i-bodies targeting complex membrane proteins identified by phage display screening

Discovery on Target, Boston 2016: Antibodies Against Membrane Protein Targets
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AdAlta is developing a new technology platform that produces unique proteins known as i-bodies, that mimic the shape of shark antibody binding domain and engineers their key stability features into a human protein, for therapeutic intervention in disease.

The single domain antigen binding region of shark antibodies is extremely stable and has a long binding loop not present in either human or next generation antibodies.

**Advantages of i-bodies**

- High target specificity and high affinity for their target
- Small proteins; 10% the size of a typical human antibody
- Highly stable to proteases, high temperatures and low pH
- Long loop that can bind to a diverse range of therapeutically relevant targets including those that are difficult for current antibody therapies
- **Human protein** – reduced risk of immune response
i-bodies: human single domains
Because of the long binding loop of the i-body, that is lacking in traditional antibodies, i-bodies recognise and bind to a diverse range of different therapeutically-relevant targets including those that are difficult/intractable to access by current antibody therapies such as G-protein coupled receptors (GPCRs) and ion channels.

The small physical size and stable properties of i-bodies provides advantages for tissue and organ penetration as well as multiple delivery routes.

As a result of their small size and exceptional stability i-bodies can serve as building blocks to engineer therapeutics with tailored pharmacokinetic properties.

Can easily engineer unique differentiated i-body products in a variety of formats including monospecific and bispecifics as well as i-body drug conjugates (IDCs), thus tailoring them for different therapeutic purposes.
i-body monomeric and extremely stable to proteases, high temperatures and low pH

i-bodies are extremely stable at high temperatures, including boiling, while still retaining their activity. Here we show we can reduce a number of *E. Coli* proteins heating above 60°C.

On heating and re-cooling the i-body we show by Circular dichroism spectroscopy that the b-sheet of the i-body unfolds and re-folds. This process was repeated eight times.

The i-body is a monomeric protein.
i-bodies combine benefits of small molecules and conventional antibodies

<table>
<thead>
<tr>
<th></th>
<th>Small Molecule</th>
<th>Conventional Antibody</th>
<th>AdAlta i-body</th>
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</thead>
<tbody>
<tr>
<td>High selectivity-specificity</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Low toxicity: no off target effects</td>
<td></td>
<td>●</td>
<td>●</td>
</tr>
<tr>
<td>Cavity binding and new epitopes</td>
<td>●</td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>Stability</td>
<td>●</td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>Alternative routes of administration</td>
<td>●</td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>Easy to manufacture</td>
<td>●</td>
<td></td>
<td>●</td>
</tr>
<tr>
<td>Speed &amp; risk of development</td>
<td></td>
<td>●</td>
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i-bodies offer a new and potentially more effective approach to the treatment of a wide range of human diseases.
Fibrosis: unmet medical need with multiple indications

- Developing i-bodies as improved therapies for the treatment of fibrosis
  - a condition that is prevalent in 45-50% of all diseases
- Fibrosis can occur in many tissues of the body as a result of inflammation or damage
  - it can result in scarring of vital organs causing irreparable damage and eventual organ failure
- AdAlta’s initial focus is on lung fibrosis

Collectively fibrosis represents a large unmet clinical need
CXCR4 is involved in fibrosis and other disease states

CXCR4 is important in maintaining stem cells in bone marrow with Mozobil (AMD3100) approved for single use.

HIV-1 uses CXCR4 as a co-receptor for viral entry into host cells and CXCR4 has been associated with more than 23 types of cancers.

CXCR4 has more recently been recognised as a critical player in development of a number of areas of fibrosis including:

- Lung
- Kidney
- Heart
- Eye
- Skin
Selecting i-bodies against CXCR4

Large diverse synthetic library of 2 billion i-body protein compounds that can bind to a broad range of therapeutically relevant targets.

i-body identified by rapid screening on CXCR4 lipoparticles

i-body affinity matured to enhance target binding and generate lead i-body candidate

Manufactured in microbial systems; more cost-effective and easier than conventional monoclonal antibodies. Potential for direct peptide synthesis.
Round 1 affinity maturation

Single point mutations were made in the CDR1 and CDR3 binding regions only of the lead i-body identified

The sequences of i-bodies identified above demonstrated binding to CXCR4 lipoparticles.

