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In vitro Activity of Sparfloxacin and Its Combination with Efflux Pump Blockers Against Methicillin-Resistant *Staphylococcus aureus* and *Escherichia coli*

Rahima Tahir[1], Neha Farid[1], Khansa Syed[1], Misbah Mohammad Hanif[1], Asma Bashir[1], Kashif Ali[1]

[1]*Department of Biosciences, Faculty of Life Sciences, Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST) University, Karachi, Pakistan.*

ABSTRACT

Background: Multi-drug resistance has emerged as a significant global health challenge that has resulted in the occurrence of numerous diseases. This paper highlighted the potential of Sparfloxacin as a solution to antimicrobial resistance when used alone or in combination with efflux pump blockers. Sparfloxacin is a fluoroquinolone antibiotic that inhibits the bacterial DNA gyrase, thereby preventing DNA replication. The purpose of this study was to determine the efficacy of Sparfloxacin against MRSA and *E. coli*, and whether it could be used either solely or in combination with the efflux pump blockers to overcome multidrug resistance.

Methods: The study targeted two pathogens, Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli (E. coli)*. The research methods included the determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of Sparfloxacin solely, and in combination with Tamoxifen and Verapamil. The study was conducted over a time period of one year and the study design was basic.

Results: The effective concentration of Sparfloxacin was found to be 0.001µg/ml for MRSA and 5µg/ml for *E. coli*. Whereas, when used in combination with the efflux pump blocker, the effective concentration was found to be 0.31µg/ml with 128µg/ml of Tamoxifen for both the microbes. Verapamil with Sparfloxacin (0.31µg/ml) was found to be effective with 24µg/ml as MBC, and 12µg/ml as MIC value against Methicillin-Resistant *Staphylococcus aureus*. For *E. coli*, MBC value of Verapamil with Sparfloxacin (20µg/ml) was found to be 166.66µg/ml.

Conclusion: The study suggested that Sparfloxacin could be a potential solution to multi-drug resistance even when used solely, unlike other antibiotics, which show better efficacy at higher concentrations. Tamoxifen and Verapamil can be used with other antibiotics to enhance their efficacy against the multidrug-resistant strains.

Keywords: Sparfloxacin, Antimicrobial Resistance, *Escherichia coli*, Minimum Inhibitory Concentration.

Corresponding Author:
Miss Neha Farid,
Department of Biosciences, Faculty of Life Sciences
Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST) University,
Karachi, Pakistan.
Email: neha_farid@hotmail.com
ORCID: https://orcid.org/0000-0003-4051-4674
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INTRODUCTION

Multi-drug resistance (MDR) is a growing problem that poses a significant challenge to public health worldwide. Methicillin-Resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli* have become antimicrobial resistant and emerged as superbugs that are difficult to treat [1, 2]. One of the mechanisms that pathogens use to develop resistance is through efflux pumps. These pumps function as channels to flush out antibiotics from the bacterial cell, thereby reducing their effectiveness [3, 4].

Sparfloxacin is a member of the fluoroquinolone class of antibiotics. Its mechanism of action involves the inhibition of bacterial DNA gyrase, an essential enzyme required for DNA replication in bacteria [5]. This results in DNA damage, ultimately leading to bacterial cell death. Efflux pump blockers are compounds that can inhibit the activity of efflux pumps, thereby increasing the efficacy of antibiotics within the bacterial cell [6, 7]. Tamoxifen and Verapamil have been reported to inhibit the activity of efflux pumps. Tamoxifen is a non-steroidal, selective estrogen receptor modulator (SERM) medication that is used to treat breast cancer in both men and women [8,9]. Verapamil is a calcium channel blocker and is a derivative of phenylethylamine. It works by attaching to the cytochrome P450 system and blocking the voltage-dependent calcium channels [10]. Efflux pump inhibitors are being used in multiple studies to overcome antimicrobial resistance [11, 12].

Combination of efflux pump blockers with Sparfloxacin is a promising approach to solve the problem of multi drug resistance. The study's objective was to assess the effect of Sparfloxacin solely, and in combination with the efflux pump blockers, Tamoxifen and Verapamil against the MDR pathogens by determining the effective concentration of the antibiotic and that of efflux pump blockers in combination with the antibiotic.

