Turk J Pharm Sci 2021;18(1):68-74
DOI: 10.4274/tjps.galenos.2019.23500

ORIGINAL ARTICLE

Repurposing of Tamoxifen Against the Oral Bacteria

Tamoksifenin Oral Bakterilere Karşı Yeniden Konumlandırılması

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ABSTRACT

Objectives: Tamoxifen (TAM), which is used for treating breast cancer, has exhibited another important function as an antimicrobial agent. The objective of this study is to investigate the antibacterial action of TAM against the bacteria present in the human oral cavity.

Materials and Methods: The bacteria present in the human oral cavity were isolated from healthy individuals. Different concentrations of TAM were tested against the isolated bacteria. Additionally, bactericidal and bacteriostatic effects of TAM were also determined.

Results: Out of 23 isolated bacteria, a greater number of Gram-positive bacteria were highly susceptible to the low concentrations of TAM than Gram-negative bacteria. *Kytococcus sedentarius*, which is Gram-positive bacterium, and *Pseudomonas stutzeri*, which is Gram-negative bacterium, needed a high minimum inhibitory concentration value of TAM (2.5 mg/mL) to be inhibited by TAM's bacteriostatic action. Resistance to TAM was also observed in three strains of Gram-positive and four strains of Gram-negative bacteria.

Conclusion: TAM has shown a potential antibacterial effect against the bacteria present in the oral cavity, especially against Gram-positive bacteria. This effect is mostly bacteriostatic. This study also found bacterial resistance toward TAM.

Key words: Bacteria, oral cavity, tamoxifen, Gram-positive, Gram-negative

ÖZ

Amaç: Meme kanserinin tedavisinde kullanılan tamoksifenin (TAM), antimikrobiyal ajan olarak önemli bir etkisi daha vardır. Bu çalışmanın amacı, TAM'nin insan ağız boşluğunda bulunan bakterilere karşı antibakteriyel etkinliğini araştırmaktır.

Gereç ve Yöntemler: İnsan ağız boşluğunda bulunan bakteriler sağlıklı bireylerden izole edildi. İzole edilen bakterilere karşı farklı TAM konsantrasyonları test edildi. Ek olarak, TAM'nin bakterisidal ve bakteriyostatik etkileri de belirlendi.

Bulgular: İzole edilen 23 bakteriden Gram-negatif bakterilere kıyasla daha fazla sayıda Gram-pozitif bakterinin, düşük TAM konsantrasyonlarına yüksek oranda duyarlı olduğu saptandı. Gram-pozitif bakteri olan *Kytococcus sedentarius* ve Gram-negatif bakteri olan *Pseudomonas stutzeri*, TAM'nin bakteriyostatik etkisiyle inhibe edilebilmeleri için yüksek bir minimum inhibitör konsantrasyon değerine (2,5 mg/mL) ihtiyaç duydukları saptandı. TAM'ye direnç, üç Gram-pozitif ve dört Gram-negatif bakteri suşunda da gözlenmiştir.

Sonuç: TAM, özellikle Gram-pozitif bakteriler olmak üzere ağız boşluğunda bulunan bakterilere karşı potansiyel antibakteriyel etki göstermiştir. Bu etki çoğunlukla bakteriyostatiktir. Bu çalışmada aynı zamanda TAM'ye karşı bakteriyel dirençte gösterilmiştir.

