Oral Administration of the Broad-Spectrum Antibiofilm Compound Toremifene Inhibits Candida albicans and Staphylococcus aureus Biofilm Formation In Vivo

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We here report on the in vitro activity of toremifene to inhibit biofilm formation of different fungal and bacterial pathogens, including Candida albicans, Candida glabrata, Candida dubliiniensis, Candida krusei, Pseudomonas aeruginosa, Staphylococcus aureus, and Staphylococcus epidermidis. We validated the in vivo efficacy of orally administered toremifene against C. albicans and S. aureus biofilm formation in a rat subcutaneous catheter model. Combined, our results demonstrate the potential of toremifene as a broad-spectrum oral antibiofilm compound.

Biofilm formation is a key process in many microbial infections, including those of the oral cavity, the gastrointestinal tract, the urinary tract, and various wound tissues. It is estimated that 60% (Centers for Disease Control and Prevention) to 80% (National Institutes of Health) of all microbial infections are biofilm related. Additionally, numerous nosocomial biofilm infections arise from the increased use of implanted medical devices, like intravascular catheters, which are a preferred niche for microbial cell adherence (1). Such catheters frequently become colonized with pathogenic Candida or Staphylococcus spp., especially in intensive care units (2, 3). Biofilm-related infections are associated with a high mortality rate, and therefore, the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the Infectious Diseases Society of America (IDSA) suggest that infected medical devices be removed when possible (4, 5). However, removal of infected devices in less accessible locations, such as orthopedic joints or heart valves, or in patients with a reduced health condition might be impossible (2, 6, 7). Unfortunately, in the case of fungal biofilm infections, most antifungal drugs show only limited antibiofilm activity, with echinocandins (e.g., caspofungin) and liposomal formulations of amphotericin B being the most effective (2). Antifungal lock therapy to apply these compounds can be successful but is restricted to devices with an internal space. For treatment of other devices and for systemic treatments, these compounds need to be administered intravenously, as they are not absorbed after oral administration (5, 8).

To combat bacterial biofilm infection, the major therapeutic strategy is the use of antibiotics. However, the intrinsic and adaptive resistance of bacterial biofilms to current antibiotics, as well as to host immune clearance mechanisms, has led to a growing problem in health care settings. Staphylococcus aureus is a major human pathogen and is one of the most common pathogens in biofilm-associated device infections (9, 10). Traditional antibiotic therapy has been limited due to resistance development, and to a lack of host immune clearance mechanisms. Staphylococcus aureus is a major human pathogen and is one of the most common pathogens in biofilm-associated device infections (9, 10). Traditional antibiotic therapy has been limited due to resistance development, and to a lack of host immune clearance mechanisms. Staphylococcus aureus is a major human pathogen and is one of the most common pathogens in biofilm-associated device infections (9, 10). 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in vitro. In addition, we translated these results in vivo and show activity of toremifene against C. albicans and S. aureus biofilm formation in a rat subcutaneous catheter model (21), importantly, via simple oral administration.

We used the BIC-2 value (minimal concentration of the compound that inhibits biofilm formation 2-fold) to assess the antibiofilm activity of toremifene (TCI Europe, Zwijndrecht, Belgium) for different fungal and bacterial species (Table 1). As a control treatment, we included caspofungin. The in vitro antibiofilm activities of toremifene and caspofungin against Candida spp. were assayed in RPMI 1640 medium and quantified with the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) assay (22). Briefly, toremifene (0.78 to 100 μM, 0.5% dimethyl sulfoxide [DMSO] background) was added during the adhesion (1 h at 37°C) and biofilm formation (24 h at 37°C) phases. Afterwards, biofilms were washed with phosphate-buffered saline (PBS) and quantified with XTT as described previously (15). XTT can be metabolized within 1 h by all fungal species tested, in contrast to cell titer blue (CTB), which was used in our initial study (15). We observed comparable in vitro antibiofilm activities of toremifene against C. albicans, C. glabrata, Candida dubliniensis, and Candida krusei, albeit less potent than those of caspofungin (Table 1). Subsequently, three clinical isolates of C. albicans (2CA, 10CA, and 15CA) that form high-persistence biofilms (23) were assessed. Persistor cells can survive high doses of an antimicrobial agent and partly explain the recalcitrance of chronic infectious diseases against antimicrobial therapy (24, 25). Interestingly, C. albicans CA2 is susceptible to toremifene, whereas C. albicans CA10 and CA15 are more resistant (*P < 0.01 and *P < 0.0001), respectively, by unpaired two-tailed Student’s t test). The activity of toremifene against planktonic C. albicans cells was assayed according to the CLSI M27-A3 protocol (26). The MIC-2 (i.e., the MIC of the compound that reduces growth by 2-fold relative to the results for the growth control [0.5% DMSO]) for toremifene against C. albicans is 49.7 ± 10.1 μM (mean ± standard error of the mean [SEM]), which is comparable to its BIC-2 value against C. albicans (36 ± 2 μM) (Table 1). The latter observation indicates that toremifene has no biofilm-specific activity and does not interfere specifically with the biofilm formation process. The in vitro antibiofilm activities of toremifene against bacterial spp. were assayed using a Calgary biofilm device (Nunc-Immuno TSP [transferable solid-phase] replicator; VWR International). To this end, biofilms were grown on the polystyrene pegs of the Calgary biofilm device for 24 h at 37°C in the presence of a range of concentrations of toremifene (0 to 200 μM in a 0.5% DMSO background for Staphylococcus spp. and a 1% DMSO background for Pseudomonas aeruginosa). Next, the biofilms were disrupted and cells were collected in recovery medium using sonication, after which the number of viable cells was assessed by plate counting (27). Our results indicate that toremifene prevents in vitro biofilm formation of Staphylococcus epidermidis and S. aureus, as illustrated by their low BIC-2 values, whereas the activity of toremifene against P. aeruginosa biofilm formation was approximately 10-fold less (Table 1).