METHODS

Five strains each of Methicillin Resistant *Staphylococcus aureus* and *Escherichia coli* isolated from clinical samples were used in the study. Both of the bacterial species exhibited multi-drug resistance against the antibiotics. The study was conducted in the Department of Biosciences, SZABIST University, over a time period of one year. Sparfloxacin was used as an antibiotic, whereas Tamoxifen, and Verapamil were used as the efflux pump blockers. All the chemicals were purchased from Sigma-Aldrich and Oxoid. The study was approved from the Institutional Ethical Review Board (IERB) with Reference No. IERB (20)/SZABIST-KHI(BIO)/250053.

For MIC, a two-fold serial dilution of the drugs was done, and the culture was added adjusted to 0.5 McFarland Turbidity Index, and then incubated for 24 hours at 37°C [13]. The tubes were checked for turbidity. For MBC, the tubes of MIC having no or less turbidity were then swabbed on the Muller Hinton Agar followed by incubation at 37°C for 24 hours [14,15].

RESULTS

Table 1: Bactericidal Activity of Sparfloxacin

SPARFLOXACIN (ANTIBIOTIC)					
Methicillin-Resistant *Staphylococcus aureus*					
Concentrations	**Strain 01**	**Strain 02**	**Strain 03**	**Strain 04**	**Strain 05**
10µg/ml	100	100	100	100	100
5µg/ml	100	100	100	100	100
2.5µg/ml	100	100	100	100	100
1.25µg/ml	100	100	100	100	100
0.62µg/ml	100	100	100	100	100
0.31µg/ml	100	100	100	100	100
0.155µg/ml	100	100	100	100	100
0.077µg/ml	100	100	100	100	100
0.038µg/ml	100	100	100	100	100
0.019µg/ml	100	100	100	100	100
0.009µg/ml	100	100	100	100	100
0.004µg/ml	100	100	100	100	100

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0.002µg/ml	100	100	100	100	100
0.001µg/ml	100	100	100	100	100
0.0006µg/ml	75	75	75	75	75
Escherichia coli					
Concentration	**Strain 01**	**Strain 02**	**Strain 03**	**Strain 04**	**Strain 05**
20µg/ml	100	100	100	100	100
10µg/ml	100	100	100	100	100
5µg/ml	100	100	100	100	100
2.5µg/ml	75	75	75	75	75

Values refer to the percent inhibition of bacterial growth in the presence of Sparfloxacin

The efficacy of Sparfloxacin against Methicillin-Resistant *Staphylococcus aureus* was initially evaluated for the MIC value ranging from 0.31µg/ml to 0.0006µg/ml through two-fold serial dilutions. Subsequently, after the same incubatory period, it was noted that there was a slight turbidity in the tube that contained 0.0006µg/ml of the antibiotic. All ten tubes were further tested for Minimum Bactericidal Concentration, and it was determined that no bacterial growth was observed up to the concentration of 0.001µg/ml. Thus, based on the obtained results, it was concluded that 0.001µg/ml represents the MBC, while 0.0006µg/ml represents the MIC. All five strains of MRSA exhibited uniform results.

The effectiveness of the antibiotic against *Escherichia coli* was assessed through a range of concentrations from 20µg/ml to 0.312µg/ml. Following the Minimum Inhibitory Concentration test, it was noted that the first four tubes ranging from 20µg/ml to 2.5µg/ml were clear, indicating the absence of bacteria in the presence of the antibiotic. To determine the Minimum Bactericidal Concentration, the tubes were assessed for bacterial growth following the incubation period. The results indicated that there was no bacterial growth for Sparfloxacin concentrations between 20µg/ml to 2.5µg/ml, while moderate bacterial growth was observed at concentrations of 1.25µg/ml and 0.625µg/ml. Thus, it was inferred that the concentration of 1.25µg/ml represents the Minimum Inhibitory Concentration, while the concentration of 2.5µg/ml represents the Minimum Bactericidal Concentration. The results have been summarized in **Table 1.**

Table 2: Antimicrobial Activity of Sparfloxacin with Tamoxifen at Various Concentrations Against Methicillin-Resistant *Staphylococcus aureus*

