Challenges in Developing a Neutralizing Antibody Assay for a Cyno Toxicology Study

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Drug X

- Drug X is being evaluated as a potential therapeutic for metabolic disorders, such as diabetes, obesity, and severe lipodystrophy
- The *E. coli* expressed protein is methionated at the N-terminus
- 16.5KD human protein, site-specifically PEGylated with 40K linear PEG at residue 111, where the native amino acid has been exchanged for a synthetic amino acid, para-acetylphenylalanine, or pAF
- The protein has been associated with varied magnitude incidence of immunogenicity (0%-100%)
- Varied incidence may be attributed to multiple factors
Toxicologic Study Design

• DIO Cynomolgus Monkey
  ▪ Fed high fat chow for 6 weeks

• Two dose groups
  ▪ 0.3mg/kg and 1.0mg/kg

• Once weekly dosing, final dose on Day 42

• PK sample collection out to Day 88

• ADA sample collection out to Day 88
  ▪ 46 Day washout period

• Clinical pathology, histopathological, and PD markers monitored
  ▪ Lean muscle vs. fat mass loss
  ▪ Food consumption volume and duration
  ▪ Circulating triglycerides
Antibody Response to Weekly SC Administration

RLU and Titer Values for QW Dosing

- **RLU**
- **Titer**

Group 2: Ind #6, Predose
Group 2: Ind #6, D21
Group 2: Ind #6, D49
Group 2: Ind #6, D64
Group 2: Ind #8, D21
Group 2: Ind #8, D35
Group 2: Ind #8, D63
Group 2: Ind #15, Predose
Group 2: Ind #15, D21
Group 2: Ind #15, D49
Group 2: Ind #15, D64
Group 2: Ind #24, D14
Group 2: Ind #24, D35
Group 2: Ind #24, D63
Group 2: Ind #24, D70
Group 2: Ind #10, Predose
Group 2: Ind #10, D21
Group 2: Ind #10, D49
Group 2: Ind #10, D64
Group 2: Ind #10, D88
Group 2: Ind #13, D14
Group 2: Ind #13, D35
Group 2: Ind #13, D63
Group 2: Ind #13, D70
Group 3: Ind #21, Predose
Group 3: Ind #21, D21
Group 3: Ind #21, D49
Group 3: Ind #21, D64
Group 3: Ind #21, D88
Group 3: Ind #24, D14
Group 3: Ind #24, D35
Group 3: Ind #24, D63
Group 3: Ind #24, D70
Group 3: Ind #30, D14
Group 3: Ind #30, D35
Group 3: Ind #30, D63
Group 3: Ind #30, D70
## Characterization of Antibody Response

<table>
<thead>
<tr>
<th>Assay Format</th>
<th>Confirmation</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Cell-Based</td>
<td>• WT</td>
</tr>
<tr>
<td>• Plate-Based</td>
<td>• Non-PEGylated</td>
</tr>
<tr>
<td></td>
<td>• PEGylated</td>
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</tbody>
</table>

### Neutralizing Ab Assay Considerations

<table>
<thead>
<tr>
<th>PC Sensitivity</th>
<th>Drug Tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>• PAb vs. Mab</td>
<td>• Significant [drug] on board</td>
</tr>
<tr>
<td>• Commercial or Custom?</td>
<td>• Tox study – not ideal for NAb</td>
</tr>
</tbody>
</table>

### Assay Format Details

- **Cell-Based**
- **Plate-Based**

### Confirmation Details

- **WT**
- **Non-PEGylated**
- **PEGylated**
### Neutralizing Antibody Assay Development

#### Format
- Plate-based assay
- Support accelerated timeline

#### Confirmation / Detection
- PEGylated
- pAF-incorporated
- WT
  - Human
  - and cyno

#### PC
- R&D PAb
Assay Format – Round One

- Method development with Drug X
- Low signal to noise
- Low to Zero Drug Tolerance

Format I – coat with Drug X
Format II – coat with receptor
Amgen Method (Gupta, et. al.)
Assay Format – Round Two

Screening Ratio (with Drug X – Sample/Control RLU) = Sample RLU/Drug (D) Control
Specificity Ratio (no Drug X - Sample/Control RLU) = Sample RLU/Max Signal Control (M)

Poor PC Inhibition – No Increase in Signal Observed
Assay Format – Round Three

Max Signal

NAb inhibits binding

Problem: Excess Drug interferes significantly and reduces signal false positive results

• Good signal to noise with Drug X and WT Protein
• Assay not reproducible with Drug X
• Sensitivity is 2 μg/mL with WT Protein
• Drug Tolerance is an issue
Significant Interference Due to Drug

Signal (RLU)

ug/mL anti-Drug X pAb

No Drug X
10ug/mL Drug X
5ug/mL Drug X
2.5ug/mL Drug X
1.25ug/mL Drug X
0.625ug/mL Drug X

Negative
Positive
False Positives


drug concentration in ug/mL:
- 0
- 0.625
- 1.25
- 2.5
- 5
- 10
- 20

Drug positive:
No Drug X
2.5ug/mL Drug X
1.25ug/mL Drug X
0.625ug/mL Drug X
5ug/mL Drug X
10ug/mL Drug X
20ug/mL Drug X