In conclusion, we demonstrate in vitro activity of toremifene against biofilm formation of different fungal and bacterial pathogens, including Candida and Staphylococcus spp. In view of the inhibitory activity of toremifene against the fungus C. neoformans and the Ebola virus reported previously (17, 28), our data further highlight the broad-spectrum antibiofilm activity of toremifene.

Next, we translated these in vitro toremifene data against C. albicans and S. aureus to a relevant in vivo rat subcutaneous catheter model (21). Animal experiments were approved by the ethical committee of KU Leuven (project P125/2011) and animals were maintained in accordance with the KU Leuven animal care guidelines. We used a low toremifene dose with reported antiancer activity in rats (29–31), i.e., 3 mg/kg of body weight/day. Several studies used considerably higher doses of toremifene in rodents, ranging from 10 to 2,000 mg/kg/day (32–35). However, due to the limited solubility of toremifene in the vehicle solution (data not shown), 3 mg/kg/day was the highest feasible dose that could be tested in our experimental setup. The experimental setup of the in vivo experiment was similar to those of previously reported studies (36, 37). Briefly, nine catheter fragments, infected with C. albicans SC5314 (5 × 10⁶ cells/ml) or S. aureus SH1000 (1 × 10⁶ cells/ml) by static incubation in RPMI 1640 medium (90 min at 37°C), were implanted on the lower back of immunosuppressed female Sprague-Dawley rats after washing twice with PBS (21). The biofilm burdens on catheters after the adhesion period were measured by obtaining CFU counts from three catheters, showing 1,022 ± 204 adhered C. albicans and 38,000 ± 4,041 adhered S. aureus cells (mean ± SEM) per catheter prior to implantation. Starting at the day of implantation, 1 ml vehicle solution (28.8 g/liter polyethylene glycol 3000, 1.97 g/liter Tween 80, and 8.65 g/liter NaCl) with and without toremifene (0.6 mg/ml in vehicle, or 3 mg/kg/day) was given by oral gavage daily for 7 days. Several studies used considerably higher doses of toremifene in rodents, ranging from 10 to 2,000 mg/kg/day (32–35). However, due to the limited solubility of toremifene in the vehicle solution (data not shown), 3 mg/kg/day was the highest feasible dose that could be tested in our experimental setup. The experimental setup of the in vivo experiment was similar to those of previously reported studies (36, 37). Briefly, nine catheter fragments, infected with C. albicans SC5314 (5 × 10⁶ cells/ml) or S. aureus SH1000 (1 × 10⁶ cells/ml) by static incubation in RPMI 1640 medium (90 min at 37°C), were implanted on the lower back of immunosuppressed female Sprague-Dawley rats after washing twice with PBS (21). The biofilm burdens on catheters after the adhesion period were measured by obtaining CFU counts from three catheters, showing 1,022 ± 204 adhered C. albicans and 38,000 ± 4,041 adhered S. aureus cells (mean ± SEM) per catheter prior to implantation. Starting at the day of implantation, 1 ml vehicle solution (28.8 g/liter polyethylene glycol 3000, 1.97 g/liter Tween 80, and 8.65 g/liter NaCl) with and without toremifene (0.6 mg/ml in vehicle, or 3 mg/kg/day) was given by oral gavage daily for 7 days. Six (C. albicans experiment) or 4 (S. aureus experiment) rats were treated with toremifene, and 4 rats (both experiments) were treated with the vehicle solution. Afterwards, rats were euthanized and biofilm cells were dissociated from the removed catheters by sonication and vortexing and quantified by counting CFU.

