Purification of Human Hemoglobin and Drug Conjugation for Liver Targeting

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BPI West, San Francisco
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Flexible, modern facility

- cGMP biomanufacturing facility
  - 136,000 ft\(^2\) (12,800 m\(^2\))
  - Flexible clinical and commercial production suites
- cGMP warehouse and offices
  - 30,000 ft\(^2\) (2,800 m\(^2\)) warehouse with cold storage
  - 14,000 ft\(^2\) (1,300 m\(^2\)) administrative offices

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- Canada-US border in 1 hour
- Highly skilled and educated workforce
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- Cell Line
- Upstream and Downstream Processes
- Analytical Methods

cGMP Manufacturing Services
- Upstream Production
- Downstream Purification
- Aseptic Fill/Finish

Support Services
- Quality Control
- Quality Assurance
- Project Management
- Technology Transfer
Hemoglobin-Drug Conjugate Preparation from RBC Hemoglobin

Red Blood Cells (RBCs) → Purification → Hemoglobin (Hb) → Drug Conjugation → Cleavable Drug → Hemoglobin-Drug Conjugate (HDC)
• Hemoglobin-Haptoglobin (Hb-Hp) is naturally cleared predominantly to the liver

• Liver macrophages (Kupffer cells), via CD163, the Hb-Hp scavenger receptor

• Over 1.5 g Hb binding capacity
Selective tissue targeting of radiolabeled hemoglobin in rat by single photon emission computed tomography (SPECT/CT)

3 h post-injection of 10 µg $^{99m}$Tc-Hb (0.5 mCi)
Liver cancer: ~700,000 deaths/year
5-year survival rate: 15%
Floxuridine is an approved anti-cancer drug with a narrow therapeutic index
Hepatic arterial infusion (by pump) of floxuridine is used in the treatment of:
  - Hepatocellular carcinoma
  - Colorectal cancer liver metastases

- Easy to administer
  - Standard IV
- High liver uptake
  - Lower dose required
  - Lower toxicity
- Binds to haptoglobin
- High drug load
Hemoglobin and HDCs present unique challenges:

- **Scale** – grams to kilograms of hemoglobin (Hb)
- **Purity** – removal of red blood cell and plasma components
  - isolation of a single Hb sub-type (HbA₀)
- **Safety** – inactivation and removal of potential blood-borne pathogens
- **Stability** – Hb is a tetrameric (α₂β₂) globular protein with four heme groups
  - Heme groups contain readily oxidized iron
- **Drug Conjugation** – high drug payload required
  - effect on haptoglobin and receptor binding
  - stable in circulation, cleavable inside target cells
  - applicable to a range of drugs
- **Analysis** – assays specialized for Hb, drug intermediate and HDC characterization
Hemoglobin separation from red blood cells

- Expired RBCs
- RBC Pooling
- Washing
- Lysis
- Pasteurization
- Concentration
- Stroma-free Hb

Hemoglobin chromatography

- pH/conductivity Adjustment
- Anion Exchange
- pH Adjustment
- Cation Exchange
- Nanofiltration
- >99% HbA₀

Drug activation and conjugation

- Nucleoside
- Phosphorylation
- Nucleotide
- Activation
- Conjugation
- Hemoglobin-Drug Conjugate
Hemoglobin Isolation from RBCs

- Tested and expired red blood cells (RBCs) from FDA licensed blood collection centers
- Hollow fiber filtration for plasma component removal and concentration adjustment
- RBC lysis by 1:1 WFI dilution
- Hollow fiber separation of RBC membranes from Hb and RBC proteins
- CO charging to stabilize Hb against oxidation:
  \[
  \text{Hb} + \text{O}_2 \rightarrow \text{OxyHb}(\text{Fe}^{2+}) \rightarrow \text{MetHb}(\text{Fe}^{3+}) \rightarrow \text{Denaturation}
  \]
- Viral inactivation by pasteurization (10 hours at 62°C, \(\geq 4.5 - \geq 5.5 \log_{10}\) virus reduction)
- Pasteurization also precipitates non-Hb components
- Solids removal by depth filtration, CO charging
RBC proteins, membrane lipids and plasma proteins are reduced to acceptance limits

