A Potent Solution to a Low Affinity Problem

Potency Release Methods for an NK Cell Targeted Cytokine

CASSS CMC North American Strategy Forum
18-19 July 2022

Morgan Wilson
On behalf of the Inhibrx CMC Team
INBRX-121 – Path to Potency Assays

Topics to Cover

Mechanism of Action
- INBRX-121 components and mechanism of action

INBRX-121 Assays
- Out-of-the-gate challenges
- Assay development strategy for a Clinical Phase 1 program

INBRX-121 Cell-Based Bioassay
- Key functional considerations of INBRX-121 and cell line design
- Cell line characterization strategy
- Pre-validation de-risking and assay performance

INBRX-121 ELISA
- Concept and development strategy
- Pre-validation de-risking and assay performance

Early Structure/Function Assessment
- Hinge cleaved INBRX-121
INBRX-121 Introduction

Components

- High-affinity single-domain antibody (sdAb) targeting NKp46 combined with an engineered IL-2 variant with reduced affinity* for the IL-2 receptor
- IgG1 Fc with ablated effector function

MOA

- The large affinity difference between the sdAbs and IL-2-X ensures specific modulation of intended target cells through cis-signaling, specifically enhancing NK cells without impacting the T cell subsets or vascular endothelial cells

*Discussed in detail on following slide
Immediate Challenges

Low Affinity (Detuned) IL-2-X

**Problem Statement**

A detuned IL-2 is challenging to model with bioassays

**Challenge**

No commercial/off-the-shelf options

**Solution**

Design INBRX-121-specific potency cell line

**Challenge**

Low affinity IL-2 difficult to bind

**Solution**

Screen various approaches to binding assays

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**NKp46 Binding (High Affinity)**

**IL-2-X Receptor Binding (Low Affinity)**

**PBMC Affinity**

- NK Cells
- CD8 T cells
- Tregs
- CD4 T Cells
- B Cells
- CD14+ Cells
- NK-T Cells

Angelica Sanabria

**CD25**

- INBRX-121
- WT-IL-2

- $K_d = 0.046 \text{ nM}$

**CD122**

- INBRX-121
- WT-IL-2

- $K_d = 3.4 \text{ nM}$

**CD122/132**

- INBRX-121
- WT-IL-2

- $K_d = 1.5 \text{ nM}$

- $K_d = 50 \text{ nM}$

Abraham Hussain

**Cell-Based Bioassay**

**ELISA**
**Assay Strategy**

*Clinical Phase 1 Program*

**Cell Line Generation**
- Clone selection
- Characterization
- Banking

**Cell-Based Bioassay**
- Development
- DOE/Robustness
- Pre-qualification

**Functional ELISA**
- Development
- DOE/Robustness
- Pre-qualification

*Time prior to assay validation*

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**Functional Considerations**

Both assays must be able to model:
- NKp46 binding
- IL-2-X functional region
- Complete molecule

Potential PTMs / molecule variants should be considered during assay design
INBRX-121 Cell-Based Bioassay

Problem Statement
No commercial/off-the-shelf options

Solution
Design novel INBRX-121-specific potency cell line
Key Functional Considerations for Cell-Line Design

**NKp46 Targeting**

- Binding of NKp46-specific sdAb part of INBRX-121 to PBMC subpopulation in healthy human donor blood

**IL-2 Signal Transduction and Specificity**

- Downstream pSTAT5 signaling is driven by IL-2 binding

- Specificity of NKp46 targeting combined with IL-2-X signaling

- NK cell-specific pSTAT5 signaling is the result of:
  1. NKp46 targeting
  2. Detuned IL-2-X binding to its receptor

**PBMC Binding**

- $K_d = 0.046 \text{ nM}$

**pSTAT5 Signaling**

- $EC_{50} = 0.245 \text{ nM}$
Cell Line Design and Generation

Commercial Cell Line

- HEK-Blue™ IL-2 cells from InvivoGen

Augmented INBRX-121-Specific Cell Line

- Addition of NKp46 “Dummy Receptor”

