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RESEARCH ARTICLE

Repurposing of the Tamoxifen Metabolites to Treat Methicillin-Resistant *Staphylococcus epidermidis* and Vancomycin-Resistant *Enterococcus faecalis* Infections

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ABSTRACT Repurposing drugs provides a new approach to the fight against multidrug-resistant (MDR) bacteria. We have reported that three major tamoxifen metabolites, *N*-desmethyltamoxifen (DTAM), 4-hydroxytamoxifen (HTAM), and endoxifen (ENDX), presented bactericidal activity against *Acinetobacter baumannii* and *Escherichia coli*. Here, we aimed to analyze the activity of a mixture of the three tamoxifen metabolites against methicillin-resistant *Staphylococcus epidermidis* (MRSE) and *Enterococcus* species. MRSE (n = 17) and *Enterococcus* species (*Enterococcus faecalis* n = 8 and *Enterococcus faecium* n = 10) strains were used. MIC of the mixture of DTAM, HTAM, and ENDX and that of vancomycin were determined by microdilution assay. The bactericidal activity of the three metabolites together and of vancomycin against MRSE (SE385 and SE742) and vancomycin-resistant *E. faecalis* (EVR1 and EVR2) strains was determined by time-kill curve assays. Finally, changes in membrane permeability of SE742 and EVR1 strains were analyzed using fluorescence assays. MIC_{90} of tamoxifen metabolites was 1 mg/liter for MRSE strains and 2 mg/liter for *E. faecalis* and *E. faecium* strains. In the time-killing assays, tamoxifen metabolites mixture showed bactericidal activity at 4× MIC for MRSE (SE385 and SE742) and at 2× MIC and 4× MIC for *E. faecalis* (EVR1 and EVR2) strains, respectively. SE385 and EVR2 strains treated with the tamoxifen metabolites mixture presented higher membrane permeabilization. Altogether, these results showed that tamoxifen metabolites presented antibacterial activity against MRSE and vancomycin-resistant *E. faecalis*, suggesting that tamoxifen metabolites might increase the arsenal of drug treatments against these bacterial pathogens.

IMPORTANCE The development of new antimicrobial therapeutic strategies requires immediate attention to avoid the tens of millions of deaths predicted to occur by 2050 as a result of MDR bacterial infections. In this study, we assessed the antibacterial activity of three major tamoxifen metabolites, *N*-desmethyltamoxifen (DTAM), 4-hydroxytamoxifen (HTAM), and endoxifen (ENDX), against methicillin-resistant *Staphylococcus epidermidis* (MRSE) and *Enterococcus* spp. (*E. faecalis* and *E. faecium*). We found that the tamoxifen metabolites have antibacterial activity against MRSE, *E. faecalis*, and *E. faecium* strains by presenting MIC_{90} between 1 and 2 mg/liter and bactericidal activity over 24 h. In addition, this antibacterial activity is paralleled by an increased membrane permeability of these strains. Our results showed that tamoxifen metabolites might be potentially used as a therapeutic alternative when treating MRSE and *E. faecalis* strains in an animal model of infection.

KEYWORDS *Enterococcus*, Gram-positive, repurposing, *Staphylococcus*, tamoxifen, antibacterial, metabolite

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Staphylococcus epidermidis and *Enterococcus* spp. are common health care-associated pathogens in different infections, causing significant morbidity, mortality, and/or health care costs (1–3). Glycopeptides are among the recommended treatments for the infections caused by methicillin-resistant *S. epidermidis* (MRSE) and ampicillin-resistant *Enterococcus* spp. (1, 4). However, the emergence of isolates with reduced susceptibility to vancomycin, teicoplanin, linezolid, and daptomycin have commonly been reported (1, 5–7). Therefore, it is important to increase the arsenal of antimicrobial agents and to find drugs active against MRSE and *Enterococcus* spp. with reduced susceptibility to glycopeptides.