<table>
<thead>
<tr>
<th>Units</th>
<th>Endotoxin</th>
<th>PE</th>
<th>PI</th>
<th>PS</th>
<th>CA</th>
<th>HSA</th>
<th>Spectrin</th>
<th>Glyco-</th>
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</thead>
<tbody>
<tr>
<td>Targets</td>
<td>≤0.06</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;0.1</td>
<td>&lt;0.36</td>
<td>&lt;0.1</td>
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<tr>
<td>Lot A</td>
<td>≤0.06</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>1.8</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
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<tr>
<td>Lot B</td>
<td>≤0.06</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>2.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
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<tr>
<td>Lot C</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>1.4</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.1</td>
<td>&lt;0.01</td>
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</table>

PE = Phosphatidyl Ethanolamine, PI = Phosphatidyl Inositol, PS = Phosphatidyl Serine
Measured by solid-phase extraction followed by RP-HPLC quantification

CA = carbonic anhydrase, HSA = human serum albumin, spectrin and glyco- phorin
Hemoglobin Chromatographic Purification

Stroma-free Hb

pH/conductivity Adjustment

Anion Exchange

pH Adjustment

Cation Exchange

Nanofiltration

>99% HbA₀

Anion Exchange Displacement Chromatography

- High pH, low ionic strength, low flow rate
- Acidic impurities bind with high affinity, displacing and eluting Hb and basic impurities

Cation Exchange Displacement Chromatography

- Low pH, low ionic strength, low flow rate
- Basic impurities bind with high affinity, displacing and eluting Hb

- Nanofiltration (≥5.0 - ≥5.5 Log₁₀ virus reduction)
- CO charging for stabilization
Purification of Hemoglobin by Displacement Chromatography

**Anion Exchange Step**

Hb with basic and acidic proteins, including multiple Hb subtypes

Final eluate is free of acidic proteins

**Cation Exchange Step**

Load eluant from anion exchange step

Final eluate is >99% HbA₀
Compared to conventional adsorption/elution chromatography, displacement chromatography provides:

- Higher yield
- 10-20x higher Hb recovery / mL resin
- Lower solution requirements.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Self-Displacement</th>
<th>Bind/Elute</th>
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<tbody>
<tr>
<td>Hb mass recovered</td>
<td>1000 g</td>
<td>1000 g</td>
</tr>
<tr>
<td>Column volume</td>
<td>5 L Anion + 3 L Cation</td>
<td>51 L</td>
</tr>
<tr>
<td>Hb mass loaded</td>
<td>1234 g</td>
<td>1754 g</td>
</tr>
<tr>
<td>Hb recovery</td>
<td>81%</td>
<td>57%</td>
</tr>
<tr>
<td>Hb recovery/mL resin</td>
<td>200-300 mg/mL</td>
<td>20 mg/mL</td>
</tr>
<tr>
<td>Running buffer required</td>
<td>132 L</td>
<td>925 L</td>
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</table>
Displacement Chromatography Scalability

- Anion and cation exchange displacement chromatography steps provide identical purity at laboratory (8 mL columns) to commercial (7-16 L columns) scale

### Anion Exchange Step

<table>
<thead>
<tr>
<th>Load Mass</th>
<th>Load / mL Resin</th>
<th>pH</th>
<th>Conductivity</th>
<th>Column L x D</th>
<th>Flow Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6 g</td>
<td>210 mg/mL</td>
<td>8.9</td>
<td>0.5 mS</td>
<td>10 x 1 cm</td>
<td>1 cm/min</td>
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<tr>
<td>3200 g</td>
<td>200 mg/mL</td>
<td>8.8</td>
<td>0.3 mS</td>
<td>10 x 45 cm</td>
<td>1 cm/min</td>
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</tbody>
</table>

### Cation Exchange Step

<table>
<thead>
<tr>
<th>Load Mass</th>
<th>Load / mL Resin</th>
<th>pH</th>
<th>Conductivity</th>
<th>Column L x D</th>
<th>Flow Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 g</td>
<td>308 mg/mL</td>
<td>7.5</td>
<td>0.5 mS</td>
<td>9.5 x 1 cm</td>
<td>0.6 cm/min</td>
</tr>
<tr>
<td>1700 g</td>
<td>254 mg/mL</td>
<td>7.6</td>
<td>0.4 mS</td>
<td>9.5 x 30 cm</td>
<td>0.6 cm/min</td>
</tr>
</tbody>
</table>
Hemoglobin Purity
Before Drug Conjugation