Anahtar kelimeler: Bakteri, ağız boşluğu, tamoksifen, Gram-pozitif, Gram-negatif

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Received: 01.10.2019, **Accepted:** 12.12.2019

INTRODUCTION

As a member of selective estrogen receptor modulators, tamoxifen (TAM) is mainly used in the treatment and prevention of estrogen-positive breast cancer.[1,2] It was first introduced by AstraZeneca, UK as a more effective therapy for estrogen-positive breast cancer in women of Pakistan and Australia.[3] After more than four decades, TAM is considered as a golden drug for the treatment of breast cancer and extending the lives of approximately 500,000 women every year worldwide.[3-5] The chemopreventive usage of TAM is another approach approved by the US Food and Drug Administration to protect women from breast cancer for at least five years.[4,6] After two years of postoperative application, a study also found that TAM had the ability to reduce mortality resulting from coronary heart disease.[7]

The potential antimicrobial activities of TAM against various pathogenic organisms have prompted researchers to conductdrug repurposing.[8] Various studies have confirmed the direct and indirect antibacterial activities of TAM against different types of bacteria. TAM has shown a direct inhibitory action on the growth of *Staphylococcus aureus* and methicillin-resistant *S. aureus* (MRSA) strains,[9] and also against *Mycobacterium tuberculosis*.[10,11] The growth of *Enterococcus faecium* and its pathogenesis was found to be reduced by TAM after its administration in a *Galleria mellonella* infection model.[12] Meanwhile, the indirect antibacterial action of TAM was observed when it enhanced the activity of immune cells represented by neutrophils against various pathogenic bacteria such as MRSA, *Pseudomonas aeruginosa*, and *Escherichia coli* through the agonist activity of the G protein-coupled estrogen receptor.[13] Moreover, the antibacterial action of standard antibiotics such as polymyxin Bis also increased against various bacteria after combination with TAM.[14]

The treatment of breast cancer by TAM usually takes a longer period that could extend up to five years;[3] therefore, the normal bacterial flora of the human body, such as in the oral cavity, could be affected by the potential antibacterial activity of TAM. Hence, the objective of this study is to investigate the *in vitro* antibacterial activity of TAM against the oral flora of the human body.

MATERIALS AND METHODS

Chemicals

TAM citrate was purchased from Ebewe Pharma, Austria. Müller Mueller-Hinton agar (MHA) and Müller Mueller-Hinton broth (MHB) were purchased from HiMedia, India.

Isolation of bacteria

The swab samples were collected from the oral cavity of the healthy volunteers by sterilized cotton swabs. Then, the collected samples were cultured on blood agar and MacConkey agar (HiMedia, India). Inoculated media were incubated at 37°C for 24 hours for growing suspected bacteria. Diagnosis of the isolated bacteria was performed by the Vitek® 2 system using Vitek® 2 YST ID diagnostic cards for Gram-positive and Gram-negative bacteria (BioMérieux, France).

Determination of minimum inhibitory concentration (MIC) of TAM

The MIC of TAM was determined by the methods of dilution antimicrobial susceptibility tests for bacteria that grow aerobically as mentioned by CLSI-M07-A10 (2015).[15] A broth culture of isolated bacteria was prepared by the selected inoculum bacteria in MHB and was incubated at 37°C for 24 hours. The turbidity of growing bacterial cells was adjusted with 0.5 McFarland standard to contain approximately $1x10^8$ CFU/mL. Serial concentrations of TAM (5, 2.5, 1.25, and 0.625 mg/mL) were prepared from a stock solution (10 mg/mL). Plastic microdilution plates (96-well plates) were used to determine the MIC values of TAM. Each well of the plate received 50 µL from the standard count of each bacterial suspension and 100 µL from MHB, followed by adding 50 µL of specific concentration of TAM. Several controls were used within a microdilution plate, including MHB with only bacteria, MHB without bacteria, and MHB with only TAM. The inoculated plate was incubated at 37°C for 24 hours. The results were visually read observed as the presence or absence of bacteria growth.

Bactericides and the bacteriostatic effect of TAM were determined by re-culturing the growth-inhibited bacteria from the microdilution plate on MHA and then incubating the same at 37°C for 24 hours. The growth of inhibited bacteria indicated bacteriostatic action, whereas the absence of growth showed bactericidal effects.

Ethical approval

The study was ethically approved by the local Ethics Committee of the College of Medicine of the University of Karbala in July 20, 2019.

