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Mutant Selection Windows of Azalomycin F_{5a} in Combination with Vitamin K_3 against Methicillin-Resistant *Staphylococcus aureus*

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Abstract

Azalomycin F_{5a}, a 36-membered macrocyclic lactone isolated from several streptomyces strains, presented remarkable anti-methicillin-resistant *Staphylococcus aureus* (MRSA) activities. To improve its anti-MRSA potential and to evaluate the probability of MRSA resistant to it before development, the anti-MRSA activities of azalomycin F_{5a} in combination with vitamin K_3 were first evaluated using checkerboard assay. Then the minimal concentration inhibiting colony formation by 99% (MIC_{99}) and mutant prevention concentration (MPC) of azalomycin F_{5a} alone and in combination with vitamin K_3 against MRSA were determined using agar plates with linear antimicrobial concentration decrease. The fractional inhibitory concentration indexes (FICIs) of 0.25 - 0.50 showed the synergistic activity of azalomycin F_{5a} in combination with vitamin K_3. The mutant selection windows (MSWs, MIC_{99}-MPC) of azalomycin F_{5a} alone against MRSA tested were 2.07 - 6.40 μg/mL, and the MPCs of azalomycin F_{5a} in combination with vitamin K_3 against MRSA tested were 1.60 - 3.20 μg/mL. These indicated that the MPCs of azalomycin F_{5a} in combination could drop down to below its MIC_{99} alone. According to the hypothesis of MSW, the narrower MSWs of azalomycin F_{5a} alone, even closed MSWs in combination with vitamin K_3, together with their synergistic anti-MRSA activities, indicated that azalomycin F_{5a} had a good potential to develop as a new antimicrobial agent.

Keywords

MRSA, Azalomycin F_{5a}, Mutant Selection Windows, Combination, Antibiotic Resistance

1. Introduction

Antimicrobials are a crucial defense against bacterial infections, while they also promote

the evolution of bacteria, and even lead bacteria to be resistant to themselves. Now, infections from resistant bacteria are too common, and some pathogens have even become resistant to multiple types of antibiotics. As antimicrobial resistance seriously threatened human health, many strategies involving the development of new antibiotics, combination therapy with several antibiotics, the revival of old antimicrobials and the optimal use of clinic antibiotics were already put forward to fight or delay resistance [1] [2]. Among them, discovering new antibiotics is still an important one to fight antibiotic resistance, while resistance would empirically emerge shortly after a new antibiotic is used.

Based on this, the hypotheses of mutant prevention concentration (MPC) and mutant selection window (MSW) were put forward [3] [4] [5]. These theories suggested that the antimicrobial concentration should avoid falling within MSW to prevent resistant mutant, and an antimicrobial concentration above its MPC would rarely enrich the growth of resistant subpopulations. As it is unavoidable for the antimicrobial concentration to drop down to below MPC, the narrower the MSW of antibiotics against pathogenic bacteria is, the less the probability of pathogenic bacteria being resistant to it is. Thus, the MSWs and MPCs of many antibiotics were recently determined to discover new antibiotics that pathogenic microorganisms are more difficultly to be resistant to [6] [7] [8]. Although the correlation observed between MIC and MPC was low (r^2 = 0.39) [5] [9], can we find high correlation between the proportion of two antimicrobials and their MIC_{99}s and MPCs in combination when their MIC_{99} and MPC alone were known? If find, we can use it to predict the MPCs and the perfect ratio of two antimicrobials in combination.

