Development and validation of neutralising anti-drug antibody (Nabs) assays

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• Anti-drug antibody and neutralising antibody responses

• Three programmes of work:
  – Two cell-based assays (CBA)
  – One competitive ligand-binding assay (LBA)

• Challenges we have faced and the approaches we have chosen and been asked to adopt
Anti-Drug Antibodies (ADA)

- Biological therapeutic molecules can elicit immune responses
- Anti-drug antibodies (ADAs) can have inconsequential effects or lead to efficacy or safety issues particularly if they are neutralising in nature
  - Neutralising (Anti-drug) Antibodies (NAbs) –
    - Efficacy: neutralise drug effects
    - Safety: neutralise homologous, non-redundant, endogenous protein
- Detecting NAb s important part of drug development strategy
  - Pre-clinical: interpretation of toxicology data
  - Clinical: interpretation of PK, PD and safety data
- ADA - frequently an immunoassay format
- NAb - ‘functional’ cell-based assay is recommended although not always possible
Assay requirements

• Positive control antibody
• Sensitivity
  – Related to ADA
• Cut point
• Specificity
• Precision
• Drug tolerance
  – Long ½ life / confirmatory assay
• Dilution
• Robustness
• Positional variation
• Confirmable

Gupta et al 2007
Recommendations for the design, optimization, and qualification of cell-based assays for the detection of neutralizing antibody responses elicited to biological therapeutics
Journal of Immunological Methods 321 p1-18

Gupta et al 2011
Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody responses elicited against biological therapeutics
Journal of Pharmaceutical and Biomedical Analysis 55 p878-888
• Functional cell-based assay for drug effect
• Drug effect:
  – Direct – induces biological effect (proliferation/cytotoxicity/pathway activation/protein or gene expression)
  – Indirect - antagonism/agonism of normal biological process (receptor/ligand binding)
• Blocked by NAb
Cell-based vs Ligand binding assay

- Competitive ligand binding assay for drug
- Receptor or ligand is coated onto plates
- Labelled drug is added

- Blocked by NAb

Drug molecule

Drug molecule + NAb

Drug target/receptor
Pros and cons

- Cell-based assays (if available) are generally preferred
- But they do require a good understanding of the drugs mode of action and biology
- They require appropriate cell lines
- Can take longer to develop and validate
- Often are more susceptible to matrix effects and to drug interference
- Generally are less precise and robust than LBA

- Ligand binding assays are (generally) developed more rapidly and more robust
- Have shorter assay times
- More likely to meet validation criteria

- However may not represent a true functional read out for Nabs

- Finco et al 2011 Comparison of competitive ligand-binding assay and bioassay formats for the measurement of neutralizing antibodies to protein therapeutics Journal of Pharmaceutical and Biomedical Analysis 54 (351–358)
Example 1

- Drug: Hormone recently shown to possess vasodilatory properties, assessed as a therapy for acute heart failure (AHF)
- Quotient are supporting the PK, ADA and NAb
Assay Format

- Drug binds to a G protein-coupled receptor (GPCR) on cells
- This initiates an intracellular signalling pathway, increasing cAMP accumulation within cells
- NAb in sample binds to Drug to prevent receptor binding, pathway activation and therefore cAMP accumulation
HTRF Assay

- Drug NAbs are detected using a cell-based HTRF method from CisBio
- HTRF: homogenous time-resolved FRET
- FRET: Förster Resonance Energy Transfer
Assay Format

High cellular cAMP

Processed HTRF ratio = \[ \frac{\text{emission at 665 nm}}{\text{emission at 620 nm}} \times 10,000 \]

The processed HTRF ratio will increase as intracellular cAMP decreases
cAMP Standard Curve

Processed HTBF ratio (665/620 nm x 10,000) vs. cAMP concentration (nM)
Drug Standard Curve

- Cells were incubated with increasing concentrations of Drug
Screening Cut Point
Assessing whether a sample is positive for neutralising effect

• 51 individual serum samples incubated with cells and a fixed Drug concentration on 6 occasions
• The screening cut point (SCP): 95\textsuperscript{th} percentile - theoretically detect 5% false positive samples
• The assay cut point factor (CPF): Plate cut point / mean NC
• The mean of the assay CPF (1.13) was used to calculate plate cut points for subsequent assays: Mean NC*CPF