Statistical analysis

Data of all the tests were expressed as mean ± standard deviation. The values were statistically analyzed with One-Way ANOVA using Microsoft Excel for Windows version 10. The minimum level of p value >0.05 was considered as statistically significant.

RESULTS

In total, 23 isolated strains of bacteria were diagnosed after culturing the swab samples from the oral cavity. There were 13 strains of Gram-positive and 10 strains of Gram-negative bacteria. Most of the Gram-positive bacteria were highly susceptible to the low concentration of TAM as compared to the Gram-negative bacteria. *Kytococcus sedentarius* as one Gram-positive and *Pseudomonas stutzeri* as one Gram-negative bacterium needed higher concentrations of TAM (MIC: 2.5 mg/mL) to be inhibited by the same. Additionally, the effect of TAM on these two types of bacteria was determined as bacteriostatic action (Table 1 and 2).

The effective low concentrations of TAM on susceptible bacteria, which showed MIC at 0.625 mg/mL, were mostly determined as bacteriostatic action. This bacteriostatic action was clearly shown in the five isolated strains of Gram-positive and in the two strains of Gram-negative bacteria, whereas TAM

demonstrated bactericidal action against three strains of Gram-positive and one strain of Gram-negative bacteria at the same concentration (Table 1 and 2).

The resistance to TAM was observed in three strains of Gram-positive bacteria, namely *Granulicatella elegans*, *Kocuria kristinae*, and *K. varians*, and in four strains of Gram-negative bacteria, namely *Acinetobacter haemolyticus, E. coli*, *Enterobacter cloacae*, and *Klebsiella pneumonia* (Table 1 and 2).

DISCUSSION

TAM is an effective drug for the treatment of breast cancer in both women and men.[3,4] A clinical trial study conducted worldwide demonstrated that TAM can reduce the incidence of breast cancer by 50% in high-risk pre- and post-menopausal women.[6] It has also been used as an adjuvant therapy with an efficacy of more than five years for treating postmenopausal, node-positive, and estrogen or progesterone receptor-positive women since the mid-1980s.[3,6] Hence, TAM is commonly used by more than 7 million patients in a year and had saved the lives of approximately 500,000 women.[3,4,6] In addition to the antagonist binding property of TAM with estrogen receptor to prevent the development of breast cancer.[4,6] It also has another mechanism to prevent this type of cancer via the stimulated production of transforming growth factor (TGF)-calmodulin and protein kinase C, and also by blocking the angiogenesis process by lowering the production of IGF-1 and TGF.[16]

Table 1. The minimum inhibitory concentration of TAM in Gram-positive bacteria

No	Bacteria	MIC value (mg/mL)	MBC
1	*Dermacoccus nishinomiyaensis*	0.625	-
2	*Gemella sanguinis*	0.625	+
3	*Granulicatella adiacens*	0.625	-
4	*Granulicatella elegans*	R	+
5	*Helcococcus kunzii*	0.625	+
6	*Kocuria kristinae*	R	+
7	*Kocuria rosea*	1.25	+
8	*Kocuria varians*	R	+
9	*Kytococcus sedentarius*	2.5*	-
10	*Leuconostoc mesenteroides* ssp. *cremoris*	0.625	-
11	*Staphylococcus aureus*	0.625	+
12	*Staphylococcus pseudintermedius*	0.625	+
13	*Staphylococcus warneri*	0.625	+

*Significant difference between bacterial species at p<0.05, MIC: The minimum inhibitory concentration, TAM: Tamoxifen, MBC: Minimum bactericidal concentration