Combo A- (Tamoxifen and Sparfloxacin at 1.25 µg/ml)										
Concentration of Tamoxifen	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
256 µg/ml	100	100	100	100	100	100	100	100	100	100
128 µg/ml	75	100	75	100	75	100	75	100	75	100
64 µg/ml	50	100	50	100	50	100	50	100	50	100
32 µg/ml	25	100	25	100	25	100	25	100	25	100
Combo B- (Tamoxifen and Sparfloxacin at 0.62 µg/ml)										
Concentration of Tamoxifen	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
256 µg/ml	100	100	100	100	100	100	100	100	100	100
128 µg/ml	50	100	50	100	50	100	50	100	50	100
64 µg/ml	25	100	25	100	25	100	25	100	25	100
32 µg/ml	25	100	25	100	25	100	25	100	25	100
Combo C- (Tamoxifen and Sparfloxacin at 0.31 µg/ml)										
Concentration of Tamoxifen	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
256 µg/ml	100	100	100	100	100	100	100	100	100	100
128 µg/ml	100	100	100	100	100	100	100	100	100	100
64 µg/ml	100	75	100	75	100	75	100	75	100	75
32 µg/ml	100	75	100	75	100	75	100	75	100	75
16 µg/ml	75	75	75	75	75	75	75	75	75	75

Values refer to the percent inhibition of bacterial growth in the presence of Sparfloxacin with Tamoxifen

For the combination studies, Tamoxifen was used at concentrations ranging from 256µg/ml to 2µg/ml. Three concentrations of Sparfloxacin (1.25µg/ml, 0.62µg/ml, and 0.31µg/ml) were tested with all the concentrations of Tamoxifen to evaluate their antimicrobial efficacy.

The results showed that Sparfloxacin at a concentration of 1.25µg/ml was effective against all five strains of MRSA when used in combination with Tamoxifen at a concentration of 256µg/ml. As evident from the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) assays, which showed no bacterial growth. Therefore, the MIC of Tamoxifen with Sparfloxacin (1.25µg/ml) was determined as 128µg/ml, and the MBC value as 256µg/ml. These results were consistent across all five strains of MRSA. Similarly, at a concentration of 0.62µg/ml of Sparfloxacin, the bactericidal activity was found at 256µg/ml, which is inhibitory at 128µg/ml of Tamoxifen.

However, when tamoxifen was used with 0.31µg/ml of Sparfloxacin, bactericidal concentrations were observed at both 256 µg/ml and 128 µg/ml, with inhibition at 64 µg/ml. Therefore, the MIC and MBC against Sparfloxacin (0.31 µg/ml) are 128 µg/ml and 64 µg/ml, respectively.

These results indicate that the most effective concentration of the antibiotic is 0.31 µg/ml. Additionally, it can be inferred that the low concentrations of antibiotics can achieve higher efficacy when used in conjunction with efflux blockers at high concent rations, as shown in **Table 2.**

Table 3: Antimicrobial Activity of Sparfloxacin with Tamoxifen at Various Concentrations Against *Escherichia coli*

Combo 1 (Tamoxifen and Sparfloxacin at 20µg/ml)				
Concentration of Tamoxifen	***Escherichia coli* 01**		***Escherichia coli* 02**	
	MIC	MBC	MIC	MBC
500µg/ml	100	100	100	100
250µg/ml	75	75	75	75
125µg/ml	50	50	50	50
62.5µg/ml	50	25	50	25
31.25µg/ml	25	25	25	25
Combo 2 (Tamoxifen and Sparfloxacin at 10µg/ml)				
Concentration of Tamoxifen	***Escherichia coli* 01**		***Escherichia coli* 02**	
	MIC	MBC	MIC	MBC
500µg/ml	75	75	75	75
250µg/ml	50	25	50	25
125µg/ml	50	0	50	0
62.5µg/ml	50	0	50	0
31.25µg/ml	25	0	25	0
Combo 3 (Tamoxifen and Sparfloxacin at 5µg/ml)				
Concentration of Tamoxifen	***Escherichia coli* 01**		***Escherichia coli* 02**	
	MIC	MBC	MIC	MBC
500µg/ml	50	75	50	75
250µg/ml	50	50	50	50
125µg/ml	50	25	50	25
62.5µg/ml	50	25	50	25
31.25µg/ml	25	25	25	25