Different approaches can be used to find new antibacterial agents, such as repurposing drugs. Anticancer drugs, such as tamoxifen, have demonstrated antibacterial activity against *Acinetobacter baumannii*, *Escherichia coli*, and *Staphylococcus aureus* (8–10). This antimicrobial activity might result from cytochrome P450-mediated tamoxifen metabolism releasing three major metabolites, *N*-desmethyltamoxifen (DTAM), 4-hydroxytamoxifen (HTAM), and endoxifen (ENDX) (11).

Few studies have investigated the activity of these metabolites against infectious agents (9, 12–15). One of them, HTAM, has been reported to act as a weak base to protect cells and mice against lethal Shiga toxin 1 (STx1) or Shiga toxin 2 (STx2) toxicosis (9) and to be active against *Plasmodium falciparum* and *Cryptococcus neoformans* var. *grubii* (13, 14). HTAM has also presented activity when used in monotherapy against *Mycobacterium tuberculosis* (MIC_{50} ~2.5 to 5 mg/liter) and in combination with rifampin, isoniazid and ethambutol being the most active at 10 and 20 mg/liter of HTAM (15). Moreover, the activity of ENDX was studied against *C. neoformans* var. *grubii* with MIC of 4 mg/liter (14).

A previous study from our research group showed that the mixture of DTAM, HTAM, and ENDX exhibited MIC_{50} values of 8 and 16 mg/liter against clinical isolates of *A. baumannii* and *E. coli*, respectively (16), whereas their activity against Gram-positive bacteria remains unknown. The objective of this study is to investigate the activity of tamoxifen metabolites against MRSE and *Enterococcus faecalis* with reduced susceptibility to vancomycin.

RESULTS

Antimicrobial activity of tamoxifen and tamoxifen metabolites. Tamoxifen, tamoxifen metabolites, separately and in mixture, and vancomycin were tested against clinical strains of MRSE, *E. faecalis*, and *Enterococcus faecium*. The MIC_{50} and MIC_{90} values are detailed in Table 1. The MICs of tamoxifen, tamoxifen metabolites mixture, and vancomycin for MRSE strains ranged from 2 to 4 mg/liter, 0.5 to 2 mg/liter, and 0.5 to 4 mg/liter, respectively, while those for *E. faecalis* strains ranged from 2 to >32 mg/liter, 1 to 2 mg/liter, and 1 to 128 mg/liter, respectively, and those for *E. faecium* strains ranged from 2 to 4 mg/liter, 1 to 2 mg/liter, and 0.5 to 1 mg/liter, respectively. The MIC_{50} and MIC_{90} of tamoxifen were 2 and 4 mg/liter (for MRSE strains), 8 and >32 mg/liter (for *E. faecalis* strains), and 4 mg/liter (for *E. faecium* strains). The MIC_{50} and MIC_{90} for DTAM, HTAM, and ENDX for the three pathogens ranged from 2 to 32 mg/liter. When these three metabolites were grouped together, their MIC_{50} and MIC_{90} were 1 and 2 mg/liter, respectively, for MRSE and *E. faecalis* and 1 and 2 mg/liter, respectively,

TABLE 1 MICs effective for ≥50% and ≥90% of isolates tested (MIC_{50} and MIC_{90}) of tamoxifen, tamoxifen metabolites, and vancomycin for *S. epidermidis* and *Enterococcus* spp.[a]

		TAM (mg/liter)		DTAM (mg/liter)		HTAM (mg/liter)		ENDX (mg/liter)		MET (mg/liter)		Vancomycin (mg/liter)	
Pathogen	*n*	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
S. epidermidis	17	2	4	2	4	8	8	4	8	**1**	**1**	2	4
E. faecalis	8	8	>32	4	4	8	32	8	16	**2**	**2**	2	128
E. faecium	10	4	4	2	4	8	8	4	8	**1**	**2**	1	1

[a]MET: tamoxifen metabolites, 4-hydroxytamoxifen (HTAM), *N*-desmethyltamoxifen (DTAM), endoxifen (ENDX) mixture. TAM, tamoxifen; VAN, vancomycin. *Enterococcus* spp., *E. faecalis* and *E. faecium*.

