majority of antibiotics [5,7] and are responsible for the majority of complicated cases of Staphylococcus infections. MRSA is by definition any strain of *S.aureus* bacterium that has developed resistance to beta-lactam antibiotics which include penicilllins (meticillin, dicloxacillin, nafcillin, oxacillin etc) and cephalosporins. Resistance to erythromycin, clindamycin, tetracycline, aminoglycosides, and chloramphenicol has been reported with MRSA strains [8]. The major determinant of MRSA resistance to beta-lactam antibiotics is chromosomally mediated mecA gene which involves production of an unusually low affinity penicillin binding proteins PBP2 and allows peptidoglycan synthesis even in the presence of β-lactam antibiotics [9,10]. The gene *mecA* is carried on a mobile genetic element called "staphylococcal cassette chromosome *mec*" (SCC*mec*) [11]. Community- and hospital-acquired MRSA evolve upon acquisition of Staphylococcal cassette chromosome SCC*mec* [12]. Hospital MRSA contain one of four SCC*mec* types (I–IV), while the community-acquired MRSA is associated with the acquisition of SCC*mec* IV [13], the smallest element and one that confers only resistance to β-lactams. However community MRSA are genetically more diverse than hospital MRSA because of the increased frequency of acquisition of SCC*mec* IV compared with other SCC*mec* types [14]. The horizontal transfer of virulence genes, although infrequent, is epidemiologically associated with the emergence of new virulent strains of MRSA and representing a major and constantly changing clinical challenge [15].

MRSA infection markedly increases the morbidity and mortality in hospitalized patients [16,17]. MRSA is especially troublesome in hospitals. MRSA infections are responsible for more deaths in USA each year than AIDS (2007, Wahigton Post), in 1999 there was 127000 cases in hospitals and 278000 for 1999-2008 in USA. Hospitalization costs associated with MRSA infections are substantially greater than those associated with methicillin-sensitive *S. aureus* (MSSA) infections, and MRSA has wider economic effects that involve indirect costs to the patient and to the society [18]. Patients with compromised immune system are at a significantly risk of symptomatic secondary infection. MRSA may progress substantially within 24-48 hours of initial topical symptoms. After 72 hours MRSA can take hold in human tissues and eventually become resistant to the treatment. About 75% of community-associated (CA-MRSA) are localized to skin and soft tissue and usually can be treated effectively. However some CA-MRSA strains displays enhanced

virulence, spreading more rapidly and causing illness much more severe than traditional healthcare hospital-acquired associated (HA-MRSA) infections [19]. They affect vital organs and led to widespread infections (sepsis), toxic shock syndrome and necrotizing ("flash eating") pneumonia. This is thought to be due to toxins carried by CA-MRSA strains such as PVL and PSM, though PVL was recently found to not be a factor in a study by National Institute of Allergy and Infection Diseases (NIAID) and the NIH. CA-MRSA is more easily treated, though more virulent than HA-MRSA. CA-MRSA apparently did not evolve de novo in community, but represents a hybrid between MRSA and HA-MRSA. It is not known why some healthy people develop CA-MRSA skin infection that are treatable whereas other infected with the same strain develop severe infections or die.

In the UK, where MRSA is commonly called "Golden Staph", the most common strain of MRSA are EMRSA15 and EMRSA16. EMRSA 16 is the best described epidemiologically, the full genomic sequence of this strain has been published [20]. Diagnosis technique include Real-time PCR and Quantitative PCR and increasingly beign employed in clinical laboratories for the rapid detection and identification of MRSA strains [21].

The number of effective antibiotics has been reduced by the emergence of resistance to penicillin, methicillin, and, more recently, vancomycin [20]. A highly vancomycin-resistant mutant of *S. aureus* continuing to grow and synthesize peptidoglycan in the presence of vancomycin (50 mg/ml) was isolated and described by Sieradzki et al [22]. It is not excluded that the appearance of vancomycin resistance among clinical isolates of enterococci has emerged from transfer of the resistance genes to highly virulent strains of MRSA with obvious dire implications for chemotherapy. Both CA-MRSA and HA-MRSA are resistant to traditional anti-staphylococcal antibiotics, such as cephalexin etc. CA-MRSA has a greater spectrum of antimicrobial susceptibility including to sulfo drugs (like co-trimoxazole, trimethropin-sulfamethoxazole), tetraciclins (doxyciclline and minocycline) and clyndamicin, but drug of the choice for treating CA-MRSA is now believed to be vancomycin according to a Henry Ford hospital study. HA-MRSA is resistant even to these antibiotics and often is susceptible only to vancomycin. Never drugs such as linelozyd (belonging to the newer oxezolinones class) and daptomicin, are effective gains both for CA-MRSA and HA-MRSA. Vancomycin and teicoplanin are glycoprotein antibiotics used to treat MRSA infections. Several newly discovered strains of MRSA show antibiotic resistance even to vancomycin and teicoplanin. These new evolution of the MRSA bacterium have been dubbed vancomycin intermediate-resistant *S. aureus* (VIRSA) [23]. Linelozid, quinupzistin/ daptomycin and tigecyclin are used to treat more severe infections that do not respond to glycopolypeptides such as Vancomycin [24].