Affinities of the two highlighted sequences were 30nM and 25nM.

The first round of affinity maturation improved affinities from 700nM to 25nM with single point mutations.
Round 2 affinity maturation

A mutant library was made changes from the two lead candidates from affinity maturation round 1.

Mutant binders in round 2 affinity maturation were ranked with regards to their off rate.

I-bodies were identified with improvements in the second round of affinity maturation from 20nM to 1nM
Sequences of affinity matured CXCR4 binding i-bodies

The affinity maturation process allowed identification of residues in the CDR1 and CDR3 that were important for binding to CXCR4.
Family of CXCR4 binding i-bodies

i-bodies from the affinity maturation process were evaluated in several *in vitro* assays including
- β-arrestin BRET assay
- Calcium flux assay
- cAMP assay

1-3 single point mutations could dramatically effect the β-arrestin activity, while still having the same affinity for the receptor CXCR4

None of the i-bodies identified had any effect on calcium flux

<table>
<thead>
<tr>
<th>Protein</th>
<th>Affinity to CXCR4 (nM)</th>
<th>IC50 (nM) in b-Arrestin BRET assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADCX-99</td>
<td>700</td>
<td>No activity</td>
</tr>
<tr>
<td>AD-320</td>
<td>21.8</td>
<td>1419</td>
</tr>
<tr>
<td>AD-245</td>
<td>14.88</td>
<td>~uM</td>
</tr>
<tr>
<td>AD-126</td>
<td>14.75</td>
<td>610</td>
</tr>
<tr>
<td>AD-466</td>
<td>13.83</td>
<td>6322</td>
</tr>
<tr>
<td>AD-661</td>
<td>9.53</td>
<td>1544</td>
</tr>
<tr>
<td>AD-523</td>
<td>8.55</td>
<td>12713</td>
</tr>
<tr>
<td>AD-613</td>
<td>7.83</td>
<td>1826</td>
</tr>
<tr>
<td>AD-1121</td>
<td>7.21</td>
<td>796</td>
</tr>
<tr>
<td>AD-920</td>
<td>7.18</td>
<td>~uM</td>
</tr>
<tr>
<td>AD-746</td>
<td>5</td>
<td>741</td>
</tr>
<tr>
<td>AD-114</td>
<td>4.85</td>
<td>164</td>
</tr>
<tr>
<td>AD-272</td>
<td>1.6</td>
<td>861</td>
</tr>
</tbody>
</table>
Panel of i-bodies against CXCR4 specific and have *in vitro* activity

When tested against 167 GPCRs, the 10 affinity matured i-bodies specifically antagonized CXCR4. There was no agonist activity on any of the GPCRs.

The i-bodies were evaluated in a number of *in vitro* assays. In this assay we demonstrated that a panel of i-bodies with affinity to CXCR4 <20nM could inhibit inhibition of entry of the HIV virus.

REF: Griffiths et al JBC June 2016
Epitope mapping of three i-bodies to CXCR4, demonstrated that residue E262 was common to all i-bodies. E262 which is deep in groove of CXCR4, also binds AMD3100. All three i-bodies had unique binding sites reflecting their different functionalities.
Homology model of i-body and CXCR4

Side chain CDR3 contact residues also demonstrate that the long loop of the i-body, binds deep in the ligand binding pocket of CXCR4

REF: Wescott et al PNAS 2016
i-bodies do not mobilize stem cells \textit{in vivo}.

i-bodies do not mobilize stem cells in mouse model (left) or humanised mouse model (right) of stem cell mobilization, unlike the small molecule AMD3100.