TABLE 2 Checkerboard analysis of tamoxifen metabolites combinations against *S. epidermidis* SE385 and SE742 strains and *E. faecalis* EVR1 and EVR2 strains[a]

Strain	FICI (DTAM + HTAM)	FICI (DTAM + ENDX)	FICI (HTAM + ENDX)
S. epidermidis SE385	1	0.6	1
S. epidermidis SE742	0.75	0.75	1
E. faecalis EVR1	0.75	0.75	1
E. faecalis EVR2	1	0.75	0.56

[a]FICI, fractional inhibitory concentration index; DTAM, *N*-desmethyltamoxifen; HTAM, 4-hydroxytamoxifen; ENDX, endoxifen.

for *E. faecium*. In the case of vancomycin, the MIC_{50} and MIC_{90} were 2 and 4 mg/liter (for MRSE strains), 2 and 128 mg/liter (for *E. faecalis* strains), and 1 mg/liter (for *E. faecium* strains). Of note, the checkerboard assay analysis showed that all different combinations between two tamoxifen metabolites have a slight increase in the inhibitory activity from the additive of both tamoxifen metabolites combined, with a fractional inhibitory concentration index (FICI) between 0.56 and 1 (Table 2). These results showed that the mixture of tamoxifen metabolites presented higher antibacterial activity than their prodrug tamoxifen and vancomycin against MRSE and *Enterococcus* species strains.

Time-kill curves. Using time-kill assays, we examined the bactericidal activity of tamoxifen metabolites and vancomycin against MRSE SE385 and SE742 strains and vancomycin-resistant *E. faecalis* EVR1 and EVR2 strains (Fig. 1). The MICs and MBCs of tamoxifen metabolites and vancomycin for these strains are summarized in Table 3. Tamoxifen metabolites at 4× MIC showed bactericidal activity for both MRSE strains, whereas at 1× MIC and 2× MIC they were not bactericidal (Fig. 1A). In the case of *E. faecalis* EVR1 and EVR2 strains, tamoxifen metabolites at 2× MIC and 4× MIC showed bactericidal activity for both strains (Fig. 1B). For SE385, SE742, and EVR2 strains, a regrowth has been particularly observed at 24 h in the presence of tamoxifen