- Hb Purity: >99.0% HbA₀ (range 99.3-100%) by analytical anion exchange HPLC

Analytical anion exchange chromatography:
Purified HbA₀

- <1% MetHb (oxidized Hb, range 0-0.7%) by spectrophotometry (COOXimetry)
Drug Activation and Conjugation

Nucleoside Activation and Conjugation Chemistry

1. POCl₃, TMP, H₂O
2. pH 3 hydrolysis
3. CHCl₃ extraction
4. IEX free acid conversion

Ribavirin (RBV)
(or other nucleoside)

RBV 5’-monophosphate (RBV-P)

1. CDI, Imidazole, DMF (anhydrous)
2. EtOH/ether precipitation

Hb Conjugation

Hb Conjugation

1. Purified CO-Hb, pH 9.5
2. UF/DF

Hb-N

Hemoglobin-RBV Conjugate
Hazardous Materials Containment

- 2-person isolator
- Classifiable to levels C, B or A
- Located inside an isolated Class C area
- Containment of hazardous reagents
- Safe exhaust capability for CO gas
- Containment during material transfer and sanitization, and waste flow
Hb-FUdR: Drug Load

10 ± 2 FUdR per Hb tetramer

- ESI-MS: α and β globin chains with single or multiple FUdR-P groups
- Acid phosphatase cleavage of FUdR and RP-HPLC analysis
- Inorganic phosphate quantification (Ames method)
- $^{31}$P NMR measurement of the phosphoramide linkage
Hb-FUdR: Haptoglobin Binding

Size Exclusion Chromatography of HDC

Hb

Nucleoside analogue

HDC

Haptoglobin (Hp)

Hp-HDC Complex
Acid phosphatase cleaves FUdR from Hb-FUdR

Cleaved FUdR activity is the same as free FUdR

- Acid phosphatase cleaves FUdR from Hb-FUdR
- Cleaved FUdR activity is the same as free FUdR
Hb-FUdR Activity: Hepatocellular Carcinoma Model

Primary Liver Cancer Orthotopic Implantation Model

- Balb/c mice, HepG2 liver tumor line, orthotopic implant into the liver
- Twice weekly dosing for 6 weeks

• Tumors confirmed as hepatocellular carcinoma by histopathology
• Hb-FUdR suppressed tumor growth in 7/10 animals
• Equal dose of free floxuridine was ineffective (3/10)
• No significant adverse clinical signs
• No weight loss in animals treated with Hb-FUdR

Percent Animals with No Measureable Tumor

PBS: 20%
Floxuridine Alone: 30%
Hemoglobin-Floxuridine: 70%
Hb-FUdR Activity: Colorectal Cancer Liver Metastasis Model

**Colorectal Cancer Liver Metastasis Model**

- NCr nude mice, human HCT-116-derived tumor cells transfected with GFP (MetaMouse)
- Orthotopic implant into the ascending colon
- Twice weekly dosing for 5 weeks

**Untreated (PBS)**

**Treated (FUdR or Hb-FUdR)**

- Hb-FUdR inhibited primary colon tumor growth (volume) at all doses relative to untreated mice
Hemoglobin and Drug Conjugate Summary

- **Hb purification process:**
  - GMP process (1 g to multi-kg scale)
  - 2-stage displacement chromatography, using 3-5 L of resin per kg of purified Hb
  - >99% pure HbA₀ isolated from other Hb subtypes and RBC proteins
  - Hb oxidation prevented by CO control in the process stream atmosphere

- **Drug conjugation:**
  - GMP process for small molecule drug activation and protein conjugation
  - 10 drug molecules per Hb, releasable and active
  - Improved *in vitro* and *in vivo* activity compared to free drug in models of liver cancer and viral hepatitis
  - Phase I clinical trial in liver cancer FDA-approved for Hb-FUdR (TBI 302)
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