Table 2. The minimum inhibitory concentration of TAM in Gram-negative bacteria

No	Bacteria	MIC value (mg/mL)	MBC
1	*Acinetobacter haemolyticus*	R	+
2	*Aeromonas salmonicida*	1.25	+
3	*Burkholderia cepacia*	0.625	-
4	*Escherichia coli*	R	+
5	*Enterobacter cloacae* complex	R	+
6	*Klebsiella pneumoniae* ssp. *pneumoniae*	R	+
7	*Pseudomonas fluorescens*	1.25	-
8	*Pseudomonas stutzeri*	2.5*	-
9	*Ralstonia insidiosa*	0.625	+
10	*Sphingomonas paucimobilis*	0.625	+

*Significant difference between bacterial species at p<0.05, MIC: The minimum inhibitory concentration, TAM: Tamoxifen

In recent times, the resistance of bacteria to the most common antibacterial agents has increased progressively because of the massive overuse of these types of agents.[17] The misuse and overprescription of antibacterial agents is considered as the most important factor that has contributed to the rise of resistant bacterial strains for such type of agents.[18] The bacteria present in the oral cavity have also developed drug resistance toward many common types of antibiotics because of the genetic changes in their genomic structure.[19] The resistance of the oral bacteria to erythromycin due to the activity of *mef* and *erm* (B) genes is one example of such antibiotic resistance.[20] However, the list of antibiotics has hardly changed in four decades, and most of the pharmaceutical companies have left the antibiotic field due to the absence of a new class of antibacterial agents.[17] Hence, antibiotic resistance is emerging as one of the modern crises, and now is the right time for a global commitment to develop new antibacterial drugs.[17,21] The repurposing of existing drugs can be introduced as a solution to resolve the limited number of antimicrobial agents and for the enhancement of the treatment of most severe bacterial and fungal infections.[22] The repurposing process is usually employed to discover a new therapeutic action of a specific drug to add to its previously known usage.[8] TAM is one of those drugs, whose potential antimicrobial effect has been determined by many studies. These studies have presented promising data to repurpose the use of TAM from an anticancer drug into an antimicrobial agent.[9-12,23] Low side-effect profile and cheaper price are other important characteristics that could encourage the continued usage of TAM for the treatment of cancer and microbial infections.[6]

Out of 23 strains of the oral bacteria isolated in this study, 19 of them revealed susceptibility to TAM with variable MIC values. Alow concentration of TAM (7.1 µg/mL) exhibited moderate antibacterial effect against *M. tuberculosis*,[23] whereas a high concentration of TAM (MIC_{50}: 5-10 mg/mL) is required to suppress its growth.[12] However, drug-sensitive strains of *M. tuberculosis* could be inhibited by low concentrations of TAM (3.125-6.25 µg/mL) as compared with drug-resistant strains (6.25-12.5 µg/mL).[10] Antibacterial effects of TAM were also recorded against various other types of bacteria such as *E. faecium* and *S. aureus*[9,11] and also against more drug-resistant bacterial strains such as MRSA and *M. tuberculosis*.[9,10] The chemical derivatives of TAM have also shown potent antibacterial action. 4-hydroxytamoxifen,

ametabolic derivative of TAM, exerted an inhibitory action against *M. tuberculosis* with an MIC_{50} value of 5-10 mg/mL.[12] The newly synthesized triaryl butane, which is an analog of TAM, exhibited antibacterial activity against Gram-positive and Gram-negative food-borne pathogens, such as *Listeria monocytogenes*, *Listeria ivanovii*, *Enterococcus faecalis*, *S. aureus,* and *E. coli*.[24] Triphenylethylene, which is considered as a backbone of TAM, has also shown antibacterial activity against various types of pathogens including bacteria.[8] Otherwise, TAM had shown synergistic action with many known antibiotics to make them more effective against pathogenic bacteria as with polymyxin B against the polymyxin-resistant *P. aeruginosa*, *K. pneumoniae*, and *Acinetobacter baumannii*[14,25] and the three first-line antibiotics (rifampin, isoniazid, and ethambutol) against *M. tuberculosis*.[12] The activity of chitosan against *E. coli* and *Staphylococcus* spp. was increased in the presence of TAM when they were prepared in the nano-fiber polycaprolactone structure.[26] Moreover, TAM can increase the defensive ability of the immune cells. This process is well proven when the bactericidal activity of neutrophils is increased by TAM against various bacteria such as MRSA, *P. aeruginosa*, and *E. coli*. TAM can also enhance the bacterial clearance by this immune cell with 2.4-4.2 log reductions in bacterial counts in several types of tissue samples.[13] Intracellular tuberculosis in macrophages was also decreased after its treatment with TAM in a dose-dependent manner.[10]