...Graph showing signal (RLU) vs. concentration (ug/mL) for different levels of Drug X pAb (anti-Drug X antibodies). The graph highlights the interference caused by various concentrations of Drug X on the signal measurement.
Assess SE and Protein A Purification

MW cutoff filters
• Acidify sample – dissociate bound antibody from Drug X or endogenous
• Filter through 100K filter to retain antibody and buffer exchange
• Assay

Protein A purification (protein A pipette tips)
• Acidify sample – dissociate bound antibody from Drug X or endogenous
• Filter through 100K filter to retain antibody
• Protein A purify sample
• Assay
100 kDa Filters with high salt elution buffer did not remove excess Drug X

Cyno Plasma
No anti-Drug X Polyclonal Antibody

Cut Point

<table>
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<tr>
<th>Concentration</th>
<th>ARX328</th>
<th>ARX328</th>
<th>ARX328</th>
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<tbody>
<tr>
<td>10 ug/mL</td>
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</table>

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Protein A purification required a gentle elution with high salt vs acid elution.

[Bar chart showing the comparison between gentle elution and acid elution at different pH levels. The chart indicates that gentle elution yields higher antibody production compared to acid elution at pH 2.8, with a cut point identified for selection.]
The polyclonal antibody was a poor positive control.
Sensitivity was greatly improved with the Epitomics monoclonal antibody supernatant.
0.5 ug/mL mAb can tolerate <250 ng/mL Drug X and 5 ug/mL mAb can tolerate >16 ug/mL Drug X
nAb Run #1 – cut point based on NC not a panel

Plate specific Cut Point

Group 2 (0.3 mg/kg SC)

Group 3 (1.0 mg/kg SC)
Assay Format – Round Four
Final Method

Acidification with gentle elution buffer
Protein A purification

Anti-Drug X NAb

Ruthenylated drug*

Biotinylated human Drug X receptor chimera

Ruthenylated drug*

* Human WT met-protein X, human pAF Drug X, cyno met-protein X
Cut Point for Each Form of Drug

<table>
<thead>
<tr>
<th>Panels</th>
<th>Human WT</th>
<th>Human pAF</th>
<th>Cyno WT</th>
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<tbody>
<tr>
<td>Gp1, n=1, 2116h</td>
<td>355</td>
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<tr>
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<td>128</td>
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<tr>
<td>STDEV*1.645</td>
<td>80</td>
<td>210</td>
<td>1325</td>
</tr>
</tbody>
</table>

Cut point (5% FP) 334 787 18782
Req % drop in cut point 19.2 21.1 6.60
Percent drop in signal compared to pre-dose

- human WT
- human pAF
- cyno WT

- Human WT cut point
- Human pAF cut point
- Cyno WT cut point
Pharmacodynamic Markers

- Drug was well tolerated overall, in terms of all clinical pathology and histopathological markers
- Statistically significant loss or decrease in each of the following:
  - Food consumption volume
  - Duration of food consumption
  - Body weight
  - Brown adipose tissue
  - White adipose tissue
  - Circulating triglycerides
Summary

• Drug X, administered QW, generated a robust antibody response, detected in the presence of very high levels of drug in circulation

• Neutralizing antibodies were more difficult to detect due to issues related to drug tolerance

• Drug tolerance was addressed through implementation of acid dissociation and protein A purification

• Comparison was performed via pAF incorporated protein and WT protein to ensure detection of antibodies to Drug X as well as cross-neutralizing antibodies, those which would bind to the endogenous protein

• Although neutralizing antibodies were generated, the clinical significance is difficult to predict

• 2 of 7 animals tested had altered PK

• PD endpoints were well-maintained throughout the course of the study for all animals
Acknowledgements

• Kristine de Dios, M.S., Scientist, Ambrx, Inc.
  ▪ Bioanalytical Lead, Scientist, Preclinical Science

• Kari Cox, Ambrx, Inc.
  ▪ Associate Scientist, Cell Assay, Preclinical Science

• Lorraine Sullivan, Ph.D.
  ▪ Toxicologist, Program Manager, Preclinical (now at BioMarin)

• Valerie Leesch, Ph.D.
  ▪ Associate Scientific Director, Bioanalytical Sciences, WIL Research

• Jenifer Vija, Ph.D.
  ▪ Director, Bioanalytical Sciences, WIL Research
Thank You
WT Human Drug X NAb Data

Cut Point

Pre-Dose

Term

Gp2, n=6
Gp2, n=6
Gp2, n=6
Gp2, n=6
Gp2, n=8
Gp2, n=15
Gp3, n=10
Gp3, n=13
Gp3, n=21
Gp3, n=30
pAF Human Drug X NAb Data

Cut Point
Cyno WT Drug X NAb Data

Pre-Dose

Term

Cut Point

Gp2, n=6
Gp2, n=6
Gp2, n=6
Gp2, n=6
Gp2, n=8
Gp2, n=15
Gp3, n=10
Gp3, n=13
Gp3, n=21
Gp3, n=30