Azalomycin F_{5a} (**Figure 1**), a main macrocyclic lactone produced by *Streptomyces* sp. 211,726, was first isolated from *Streptomyces hygroscopicus* var. *azalomyceticus*, and showed broad-spectrum antimicrobial activity against gram-positive bacteria, yeast and fungi [1] [10] [11] [12]. During our research on its relative configuration [12], its anti

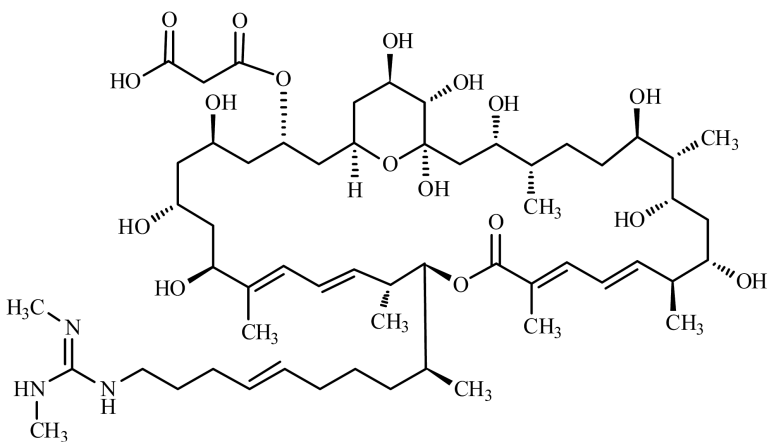

Figure 1. The structure of azalomycin F_{5a}. A main 36-membered macrocyclic lactone produced by *Streptomyces* sp. 211,726, was first isolated from *Streptomyces hygroscopicus* var. *azalomyceticus*, and showed broad-spectrum antimicrobial activity against gram-positive bacteria, yeast and fungi.

methicillin-resistant *Staphylococcus aureus* (MRSA) activities, together with those of vitamin K_3, were evaluated by us [13] [14], and the results showed that azalomycin F_{5a} and vitamin K_3 had remarkable anti-MRSA activities. These, combined with that it can eradicate MRSA biofilm and MRSA-Candida albicans complex biofilm (unpublished data), showed that azalomycin F_{5a} was a potential anti-MRSA agent. Thereby, the anti-MRSA activities of azalomycin F_{5a} in combination with vitamin K_3 were evaluated to improve its potential uses. Further, the MSWs of azalomycin F_{5a} alone and in combination with vitamin K_3 against MRSA were determined to predict the probability of MRSA resistant to it before development and to explore the synergistic strategy to prevent resistance.

2. Materials and Methods

2.1. Bacterial Strains and Medium

MRSA ATCC 33,592 (Gentamycin and methicillin-resistant) were purchased from American Type Culture Collection, Manassas, VA, USA, and three clinic isolates MRSA HK01, HK02 and HK03 (Methicillin-resistant) were friendly presented by Hainan General Hospital, Haikou, China. Bacterial inocula were prepared in Mueller Hinton Broth (MHB) at 35˚C until the OD_{600} nm value was 0.60 before use. MHB used for MIC test and Mueller Hinton Agar (MHA) used for MIC_{99} and MPC test were purchased from Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China.

2.2. Antimicrobials and Chemicals

Azalomycin F_{5a} was prepared in our laboratory according to our previous work [15], and high performance liquid chromatography analysis showed that the purity of azalomycin F_{5a} was 98.2%. Vitamin K_3 (Menadione sodium bisulfate) were purchased form Aladdin Industrial Corporation, Shanghai, China. All other chemicals used in these experiments were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.3. Measurement of MIC

The MICs of azalomycin F_{5a} and vitamin K_3 against MRSA ATCC 33,592 and three clinical isolates MRSA HK01, HK02, HK03 were respectively determined using broth microdilution method according to a standard procedure described by the Clinical and Laboratory Standards Institute (CLSI) in 2012 [16].

