HW4 due

Today:

• Midterm review
• Therapeutic protein hormones
• Discussion of project ideas
Rational design of therapeutic proteins

Case studies:
- hormone protein insulin
- hormone protein HGH
- hormone protein FSH
The case for therapeutic proteins

• Advantages over small-molecule drugs:
  – Do not have small size limitation
  – Do not require deep binding pockets
  – Higher binding selectivity and specificity

• Disadvantages over small-molecule drugs:
  – Usually not orally bioavailable
    • Cannot take orally because digestive proteases will degrade therapeutic protein
    • Must be injected by needle
  – Usually cannot pass membranes and enter cells
    • Bind cell surface receptors or extracellular proteins
Challenges for therapeutic proteins

- **Protein instability**
  - Short half-life in blood
    - Limited efficacy
    - Require frequent dosing (problems with patient compliance)
  - Short shelf-life

- **Immunogenicity**
  - If patient develops immune response to protein
    - Therapeutic protein may lose efficacy
    - Patient may experience side-effects

- **Difficult and costly to produce**
Ways to overcome challenges of...

- **Protein instability in blood**
  - Fuse therapeutic protein to another human protein (antibody Fc fragment, human serum albumin, transferrin)
    - Increases size of proteins and reduces clearance in kidneys (proteins <70kD)
  - Complete and/or increased glycosylation of protein “glycoengineering”
    - Increases protein size, stability, solubility
  - PEGylation
    - Increases protein size, stability, solubility

- **Protein instability: short shelf-life**
  - Protein engineering: mutations that stabilize during high/low temperature storage, or mutations that decrease susceptibility to proteolytic degradation

- **Immunogenicity**
  - Shield from immune response with glycosylation, PEGylation, or “humanization” of protein
Engineering hormone proteins

- Insulin
- HGH
- Fertility hormones
Engineering insulin as a therapeutic protein for diabetes
Problem: Diabetes

• Disease in which person has high blood sugar (glucose) levels

  • **Type I:** formerly “juvenile diabetes”
    – In majority of cases, autoimmune attack leads to loss of insulin-producing cells – partly genetic

  • **Type II:** formerly “adult onset diabetes”
    – In majority of cases, obesity and poor diet lead to loss of sensitivity to insulin by insulin receptor

• **Gestational diabetes:** pregnancy-induced
  – Resembles Type II diabetes in that there is a loss of sensitivity to insulin during pregnancy
Untreated diabetes can lead to more severe disease such as vomiting, dehydration, labored breathing, confusion, coma and death (especially in untreated type 1 diabetes).
**Discovery of insulin**

- First hormone discovered; highly conserved in vertebrates
- Early 1900s, scientists suggest pancreas cell secretions are involved in digestion: removal of pancreas in dogs gives them diabetes
- Scientists isolated dog pancreas secretions (containing insulin), then injected and cured dogs with diabetes
- 1922: 14-year old Leonard Thompson in hospital and dying from diabetes, was injected with purified fetal calf pancreas secretions (i.e. insulin) and cured!
- One of medicine’s most dramatic moments: a ward of ~50 diabetic, comatose, dying children were sequentially injected with insulin. Before final injections given, first children started waking up from comas.
- For decades, animal insulin was used to treat diabetic patients
- 1923: Nobel prize to F. Banting and J.J.R. Macleod for discovery of insulin
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Insulin

- Insulin is a protein hormone secreted by Beta cells in the pancreas in response to increased glucose levels (i.e. after eating a meal)
Insulin

- Secreted insulin binds the insulin receptor, which *promotes glucose uptake in a variety of cells* including muscle cells (for energy) and fat cells (for energy storage as triglycerides)
• Insulin regulates blood sugar levels to ensure that levels are never too high
• Too much glucose in blood causes symptoms of diabetes
Processing of insulin

Insulin gene encodes **preproinsulin** sequence

Cleavage of signal sequence by signal peptidase in ER yields **proinsulin**

Two endopeptidases cleave chain C to yield 51 amino acid **insulin**, ready and waiting intracellularly to be secreted by Beta cell

http://www.betacell.org/content/articleview/article_id/1/
How to make recombinant human insulin? In *E. coli*!? 

Former (1978-1986) method in *E. coli*:
- Production of A-chain and B-chain separately, as B-gal fusion proteins
- Removal of B-gal, mix, oxidize
- Inefficient
How to make recombinant human insulin? In *E. coli*!