Specific Engagement of INBRX-121

- With the addition of NKp46 extracellular and transmembrane domains, we observed a 250x difference between INBRX-121 and untargeted IL-2-X
- This allows us to effectively model the affinity difference between the sdAbs and IL-2-X

The binding of IL-2 to its heterotrimeric receptor leads to the JAK/STAT signal cascade and production of SEAP

SEAP production can be colorimetrically quantified with the addition of substrate

NKp46 extracellular and transmembrane domain was stably engineered into the HEK-Blue™ IL-2 SEAP reporter gene line from InvivoGen

With the addition of NKp46 extracellular and transmembrane domains, we observed a 250x difference between INBRX-121 and untargeted IL-2-X

This allows us to effectively model the affinity difference between the sdAbs and IL-2-X

Additional details and cell line history can be found in the appendix
Cell Line Characterization

<table>
<thead>
<tr>
<th></th>
<th>RCB</th>
<th>MCB*</th>
<th>WCB*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence transgene</td>
<td></td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma, Sterility, Human Virus Panel Screening (External GMP testing)</td>
<td></td>
<td>Y</td>
<td></td>
</tr>
<tr>
<td>Post-banking functional check (1 week)</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Post-banking functional check (1 month)</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Functional assessment every 5 passages (to p25), 50-200% linear recovery and repeatability</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>NKp46 receptor density quantification every 5 passages (to p25)</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>IL2 R receptor density quantification CD25, CD122, CD132, every 5 passages (to p25)</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Bank-to-bank receptor density conformity</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
</tbody>
</table>

*Freezing Media and Banking Density Optimized

Four test banks (5 vials/bank) were created to optimize:
- Cell banking density
- Freezing media
- Pre-banking harvest density

All test banks were assessed for:
- Post-thaw recovery
- Cell growth
- Assay function

Program Support: Cell Banks

Due to the novelty of the cell line, Inhibrx is the sole supplier of this critical reagent. We created a characterization strategy to de-risk both bank-to-bank performance and supply chain.

- All test banks were assessed for:
  - Post-thaw recovery
  - Cell growth
  - Assay function

- Four test banks (5 vials/bank) were created to optimize:
  - Cell banking density
  - Freezing media
  - Pre-banking harvest density

- 25% WCB bank generation (CRO)
- 25% Long-term storage, risk mitigation (External)
- Non-GMP work (Inhibrx)
- P1 assay validation, DS/DP release/stability (CRO)
INBRX-121 Cell-Based Bioassay Performance

Example Plate Layout

- For our internal assay development, robustness, and pre-qualification, we place the control on the outer rows
- This helps our team to monitor potential edge effects over time and passage number

Representative Dose Response Curves

100% Repeatability

50%, 200% Linear Recovery
De-Risking CBBA Prior to Transfer and Validation

**Robustness**

**Protocol**

<table>
<thead>
<tr>
<th>Day 1</th>
<th>Add Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Add INBRX-121</td>
</tr>
<tr>
<td></td>
<td>Incubate o/n</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day 2</th>
<th>Add supernatant to detection reagent*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Develop detection</td>
</tr>
<tr>
<td></td>
<td>Read on spectrophotometer</td>
</tr>
</tbody>
</table>

*Previously optimized from the vendor's protocol

**Robustness/DOE**

- **Cell confluency:** $\pm 30\%$ of target
- **Cell density:** $\pm 20\%$ of target
- **No impact**

**Key Factors**

- **Incubation:** $\pm 10\%$ of target
  - Locked at target $\pm 2.5\%$
- **Supernatant:** $\pm 20\%$ of target
  - Locked at target
- **Incubation:** $\pm 60\%$ of target
  - Locked at target $\pm 30\%$

**Factors assessed with 50%, 100%, 200% linear recovery**

- Curve shape of target condition or factors with no impact
- Limits of assay performance observed first in 50% and 200% loss of parallelism

*Ying Li*
De-Risking CBBA Prior to Transfer and Validation

Assay Pre-qualification and Results

An in-house Inhibrx pre-qualification was performed to de-risk technical transfer and subsequent external validation:

- 2 Analysts
- 5 Linear Recovery Levels: 50%, 80%, 100%, 125%, 200%
- 2 Linearity Repeats/Analyst
- 4 100% repeats/Analyst

Assay Pre-qualification Results

Key Findings

<table>
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<th>Parameter</th>
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<tr>
<td>Intermediate Precision</td>
<td>5% RSD</td>
</tr>
<tr>
<td>Specificity</td>
<td>Assay is specific, material used:</td>
</tr>
<tr>
<td></td>
<td>- NKP46 targeting only</td>
</tr>
<tr>
<td></td>
<td>- IL-2-X only</td>
</tr>
<tr>
<td>Stability Indicating</td>
<td>Assay is stability indicating, material used:</td>
</tr>
<tr>
<td></td>
<td>- Blended Hinge-Cleaved Material</td>
</tr>
<tr>
<td></td>
<td>(See discussion on Slide 20)</td>
</tr>
</tbody>
</table>

Linear Regression

\[ R^2 = 0.988 \]

\[ Y = 1.044X - 3.634 \]

Relative Bias

Relative recovery: 98%-106%

Ying Li and Thi Ho
INBRX-121 Dual Domain ELISA

**Problem Statement**

Low affinity IL-2-X is difficult to bind or detect in a plate-based binding assay

**Solution**

Screen non-traditional approaches to binding assays
Plate-Based Assay Screening

- We considered only commercially available reagents for this assay screening
- For expediency, we used linear recovery samples to further hone our assay screening

*We screened several other assay formats and reagents. These are the only formats that generated reasonable curves*

<table>
<thead>
<tr>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Final Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coat</td>
<td>NKp46</td>
<td>CD122 x CD132 hFc</td>
</tr>
<tr>
<td>Detect</td>
<td>Biotinylated CD122 x CD132 hFc</td>
<td>Biotinylated NKp46</td>
</tr>
<tr>
<td>Secondary</td>
<td>Strep-HRP</td>
<td>Strep-HRP</td>
</tr>
</tbody>
</table>

Results

![Graphs showing assay results](image)

<table>
<thead>
<tr>
<th>Suitability</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Final Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not suitable</td>
<td>Uncontrolled upper asymptote variability</td>
<td>Not suitable Failure in 200% linear recovery</td>
<td>Promising Accurate linear recovery, limited variability</td>
</tr>
</tbody>
</table>

*Anti-IL2 mAb, Clone MAB-202*
This mouse anti-human IL-2 antibody binds to the functional region and completely blocks the binding of IL-2 to its receptor
Clone MAB-202 and IL-2 Blocking Potential

**Challenge**
How can we prove MAB-202 binds to and blocks the functional region of IL-2-X?

**Proof of Concept**
If MAB-202 is blocking, it will:
- Prevent INBRX-121 from binding to IL-2 receptor
- No downstream signaling

**Competitive Inhibition with CBBA**
The loss of STAT5-driven SEAP signal with increasing concentrations of MAB-202, which supports blocking of the functional region of IL-2

With the highest concentrations of MAB-202, complete loss of signal is observed, on par with low INBRX-121 concentrations and no-treatment negative control.

**Dual Domain ELISA**

**CBBA Refresher**
Use INBRX-121 CBBA to assess MAB-202 functional region binding

**Competitive Inhibition with CBBA**

INBRX-121 (positive control)
MAB-202 titration + INBRX-121 (EC80 concentration)

No treatment (negative control)

INBRX-121 (at an EC80 concentration) was pre-incubated with serially diluted MAB-202 prior to addition to the INBRX-121 CBBA reporter cells.