2.4. Checkerboard Assay

According to the MIC of azalomycin F_{5a} and vitamin K_3, the checkerboard method was designed to determine the fractional inhibitory concentration (FIC) indexes of azalomycin F_{5a} in combination with vitamin K_3 against MRSA ATCC 33,592 and three clinical isolates MRSA HK01, HK02 and HK03. The tests were performed on 96-well plate according to published methods [11]. Briefly, azalomycin F_{5a} and vitamin K_3 dilutions with concentrations from 8 MIC to 1/8 MIC in the horizontal or vertical direction were

obtained in a separate 96-well plate by twofold dilution method. Then, 100 μL azalomycin F_{5a} or vitamin K_3 dilutions with different concentrations were respectively added into the corresponding wells in another plate, and to create many different combinations with azalomycin F_{5a} and vitamin K_3 concentrations from 4 MIC to 1/16 MIC. Simultaneously, columns 11 and 12 only contained MHB with MRSA strain concentration of 5×10^5 CFU/mL were used as blank controls. The plate was incubated at 35˚C for 24 h. When the microbial growth in the well of blank controls was sufficient, the MIC of each sample was determined as the lowest concentration visibly inhibited the microbial growth. If necessary 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) stain method was used to clearly observe the results. The MICs of azalomycin F_{5a} and vitamin K_3 alone were respectively determined in row A and in column 1, and the MICs of azalomycin F_{5a} in combination with vitamin K_3 were determined from wells B2 to H8.

The fractional inhibitory concentrations (FICs) were calculated as follows: FIC of azalomycin F_{5a} = MIC of azalomycin F_{5a} in combination/MIC of azalomycin F_{5a} alone, and FIC of vitamin K_3 = MIC of vitamin K_3 in combination/MIC of vitamin K_3 alone. The FIC index (FICI) was defined as the FIC of azalomycin F_{5a} added to the FIC of vitamin K_3. The effect of azalomycin F_{5a} in combination with vitamin K_3 against MRSA was interpreted as follows: Synergy, FICI ≤ 0.5; antagonism, FICI ≥ 4.0; and indifferent, 0.5 < FICI > 4.0.

2.5. MIC_{99} of Azalomycin F_{5a} Alone and in Combination with Vitamin K_3

According to the MIC of azalomycin F_{5a} and vitamin K_3 alone or in combination, their minimal concentrations that inhibit colony formation by 99% (MIC_{99}s) against MRSA ATCC 33,592 were determined by utilizing linear antimicrobial concentration decrease (20% per sequential decrease) from MIC, plus a replicate. Referring the methods as described in previous papers [17] [18], the colonies growing on the plates contained different antimicrobial concentration were numbered, and the inhibition percentages were respectively calculated. Then, the inhibition percentage (y) was plotted against the antimicrobial concentration (x) to obtain the regression equation, and their MIC_{99}s were calculated according to this equation.

2.6. MPC of Azalomycin F_{5a} Alone and in Combination with Vitamin K_3

Using MRSA ATCC 33,592, the MPCs were determined as described elsewhere [17] [18]. Briefly, high-density cultures were prepared from overnight cultures grown in MHB, and followed by a 10-fold dilution, 6 h of incubation with shaking at 35˚C and centrifugation. A series of MHA plates containing azalomycin F_{5a} or/and vitamin K_3 with twofold dilution concentrations were inoculated with 100 μL culture containing about 5.0×10^9 colony forming unit (CFU). The plates were incubated at 35˚C for 72 h, and were screened visually for growth, and then the preliminary MPC was recorded as the lowest antimicrobial concentration that prevented bacterial growth. To estimate the exact MPC, the measurement was followed by a second determination, plus a replicate,

that utilized linear antimicrobial concentration decrease (20% per sequential decrease) from preliminary MPC.

3. Results

3.1. MICs and Checkerboard Assay

The MICs of azalomycin F_{5a} against all MRSA strains tested were 4.0 μg/mL, and those of vitamin K_3 were 16.0 μg/mL except for 32.0 μg/mL against MRSA HK03. Further, checkerboard test showed that all the FICIs of azalomycin F_{5a} in combination with vitamin K_3 against MRSA strains tested were 0.25 - 0.50 (Table 1). This indicated that azalomycin F_{5a} combined with vitamin K_3 presented synergistic anti-MRSA activities. Moreover, the MIC of azalomycin F_{5a} in combination could drop down to 0.5 - 1.0 μg/mL.