Thus, there are indications suggesting that MRSA infections increase morbidity and the risk of mortality and the role of traditional antibiotics in the management of serious infections is now being reconsidered [24]. In order to control this infection, there is a need to develop novel agents with greater

inhibitory activity against MRSA. From this point of view it is important to develop new alternate therapies based on immune response activation and study their effectiveness in their antimicrobial activities. Natural products have shown to be a potential source of antimicrobial agents, such neurohormones and immunomodulators cytokines. The discovery by Prof. A. Galoyan and coworkers of new type of cytokines of the neurosecretory hypothalamus, the proline rich polypolypeptides (PRPs), resulted in changes in understanding of the regulation of immune system in general, and especially genesis, differentiation, proliferation and mobilisation of bone marrow cells into the blood circulation. Data obtained was a basis for the establishing a new field of neurobiology – neuroendocrine immunology s have shown that Galarmin is regulator of humoral and cellular immunity, thymocyte differentiation, and myelopoiesis [25-30]. Galarmin completely restores myelopoiesis after cyclophosphamideinduced leucopenia in mice and increase survival after infection with *Pseudomonas aeruginosa*. Galarmin dramatically enhances spontaneous or fMLP- and PMA-induced oxidative burst, as well as the intracellular killing of *S. aureus* by human neutrophils and monocytes. Galarmin was also shown to protect guinea-pig and mice against *Bacillus anthracis*, even when administrated the ten fold lethal dose infection. This allowed us to suggest that Galarmin can be effectively used against multidrug-resistant strain of methicillin-resistant *S.aureus* (MRSA). Received by us experimental results indicate that Galarmin and its structural analogues Gx-NH_2 discovered by A. Galoyan and d-15 Galarmin analogue are strong remedies against MRSA infection *in vivo*. It was shown also significant influence of Galarmin on MRSA infected animal's complete blood count and plasma immunoglobulins and cytokines levels.

Material and Methods

Bacterial strains and growth conditions: MRSA ATCC 43300 strain (LGC Standards) were used in this study resistant to β-lactams and cephalosponins and susceptible to four other antibiotics. Fresh colonies of MRSA were seeded on elective saline agar (Allergen Corp, Russia) plates and grown overnight at 37°C. The MRSA bacteria were collected by centrifugation, washed, and resuspended in saline and adjusted to an optical density at 570 nm of 0.6 (about $4x10^{10}$ cfu/mL) with a spectrophotometer (KFK-2, Russia) prior to injection which gave approximately $8x10^{8}$ cfu per mouse in a volume of 200 μL. The minimal lethal dose of MRSA was previously determined following subsequent titration on mice according the optical densities (OD) of the bacterial solutions measured. The bacterial inoculum was confirmed by colony counting.

Polypeptides: Apirogenic sterile solution of the proline-rich polypeptides Galarmin (AGAPEPAEPAQPGVY), Gx-NH_2 (APEPAEPAQP) and their patented analogues d-15 Galarmin and d-Gx--NH_2 were used in this study. Galarmin was adiminstarted intramuscularly (i.m.) at the concentration range

from 0.1 to 10 μg per mouse diluted with the saline in a volume of 200 mL. The analogue d-15 were used at concentration of 16 μg.

Animals and infection model: Groups of at least ten male inbred non-linear white and C57Black/6 mice (8 to 10 weeks old weighting 22-25g) were used (Animal house of the UNESCO Chair- Life Sciences International Postgraduate Educational Center (LSIPEC), Yerevan, Armenia). Mice were housed at a temperature of 20-25°C in a room with a 12:12-h light-dark cycle for 1 week before experiments in standart ventilated *cages (up to 10 mice per cage) and* fed with commercial pelleted mouse diet and water *ad libitum*. Animals injected intraperitoneally (i.p.) with MRSA ($8x10^8$ CFU/mouse) in 200 μL of saline. Control mice were injected with the same volume of saline. At 24 h before infection, parallel and 1 h post-infection (p.i.), Galarmin and its analogues were administrated i.m. at 200 μL of saline in various concentrations to deduce sub-lethal levels in mice, and the survival rate was measured. Untreated mice received the same volume of saline without polypeptides. Each experimental group consisted of 10 animals, the survival following MRSA infection was monitored during the next 9 days. Five independent sets of experiments were performed. All surviving animals were eutanaziated by cervical dyslocation under chloral hydrat anesthesia (400 mg/kg ip). Animal housing and care were performed according to the US National Research Council's ''Guide for the Care and Use of Laboratory Animals''.