\textbf{REF: Griffiths et al JBC June 2016}
Developing i-bodies as improved therapies for the treatment of fibrosis
- a condition that is prevalent in 45-50% of all diseases

Fibrosis can occur in many tissues of the body as a result of inflammation or damage
- it can result in scarring of vital organs causing irreparable damage and eventual organ failure

AdAlta’s initial focus is on lung fibrosis

Collectively fibrosis represents a large unmet clinical need
Fibrosis is a complex disease

Inflammation

Fibrocyte migration

Deposition of collagen/ECM
CXCR4 and idiopathic pulmonary fibrosis (IPF)

Patients that rapidly progress express more CXCR4 compared to slow IPF progressors

CXCR4 +ve cells (fibrocytes) significantly elevated in stable IPF patients, and further increased during acute exacerbations

Fibrocytes not correlated with lung function but an independent predictor of early mortality

- 7.5 months with more than 5% fibrocytes
- 27 months with less than 5% fibrocytes

![CXCR4 expression increased in fast progressing IPF patient tissue](image1)

![Fibrocyte numbers predict mortality](image2)

AD-114 binds to lung tissue from patients with fibrosis

The i-body AD-114 was used for Immunohistochemical (IHC) staining of normal and diseased lung tissues to verify expression of CXCR4 *in situ*
Migration/invasion specifically reduced with IPF lung fibroblasts

i-bodies specifically inhibited migration of slow and rapid IPF fibroblast migration but did not have any effect on normal fibroblasts.

AD-114 has greater in vitro efficacy compared to the only approved therapies Nintedanib and Pirfenidone for IPF treatment.

<table>
<thead>
<tr>
<th></th>
<th>No effect on normal fibroblasts</th>
<th>Inhibits slow IPF progressors</th>
<th>Inhibits fast IPF progressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>i-body AD-114</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Nintedanib (Boehringer)</td>
<td>✗</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Pirfenidone (Roche)</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
</tr>
<tr>
<td>Other CXCR4 drug (Sanofi)</td>
<td>✔</td>
<td>✗</td>
<td>✗</td>
</tr>
</tbody>
</table>

Normal fibroblasts

Slow/stable IPF progressor fibroblasts

Rapid IPF progressor fibroblasts
i-bodies block infiltration of leukocytes

AD-114 has also been evaluated for its anti-inflammatory activity. Anti-inflammatory activity is important for preventing fibrosis.

In this model, an inflammatory stimulant known as SDF-1 is added to an air-pouch created on the back of a mouse, which results in a dramatic increase in the amount of inflammatory cells counted in the air-pouch (black column). When a single dose of AD-114 is injected into the mouse, the migration of the inflammatory cells to the air-pouch is blocked (red column).

**Figure 4. Anti-Inflammatory Air Pouch Mouse Model**

- **No stimulus**
- **With inflammatory stimulus (SDF-1)**
- **With inflammatory stimulus (SDF-1) and i-body AD-114**

**Inflammatory cell count**

- **Thousands of Inflammatory cells**
  - **0.0**
  - **500.0**
  - **1000.0**
  - **1500.0**
  - **2000.0**

**Source:** Griffiths et al, Journal of Biological Chemistry, April 2016

In this model, an inflammatory stimulant known as SDF-1 is added to an air-pouch created on the back of a mouse, which results in a dramatic increase in the amount of inflammatory cells counted in the air-pouch (black column). When a single dose of AD-114 is injected into the mouse, the migration of the inflammatory cells to the air-pouch is blocked (red column).

**REF:** Griffiths et al JBC June 2016
Bleomycin mouse model

Administration of Bleomycin is the most common animal model for the assessment of candidate drugs for the treatment of IPF. The Bleomycin treated mouse lung shows extensive collagen deposition and inflammatory cell infiltration.