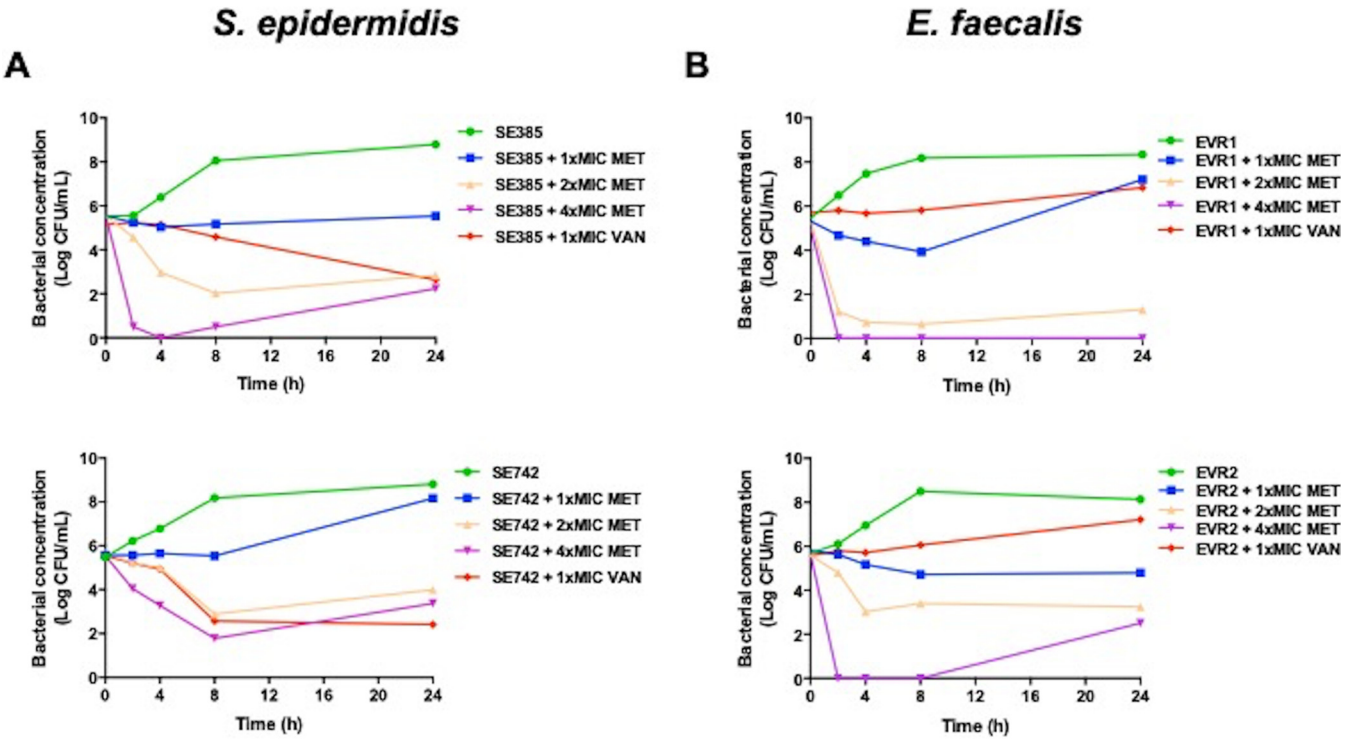

FIG 1 Antibacterial activity of tamoxifen metabolites at different concentrations against *S. epidermidis* and *Enterococcus faecalis* strains. Time-kill curves of *S. epidermidis* SE385 and SE742 strains (A) and *E. faecalis* EVR1 and EVR2 strains (B) in the presence of 1×, 2×, and 4× MIC tamoxifen metabolites and 1× MIC vancomycin for 24 h. MET, tamoxifen metabolites; VAN, vancomycin. Data are represented as mean from two independent experiments.

TABLE 3 MICs and minimal bactericidal concentrations of tamoxifen metabolites and vancomycin for *S. epidermidis* SE385 and SE742 and *E. faecalis* EVR1 and EVR2 strains[a]

	MET (mg/liter)		VAN (mg/liter)	
Strain	MIC	MBC	MIC	MBC
S. epidermidis SE385	1	2	4	4
S. epidermidis SE742	1	2	4	4
E. faecalis EVR1	2	4	128	>256
E. faecalis EVR2	1	2	128	>256

[a]MET: tamoxifen metabolites 4-hydroxytamoxifen (HTAM), *N*-desmethyltamoxifen (DTAM), endoxifen (ENDX) mixture; MBC, minimal bactericidal concentration.

metabolites at 4× MIC. Finally, vancomycin at 1× MIC was bactericidal against SE385 and SE742 strains but not against EVR1 and EVR2 strains.

***In vitro* cytotoxicity of tamoxifen metabolites.** The study of the cytotoxicity of the tamoxifen metabolites was carried out. The percentage of cell viability on the human lung epithelial cells (A549 cells) and murine macrophages (RAW 264.7 cells) incubated for 24 h with the mixture of DTAM, HTAM, and ENDX at decreasing concentrations was determined from 400 to 0 mg/liter. Only at 400 mg/liter did this mixture show reduction in the cell viability below 50%, while the rest of the concentrations showed higher cell viability, between 83% and 100% (Table S1).

