Proteins As Drugs - The Major Products of the Biotechnology Industry

• Insulin (Diabetes)
• Growth Hormone (Short Stature)
• Erythropoietin (Epo; Stimulates production of red blood cells)
• Granulocyte Colony Stimulating Factor (G-CSF; Stimulates production of a class of white blood cells)
• Thrombopoietin (Tpo; Stimulates production of platelets)
• Clotting Factor VIII, Factor IX (Hemophilia)
• Interferon (Multiple Sclerosis)
• Glucocerebrosidase (Provides the "missing" protein in Gaucher's disease)
• Anti- Her2 monoclonal antibody (overexpressed EGF receptor in breast cancer)
• Etanercept (Inflammation)
Herceptin - a monoclonal antibody against the HER2 receptor for Epidermal Growth Factor that is used to treat certain cases of breast cancer.
Activation of the EGF (Epidermal Growth Factor) Receptor Protein Tyrosine Kinase initiates division of many types of epithelial cells.
The **Human Epidermal Growth Factor Receptor (HER) Family**

**Hormones:**

- EGF
- HB-EGF
- TGFα
- Neuregulin
- HB-EGF

HER2 has no known ligand, but is activated by binding to HER1, 3, or 4 that, in turn, have bound hormones in the EGF family.
The HER2 gene

- The HER2 proto-oncogene encodes the production of a cell surface receptor protein known as the HER2 protein or receptor. This gene has homology to the rat gene neu, and is therefore sometimes referred to as HER2/neu or c-erbB-2.

- Many normal cells express a small amount of HER2 protein on their plasma membranes in a tissue-specific pattern.

- The HER2 receptor has no known ligand; however, HER2 forms heterodimers with HER1 (the epidermal growth factor receptor, EGFR), HER3 and HER4 in a complex with the ligands for these receptors.

- Heterodimer formation results in the activated HER2 receptor transmitting growth signals from outside the cell to the nucleus, thus controlling aspects of normal cell growth and division.
Amplification of the HER2 gene

• In tumor cells, errors in DNA replication may result in multiple copies of a gene on a single chromosome. This alteration is known as gene amplification.

• Amplification of the HER2 gene leads to an overexpression of HER2 protein, resulting in increased cell division and a higher rate of cell growth. HER2 gene amplification may also be associated with transformation to the cancer cell phenotype.

• Breast cancer is the most common malignancy among women in the United States, with 211,300 new cases projected for 2005.

• Amplification of the Human Epidermal growth factor Receptor 2 (HER2) gene results in HER2 protein overexpression. HER2 overexpression occurs in approximately 25% of breast cancer patients.

• HER2 protein overexpression correlates with several negative prognostic variables, including estrogen receptor-negative status, high S-phase fraction, positive nodal status, mutated p53, and high nuclear grade.

• HER2 gene amplification was found to be highly correlated with shortened disease-free survival and shortened overall survival of node-positive patients.
Structure of an antibody molecule
Herceptin - a monoclonal antibody

**Normal Cell**
In normal breast tissue cells, the HER2 gene produces a protein receptor on the cell surface. These growth factor-like receptors are thought to play a role in normal cell growth by signaling the cell to divide and multiply.

**HER2 Overexpressing Cancer Cell**
Cancerous breast tissue cells that overexpress (or overproduce) the HER2 gene produce extra protein receptors on the cell surface. The higher density of receptors triggers the cell to divide and multiply at an accelerated rate, thus contributing to tumor growth. Approximately 25-30% of all women with metastatic breast cancer overexpress the HER2 protein.

**Herceptin® (Trastuzumab)**
It is thought that Herceptin (a HER2 antibody) binds to numerous HER2 receptor sites found on the cell surface, blocking the receptor sites and possibly preventing further growth by interrupting the growth signal. As a result, the HER2 antibody may slow progression of the disease.
Man-made recombinant fusion proteins as drugs - ENBREL™

- TNFα is a naturally occurring cytokine that is involved in normal inflammatory and immune responses. It plays an important role in the inflammatory processes of rheumatoid arthritis (RA), polyarticular-course juvenile rheumatoid arthritis (JRA), and ankylosing spondylitis and the resulting joint pathology.