3.2. MIC_{99} and MPC of Azalomycin F_{5a} Alone and in Combination with Vitamin K_3

Various ratios of azalomycin F_{5a} to vitamin K_3 could be selected to determine their MIC_{99}s and MPCs in combination. Considering that Table 1 showed the ratios of azalomycin F_{5a} to vitamin K_3 in the MICs of combinations were 1:2 or 1:4 (m/m) except 1:8 or 1:16 (m/m) against MRSA HK03, the MIC_{99}s and MPCs of azalomycin F_{5a} in combination with vitamin K_3 (1:2 to 1:16), together with azalomycin F_{5a} and vitamin K_3 alone, were respectively determined. The MPC/MIC ratios, defined as the ratio of the MPC obtained to the original MIC_{99} alone, of azalomycin F_{5a} alone and in combination with vitamin K_3 (1:2 to 1:16) against MRSA ATCC 33,592 and HK03 were respectively 2.35 - 0.73 (Table 2) and 3.09 - 0.77 (Table 3). These indicated that all the MSWs of azalomycin F_{5a} were narrower, and even closed when the proportion of vitamin K_3 in combination increased up to 88.9% for MRSA ATCC 33,592 or 94.1% for MRSA HK03.

4. Discussion

4.1. MIC_{99}s and MPCs of Azalomycin F_{5a} in Combination with Vitamin K_3

Checkerboard test showed synergistic anti-MRSA activity of azalomycin F_{5a} in combination with vitamin K_3, while the potential of MRSA be resistant to azalomycin F_{5a} was

Table 1. Fractional inhibitory concentration indices (FICIs) of azalomycin F_{5a} in combination with vitamin K_3 against MRSA.

MRSA strains	Azalomycin F_{5a}				
	MICs (μg/mL)			FICIs	
	A[a]	B[b]	C	b	c
ATCC 33,592	4.0	1.0	1.0	0.375	0.50
HK01	4.0	1.0	1.0	0.375	0.50
HK02	4.0	1.0	1.0	0.375	0.50
HK03	4.0	0.5	0.5	0.25	0.375

[a]A: MICs of azalomycin F_{5a} alone. [b]B, C, b and c: The MICs (B and C) and FICIs (b and c) of azalomycin F_{5a} in combination with vitamin K_3 when the concentrations of vitamin K_3 were respectively 0.125 and 0.25 MIC.

Table 2. MIC_{99}s and MPCs of azalomycin F_{5a} alone and in combination with vitamin K_3 against MRSA[a].

Antimicrobials[b]	MIC (μg/mL)	Regression equation (r[c])	MIC_{99} (μg/mL)	MPC (μg/mL)	MPC/MIC_{99}[d]
AZF_{5a}	4.0	$y = 0.4251x + 0.0627$ (0.996)	2.18	5.12	2.35
Vit K_3	16.0	$y = 0.0916x + 0.0660$ (0.998)	10.09	32.00	3.17
AZF_{5a}/Vit K_3 (1:2)	1.0/2.0	$y = 2.0175x - 0.0947$ (0.995)	0.54	3.20	1.47
AZF_{5a}/Vit K_3 (1:4)	1.0/4.0	$y = 2.2363x + 0.0400$ (0.993)	0.42	2.56	1.17
AZF_{5a}/Vit K_3 (1:8)	ND[e]	$y = 1.4602x + 0.0382$ (0.994)	0.65	2.05	0.94
AZF_{5a}/Vit K_3 (1:16)	ND	ND	ND	1.60	0.73

[a]MRSA ATCC 33,592 was used as MRSA strain tested. [b]AZF_{5a} and Vit K_3 were abbreviations of azalomycin F_{5a} and vitamin K_3, respectively. [c]r, correlation coefficient of a binary regression equation. [d]MPC/MIC_{99} ratio is defined as the ratio of the MPC obtained to the original MIC_{99} alone. [e]ND, not tested.