Effect of tamoxifen metabolites on the bacterial cell membrane. In order to determine the mode of action of tamoxifen metabolites, we examined their effect on the membrane permeability of *S. epidermidis* (SE742 and SE385) and *E. faecalis* (EVR1 and EVR2) strains. The three tamoxifen metabolites mixture at 0.5× MIC significantly increased the membrane permeability of these strains, by 70.22%, 54.97%, 244.61%, and 86.6%, respectively (Fig. 2). This result suggests that tamoxifen metabolites affect the integrity of the bacterial cell wall of MRSE and vancomycin-resistant *E. faecalis*.

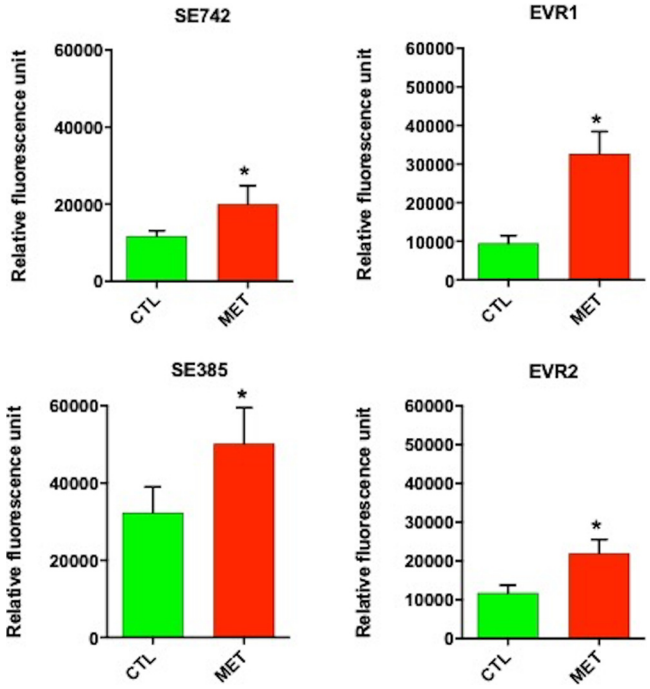

FIG 2 Tamoxifen metabolites effects on the bacterial permeability of *S. epidermidis* and *E. faecalis* strains. The membrane permeabilization of *S. epidermidis* (SE742 and SE385) and *E. faecalis* (EVR1 and EVR2) strains in absence and presence of tamoxifen metabolites (0.5× MIC) incubated for 10 min was quantified by Typhon Scanner. MET, the three tamoxifen metabolites together; CTL, control. *, $P < 0.05$: CTL versus MET.

DISCUSSION

The present study provides new data highlighting the antibacterial effect of tamoxifen metabolites against Gram-positive bacteria through the increase of bacterial membrane permeability.

The MIC_{50} of tamoxifen metabolites was 1 and 2 mg/liter against MRSE and *E. faecalis*, respectively. However, the MIC_{50} values obtained against Gram-negative bacteria *A. baumannii* and *E. coli* were 8 and 16 mg/liter, respectively (16). Obvious reasons for this difference could be the structural and molecular differences between the two classes of bacteria (17). Similar differences between Gram-negative and Gram-positive bacteria have been observed with other repurposed drugs, such as the statin (simvastatin), anthelmintics (niclosamide, oxycloznide, and closantel), and anti-inflammatory (celecoxib) drugs (18–20).

The antibacterial activity of tamoxifen metabolites at 4× MIC with MRSE began earlier than that of vancomycin. This result may be related to the ability of tamoxifen metabolites to eliminate staphylococcal biofilms as observed with the tamoxifen analogue, toremifene, compared with the reduced ability of vancomycin to penetrate biofilms (21, 22). In addition to the good antibacterial activity of tamoxifen metabolites at 4× MIC, a regrowth of MRSE strains and *E. faecium* EVR2 strain has been observed at 24 h. The MIC of tamoxifen metabolites for SE385, SE742, and EVR strains in this time-kill condition were 2, 2, and 1 mg/liter, respectively, MICs below the 4× MIC of tamoxifen metabolites concentration. Further investigations, including the determination of tamoxifen metabolites concentration during the time-kill assay, are necessary to better understand the regrowth of these strains in the presence of tamoxifen metabolites.