- Elevated levels of TNFα are found in involved tissues and fluids of patients with RA, psoriatic arthritis and ankylosing spondylitis (AS).

- Etanercept is useful in treatment of these diseases.

- ENBREL (Etanercept) is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human p75 tumor necrosis factor α receptor linked to the Fc portion of human IgG1.

- Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system. It consists of 934 amino acids.

- Etanercept binds specifically to tumor necrosis factor α (TNFα) and blocks its interaction with cell surface TNFα receptors.
Disadvantages of proteins as drugs

- Difficult to produce
  Many require production in cultured mammalian cells or transgenic animals

- Inhomogenous:
  N- and O- linked oligosaccharides
  Proteolytic cleavages

- Difficult to purify

- Unstable

- Must be injected

- Immunogenic

- Often side effects if administered systemically
Red Blood Cells

- Comprise about 45 - 47% of the volume of blood.
- Filled with the red oxygen-binding protein hemoglobin.
- Transports oxygen from the lungs to all tissues in the body.
- Transports carbon dioxide from tissues to the lungs.
Erythropoietin (Epo), the Protein that Controls Red Blood Cell Production

- PRODUCED BY THE KIDNEY IN RESPONSE TO LOW O$_2$ PRESSURE IN THE BLOOD
- BINDS TO EPO RECEPTORS ON THE SURFACE OF ERYTHROCYTE PROGENITOR CELLS IN THE BONE MARROW
- STIMULATES THESE CELLS TO DIVIDE 5 TO 7 TIMES; EACH OF THE ~30 TO 100 DAUGHTERS THEN DIFFERENTIATES INTO A RED BLOOD CELL
- USED CLINICALLY TO TREAT ANEMIA CAUSED BY KIDNEY FAILURE OR BY DISEASES SUCH AS AIDS
Epo regulates red cell mass. Epo synthesis is induced in the kidney in response to hypoxia (low oxygen in the blood).
Epo “Gene Knock-Out” mice die at embryonic day 14 of severe anemia
Epo stimulates the terminal proliferation and differentiation of erythroid progenitor cells to mature red cells.
Erythropoiesis is controlled by a life- or death decision of an erythroid progenitor that is regulated by Epo.
Structure of the Epo - EpoR Complex
Epo binding locks two Epo receptors into an “activated” dimeric conformation leading to trans-phosphorylation and activation of the JAK2 kinase.
**Signal Transduction by the Epo Receptor**

- Activated JAK2 phosphorylates up to 8 tyrosine residues on the cytosolic domain of the Epo receptor.

- Each phosphotyrosine can form the “docking site” for the SH2 domain of a signal transduction protein.

- Many of these proteins become phosphorylated and thus activated by JAK2.
Cytokine Receptors that form Hormone-Induced or Hormone-Stabilized Homo-Dimers:

- Erythropoietin (Epo)
- Granulocyte Colony Stimulating Factor (G-CSF)
- Thrombopoietin (Tpo)
- Prolactin (Prl)
- Growth Hormone (GH)

Sales of Epo are in excess of $6 billion/year and G-CSF in excess of $2 billion/year.

Epo sells for about $2,000,000,000 per kg, and is probably the most valuable substance on Earth.
Recombinant human Epo as treatment for anemia due to loss of kidney function

- More than 300,000 kidney patients around the world receive recombinant human Epo.

- Anemia due to kidney failure is the prototype of Epo deficiency.

- More than 95% of patients have a positive response and become transfusion independent.

- Recombinant human Epo, administered twice weekly, is cost effective compared to blood transfusions.

