Tolvamer Is Not Efficacious in the Neutralization of Cytotoxin in a Human Gut Model of *Clostridium difficile* Infection[†]

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The efficacy of tolvamer, a nonantimicrobial styrene derivative toxin-binding agent, in treating simulated *Clostridium difficile* infection in an in vitro human gut model was investigated. Tolvamer reduced neither the duration nor magnitude of cytotoxin activity by *C. difficile*, reflecting poor efficacy observed in recent phase III clinical trials.

Clostridium difficile infection (CDI) incidence is increasing worldwide. Effective treatment is compromised by limited therapeutic options (metronidazole and vancomycin) and frequent symptomatic recurrence (14). Although early studies suggested there is little difference between the two agents (9, 26–29), reports of metronidazole inferiority in treating severe disease have recently emerged (1, 10, 21, 22, 29). Nonantimicrobial treatments for CDI may avoid depletion of gut microflora and lessen the risk of recurrent CDI. Tolvamer is a nonantimicrobial styrene derivative that neutralizes the effects of *C. difficile* toxins A and B in vitro (7, 15, 16). Despite encouraging early in vitro, hamster model and phase II clinical trial data (7, 15–18), tolvamer failed to meet its primary endpoint of non-inferiority to vancomycin in both recently reported phase III clinical trials (6, 17).

Both in vitro and animal model systems for studying CDI pathogenesis are subject to practical and ethical drawbacks (5). We have adapted an existing validated triple-stage chemostat model of the human gut to study the interplay between antimicrobial agents, human gut microflora, and *C. difficile* (2–4, 11–13). The gut model reflects the spatial, temporal, nutritional, and physicochemical characteristics of the proximal-to-distal bowel but cannot model immunological or secretory events. Nonetheless, it has proven reflective of in vivo observations when evaluating the relative risk of antimicrobial agents to induce CDI (2, 4, 13, 25) and the efficacy of CDI treatments (3, 11, 12). We therefore evaluated the efficacy of tolvamer in treating simulated CDI in the gut model.

The model consists of three anaerobic fermentation vessels, operating at increasing alkalinity, in a top-fed weir-cascade system. Thus, the increasingly alkaline, nutrient-limited conditions found in the human gut from proximal to distal colon are reflected (19).

The gut model was prepared and inoculated, and clindamycin was used to induce *C. difficile* PCR ribotype 001 (MIC, 1 mg/liter) germination and high-level toxin production as de-

scribed previously (3, 11, 12) (period C). *C. difficile* cytotoxin titers were allowed to reach ≥ 4 relative units (RU) for at least 2 consecutive days in vessel 3 (period D) before tolvamer (GT267-224; Genzyme, MA) dosing commenced at 4 g/liter/dose three times a day for 7 days (period E). No further interventions were made thereafter until the conclusion of the experiment 6 days after cessation of tolvamer (period F). Gut microflora, *C. difficile* total counts and spores, and cytotoxin titers were quantified as described previously (11).

The effects of clindamycin on the gut microflora and *C. difficile* reflected prior gut model studies (3, 11, 12). Obligately anaerobic bacteria were adversely affected; bifidobacteria were most severely depleted, with a lesser decline in *Clostridium* spp. As previously reported, *C. difficile* remained as spores, before and during clindamycin dosing, germinating 6 days after clindamycin instillation ceased and only after concentrations decreased below the MIC for the examined strain (3). Cytotoxin reached a high titer of 5 RU 72 h after germination, and tolvamer dosing commenced 48 h thereafter (Fig. 1). *C. difficile* proliferation was not immediately curtailed by tolvamer dosing, and total counts declined only gradually to converge with spore counts by the end of the period. Similarly, cytotoxin titers increased to 6 RU after 3 days of tolvamer addition

FIG. 1. Comparative cytotoxin production profiles of *C. difficile* PCR ribotype 001 in control and tolvamer experiments with clindamycin-induced CDI in the gut model. Tolvamer instillation commenced after 2 days of high-level toxin titers of >4 RU. tid, three times a day. (Reprinted from reference 11 with permission of the publisher.)

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FIG. 2. Vero cell cytotoxicity assay. (A) Vero cell monolayer; (B) *C. difficile* cytotoxin control; (C) undiluted culture from vessel 3 on day 42; (D) 10^{-2} dilution of vessel 3 culture; (E) 10^{-3} dilution of vessel 3 culture; (F) 10^{-4} dilution of vessel 3 culture. Cytotoxin titer, 5 RU.

before declining to 4 RU by the end of dosing. Therefore, despite the high dosage of tolevamer, no reduction in duration or magnitude of cytotoxin activity compared with untreated control data was observed (Fig. 1). Tolevamer instillation did not adversely affect any gut bacterial group within the gut model, as seen in prior studies (16).

In vitro tests showed that addition of tolevamer to Vero cell monolayers prevented the cytopathic effect of the cytotoxin by five distinct *C. difficile* PCR ribotypes in a concentration-dependent fashion (data not shown). Despite these observations, instillation of high tolevamer concentrations into the gut model was not associated with a loss of *C. difficile* cytopathic effect (Fig. 2), in contrast to previous in vitro studies (15, 16). This may be explained by differing methodologies. Prior to inoculation of Vero cell monolayers, gut model samples that contained cytotoxin titers comparable to those observed in feces (8, 20) were serially 10-fold diluted, resulting in concurrent dilution of cytotoxin and tolevamer. Contrastingly, previous studies added fixed tolevamer concentrations to Vero cell monolayers inoculated with serial dilutions of *C. difficile* toxins (7, 15, 16). The latter is unlikely to reflect the interaction of *C. difficile* cytotoxins in vivo or within the gut model. Both *C. difficile* toxins are cytotoxic, although toxin B is more potent and is thought to play a greater role in *C. difficile* virulence (23, 24). Previous studies indicated that there was considerably less high-affinity binding of tolevamer and inhibition of protein synthesis in Vero cells with toxin B than toxin A (16). This may indicate that insufficient tolevamer was present to fully neutralize *C. difficile* toxins and/or drug saturation despite the high dose instilled into the gut model. The tolevamer concentrations instilled into the gut model were based on theoretical calculations (not shown), due to the lack of in vivo data; these indicated that the dosing regimen described would yield ~12 g/liter tolevamer in vessel 1. Alternatively, the interaction between tolevamer and *C. difficile* cytotoxins may not be sufficiently stable to maintain cytotoxin neutralization. Although neutralization by tolevamer was unaffected by fecal material in

preliminary in vitro assays (data not shown), binding of toxins A and B by tolevamer is by multiple weak contacts (7), and it is possible that particulate/biofilm matter within the gut model may have interfered with tolevamer binding. The paradox between the efficacy of tolevamer in simple fixed-dose cytotoxicity studies (16) versus its poor activity in the gut model underscores the need for gut-reflective systems to examine drug efficacy in CDI.

Studies of tolevamer efficacy in the hamster CDI model demonstrated increased survival and reduced recurrent disease compared with metronidazole and vancomycin (16). Furthermore, prophylactic treatment with tolevamer markedly increased survival of clindamycin-treated hamsters. Despite this and apparent efficacy in phase II clinical trials (18), an increased dose (9 g/day) of tolevamer failed to meet its primary endpoint in both phase III clinical trials (6, 17). It is possible that tolevamer may neutralize lower toxin concentrations and could be used prophylactically, but this remains speculative.

In summary, lack of tolevamer efficacy in this gut model supports poor in vivo results in phase III trials.

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