We here report on the in vivo activity of toremifene to inhibit biofilm formation of different fungal and bacterial pathogens, including Candida albicans, Candida glabrata, Candida dubliniensis, 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.
in vitro. In addition, we translated these results in vitro 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. Persister 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 < 0.00001*, respectively, by unpaired two-tailed Student’s *t* test). The activity of toremifene against planktonic *C. albicans* cells was assessed 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 anticancer 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 x 10^6 cells/ml) or *S. aureus* SH1000 (1 x 10^6 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*).
toremifene doses (200 and 240 mg/day) in humans showed higher than the toremifene dose used in this study (3 mg/kg/day) (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 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 antibiotic 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.

FIG 1 In vivo effect of toremifene on C. albicans (left) and S. aureus (right) biofilm formation on subcutaneous implanted catheters in rats. Female Sprague-Dawley immunosuppressed rats were treated for 7 days with toremifene (3 mg/kg of body weight/day; n = 6 and n = 4 rats for the C. albicans and S. aureus experiment, respectively) or vehicle solution (control; n = 4) by oral gavage after subcutaneous implantation of C. albicans- and S. aureus-infected catheters. Biofilm formation on the catheters was evaluated after 7 days by CFU counting. Error bars show SEMs. Unpaired two-tailed Student’s t test was used for statistical analysis. ***, P < 0.001; **, P < 0.01.

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