Table 3. MIC_{99}s and MPCs of azalomycin F_{5a} alone and in combination with vitamin K_3 against MRSA[a].

Antimicrobials[b]	MIC (μg/mL)	Regression equation (r[c])	MIC_{99} (μg/mL)	MPC (μg/mL)	MPC/MIC_{99}[d]
AZF_{5a}	4.0	$y = 0.4929x - 0.0325$ (0.999)	2.07	6.40	3.09
Vit K_3	32.0	$y = 0.0630x - 0.0440$ (0.998)	16.41	81.92	4.99
AZF_{5a}/Vit K_3 (1:2)	ND[e]	ND	ND	3.20	1.54
AZF_{5a}/Vit K_3 (1:4)	ND	ND	ND	3.20	1.54
AZF_{5a}/Vit K_3 (1:8)	0.5/4.0	ND	ND	2.56	1.24
AZF_{5a}/Vit K_3 (1:16)	0.5/8.0	ND	ND	1.60	0.77

[a]MRSA HK03 was used as MRSA strain tested. [b]AZF_{5a} and Vit K_3 were abbreviations of azalomycin F_{5a} and vitamin K_3, respectively. [c]r, correlation coefficient of a binary regression equation. [d]MPC/MIC_{99} ratio is defined as the ratio of the MPC obtained to the original MIC_{99} alone. [e]ND, not tested.

unable to known in combination. Considering resistance would empirically emerge shortly after a new antibiotic is used, new antibiotics that MRSA be less resistant to was more value to develop. Thereby, the MIC_{99}s and MPCs of azalomycin F_{5a} and vitamin K_3 alone and in combination were determined according the hypotheses of MPC and MSW [5] [18]. Different concentrations of two antimicrobial agents in combination would lead to different MPCs and MIC_{99}s, while different combinations with reasonably designed ratios of two agents were rarely determined. Based on the concentration ratios of azalomycin F_{5a} to vitamin K_3 in combinations of above checkerboard test, the MPCs and MIC_{99}s of different combinations presented synergistic anti-MRSA activity were first determined for the best combination that MRSA was most difficult to be resistant to. The results showed that the best ratio of azalomycin F_{5a} to vitamin K_3 was 1:8 against MRSA ATCC 33,592 and 1:16 against MRSA HK03 in combination (Table 2 and Table 3), while that of vitamin K_3 to azalomycin F_{5a} was 2:1 against MRSA ATCC 33,592 and 4:1 against MRSA HK03 in combination.

To better understand the experimental results, researches on MPCs, MIC_{99}s and MSWs of azalomycin F_{5a} alone and in combination with vitamin K_3 were further carried forward using the schematic representation of their mutant selection windows

(Figure 2). The MPCs (Figure 2(a)) of azalomycin F_{5a} decreased along with the proportion of vitamin K_3 increased in combinations, and even those were less than the MIC_{99}s of azalomycin F_{5a} alone when the proportion of vitamin K_3 in combination increased up to 88.9% for MRSA ATCC 33,592 or 94.1% for MRSA HK03. Similarly, the MPCs (Figure 2(b)) of vitamin K_3 could also drop down to below its MIC_{99} alone when the proportion of azalomycin F_{5a} increased up to 33.3% for MRSA ATCC 33,592 or 20.0% for MRSA HK03. These will provide vitamin K_3 larger dose range to prevent resistance and to decrease its adverse effects in clinic use. Deduced from the report [19], azalomycin F_{5a} might lead a leakage of cellular substances by acting cell-membrance of *S. aureus*, while the synergistic anti-MRSA mechanism of azalomycin F_{5a} and vitamin K_3 were not made clear, and was value to further research.