Statistical analysis: In every experiment, ten mice were used for each agent at each concentration. Data were expressed as mean ± S.D. of five independent sets of experiments (n = 5). Statistical processing of the results was performed using Statistical Package SPSS11. Comparative analysis was performed using parametric univariate analysis ANOVA, multiple comparisons performed using Scheffe test for linear contrasts and paired Student's t-test. The level of significance was defined at $P \leq 0.05$.

Results

For the first sets of experiments Galarmin and Gx-NH_2 were adiminstrated at a concentration of 1 μg per mice two-fold – 24 h b.i. and parallel to infection process ($8x10^8$ cfu). Received data (Table 1A) suggests that in this mode of administration Gx-NH_2 showed remarkable protective effect by increasing the overall survival rate up to 30% as compared to untreated group, however Galarmin in this case doesn't affect survival rate. Therefore in further experimental design we used smaller concentration of Galarmin administrating 120-24h b.i in a concentration range from 0.01-0.1 μg. As it can seen from the Table 1B the most significant protective effect produced the concentration of Galarmin 0.01 μg injected 24 h b.i. while early administration of polypeptides (120 h b.i.) failed to produce significant protection.

We could conclude therefore that Galarmin and Gx-NH_2 are potential protective compounds against MRSA infection *in vivo*. Particularly protection by Galarmin requires smaller concentration of polypeptides and administration

24 h prior to infection process, while Gx-NH_2 manifests its beneficial effect when injected simultaneously and prior to infection process at greater concentrations. Upon received results we have investigated the protective activity of Galarmin for prophylaxis of MRSA (ATCC 43300) infection at a concentration range from 0.1 to 1 μg injected 24 h b.i. Received by us experimental results as compared to untreated and Gx-NH_2 group are presented on Fig. 1.

Table 1. *Survival rate of MRSA infected mice following i.m. administration of Galarmin and Gx-NH_2 at different concentrations and time-period as compared to untreated group*

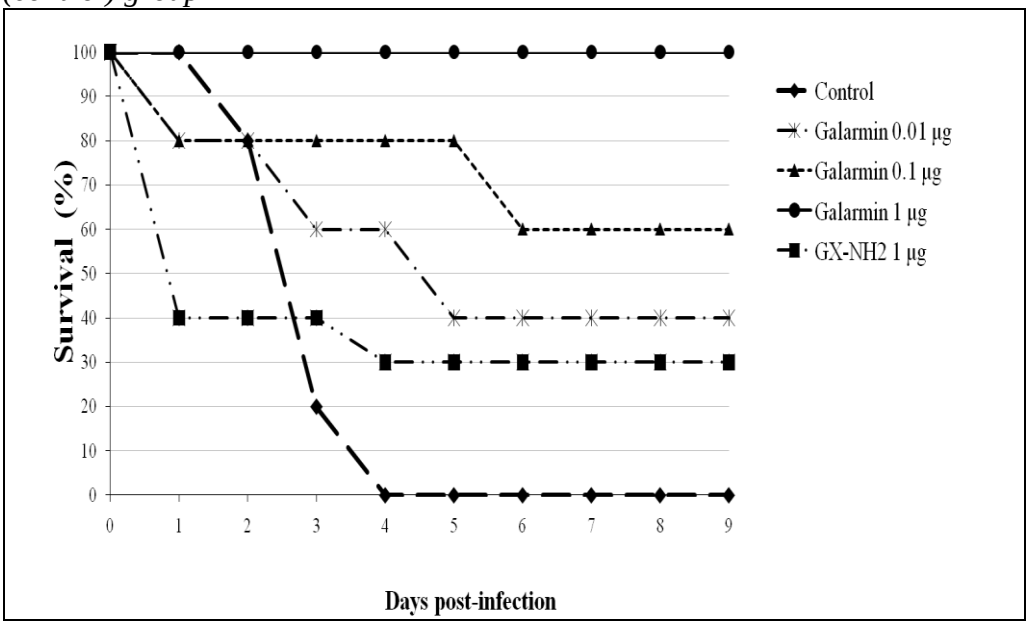
Agent	Concentration of agent (μg/mice)	Administration mode	Survival rate
Untreated	-	-	20
Galarmin	1	24 h b.i. and parallel	23
Gx-NH_2	1	24 h b.i. and parallel	50

A

Agent	Concentration of agent (μg/mice)	Administration mode	Survival rate
Untreated	-	-	60
Galarmin	0.01	120 h b.i.	60
Galarmin	0.1	120 h b.i.	40
Galarmin	0.01	24 h b.i.	100

B

Figure 1. *Survival rate of MRSA infected mice following i.m. administration of Galarmin and Gx-NH_2 at different concentrations as compared to untreated (control) group*



Protective activity of novel analogue d-15 Galarmin have been studied as well for the concentration of 16 μg injected 24, 8 and 5h before MRSA infection. Figures 2 and 3 represent survey of outcome of MRSA-infected mice following Galarmin and d-15 administration.