Ying Li

**Goat anti-mouse Fc HRP**
MAB-202 (Mouse anti-human IL2)
INBRX-121

**Use INBRX-121 CBBA to assess MAB-202 functional region binding**

**INBRX-121**
IL-2R
NKp46 DR

**STAT5**
SEAP

**Challenge**
Proof of Concept
### INBRX-121 NKp46xIL2 Binding ELISA Performance

#### Example Plate Layout

- For our platform ELISAs, we used a more traditional plate layout
- Pseudo replicates are critical to reduce the inherent variability likely caused by the weak binding interactions of INBRX-121 with MAB-202

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<th>10</th>
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<tbody>
<tr>
<td>A</td>
<td>STD – Pseudo Rep 1</td>
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<tr>
<td>B</td>
<td>Sample 1 – Pseudo Rep 1</td>
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<tr>
<td>C</td>
<td>Sample 2 – Pseudo Rep 1</td>
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<tr>
<td>D</td>
<td>CTRL – Pseudo Rep 1</td>
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<tr>
<td>E</td>
<td>STD – Pseudo Rep 2</td>
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<tr>
<td>F</td>
<td>Sample 1 – Pseudo Rep 2</td>
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<tr>
<td>G</td>
<td>Sample 2 – Pseudo Rep 2</td>
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<tr>
<td>H</td>
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#### Representative Dose Response Curves

- **100% Repeatability**
  - %RP
    - CTRL: 97%
    - 100%-1: 105%
    - 100%-2: 109%

- **50%, 200% Linear Recovery**
  - %RP
    - CTRL: 97%
    - 50%: 47%
    - 200%: 209%
De-Risking Dual Domain ELISA Prior to Transfer and Validation

Robustness

**Protocol**

**Day 1**

- Coat NKp46 o/n
  - Block*
  - Add INBRX-121 *
  - Add MAB-202 *
  - Add 2° *
  - Add TMB*
  - Add TMB Stop
  - Read on spectrophotometer

**Day 2**

- Coat NKp46 o/n
  - Block*
  - Add INBRX-121 *
  - Add MAB-202 *
  - Add 2° *
  - Add TMB*
  - Add TMB Stop
  - Read on spectrophotometer

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**Robustness/DOE**

- NKp46 concentration, + 20% target
- NKp46 lot-to-lot
- Coating, o/n up to 3 days
- INBRX-121 2° incubation, + 20% target
- MAB-202 concentration, + 20% target
- MAB-202 2° incubation, + 20% target
- 2° concentration, + 20% target
- 2° incubation, + 20% target

*Wash prior to addition

**Factors assessed with 50%, 100%, 200% linear recovery**

Inhibrx platform – previously optimized

No impact
De-Risking Dual Domain ELISA Prior to Transfer and Validation

Assay Pre-qualification and Results

An in-house Inhibrx pre-qualification was performed to de-risk technical transfer and subsequent external validation:

- 2 Analysts
- 5 Linear Recovery Levels: 50%, 80%, 100%, 125%, 200%
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Linear Regression

\[ R^2 = 0.954 \]

\[ Y = 1.014X + 2.464 \]

Relative Bias

Relative recovery: 99%-107%

Ying Li and Thi Ho

R squared 0.9542

Expected

Measured

Spike Level (%)

Relative Recovery(%)
INBRX-121 Early Structure/Function
Structure-Function: Hinge Cleaved Samples

Concept

Due to program stage (pre-clinical), no relevantly stressed materials were available:

- Therefore, based on the MOA of INBRX-121, we decided to proteolytically cleave it at the hinge with IdeS
- Fully cleaved material was then blended with untreated INBRX-121 (study control)

Results

CBBA

<table>
<thead>
<tr>
<th>INBRX-121 25% HC</th>
<th>INBRX-121 50% HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="CBBA Graph" /></td>
<td><img src="image2" alt="CBBA Graph" /></td>
</tr>
</tbody>
</table>

ELISA

<table>
<thead>
<tr>
<th>INBRX-121 25% HC</th>
<th>INBRX-121 50% HC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3" alt="ELISA Graph" /></td>
<td><img src="image4" alt="ELISA Graph" /></td>
</tr>
</tbody>
</table>

Results Summary

<table>
<thead>
<tr>
<th></th>
<th>CBBA %RP Avg N=2</th>
<th>ELISA %RP Avg N=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% HC</td>
<td>67%</td>
<td>70%</td>
</tr>
<tr>
<td>50% HC</td>
<td>51%</td>
<td>53%*</td>
</tr>
</tbody>
</table>