4.2. Correlation between the Ratio of Azalomycin F_{5a}/Vitamin K_3 and the Sum of Their MPC/MIC_{99}s in Combination

As the MPCs of one in combination will change as those of another, is there a certain correlation between the proportions of two antimicrobials and their MPCs in combinations? To understand it, experimental data shown in Table 2 and Table 3 were further analyzed. The correlation between the ratio of azalomycin F_{5a}/vitamin K_3 (y) and the sum of the $MPC/MIC_{99Alone}$ (x) in combination was discovered, and can be respectively expressed as two binary regression equations are $y = 0.0577x^2 - 0.1752x + 2.345$ ($r = 0.91$) to MRSA ATCC 33592 and $y = 0.3018x^2 - 1.8019x + 4.588$ ($r = 0.91$) to MRSA HK03. Using these two regression equations, we may predict the MPCs of azalomycin F_{5a} in combinations with different proportional vitamin K_3, and deduce the perfect ratio of azalomycin F_{5a} and vitamin K_3 in combination. Moreover, the correlation between

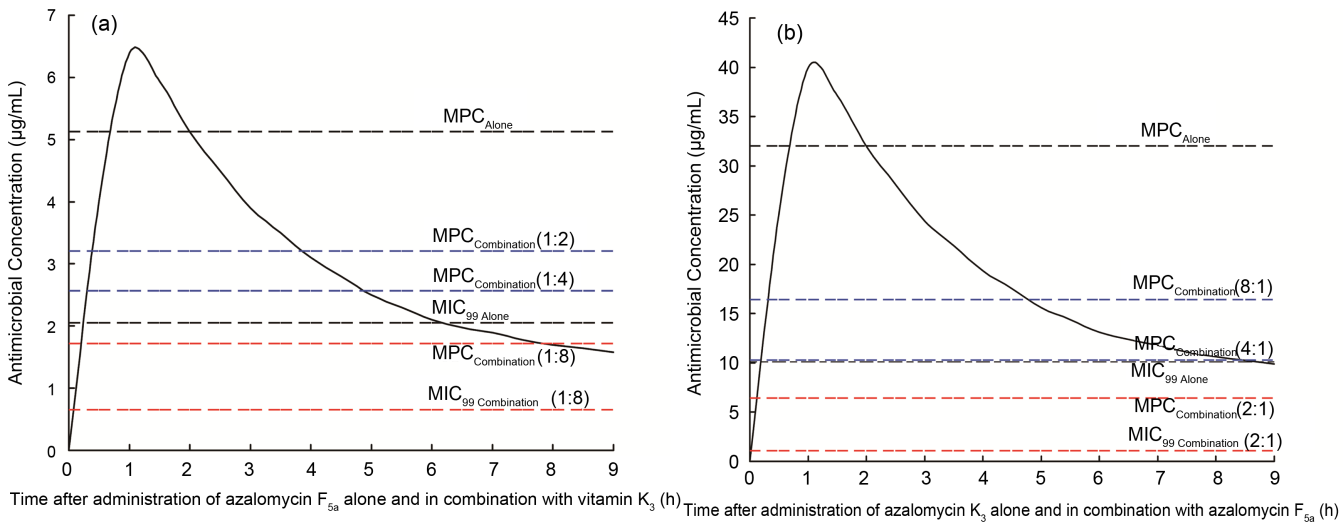

Figure 2. Schematic representation of the MPCs and MIC_{99}s of azalomycin F_{5a}-vitamin K_3 against MRSA. The MPCs and MICs of azalomycin F_{5a} alone or in combination (a) and those of vitamin K_3 (b) were determined with agar plate data utilizing linear antimicrobial concentration decrease. *x*-axis represented time (h) after administration of azalomycin F_{5a} or vitamin K_3; *y*-axis simulated the antimicrobial concentration (μg/mL). Dashed lines, MIC_{99} and MPC. $MIC_{99Alone}$ and MPC_{Alone} were the MIC_{99} and MPC of azalomycin F_{5a} or vitamin K_3 alone, and $MIC_{99Combination}$ and $MPC_{Combination}$ were the MIC_{99} and MPC of azalomycin F_{5a} or vitamin K_3 in combination.

the ratio of two antimicrobials and the sum of the $MPC/MIC_{99Alone}$ in combination to a certain pathogenic microorganism may be correlative although that observed between MIC and MPC was low ($r^2 = 0.39$) [5] [20].