Oral administration of 3 mg/kg/day of toremifene resulted in 56% fewer C. albicans biofilm cells retrieved from the catheter fragments than for the control treatment (5,158 ± 881 CFU versus 11,682 ± 282 CFU for toremifene and the control treatment, respectively; *P = 0.0004) (Fig. 1, left). Similarly, oral administration of toremifene resulted in 57% fewer S. aureus biofilm cells retrieved from the catheter fragments than for the control treatment (5,158 ± 881 CFU versus 11,682 ± 282 CFU for toremifene and the control treatment, respectively; *P = 0.0004) (Fig. 1, right).

### Table 1 Minimal BIC-2 values of toremifene and caspofungin against fungal and bacterial pathogens

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference or source</th>
<th>Mean BIC-2 ± SEM (μM) ofa</th>
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<tbody>
<tr>
<td>Candida albicans SC5314</td>
<td>46</td>
<td>32 ± 3</td>
</tr>
<tr>
<td>Candida albicans CA2</td>
<td>23</td>
<td>36 ± 2</td>
</tr>
<tr>
<td>Candida albicans CA10</td>
<td>23</td>
<td>85 ± 8</td>
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<tr>
<td>Candida albicans CA15</td>
<td>23</td>
<td>97 ± 3</td>
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<tr>
<td>Candida glabrata BG2</td>
<td>47</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Candida dubliniensis NCPF 3949</td>
<td>48</td>
<td>23 ± 4</td>
</tr>
<tr>
<td>Candida krusei IHEM 6104</td>
<td>BCCMa</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>49</td>
<td>46 ± 7</td>
</tr>
<tr>
<td>UCBPP-PA14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis CH</td>
<td>50</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Staphylococcus aureus SH1000</td>
<td>51</td>
<td>3.5 ± 0.7</td>
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a BIC-2 values were determined by XTT for Candida spp. and by counting CFU for P. aeruginosa and Staphylococcus spp. n = 3 independent biological replicates. BIC-2, biofilm inhibitory concentration that inhibits biofilm formation 2-fold; Tore, toremifene; CAS, caspofungin; ND, not determined; NA, not applicable.

b BCCM, Belgian Coordinated Collections Of Microorganisms/IHEM (Brussels, Belgium).
tried from the catheters than for the control treatment (2,441,701 ± 638,290 CFU versus 5,707,540 ± 468,832 CFU for toremifene and the control treatment, respectively; P = 0.0062) (Fig. 1, right). These data indicate that toremifene (3 mg/kg/day) is active in vivo against C. albicans and S. aureus biofilm formation upon oral administration. The efficacy of 3 mg/kg/day of the reference antifungal agent caspofungin against C. albicans biofilms upon intravenous injection was previously demonstrated in a similar in vivo model (37). Even upon dosing rats at 50 mg/kg caspofungin orally, no caspofungin could be detected in serum because of low oral bioavailability (<1%) (38). Hence, caspofungin can only be applied by intravenous injection.

Note that the minimal dose of toremifene resulting in 50% death (i.e., 50% lethal dose [LD₅₀]) in rats is 3,000 mg/kg (toremifene datasheet sc-253712 [http://datasheets.scbt.com/sc-253712.pdf]; Santa Cruz Biotechnology, Dallas, TX, USA), i.e., 1,000-fold higher than the toremifene dose used in this study. The commonly used dose of toremifene in humans for treating ER⁺ breast cancer is 60 mg daily (16, 39). However, several clinical studies with higher toremifene doses (200 and 240 mg/day) in humans showed no significant increase in toxic side effects compared to the standard dose of 60 mg toremifene daily (40–45). The latter studies confirm that the toremifene dose used in this study (3 mg/kg/day) is achievable in humans. Toremifene (60 to 240 mg daily) is in general well tolerated, and adverse side effects comprise mainly hot flashes, sweating, nausea, vaginal discharge, dizziness, edema, vomiting, and vaginal bleeding. In addition, an elevated risk of thromboembolic events, endometrial cancer (higher for tamoxifen treatment), and a prolongation of the QT interval is noticed in some cases when using ER modulators such as tamoxifen and toremifene. Besides these adverse side effects, tamoxifen and toremifene have positive effects on serum lipid levels (decreased cholesterol) and bone mineral density (16, 44, 45).

In conclusion, toremifene is a broad-spectrum antibiofilm compound that prevents C. albicans and S. aureus biofilm formation in vivo upon oral administration. The good oral bioavailability of toremifene makes toremifene a valuable systemic alternative candidate for treating biofilm-associated fungal and bacterial device infections.

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