* Estimated relative potency due to lack of parallelism

Ying Li

25% INBRX-121 Hinge Cleaved + 75% INBRX-121 Study Control

50% INBRX-121 Hinge Cleaved + 50% INBRX-121 Study Control

Fully Hinge Cleaved INBRX-121

Yao Chen
## INBRX-121 Assays Ready for Phase 1 Validation

### Current Status

**POC (Proof of Concept)**
- **Optimization**
- **Pre-Qualification**
- **Technical Transfer**
- **Assay Establishment**
- **CBBA**
- **ELISA**
- **CRO**
- **Phase 1 Validated Assay**

### Key Designations

<table>
<thead>
<tr>
<th>Key Designations</th>
<th>CBBA: <em>NKp46 x IL2 QuantiBlue Reporter Gene</em></th>
<th>Dual Domain ELISA: Anti-IL2</th>
</tr>
</thead>
</table>

### Pre-Qualification

**Key Findings**
- 5% RSD
- Specific to intact INBRX-121
- Stability indicating

**Critical Reagents**

**De-Risked**
- Cell line characterization complete
- Cell line licensing complete
- Vector licensing complete
- Sterility, mycoplasma, human virus panel testing complete

**Documentation**

**Enabled Technical Transfer** (2 sites)
- Protocol complete
- MDR complete
- Cell Banking and Characterization Document complete

**Validation**

Planned Q4 2022

**Planned Q3 2022**
Next Steps for INBRX-121 Potency Assays

The CBBA and Dual Domain ELISA will be used for release and stability testing under GMP conditions. Using these assays we will:

**Stage 1**
Monitor changes to INBRX-121 while on stability

**Stage 2**
Understand the impact of minor forms of INBRX-121

**Theoretical Minor Forms**

**Half Molecules**
- IL-2-X
- + IL-2-X

**Homodimers**
- Knob-knob
- Hole-hole

To date, no half-molecules or homodimers have been observed with current INBRX-121 single cell clone material

**Potential Impact**

*In vitro NK cell Proliferation (Human)*

This is an example of potential limitations of the CBBA

- Both assays will be used in parallel for further product characterization, and to understand assay limitations
- If one assay is insensitive to molecule change, we have an orthogonal method as an alternative

**Chelsie Macedo**

Healthy human donor blood was labeled with a proliferative dye and treated with INBRX-121. NK cells were identified with specific cell lineage markers and NK proliferation was quantified via flow cytometry

1x NKp46 with IL-2-X
1x NKp46 w/IL-2-X
Untargeted IL-2-X
Appendix
INBRX-121 Cell-Based Bioassay

Cell Line Generation History

- The NKp46 x IL-2 QuantiBlue reporter gene cell line is a weakly adherent HEK293 line
- Upon IL-2 stimulation, HEK-Blue™ IL-2 cells trigger the activation of STAT5 and the subsequent secretion of SEAP. The levels of STAT5-induced SEAP can be readily monitored using QUANTI-Blue™
- Insert: NKp46 signal peptide, extracellular domain and transmembrane domain. Intracellular domain was replaced with a fluorescent compound to prevent NKp46 driven intracellular signaling
- Cells were transfected using a commercially available protocol
- Transfected cell pools were then harvested and labeled with a conjugated antibody according to the target of interest. Cells single sorted into 96-well culture plates (confirmed visually 1 cell/well) based on expression
- Clonal cell cultures were expanded and were screened for citrine expression. ICD fluorescent compound positive clones were expanded into flasks. Selected cell lines were tested for NKp46 expression and reporter gene function.

Cell Line Media and Culturing Conditions

Growth Media for NKp46 transfected cells

- DMEM-based with FBS and selection antibiotics
*Other lots/vendors have been tested with this cell line – no differences in expression or performance were observed

Seeding Density for Culturing

- 2-, 3-, and 4-day culturing conditions were established

Seeding Density for Cell Banking

- 2-, and 3-day culturing conditions were established
- These conditions differ from regular culture conditions and were optimized for bank consistency and post-thaw recovery