The results of this study have shown that Gram-positive bacteria are more susceptible to TAM than Gram-negative bacteria. The resistance rate in Gram-negative bacteria was also found to be higher (36-73%) toward many antibiotics as compared to that in Gram-positive bacteria.[27] Generally, the resistance of Gram-negative bacteria is clearly identified toward various types of antibiotics because of its cell wall components, which make them a formidable barrier against any dangerous materials.[28] The inhibition of function of the bacterial cell membrane is the proposed mechanism of TAM action against bacteria.[23,24,29] This type of antibacterial action is mostly related to the hydrophobicity of TAM because of the presence of alkyl groups that are attached to the amino group in its structure.[9] Ultrastructural alterations in the components of the cell membrane of *Bacillus stearothermophilus,* which cause bacterial cell killing after treatment with TAM shows evidence that TAM is a membrane-active drug.[29] This type of alteration, which leads to high K^+ and Na^+ efflux from bacterial cells and causes severe damage in the inner and outer membranes, is also recognized after the treatment of *E. coli* and *L. ivanovii* with an analog of TAM (triaryl butane).[24] The mitochondrial membrane of *M. tuberculosis* was also observed to be collapsing by the ionic protonophore uncouplers of TAM and its lipophilic nature.[23]

Seven of the isolated bacteria, including three types of Gram-positive and four types of Gram-negative bacteria, in this study showed resistance to TAM. This resistance may related to the modification of the lipid or protein composition of the outer membrane of bacterial cells as observed in most of the antibiotic-resistant bacteria.[28] Other membrane modifications may include changes in the action of efflux pumps, the expression of various drug-deactivating agents, and proteolytic degradation.[30]

As one of four members of Gram-negative bacteria that was resistant to TAM, *A. haemolyticus* also showed resistance against other antibiotics. This bacterium, which usually causes nosocomial infections and is frequently isolated from the intensive care unit (ICU) of the hospitals, is emerging to be one of the bacteria that are to most of antimicrobial agents.[31] Isolates of *A. haemolyticus* from patients with immunocompromised status revealed a high level of resistance toward a wide range of antibiotics, including ampicillin-sulbactam, ampicillin, aztreonam, cefuroxime, and ceftazidime.[32] This resistance was also observed among isolated strains from patients receiving treatment at ICU against ciprofloxacin, cefepime, ceftazidime, piperacillin, and amikacin.[31,33] Like clinical isolates, *A. haemolyticus* isolated from the natural environment also showed resistance toward antibiotics such as cefotaxime and ceftazidime.[34] However, the resistance of *A. haemolyticus* mainly depends on its acquisition of beta-lactamase and cephalosporinase enzymes, whereas resistance to quinolone is related to the mutations in *gyrA* and/or *parC* genes.[35]

E. coli was a second Gram-negative bacterium that was resistant to TAM. This type of bacterium is usually sensitive to almost all the relevant antibiotics, but it also has the ability to acquire resistance genes from other species of bacteria via horizontal gene transfer.[36] Another source of multiple antibiotic resistance may result from the change in amino acids of *mar* locus regulator or mutation in the operator-promoter region *marO* of the bacterium.[37] In total, 137 *E. coli* isolates extracted from the cases of urinary tract infection (UTI) exhibited a high resistance (51.1-94.3%) toward ten types of antibiotics,[38] whereas 17 non-pathogenic *E. coli* strains extracted from different sources revealed multiple antibiotic resistance because of the genes carried by class 1 and class 2 integrons.[37] However, the transfer of resistance genes acquired by *E. coli* could result from plasmids and from other mobile genetic elements, such as transposons and gene cassettes in class 1 and class 2 integrons.[36]