Current (1986-now) method in *E. coli*:
- Production of proinsulin fused to tryptophan synthase
- Tryptophan synthase removal with cyanogen bromide
- Trypsin and carboxypeptidase to proteolytically cleave chain C
Advantages of recombinant insulin over animal-derived insulin

• Recombinant protein is human insulin
  – Bovine insulin has 3 amino acid differences
  – Porcine insulin has 1 amino acid difference
  – Animal insulin have small contaminants of glucagon and somastatin

• 100% E. coli cells produce recombinant insulin
  – Animal pancreas glands are only 1% “islet cells” and only a fraction of islet cells are Beta-cells containing insulin
  – In 1981: a 40,000 liter bacterial fermentation completed

• Potential issue of disease transmission in animal-derived insulin
Problems with recombinant insulin: rates of diffusion

- Insulin can form dimers and hexamers
- Diffusion rates can depend on insulin oligomeric state
- Need for fast diffusion (fast-acting)
- Need for slow diffusion (long-lasting)

Insulin hexamer, stabilized by zinc ion
Structure-based engineering of fast-acting insulin analogs

- Mutations to sterically disrupt hexamer formation, promoting monomer formation and faster adsorption – for use at meal times
- Insulin lispro: Pro28Lys and Lys29Pro mutations
- Insulin aspart: Pro28Asp mutation
- Insulin glulisine: Asn3Lys and Lys29Glu mutations
Engineering of long-lasting insulin analogs

- Mutations maintain constant, low-levels of plasma insulin
- Insulin glargine: Asn21Gly and +Arg31,Arg32 mutations
  - Arg31,Arg32 increases protein pl from pl~5 to pl~7 (net charge=0 at injected pH) and makes protein more insoluble = slow diffusion, steady absorption
  - Asn21Gly enhances stability (note error in figure saying Ala21, not Asn21!)
Engineering of long-lasting insulin analogs

- Modification to maintain constant, low-levels of plasma insulin
- Detemir insulin: Lys29 fatty acid acylated
  - Promotes reversible binding of Detemir insulin to albumin, delaying its absorption from the injection site (subcutaneous tissue)
- Lys29 PEGylation: enhanced stability
Current challenges for insulin protein therapy

• Patient compliance:
  – Glucose level monitoring
  – Needle injections of insulin

• Future:
  – More protein engineering / optimization?
  – Alternate routes of delivery:
    • Pulmonary route: inhaler
    • “Artificial pancreas”: implantable devise with glucose monitor and automatic infusion of insulin

“The trials and tribulations of producing the first genetically engineered drug” Nature Reviews Drug Discovery 2003

“New horizons – alternative routes for insulin therapy” Nature Reviews Drug Discovery 2002
Engineering human growth hormone (HGH) as a therapeutic protein for growth disorders
HGH stimulates bone and muscle growth

- Until 1985, HGH was extracted from the pituitary glands of cadavers
  - Taken off market after reported cases of Creutzfeldt-Jakob disease (infectious prions derived from cadavers)

- Currently, recombinant HGH is used to treat children’s growth disorders

- Recombinant HGH is abused by athletes to increase muscle growth
HGH has many variants

**Figure 1.** Primary structure of HGH and its isoforms. The main chain represents 22K-GH (GH-N). The sequence indicated by the bold line from residue 32 to 46 is deleted in 20K-GH. The black dot at the amino terminus denotes the acyl (probably acetyl) group in N-acylated GH. The two asterisks denote the deamidated residues in desamido-GH forms. The amino acid designations next to the main chain denote the residues that are changed in placental GH (GH-V). The tree structure at residue 140 indicates the glycosylation site in glycosylated GH-V. [Reproduced from G. Baumann: Growth hormone heterogeneity: genes, isoformes, variants, and binding proteins. Endocr Rev 12:424–449, 1991 (20), with permission. © The Endocrine Society.]

**TABLE 2.** Estimated average proportions for GH isoforms in human blood 15–30 min after a secretory pulse

<table>
<thead>
<tr>
<th>Type</th>
<th>Proportion</th>
</tr>
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<tbody>
<tr>
<td>Monomeric GH</td>
<td></td>
</tr>
<tr>
<td>22K-GH</td>
<td>45%</td>
</tr>
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<td>20K-GH</td>
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<td>Acidic GH (desamido-, acylated, and glycosylated GH)</td>
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Recombinant HGH is the 22K-GH form

- Recombinant HGH, the 22K-GH form, is produced in *E. coli*

- Detection of illegal HGH abuse involves measurement of ratio of 22K-to-20K form
  - Ratio is very consistent 9:1 in most humans
  - Excess 22K form indicates abuse
  - 20K-GH is alternatively spliced variant

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Engineering FSH as a therapeutic protein for infertility
Infertility

• Infertility affects up to 10-15% of couples
• A common cause of infertility is a lack of ovulation
• First line of treatment is usually clomiphene, a.k.a. Clomid, a small molecule inhibitor of the estrogen receptor

![Clomid molecule](image)

• By blocking the estrogen receptor in the hypothalamus, the body perceives a low level of estrogen and continues to produce several reproductive protein hormones (gonadotropins: FSH, LH, and CG)
• If Clomid doesn’t work and the body doesn’t produce the hormones, then hormone protein therapy is advised
Follicle Stimulating Hormone

- FSH, Follicle Stimulating Hormone, is produced by the pituitary gland.
- FSH stimulates the growth of follicles in the ovary.
- FSH works in concert with Leutinizing Hormone (LH), which causes the release of an egg (ovulation).
FSH for therapeutic use

- Natural FSH isolated from urine
- Recombinant FSH
  - Potential to engineer and optimize features