- Mice received intratracheal instillation of Bleomycin at 2U/kg/mouse
- Groups treated starting on Day 0 of the study 1 hr prior to Bleomycin installation, with selected test compounds
- i-body was dosed at three levels, 1mg/kg, 10mg/kg and 30mg/kg daily
- At Day 4 the number of fibrocytes (CXCR4+, Col1+ and CD45+ cells) in the lungs were measured by flow cytometry, RNA levels of collagen and collagen content were measured
- At Day 19 whole lungs were assayed for Hydroxyproline and histology (Masson’s Trichrome) and Ashcroft score completed to analysis collagen content (Ashcroft, T., J.M. Simpson, and V. Timbrell. 1988. J. Clin. Pathol. 41:467-470). Body weights were also evaluated
AD-114 reduces fibrocytes in the Bleomycin mouse model

Mice challenged with Bleomycin and treated with AD-114 had reduced levels of fibrocytes in their lungs when compared to the mice treated with the negative control i-body.
Collagen gene expression reduced with i-body AD-114

- RNA extracted and analyzed for collagen gene expression
- Both COL1A1 and COL3A1 reduced in mice treated with i-body AD-114 in the Bloemycin mouse model
- The negative control i-body had no effect on either COL1A1 or COL3A1
Collagen content reduced with i-body AD-114

- Collagen-1 content was measured by Sircol assay
- AD-114 reduced the level of collagen protein deposited in the lung of Bleomycin-treated mice
- Negative control i-body had no effect on collagen deposition
AD-114 prevents lung fibrosis in disease models

Extensive pre-clinical AD-114 studies have demonstrated positive \textit{in vitro} (in the lab) and \textit{in vivo} (in animals) data

- Normal lung tissue
- IPF lung tissue (lung disease mouse model)
- IPF lung tissue + AD-114 dosed for 21 days (lung disease mouse model)

AD-114 reduces collagen content and inflammatory cell infiltration and demonstrates a similar architecture to that of the normal lung in the Bleomycin mouse model.
AD-114 prevents lung fibrosis in disease models

AD-114 reduction in collagen deposition confirmed by quantitation using Ashcroft scores

Loss of body weight due to Bleomycin is prevented with daily dosing of i-body AD-114 10mg/kg
AD-114 inhibits key features of the fibrogenic pathway with novel MOA

Modulate aspects of inflammation
Block fibrocyte recruitment into the damaged lung
Reduce ECM deposition during tissue remodeling
Developing i-bodies as improved therapies for the treatment of fibrosis
- a condition that is prevalent in 45-50% of all diseases

Fibrosis can occur in many tissues of the body as a result of inflammation or damage
- it can result in scarring of vital organs causing irreparable damage and eventual organ failure

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Collectively fibrosis represents a large unmet clinical need
AD-114 prevents eye fibrosis

- Mouse choroidal neovascularization model: laser burn to the retina
  - Induces subretinal haemorrhage
  - Contraction of retinal tissue
  - Alteration in microglia and glial response
  - Alteration in gene expression

- IVT injection of single dose of i-body
  - Improves retinal retraction and reduces lesion size
  - Fibrosis gene expression reduced

AD-114 reduces contraction and lesion size in eye fibrosis mouse model

AD-114 improves all outcomes.
AdAlta summary

- Powerful proprietary technology platform to develop a pipeline of i-bodies for the treatment of a wide range of human diseases
  - Extreme stability of i-body similar to single domain shark antibody
  - Long loop of i-body binds deep in GPCR pocket and has functional activity

- Advanced lead candidate AD-114 with significant pre-clinical validation
  - has specificity for diseased human tissue with effects only shown on IPF tissue and no effects displayed on normal lung tissue nor any evidence of off target effects;
  - is more effective than existing IPF approved drugs showing greater in vitro efficacy compared to the only approved therapies Nintedanib and Pirfenidone;
  - demonstrates both anti-fibrotic and anti-inflammatory effects in multiple animal models in multiple areas of fibrosis; and
  - is a novel mechanism of action for fibrosis making AD-114 a potential “first in class” therapy.
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