Values refer to the percent inhibition of bacterial growth in the presence of Sparfloxacin with Tamoxifen

For combination testing against *Escherichia coli*, Tamoxifen was tested at concentrations ranging from 500µg/ml to 3.90µg/ml. Three sets of combinations were prepared using Sparfloxacin with concentrations of 20µg/ml, 10µg/ml, and 5µg/ml. With 20µg/ml of Sparfloxacin, the minimum bactericidal concentration of Tamoxifen was observed at 500 µg/ml, and MIC at 250µg/ml. Tamoxifen, when used at the same concentrations against Sparfloxacin (10µg/ml), 500µg/ml was interpreted as the Minimum Inhibitory Concentration (MIC). Similar results were found when Tamoxifen was tested with 5µg/ml of Sparfloxacin. The findings have been tabulated in **Table 3.**

Table 4: Antimicrobial Activity of Sparfloxacin with Verapamil Against Methicillin-Resistant *Staphylococcus aureus*

A-(Verapamil and Sparfloxacin at 1.25µg/ml)										
Concentration of Verapamil	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
24µg/ml	100	100	100	100	100	100	100	100	100	100
12µg/ml	100	100	100	100	100	100	100	100	100	100
6µg/ml	100	100	100	100	100	100	100	100	100	100
3µg/ml	100	100	100	100	100	100	100	100	100	100
1.5µg/ml	100	75	100	75	100	75	100	75	100	75
B-(Verapamil and Sparfloxacin at 0.625µg/ml)										
Concentration of Verapamil	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
24µg/ml	100	100	100	100	100	100	100	100	100	100
12µg/ml	50	100	50	100	50	100	50	100	50	100
6µg/ml	50	100	50	100	50	100	50	100	50	100
3µg/ml	25	100	25	100	25	100	25	100	25	100
C-(Verapamil and Sparfloxacin at 0.31µg/ml)										
Concentration of Verapamil	**MRSA 01**		**MRSA 02**		**MRSA 03**		**MRSA 04**		**MRSA 05**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
24µg/ml	100	100	100	100	100	100	100	100	100	100
12µg/ml	75	100	75	100	75	100	75	100	75	100
6µg/ml	75	100	75	100	75	100	75	100	75	100
3µg/ml	50	100	50	100	50	100	50	100	50	100
1.5µg/ml	0	50	0	50	0	50	0	50	0	50

Values refer to the percent inhibition of bacterial growth in the presence of Sparfloxacin with Verapamil.

Verapamil was the other efflux pump inhibitor to be used in combination with Sparfloxacin at various concentrations. For antimicrobial testing against MRSA, Verapamil was tested at concentrations ranging from 24µg/ml to 1.5µg/ml, while Sparfloxacin was tested at concentrations of 1.25µg/ml, 0.625µg/ml, and 0.31µg/ml. The most effective concentration of Verapamil with Sparfloxacin (0.31µg/ml) was found to be as MBC value of 24µg/ml and MIC value of 12µg/ml against Methicillin-Resistant *Staphylococcus aureus* as shown in **Table 4.**

Table 5: Antimicrobial activity of Sparfloxacin with Verapamil against *Escherichia coli*

A-(Verapamil and Sparfloxacin at 20µg/ml)				
Concentration of Sparfloxacin	***Escherichia 01***		***Escherichia 02***	
	MIC	**MBC**	**MIC**	**MBC**
166.66µg/ml	100	100	100	100
83.33µg/ml	75	50	75	50
41.65µg/ml	75	50	75	50
20.825µg/ml	75	50	75	50
10.4125µg/ml	50	50	50	50
B-(Verapamil and Sparfloxacin at 10µg/ml)				
Concentration of Sparfloxacin	***Escherichia 01***		***Escherichia 02***	
	MIC	**MBC**	**MIC**	**MBC**
166.66µg/ml	75	50	75	50

83.33µg/ml	75	50	75	50
41.65µg/ml	75	50	75	50
20.825µg/ml	75	50	75	50
10.4125µg/ml	50	50	50	50
C-(Verapamil and Sparfloxacin at 5µg/ml)				
Concentration of Sparfloxacin	***Escherichia 01***		***Escherichia 02***	
	MIC	**MBC**	**MIC**	**MBC**
166.66µg/ml	50	75	50	75
83.33µg/ml	50	75	50	75
41.65µg/ml	50	75	50	75
20.825µg/ml	50	75	50	75
10.4125µg/ml	25	50	25	50

Values refer to the percent inhibition of bacterial growth in the presence of Sparfloxacin with Tamoxifen.

