

INFECTIOUS DISEASE

Response to Comment on “A small-molecule antivirulence agent for treating *Clostridium difficile* infection”

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Ebselen’s antivirulence activity in *Clostridium difficile* infection is likely due to multiple modes of action, but the contribution of each to its efficacy remains unclear.

We would like to respond to the Technical Comment by Beilhartz *et al.* (1), outlining new data expanding the possible mechanisms of action of ebselen in blocking disease pathology induced by *Clostridium difficile*. We commend the authors of the Technical Comment for their work and are pleased to see that another group has independently identified this potentially important lead molecule from a screen for compounds that block pathogenesis of *C. difficile*. We also appreciate the efforts that the authors have undertaken to further our understanding of the mechanism of action of ebselen.

The reported finding regarding the activity of ebselen against the glycosyltransferase domain (GTD) is interesting and has brought to our attention the issue of different buffer conditions used for the in vitro assay. We did not appreciate this potential additional mode of action of ebselen, likely because of the confounding effects of dithiothreitol (DTT) in our GTD assay. The newly presented data in Fig. 1G in the Technical Comment show that, in the absence of DTT, ebselen can inhibit GTD activity, suggesting that this mechanism of action may, at least in part, be responsible for some of its activity in vivo. However, we would like to point out that the potency of ebselen against the GTD activity in the absence of DTT is more than 100 times lower [the median inhibitory concentration (IC₅₀) of the wild-type GTD domain in Fig. 1D is 790 nM] than its potency for the cysteine protease domain (CPD; referred to as the autoprocessing domain or APD in the Technical Comment). The IC₅₀ of the CPD reported by us in Fig. 2C of our original paper (2) was 6.9 nM. Beilhartz *et al.* found that the inhibition of the GTD domain was noncovalent and reversible. Although comparing in vitro IC₅₀ data requires caution because of potential differences of potency under different physiological conditions, it remains unclear how ebselen would effectively block GTD activity during a native infection in vivo. Ultimately, this will require animal studies with compounds that do not have inhibitory activity against the CPD but that still effectively inhibit the GTD activity. We are actively working to identify potent CPD-specific inhibitors with suitable in vivo properties to allow studies in the mouse model of *C. difficile* infection.

The authors also present data using a mutant version of full-length toxin B (TcdB) with all nine native cysteine residues mutated to test whether our report of ebselen’s ability to covalently modify cysteine residues in the CPD (2) contributes to its mechanism of toxin inhibition. The data presented in Fig. 1C of the Technical Comment show that ebselen has similar EC₅₀ (median effective concentration) values in the cell-rounding assay using the wild-type and Cys-less mutant

versions of the toxin added at each of the toxin’s EC₉₉ concentrations. This is indeed an interesting result and supports our original suggestion that ebselen has additional protective activity that is not related to its inhibition of the CPD activity (2). However, we would like to provide our perspective on these data. Specifically, we would like to point out that, as shown in Fig. 4D of our original paper (2), mutation of the TcdB such that the CPD is no longer able to process the GTD (L543A mutation) results in a toxin with markedly reduced ability to induce cell rounding (that is, complete cell rounding requires 24 hours for the mutant versus 2 hours for the wild-type toxin). Both the L543A mutant and the CPD cysteine active-site mutant (C698A) have been reported by others to have substantially delayed toxicity in toxigenic models of infection (3, 4). In the Technical Comment, Beilhartz *et al.* report a 20-fold increase in the EC₉₉ toxin concentration used for the Cys-less mutant as compared to the wild-type toxin (fig. S1; 0.5 pM for wild-type and 10 pM for Cys-less TcdB) (1). What remains unclear is how a toxicity that requires a higher dose of toxin or manifests itself over a delayed time frame in a cell culture model is relevant to the toxicity induced in a native infection in vivo. Thus, it is not clear whether this residual toxicity of the mutant, which can be blocked by ebselen (and is independent of the CPD activity), is important for pathogenesis in vivo or whether tissue damage is mainly mediated by the CPD-dependent activity, which is required for rapid and efficient induction of cell rounding in vitro. Ultimately, generation of a *C. difficile* strain that harbors the catalytically dead CPD in both TcdA and TcdB will need to be used in a clinically relevant animal model of *C. difficile* infection to gain a full understanding of the contributions of the CPD activity to pathogenesis. In the absence of these data, it is not possible to make any absolute claims about the importance of the CPD activity for pathogenesis.

Ultimately, the data presented in the Technical Comment provide evidence suggesting that, in addition to ebselen’s ability to inhibit the CPD and its potential to act as an antioxidant, it may have beneficial effects for the treatment of *C. difficile* infection due to its inhibition of the GTD. However, as a CPD inhibitor, ebselen is irreversible and 100 times more potent than as a GTD inhibitor. Ebselen’s potency in the mouse model tracks with its potency against the CPD and its ability to block release of the GTD in vivo. Thus, it cannot be ruled out that ebselen is, at least partially, able to protect tissue from damage as a result of its activity against CPD. Given that ebselen has been shown to be safe in human clinical trials, the results in the Technical Comment further confirm that it should be explored as a therapeutic agent due to multiple potential modes of action that all may be required for the effective treatment of *C. difficile* infection.

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