In this study, we showed that the three tamoxifen metabolites together produced an increase in membrane permeability of MRSE and vancomycin-resistant *E. faecalis* strains. It is known that the mechanism of action of tamoxifen, the prodrug of DTAM, HTAM, and ENDX in fungi, is related to the binding to calmodulin (23, 24). Additionally, Scott et al. showed that HTAM might inhibit the phospholipase D in *Pseudomonas aeruginosa* (25). Future studies on the mechanism of action used by tamoxifen metabolites against Gram-positive bacteria and on their therapeutic efficacy in animal experimental models of infection would be of interest. In addition to being antibacterial against Gram-positive bacteria, tamoxifen and its metabolites would have two properties that are advantageous for the treatment of bacterial infections. First, these drugs have excellent bioavailability and, therefore, can be administered orally (26), and second, they would induce the killing activity of macrophages and neutrophils similarly to that observed against Gram-negative bacteria (8).

In conclusion, these results suggest that tamoxifen metabolites are potential antimicrobial agents for use against MRSE and vancomycin-resistant *E. faecalis*, respectively, and they may, after further development, become a possible option for the treatment of infections by MRSE and vancomycin-resistant *Enterococcus* spp.

MATERIAL AND METHODS

Bacterial strains. Seventeen MRSE and 18 *Enterococcus* species (*E. faecalis* $n = 8$, *E. faecium* $n = 10$) clinical isolates from blood cultures, characterized previously (27, 28), were used in this study. MIC susceptibility breakpoint of vancomycin for both pathogens was determined according to the standard recommendations of the Clinical and Laboratory Standards Institute (CLSI) being susceptible at $\leq$4 mg/liter and resistant at $>$4 mg/liter (29).

Antimicrobial agents and reagents. Standard laboratory powders of tamoxifen, HTAM, DTAM, ENDX, and vancomycin (Sigma, Spain) were used. The mixture of HTAM, DTAM, and ENDX was dissolved in dimethyl sulfoxide (DMSO) in equal concentrations.

***In vitro* susceptibility testing.** MICs of HTAM, DTAM, and ENDX separately and in mixture, tamoxifen, and vancomycin against MRSE and *Enterococcus* species strains were determined in two independent experiments by broth microdilution assay according to CLSI guidelines (29). The initial bacterial inoculum of 5×10^5 CFU/ml for each strain cultured in Mueller-Hinton Broth (MHB) (Sigma, Spain) was used in a 96-well plate (Deltlab, Spain) in the presence of HTAM, DTAM, and ENDX separately and in mixture (at same concentration), tamoxifen, and vancomycin and incubated for 24 h at 37°C. *E. faecalis* ATCC 29212 and *S. aureus* ATCC 29213 strains were used as control strains. MIC_{50} and MIC_{90}, respectively, were determined.

Checkerboard assay. The assay was performed on a 96-well plate as described previously (30). The tamoxifen metabolite DTAM (metabolite 1), HTAM (metabolite 2), or ENDX (metabolite 3) was 2-fold serially diluted along the *x* axis, whereas the corresponding combined tamoxifen metabolite was 2-fold serially diluted along the *y* axis to create a matrix, where each well consists of a combination of both agents at different concentrations. Bacterial cultures grown overnight were then diluted in saline to 0.5 McFarland turbidity, followed by 1:50 further dilution Mueller-Hinton broth and inoculation on each well to achieve a final concentration of approximately 5.5×10^5 CFU/ml. The 96-well plates were then incubated at 37°C for 24 h and examined for visible turbidity. The fractional inhibitory concentration (FIC) of metabolite 1 was calculated by dividing the MIC of metabolite 1 in the presence of metabolite 2 by the MIC of metabolite 1 alone. Similarly, the FIC of metabolite 2 was calculated by dividing the MIC of metabolite 2 in the presence of metabolite 1 by the MIC of metabolite 2 alone. The FIC index was the summation of both FIC values. FIC index values of ≤ 0.5, >0.5 to 1, >1 to <2, and ≥ 2 were interpreted as synergistic, additive, indifference, and antagonism, respectively (31). The same experiment was performed with the combination of metabolite 1 and metabolite 3 and the combination of metabolite 2 and metabolite 3.