Our study results showed that *Enterobacter cloacae* complex (ECC), which contains common nosocomial bacteria that can cause various types of infection,[39] exhibited resistance to TAM. The greatest antibiotic resistance by *E. cloacae* was against penicillin, cephalosporins (cefotaxime, ceftazidime, ceftriaxone), aminoglycosides, colistin, and fluoroquinolones.[40,41] Moreover, a study recorded an emerging resistance of ECC to a new generation of carbapenems.[39] The pathogenic strains of *E. cloacae* that caused bacteremia were found to be resistant against cephalothin and ampicillin and a smaller number to these strains against aminoglycosides.[42] However, this bacterium becomes resistant by acquiring resistance genes just like the other members of Gram-negative bacteria.[39]

K. pneumoniae, which causes various nosocomial infections, sepsis in neonates and bacteremia,[43,44] is another bacterium of the Gram-negative group that showed resistance against

TAM. A study conducted from 1998 to 2010 in the USA also showed such resistance of *K. pneumoniae* against a wide range of antibiotics.[45] The emergence of such types of multidrug-resistant bacteria have been increasing nowadays because of their production of extended-spectrum beta-lactamases[43] and a mutation in the *mgrB* regulatory gene that lead to resistance against colistin.[46]

Our results also exhibited resistance of three isolates of Gram-positive bacteria, namely *Granulicatella elegans, K. kristinae* and *K. varians*, and *G. elegans*, to TAM. These bacteria are related to the nutritionally variant *streptococci* that is usually found as one of the oral flora with an ability to cause infections and endocarditis under some conditions.[47,48] It is considered as the most sensitive species to most antibiotics than other species of its genus, especially *G. adiacens*.[49,50] The resistance of *G. elegans* to macrolide and beta-lactam antibiotics was recently noticed because of the presence of *erm* and *mef* genes.[47,49,51] This resistance could be increased in the case of biofilm formation by this bacterium.[52]

Our results also showed that the two isolates of *Kocuria* spp., namely *K. kristinae* and *K. varians*, were resistant to TAM. This Gram-positive *cocci* bacterium is mostly non-pathogenic, and it mostly causes infection in the patients with immunocompromised status such as those with cancer diseases.[53,54] Antibiotic resistance had been hardly recognized in this bacterium because of very limited available data.[53] Thus, the absence of useful guidelines for determining the antibiotic resistance of *Kocuria* spp. makes the susceptibility test necessary according to the *Staphylococcus* guidelines.[54] However, *K. kristinae* was found to be more resistant to antibiotics than *K. varians*. All the isolates of *K. varians* extracted from patients with endophthalmitis were found to be sensitive to all the tested antibiotics, whereas *K. kristinae* showed resistance against amikacin and cefazolin.[55] Other isolates of *K. kristinae* from patients with UTI, immunosuppressive conditions, and cancer diseases also exhibited resistance against a wide range of antibiotics.[56,57] Meanwhile, all the isolates of *K. varians* from periodontitis and brain abscess exhibited sensitivity to all the tested antibiotics.[58,59]

CONCLUSION

The antibacterial action of TAM was clearly observed against oral bacteria, especially Gram-positive bacteria. The action was mostly determined as a bacteriostatic effect. The repurposing of TAM from cancer therapy to antimicrobial treatment could be encouraged by many factors; for example, TAM is a cheap drug with a few adverse effects. Although some bacteria show resistance, most of the known virulent species of isolated bacteria were found to be sensitive to TAM. This result will provide a promising view to use TAM in the treatment of infections caused by such types of bacteria.

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