A combo test of Sparfloxacin with Verapamil against *Escherichia coli* was performed in the concentrations of 20µg/ml, 10µg/ml, and 5µg/ml, whereas Verapamil was used in the concentration of 166.66µg/ml to 10.4125µg/ml. The first combo revealed the MBC value of Verapamil with Sparfloxacin (20µg/ml) as 166.66µg/ml. Whereas, when Sparfloxacin was used in the concentrations of 10µg/ml and 5µg/ml with Verapamil, bacterial growth was observed at 166.66µg/ml. Based on the obtained results, it was inferred that Verapamil demonstrated bactericidal activity in combination with a high concentration of Sparfloxacin, as shown in **Table 5.**

DISCUSSION

The study evaluated the role of Sparfloxacin and efflux pump blockers as antimicrobials against the pathogenic bacterial strains of Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Escherichia coli*. In this study, it was determined that Verapamil acts as a potent efflux pump blocker when used with a high concentration of Sparfloxacin. Whereas a lower concentration of Sparfloxacin can be used in combination with Tamoxifen. The novelty of the study lies in the combination testing of Sparfloxacin with the efflux pump blockers. It was also found that Sparfloxacin at a remarkably low concentration of 0.001µg/ml exhibits bactericidal activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Since Sparfloxacin shows an effective activity against the MDR strains without any efflux pump blocker, it is preferable to use the antibiotic in the least effective concentration, as determined to of 0.001µg/ml. We can also conclude that treating lethal infections caused by MRSA with this concentration of Sparfloxacin can be highly beneficial since it will reduce the adverse effects associated with a higher concentration of the drug.

Multidrug resistance in microbial strains has become a significant problem in the disciplines of medicine and life sciences, leading to numerous complications[16]. The use of efflux pump blockers is one of the most promising solutions to overcome antimicrobial resistance[17]. Efflux pump blockers can enhance the efficacy of the antibiotics by restoring them in the cells and preventing their movement outside the cell [18,19].

Tamoxifen is a selective estrogen receptor modulator (SERM) and has two mechanisms of action. Firstly, it works by blocking the role of E2 in cancer by competing with 17β-estradiol (E2) at the receptor site. Secondly, after metabolic activation, it binds DNA and initiates carcinogenesis [3,20]. Moreover, Verapamil is a calcium channel blocker that works by inhibiting the calcium ion influx in the cells of the cardiac conductile system [21,22]. Studies have been conducted where both Tamoxifen and Verapamil appeared to be promising efflux pump blockers. Verapamil has been shown to have a lower activity as compared to Tamoxifen due to its first-pass metabolism, which leads to its instant removal from the cell [23]. In previous studies, Verapamil and Tamoxifen are effective against *Mycobacterium tuberculosis, Pseudomonas aeruginosa*, and *Escherichia coli Staphylococcus aureus* in combination with the various antibiotics [24,25].

CONCLUSION

Efflux pump inhibitors are potential therapeutic agents that could enhance the efficacy of antibiotics against pathogens. The use of efflux pump blockers (Tamoxifen and Verapamil) in combination with Sparfloxacin serves as a stepping

stone towards further studies targeting efflux pump blockers with ineffective antibiotics to overcome multidrug resistance.

CONFLICT OF INTEREST
None

FUNDING
None

ETHICAL APPROVAL
The ethical approval obtained from the Department of Biosciences, Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST), University reference No. IERB (20)/SZABIST-KHI(BIO)/250053.

AUTHORS' CONTRIBUTIONS
RT and NF did the manuscript writing and editing; **RT and AB** did the result analysis; **KS and MMH** did the initial writing and experimentation; **NF** designed and supervised the study; **KA** did the formal analysis of the manuscript and results. All authors have reviewed the manuscript.

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