Time-kill kinetic assays. Time-kill curves of MRSE SE385 and SE742 strains with a vancomycin MIC of 4 mg/liter and *E. faecalis* EVR1 and EVR2 strains with a vancomycin MIC of 128 mg/liter were performed in duplicate as described previously (29, 30). Initial inoculums of 5.5×10^5 CFU/ml were added on 5 ml of MHB in the presence of 1×, 2×, and 4× MIC of HTAM, DTAM, and ENDX mixture and 1× MIC of vancomycin. Drug-free broth was evaluated in parallel as a control. Tubes of each condition were incubated at 37°C with shaking (180 rpm), and viable counts were determined by serial dilution at 0, 2, 4, 8, and 24 h. Viable counts were determined by plating 100 μl of control, test cultures, or the respective dilutions at the indicated times onto sheep blood agar plates (ThermoFisher, Spain). Plates were incubated for 24 h at 37°C, and after colony counts, the $\log_{10}$ of viable cells (CFU/ml) was determined. Bactericidal activity was defined as a reduction of ≥ 3 $\log_{10}$ CFU/ml at 24 h with respect to the initial inoculum.

***In vitro* toxicity of the tamoxifen metabolites.** Human lung epithelial A549 cells and murine macrophages RAW 264.7 cells were incubated with the mixture of DTAM, HTAM, and ENDX (0, 50, 100, 200, and 400 mg/liter) for 24 h with 5% CO_2 at 37°C. Prior to the evaluation of the tamoxifen metabolites cytotoxicity, A549 and RAW 264.7 cells were washed three times with prewarmed phosphate-buffered saline (PBS) 1×. Subsequently, quantitative cytotoxicity was evaluated by measuring the mitochondrial reduction activity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (32). The percentage of cytotoxicity was calculated from the absorbance at 570 nm as follows: (absorbance at 570 nm of treated cells/mean absorbance at 570 nm of untreated cells) × 100.

Membrane permeability assays. Bacterial suspensions (adjusted to optical density at 600 nm of 0.2) of SE742, SE385, EVR1, and EVR2 strains were placed on a 96-well plate, incubated with 0.5× MIC of tamoxifen metabolites mixture, and mixed in a solution of phosphate-buffered saline containing ethidium homodimer-1 (EthD-1; 1:500; Invitrogen, Carlsbad, CA, USA). After 10 min of incubation, fluorescence was monitored during 160 min using a Typhoon FLA 9000 laser scanner (GE Healthcare Life Sciences, Marlborough, MA, USA) and quantified with ImageQuant TL software (GE Healthcare Life Sciences, USA). Bacterial counts were obtained at the beginning and end of the experiment to ensure that the metabolite mixture did not present bactericidal activity against *S. epidermidis* and *E. faecalis* strains.

Statistical analysis. Group data were presented as means ± standard errors of means (SEM). Difference in membrane permeability was assessed by Student's *t* test. The SPSS (version 23.0; SPSS Inc., Armonk, NY, USA) statistical package was used.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.03 MB.

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Y.S. conceived the study and designed the experiments. A.M.C., A.V.D., M.C.L., and R.A.A. performed experiments and interpreted data. M.E.J.M. and J.P. revised the manuscript and Y.S. wrote the manuscript with the input of all the other authors.

We declare no conflicts of interest.

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