Figure 2. *Survival rate of MRSA infected mice following i.m. administration of Galarmin at concentration of 0.1-10 μg injected 24 h b.i., parallel and 24 h p.i.. (*p<0.05, **p<0.01)*

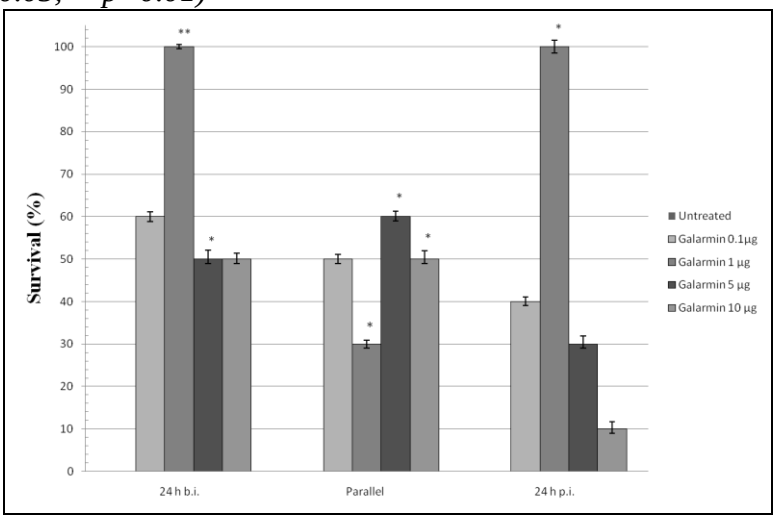
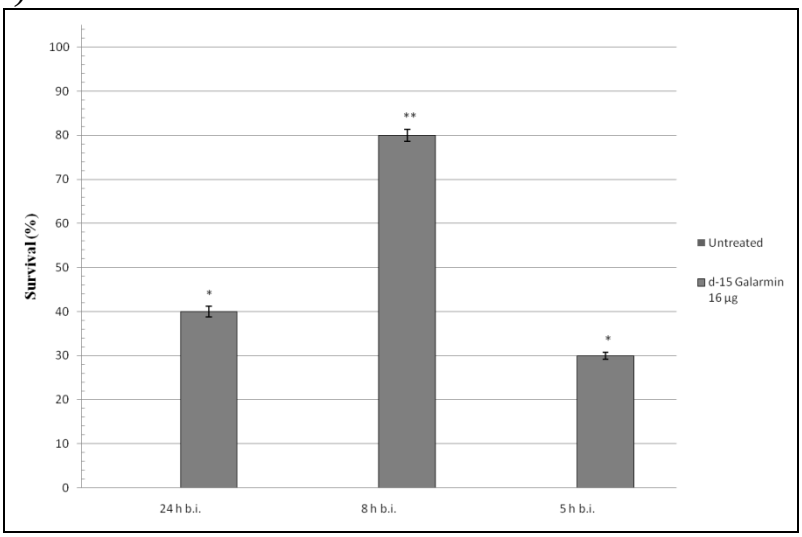


Figure 3. *Survival rate of MRSA infected mice following i.m. administration of d-15 Galarmin at concentration of 16 μg injected 24, 8 and 5 h b.i. (*p<0.05, **p<0.01)*



Conclusions

As we can see from obtained results, Galarmin administrated at the dose 1 μg per mice 24 h before infection fully protects animals from MRSA lethal

dose. Concentranions of 0.1-10 μg were also highly protective increasing the survival by 50-60%. For the parallel with infection administration of Galarmin higher concentrations (5 and 10 μg) were more efficient increasing the survival rate respectively by 60 and 50%. Galarmin at the concentration of 1 μg administrated 1h post-infection increased survival rate by 100%, smaller and higher concentration of 0.1 μg and 5 μg had significally less performance (respectively 40 and 30%), and 10 μg was uneficient. For the analogue d-15 Galarmin injected at the dose 16 μg/mice the most efficient time of administration was 8h before infection with an increase of the survival rate by 80%. Adinistration 24 and 5h before infection was less effective increasing the survival by 40 and 30% respectively. Therefore we could conclude that Galarmin is a powerful agent for MRSA prophylaxis and treatment *in vivo* and its structural analogues Gx-NH_2 and d-15 Galarmin are perspective anti-MRSA agent too.

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