• Screening positives above threshold
**Assay Sensitivity**

- NAb standard curve: Cells + fixed concentration of Drug + increasing concentrations of positive control NAb (goat polyclonal anti-drug)
- Defined as the interpolated NAb concentration corresponding to the assay cut point in six assays
- Sensitivity: 1449 ng/mL
Antibody Specificity

- NAb standard curve incubated with either Drug or “Iso-Drug” (alternate drug isoform)
- NAb inhibits Drug induced cAMP signalling
- NAb does not inhibit Iso-Drug induced cAMP signalling
- Result: the NAb is specific for Drug
Drug Tolerance / Interference

- Can the NAb be detected in the presence of Drug in serum samples?
- Serum was spiked with increasing Drug and NAb before being incubated with cells
- Results showed the method is not tolerant to any additional Drug
- This may not be a major issue as Drug is single dose and has a short half life in serum
The CisBio HTRF assay

- Easy to use, quick, low-volume (20 µL), robust and reproducible (within the confines of a cell-based assay)

- Cells as a reagent
- Matrix effect negligible (10-fold MRD)
- Good Positive Control – goat polyclonal anti-drug
- Screening with a threshold appropriate
  - Sensitivity and therefore cutpoint high (~1500 ng/mL)
  - Pre-dose vs post-dose
Example 2

- Drug: PEGylated glyco-protein hormone
- Assay: Murine myeloblastic cell line proliferates in response to Drug and endogenous non-PEGylated counterpart
Drug standard curve

- Cells + Drug in media
Neutralising assay

- Positive control NAb - AF purified Goat polyclonal anti-endogenous molecule (not PEGylated)
Effect of antibody

- Cells + Drug standard curve + positive control antibody in media
Matrix Effect

- Cells + Drug + positive control antibody + Human Serum diluted in media
• Assay Components
  – 100 µL 4x10⁴ cells/mL
  – 50 µL drug (prepared in growth media)
  – 50 µL ‘sample’ (positive control antibody in human serum +/- dilution in media)
Matrix Effect – loss of sensitivity

- Cells + Drug + ‘sample’ (positive control antibody spiked into serum and diluted in media)
Assay responds well to drug and effect is sensitive to positive control antibody

**BUT**

Human serum has a serious effect on cell growth and survival, need to dilute 50-fold – therefore sensitivity also reduced 50-fold

Investigated strategies including heat inactivation and Acid dissociation
  - No improvement

Antibody purification- Either drug-specific Affinity Purification or Protein A/G purification (i.e. magnetic beads)

Development and optimization of a cell-based neutralizing antibody assay using a sample pre-treatment step to eliminate serum interference

Krista M. McCutcheon a,*, Valerie Quarmby b, An Song b  Journal of Immunological Methods 358 (2010) 35–45
Other considerations

• Positive control antibody
  – AF purified Goat polyclonal anti-endogenous molecule (not PEGylated)
  – Is this representative of drug?
  – Anti-PEG antibodies
  – Antibodies specific to the addition of PEG not represented

• Confirmatory Assay
  – Alternative stimuli not possible in this case
  – Endogenous molecule and PEG
Example 3: Ligand binding Assay

- **Drug:** fully humanised anti-receptor “X” antibody
- **Transfer and validation of client developed assay**
- **Positive control antibody:** client-supplied Rabbit affinity purified anti-drug Fab fragment

Pre-incubate sample (positive control antibody) with biotinylated Drug

Poly-HRP-streptavidin detection solution visualised with TMB

Nickel coated plate

His-tagged Receptor
Competitive Ligand binding Assay

- Assay was sensitive (~300 ng/mL)
- Reproducible (interassay precision & accuracy < 20 %CV)
- CPF ~0.4 (competitive assay)
- Drug interferes at < 1 ng/mL - no drug tolerance & antibody therapy has long ½ life (as shown by PK)
- Confirmatory Assay – can’t do immunocompetition
  - NAb screening positive if pre-dose above cutpoint and post-dose below cutpoint (competitive assay)
  - Perform endpoint titre on both samples - positive if post-dose sample 1 whole titre > than pre-dose
- BUT is this assay truly detecting neutralising antibodies?
  - Biotin may prevent NAb binding
  - NAb could prevent streptavidin binding
  - Can antibody that doesn’t prevent receptor binding still neutralise
• Complex and Challenging
• Time is required to optimise and validate –so plan early
  – Typically very limited use!
  – Exercise pragmatism
  – Format
  – Positive control antibody
  – Sensitivity / threshold
• Need to look at data in context of PK/PD and safety data to see if it is meaningful