Although the MPC of azalomycin F_{5a} or vitamin K_3 in combination can be dropped down to below its MIC_{99} alone by increasing the proportion of another, two new MSWs ($MIC_{99Combination}$-$MPC_{Combination}$) of azalomycin F_{5a} and vitamin K_3 against MRSA ATCC 33,592 emerged (**Figure 2** and **Table 1**). That is to say, the resistant mutant is also easy to enrich when the concentrations of azalomycin F_{5a} and vitamin K_3 fall within their MSW in combination. According to the MPC and MSW hypotheses, only that no new MSW appears can completely prevent resistance when synergistic combination closed all the original MSW of each antimicrobial, while it is hardly to find the combination like this. To prevent resistant mutant and to reduce the adverse effect of antimicrobial agents, we can make an effort to discovery synergistic combinations making their MPCs decrease as much as possible. Another, we may reduce the clinical practice of combination therapy at random as possible as we can because some unfavorable combination will enrich the resistant mutant.

4.3. Some Opinions on Antimicrobial Combination and Synergistic Strategy

Antibiotic resistance, a part of natural evolution, can be significantly slowed but not be stopped. Developing new antibiotics is still an effective strategy in the battle against antibiotic resistance when we optimize the use of exist antimicrobial agents. According to the MPC and MSW hypotheses, new antimicrobials with narrow MSW will present more potential to prevent resistance. The synergistic anti-MRSA activities of azalomycin F_{5a} in combination with vitamin K_3, coupled with the narrow MSW of azalomycin F_{5a} against MRSA tested, indicated that azalomycin F_{5a} has a good potential to develop as a new antimicrobial agent [5] [18].

Combination therapy with two or more drugs is the standard treatment for infections with Mycobacterium tuberculosis, human immunodeficiency virus (HIV) and Plasmodium falciparum [2] [21] [22]. As the development of new antimicrobial agents has not kept pace with resistance, combination therapy has been considered as one strategy to delay the spread of antimicrobial resistance. Many antimicrobial combinations have been studied for synergy in vitro and in vivo, while the data on the effects of combination therapy to prevent resistance are conflicting [22]. Even more, some combination may result in high mutational frequencies, such as the combination of levofloxacin with low dose colistin [9]. Based on the above facts and our experimental data, we deduced that the validity of synergistic combination is a key to prevent or delay resistance. To analyze the probable reasons of the above conflicting evidences and to give some opinions on effective and synergistic combination, some opinions on antimicrobial combination and synergistic strategy were put forward to prevent resistance as follows:

1) Selecting remarkably synergistic and susceptible antimicrobial agents for combi-

nation: According to the MSW and MPC hypotheses, the less the MPC in combination, the larger the dosing range to prevent resistance is, and the easier maintaining antimicrobial concentration above their MPCs throughout combination therapy is. Perfectly, the MPC of each antimicrobial in combination drop down to below its MIC_{99} alone, and the $MPC_{Combination}/MIC_{99Combination}$ of each antimicrobial in combination is equal to one. That is to say, no new MSW of each antimicrobial in combination emerge when synergistic combination closes the original MSW of two antimicrobials. Thereby, we can determine the FICIs of antimicrobial combination, and select those presenting less FICIs for combination. Further, two antimicrobials that possessed different action mechanisms to the same pathogenic bacteria should theoretically present synergistic activity, and display a significantly reduced MPC in combination. Thereby, we can select antimicrobials had different action mechanisms for the synergistic evaluation of antimicrobial combination.

