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Development of an Oligonucleotide Drug Immunogenicity Assay: A Case for the Characterization of the Immune Response

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INTRODUCTION

While the immunogenicity of protein-based biologics and the characteristics of the antibody responses directed against them are well-understood, the same is not true for other categories of biologics. For example, molecules like oligodeoxynucleotides (ODN), which are typically thought to be non-immunogenic, can shift the paradigm when certain features are present.

As is seen for protein-based biologics, ODN-based therapeutics are being administered with novel technologies and formulations to tackle new and old diseases. This, in turn, renders their immunological assessment more complex and multifaceted, as those new features can all be targets of immunogenic responses.

At Altasciences, an immunogenicity assay was recently developed to support a clinical immunogenicity program for a phosphorothioate (PS) ODN toll-like receptor 9 (TLR9) agonist drug. As with most TLR9 agonists, the drug featured repeated unmethylated CpG motifs, which are also present in bacterial and viral DNA. This combination of PS backbone and CpG motifs is known to be immunogenic, and is sometimes used as a vaccine adjuvant.

Figure 1. Visual representation of a CpG motif

During assay development, a very high percentage of treatment-naive human serum lots were showing varied basal responses against the drug, with high inhibition in the confirmatory assay (upwards of 40%). An investigation was initiated to understand and characterize this immune response, which seemed to be attributed to pre-existing reactivity to ODNs.

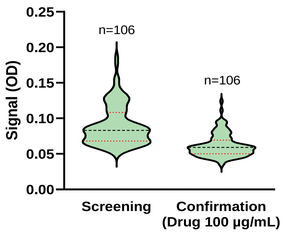

Figure 2. Comparison of screening and confirmatory signals of healthy serum lots

METHOD

The assay is an indirect enzyme-linked immunoassay (ELISA). The drug is coated on the plate, which is subsequently washed and blocked. Samples are added on the plate to allow the bindings of the anti-drug antibody (ADA), the plate is washed, and a solution of horseradish peroxidase (HRP) coupled protein A/G/L is added to the wells. After a final wash, 3,3′,5,5′-Tetramethylbenzidine (TMB) is added to detect the presence of ADAs.
As part of the investigation of the reactivity, it was first important to determine the source of the reactivity. Two sources were envisioned:

1. Non-antibody-interfering components in the serum binding to the PS backbone

To evaluate this hypothesis, the serum was depleted of antibodies using magnetic beads coupled with protein A/G as a sample pre-treatment step. Due to the high specificity of protein A/G to antibodies, a reduction in signal would mean that the observed response was indeed caused by antibodies.

2. Pre-existing/cross-reacting antibodies directed against the sequence, the PS backbone, or a combination of both. The reactivity of each component would need to be evaluated.

To evaluate hypothesis 2, different parts of the ODN were used in a competitive binding assay format to confirm whether the reactivity was directed either against the sequence only (without the PS backbone modification), to the PS backbone itself (using a random scramble sequence), or both. ODN featuring these characteristics were synthetized and used as coating, and as confirmatory reagents.

Reactivity against the PS backbone alone could suggest non-specific binding due to the "stickiness" of the sulfur atoms, but binding against every part of the ODN would suggest specific antibody response.

RESULTS

Identification of the source of reactivity

The IgG depletion by use of protein A/G-coupled magnetic beads strongly reduced the response in most human serum lots, which confirmed that the observed response was caused by antibodies.

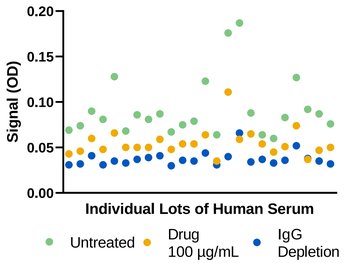

Figure 3. Comparison between untreated and confirmed (drug 100 µg/mL) IgG signal, and IgG-depleted signal

In addition to the IgG depletion and the confirmation with 100 µg/mL of the drug both showing reductions in signal, there was a meaningful correlation between the 2 approaches (R2 = 0.5). This reinforced the conclusion that the signal observed was indeed due to antibodies, as the signal reduction was proportional using both approaches.

A lack of correlation could have meant that the response observed was a mix of specific and non-specific binding, or that multiple sources of reactivity were present.

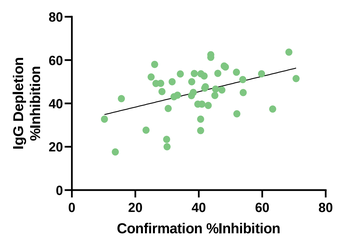

Figure 4. Correlation between IgG depletion and confirmation (drug 100 µg/mL)

Characterization of the response

The domain specificity of the antibodies was determined by inhibiting samples with the drug, the PS backbone, or the sequence. When the plate was coated with the drug, all samples tested showed an inhibition of signal when confirmation was performed using the drug. The results were more varied when confirmation was performed using the backbone or the sequence. Four lots showed some signal reduction against the backbone, but only two showed the same with the sequence. In all instances, the signal reduction was stronger with the drug than with the individual component of it.

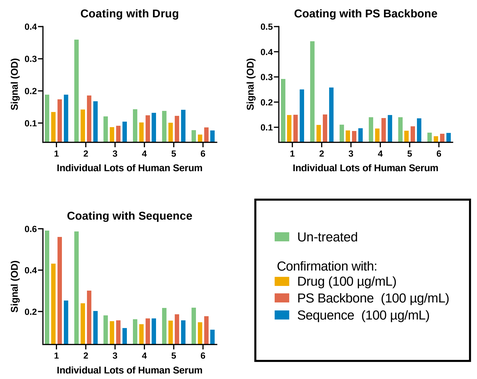

Figure 5. Evaluation of the antibody affinities of human serum lots by coating and confirmation with different parts of the drug

When the sequence was used as a coating antigen, the signal of the individual lots was overall higher, and confirmation with the sequence resulted in higher signal reduction. When the PS backbone was used for the coating, the signals were comparable to the drug, and confirmation was higher with the drug, followed by the backbone. The sequence typically resulted in poor signal reduction.

