SUPPLEMENT 2: Immunogenicity Analyses

The potential for bezlotoxumab to induce immunogenicity through 12 weeks in participants aged 1 to <18 years was evaluated as an exploratory endpoint by detecting treatment-emergent anti-drug and neutralizing antibodies to bezlotoxumab in serum at the screening visit, Week 4, and Week 12.

Methods

Whole blood samples collected for immunogenicity analysis (antibezlotoxumab antibodies, also referred to as ADA) were processed to serum, and each serum sample was split into 2 aliquots. One aliquot was sent to PPD Inc. (Richmond, Virginia, USA) and analyzed for ADA in an unblinded manner. For samples that were positive for ADA, the second aliquot was sent to the Sponsor and analyzed for neutralizing antibody (Nab) in an unblinded manner. Serum samples from placebo-treated participants were not analyzed.

A validated bridging ECL assay was used for the detection of ADA in human serum. Biotin- and sulfo-TAG-labeled bezlotoxumab were used for capture and detection of ADA. The sensitivity of the assay without drug in the screening tier was 0.496 ng/mL with a monoclonal antibody (mAb) positive control and 0.302 ng/mL with a polyclonal antibody (pAb) positive control. The sensitivity of the assay without drug in the confirmatory tier was 0.642 ng/mL with the mAb and 0.487 ng/mL with the pAb. Drug tolerance testing showed that 100 ng/mL of ADA positive control, either mAb or pAb, could be detected in the presence of ≥300 µg/mL or 100 µg/mL of bezlotoxumab, respectively.

Bioanalysis of ADA was conducted using the standard 3-tiered assay approach that consisted of screening (Tier 1), confirmatory (Tier 2) and antibody titer assessment (Tier 3). Samples that were classified as "potentially positive" in the screening tier were tested in the confirmatory tier. For confirmed ADA positive samples (based on the confirmatory assay), the immune response was further characterized for antibody titer to measure the magnitude of the response; and for NAb, to determine if the antibodies neutralized or blocked binding, of bezlotoxumab to *C. difficile* toxin B.

The NAb assay is based on the ability of ADA to block (neutralize) the critical first step in the pharmacological action of bezlotoxumab, which is binding to *C. difficile* toxin B (the in vivo target). A validated non-cell-based competitive ligand binding NAb assay was used in this study. The positive control used in the assay was a mAb. The sensitivity of the assay without the presence of drug was 369 ng/mL of the positive control. Drug tolerance testing showed that 1354 ng/mL of positive control could be detected in the presence of 20 µg/mL bezlotoxumab. Tier 2 samples that were confirmed positive in the ADA immunoassay were analyzed in the noncell-based NAb assay by the Sponsor in an unblinded manner.

Immunogenicity Results

Two of the 100 participants (42 in Cohort 1 and 58 in Cohort 2) that were evaluable for immunogenicity assessment had a low magnitude ADA positive response to bezlotoxumab. In Cohort 1, one participant was determined to be treatment-boosted positive. The participant had a baseline (Screening) titer of 5, which continued to be 5 at Day 28 and which increased to 25 at Day 84. In Cohort 2, one participant was determined to be treatment-emergent positive with a sample that tested positive at Day 84 with a titer of 1. Neither participant had any samples positive for NAb.

Three of 100 participants (3%) demonstrated positive results at baseline, but subsequent samples failed to increase in titer following dosing with bezlotoxumab; these participants were therefore considered to be nontreatment emergent positive, as the positive signal in these participants was not related to bezlotoxumab. None of the ADA positive samples from these 3 nontreatment emergent positive participants was positive for Nab.