2) As different proportion of each antimicrobial in synergistic combination would present their different MPCs, maintaining antimicrobial concentrations in vivo, especially in blood and in infection site, above their MPCs throughout combination therapy is a key for synergistic combination to prevent resistance, and this may be partly responsible for the conflicting results reported [9] [22]. Generally, drugs with similar pharmacokinetics will present consistent character in absorption, distribution, metabolism and excretion. These can decrease the proportional fluctuation of each antimicrobial in blood and in infection sites, and help to keep the concentrations of antimicrobials above their MPCs if we select antimicrobials with similar pharmacokinetics for combination. Moreover, a helpful administration to achieve the purpose is using sustained and controlled release preparations. Together with less effective dose and lager allowable range when the MPCs of each antimicrobial drop down to below their MIC_{99}s alone in synergistic combination, this can further keep excellent antibacterial effect, and remarkably decrease the side effects of each antimicrobial and the probability of new adverse toxic effects due to combination.

3) To prevent resistance, three interacted aspects include pathogenic microorganism, antimicrobial combination and human body should be systematically taken into account. As the absorption, distribution, metabolism and excretion of each antimicrobial in combination are different in human body, different proportion of each antimicrobial in synergistic combination would present their different MPC. If the antimicrobial concentration is less than its MPC in combination in the infection site or in blood of human body last for a long time, combination will accelerate the resistant mutant on the contrary. Moreover, antimicrobial concentration ranged from $MIC_{99Combination}$ to $MPC_{Combination}$ (especially to $MIC_{Combination}$) will stimulate the formation of biofilm and persisters, and this may enhance the resistance or tolerance to antimicrobials.

As we known, microorganisms in human body can promote and restrain each other to form balanced microorganism communities, and which can help us to defend pathogenic infection. Many antimicrobial agents can destruct the microorganism communities [23], and promote others growth and resistance when kill some pathogenic

microorganisms. These circumstances are unfavorable for preventing resistance and combination therapy. Simultaneously, many pathogenic microorganisms can form biofilm, complex biofilm and persisters to be resistant or tolerant to these antimicrobial agents on the contrary [24] [25] [26] [27]. Further, the side effects of antimicrobials and new ones generated from combination likely have harmful effects on our defense system sometimes, and then will weaken our defense system killing pathogenic microorganisms and eliminating toxin [23]. These above factors may be some reasons that combination therapy reported present conflicting results [9] [22].

4) Although the general benefits of combination therapy compared with single or sequential administration of antibiotics for treating bacterial infections have been difficult to conclusively demonstrate, and some clinical data have been conflicting [9] [22], we have enough reasons to believe that combination has affirmable advantages to prevent or delay the emergence of resistance during antimicrobial therapy. Some conflicting results about combination therapy will be still observed before we thoroughly make the rule of reasonable combination clear and take a variety of probable factors into account in combination. In this case, we can try our best to discovery a variety of synergistic antimicrobial agents or compounds. Further, a new antimicrobial agent together with one or more synergistic antimicrobials as a regular combination is encouraged to be approved, and even as a hybrid or multi-hybrid antibiotic such as rifamycin-quinolone [28].

5. Conclusion

Azalomycin F_{5a}, a 36-membered macrocyclic lactone, combined with vitamin K_3 showed synergistic anti-MRSA activities. Simultaneously, the MPC of azalomycin F_{5a} in combination could drop down to below its MIC_{99} alone when the proportion of vitamin K_3 increased, and the narrow and even closed MSWs like this indicated that azalomycin F_{5a} has a good potential to develop as a new antimicrobial agent. Further, the correlation between the ratio of azalomycin F_{5a}/vitamin K_3 and the sum of the MPC/MIC_{99} in combination was observed, and be expressed as a binary regression equations to a certain MRSA strain. Thus, the correlation between the ratio of two antimicrobials and the sum of the MPC/MIC_{99} was first found, which could be used to predict the MPCs and the perfect ratio of two antimicrobials in combination. Moreover, some opinions on antimicrobial combination and synergistic strategy were put forward to prevent drug resistance based on the analyses of experimental data and documents.

Conflicts of Interest

The authors declare no conflict of interest.

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