Letter to the Editor

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Reply to: "Bepirovirsen/GSK3389404: Antisense or TLR9 agonists?"

To the Editor:

Dr Andrew Vaillant has hypothesized that the principal mechanism of action of GSK3389404 (N-acetylgalactosamine [GalNAc]-conjugated antisense oligonucleotide [ASO]) and bepirovirsen (non-GalNAc-conjugated ASO with the same target sequence as GSK3389404) is through immune stimulation rather than mRNA degradation.[1] Although the relative contribution of mRNA/pregenomic RNA degradation and immune stimulation is still under investigation, we would like to make the following comments.

Dr Vaillant asserts that the mechanism of action of ASOs and small-interfering RNAs (siRNAs) is inconsistent with mRNA degradation due to the rapid selection of individual point mutations.[1] While it is true that HBV reverse transcriptase (RT) has no proofreading activity, its substitution rate is lower than that of other RT-dependent viruses, and is variable along the genome, with lower substitution rates in overlapping *vs.* non-overlapping regions.[2] The binding site of bepirovirsen and GSK3389404 is highly conserved across different HBV genotypes, where single point mutations potentially affect both the polymerase and X protein.[3]

Replication of HBV can be effectively inhibited by nucleos(t)ide analog (NA) therapy without selection of NA resistance despite long-term clinical use.[4] Consequently, the addition of an siRNA or ASO to a patient already receiving stable NA therapy with suppressed HBV replication is unlikely to lead to the rapid development of resistant infectious virus particles. Participants in the GSK3389404 study were on stable NA therapy prior to enrollment and had suppressed viral replication.[5] We note that the "saw tooth" HBsAg response observed with dosing of ARB-1467 mentioned by Dr Vaillant may be accounted for by other explanations such as drug exposure. No resistance information was provided in the reference cited.

Dr Vaillant also affirms that single mutations within the binding site will abolish activity. In the article referenced in support of this statement, double nucleotide mismatches within the central region of the binding site resulted in complete loss of activity for both siRNAs and RNase H-dependent oligonucleotides, whereas a double mutant (single nucleotide substitution in the 5' and 3'-terminal domains) reduced, but did not abolish, activity. This is consistent with mutations having differential effects depending on their location but does not support that single mutations will abolish activity. Work to characterize nucleotide substitutions within the bepirovirsen/GSK3389404 binding site is ongoing using next-generation sequencing. An *in vitro*-selected single mutation within the center of the bepirovirsen/GSK3389404 binding site conferred a 4–5-fold reduction in susceptibility;[3] the clinical significance of these results is not yet established.

We and Dr Vaillant share a mutual understanding of the secondary pharmacology of bepirovirsen by activating innate immune sensors such as toll-like receptors (TLRs). Dr Vaillant hypothesized that bepirovirsen acts as a TLR9 agonist via one CpG motif present in the DNA part of the gapmer. Typically, two CpG motifs are required for TLR9 activation in humans, while only one motif may be sufficient in rodents.[6] However, the location of the motif and composition of RNA wings may impact TLR9 activation.[6] It is therefore unclear if bepirovirsen is a *bona fide* TLR9 agonist, and *in vitro* studies indicate this is not the case.[7] If an ASO can trigger TLR9 activation, it should show elevation of key serum markers typically induced by TLR9 activation in wild-type mice or cell lines.[8] However, cytokines that are typically part of a type I immune response upon TLR9 activation, such as IL-12p40 and interferon alpha (IFN-α),[8] were not observed with bepirovirsen in wild-type C57BL/6 mice.[7] Furthermore, IL-12p40, but not IFN-α, was induced by bepirovirsen and selgantolimod, a well-characterized TLR8 agonist, in the plasma of human TLR8 knock-in mice, supporting that bepirovirsen mimics the responses of a TLR8 agonist.[7]

Dr Vaillant strongly disputed that bepirovirsen can activate TLR8 because 2'-*O*-methylation in the RNA part of the ASO would abrogate TLR8 activation. While methylation may decrease TLR8 responsiveness, there have been instances where methylated RNA can still activate TLR8 or even enhance TLR8 recognition over TLR7.[9]

In the B-Clear Phase IIb study, bepirovirsen produced robust reductions in HBsAg in participants on stable NA therapy and those not on NA.[10] In contrast, only very modest reductions have been seen with TLR agonists.[11] At the end of 24-week treatment with bepirovirsen 300 mg, approximately 30% of participants achieved HBsAg below the lower limit of detection,[10] something rarely observed with siRNAs.[12] This suggests an important contribution of the immunostimulatory properties of bepirovirsen towards the observed clinical data. Our results therefore support a primary mode of action of bepirovirsen through RNA degradation, and the immunostimulatory activity of bepirovirsen via immune sensors, such as TLR8, in the liver may be synergized with HBsAg reduction, leading to the clinical responses observed. Efforts to better understand the molecular mechanisms behind the virological and immunological responses to bepirovirsen are ongoing.

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Conflict of interest

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Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

M-F Yuen, was involved in the conception or design of the study and data acquisition. M-F Yuen, R Elston and S You were involved in data analysis or interpretation. The manuscript was reviewed and edited by all authors. All authors approved the manuscript for submission and vouch for the accuracy/completeness of the data.

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Supplementary data

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Author names in bold designate shared co-first authorship

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