These results suggest that the observed response against the drug is composed of a mix of reactivity against the sequence, the backbone, and the combination of both. In addition, the presence of signal reduction when confirming with a part of the drug that was not coated suggest some cross-reactivity or the presence of some non-specific binding.

Cut-Point Approach Evaluation

Upon confirmation that the pre-existing reactivity was caused by antibodies, it was determined that a typical three-tiered assay approach to define cut points was not possible due to the high inhibition of >80% of the individual lots. In addition, due to the high biological variation between individuals (see figure 2), a typical tiered approach could miss significant increase in signal between pre- and post-dose samples.

Therefore 2 approaches were evaluated to determine the cut points:

The Pseudo-Negative Approach – The confirmed signal is used to set the screening and titration cut-point (pseudo-negative cut-point).

- Samples screened against the pseudo-negative cut-point and positives are titered.
- This is incompatible with a confirmation cut-point.

Figure 6 shows the results of the cut-point evaluation performed in method development and the average normal and pseudo-negative cut-points, as applied to the distribution. These results demonstrate the benefit of using the pseudo-negative and how most individual lots would screen positive against it due to the high incidence of pre-existing reactivity.

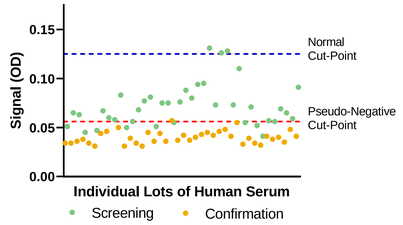

Figure 6. Data from the cut-point evaluation, showing high pre-existing reactivity and screening cut-points

The Hybrid Titration Approach – The screening signal of each post-dose sample is compared against its pre-dose signal.

- Post-dose samples signal higher than the sample specific cut-point (derived from the screening mean significant ratio (S-MSR)) are titered.
- Post-dose samples titer higher than the titration MSR are considered as treatment-boosted samples.

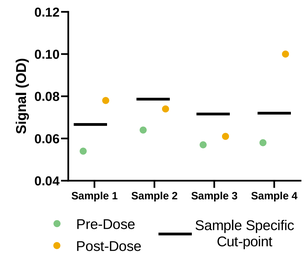

Figure 7. Illustration of the screening phase of the hybrid titration approach

Cut-Point Approach Comparison

The pseudo-negative and hybrid titration approaches were compared in their ability to detect treatment-boosted responses using the cut-point data-set to simulate treatment-boosted samples.

- The cut-point factor of the pseudo-negative approach is 1.408
- Mean significant ratio of the hybrid titration approach is 2.670

Simulated treatment boosted samples are extrapolated by computing:

Screening signal × S−MSR=Treatment Boosted Signal

These treatment boosted samples were then evaluated against the pseudo-negative cut-point in order to determine if any of them were misclassified as negative.

Following this exercise, if any "treatment-boosted" sample were to be identified as negative, it would suggest a risk of false negatives with the pseudo-negative approach.

For these assays, it was determined that any sample flagged as "treatment-boosted" in the hybrid titration approach would also be flagged as positive in the pseudo-negative approach (see figure 8), therefore confirming that the pseudo-negative approach is sufficiently sensitive for the identification of treatment-boosted samples.

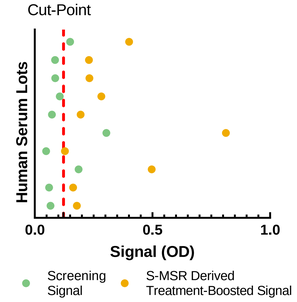

Figure 8. Pseudo-negative cut-point vs. hybrid titration approach

CONCLUSIONS

Although ODNs are typically non-immunogenic, unmethylated CpG ODNs are known to be immunogenic in nature because they are a staple of bacterial and viral DNA, something to which subjects are exposed in their daily lives, and against which they have previously mounted an immune response. It is suspected that the observed pre-existing antibodies in this project are caused by similarities between the drug- and pathogen-derived antigens.

The IgG depletion confirmed the observed reactivity that was caused by antibodies. By using various parts of the ODN drug, the binding specificity of the antibodies was explored. The results allowed us to conclude that the individual components (the sequence and the backbone) can bind antibodies, but as expected, the combination of the two components (the PS ODN drug) results in higher affinity for the detected antibodies.

As for the cut-point approaches, the hybrid titration is a novel and useful tool when the goal is to assess the immunogenicity of a drug in the presence of a predominant pre-existing reactivity. Although the pseudo-negative cut-point is a simpler approach to use when there is no added value of the hybrid approach, the latter is not reliant on the performance of the confirmatory assay. Moreover, since it better incorporates the biological variability of the population during the cut-point assessment, it is well-suited for analyzing samples in the presence of strong pre-existing reactivity and varied baseline response – a combination which can render the cut-point assessment very complex.

CLOSING STATEMENT

As biotherapeutics are becoming more complex, for example to increase their half-life and ensure efficient targeted delivery, their immunogenic profile should also be well-characterized in order to have an effective immunogenic assessment at the clinical stage. Indeed, specific features of a drug can either increase or reduce their overall immunogenicity and it is often worth exploring the source of unexpected reactivity as it allows the use of an appropriate strategy when assessing immunogenicity against a drug.