- The quality of life improves markedly for patients whose red cell number increases to near normal levels.
Generation of an Epo "expression construct" for recombinant production of Epo in a cultured mammalian cell
Transfection of Epo expression construct into cultured mammalian cells.

Random integration into chromosomal DNA
Production of Epo mRNA and protein from cloned Epo gene
The Epo “expression construct” for recombinant contains the gene encoding Dihydrofolate reductase (DHFR)
Methotrexate (MTX), an inhibitor of DHFR, selects those rare cells that have amplified their DHFR gene and thus make more DHFR protein.

These cells also have more Epo genes and make more Epo protein.
Cultured mammalian cells with multiple amplified copies of the Epo expression construct are used for commercial production of Epo.
Epo is a glycoprotein; it has four chemically linked sugar chains. Three are long and linked to an asparagine residue (N-linked). One is short and linked to a serine residue (O-linked). The composition of these sugar chains is very important.
Sialic acid
The only negatively charged sugar in glycoproteins
N-linked oligosaccharides on Epo, as in all glycoproteins, are heterogeneous, especially with respect to numbers of sialic acid units
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Glycosylation of Human Epo

• Three tetra-antennary (4 branched) N-linked oligosaccharides; maximum of $3 \times 4 = 12$ sialic acids
• One O-linked oligosaccharide with a maximum of 2 sialic acids

• Maximum of 14 sialic acids per Epo molecule

• The greater the number of sialic acids per Epo molecule:
  • Poorer binding to the Epo receptor
  • Longer half-life in the circulation
  • Overall greater potency in stimulating red cell production

• Normal Epo in blood: 3 - 9 sialic acids

• Recombinant human Epo: 9 - 14 sialic acids
Epo - a drug of abuse by athletes
Detection of recombinant Epo in urine

Figure 1 Autoradiograph of isoelectric patterns of exogenous and endogenous erythropoietin (EPO).

a: Purified commercial human urinary EPO (Sigma);
b and c: Recombinant human EPO
d: Urine from a control subject
e and f: Urine from two patients treated with recombinant EPO for post-haemorrhagic anemia
g and h: Urine from two cyclists from Tour de France 1998
NESP (Darbepoetin) - Amgen’s Hyperglycosylated Epo Analog

**rHuEPO**  \rightarrow \text{SITE- SPECIFIC MUTAGENESIS}  \rightarrow **NESP**

- **3 N- LINKED CHO CHAINS**
  - MW = 30,400
  - \sim 40\% CARBOHYDRATE
  - UP TO 14 SIALIC ACID RESIDUES
  - SERUM t_{1/2} = 8.5 hours

- **5 N- LINKED CHO CHAINS**
  - MW = 38,000
  - \sim 52\% CARBOHYDRATE
  - UP TO 22 SIALIC ACID RESIDUES
  - SERUM t_{1/2} = 25.3 hours

**LONGER SERUM HALF- LIFE; HIGHER IN VIVO BIOACTIVITY**
**LESS FREQUENT DOSING**
Larisa Lazutina will be forced to forfeit her medal in the 30K classic. AP

Johann Muehlegg has competed for Spain since 1999. AP
Olympic champions stripped of medals after positive drug tests

By Larry Siddons, Associated Press, 02/24/02

SALT LAKE CITY -- Cross-country skiers Johann Muehlegg of Spain and Larissa Lazutina of Russia were stripped Sunday of their most recent gold medals at the Salt Lake City Olympics after testing positive for a performance-enhancing drug.

A third cross-country skier, Olga Danilova of Russia, also tested positive for the same drug, darbepoetin, which is used to treat anemia by boosting the production of red blood cells that carry oxygen to muscles.

All three athletes were tossed out of the Olympics. The International Olympic Committee said Muehlegg, who won three gold medals at these games, was ordered to return his gold in the 50-kilometer classical race. But he will be allowed to keep his gold medals in the 30K freestyle and the 10K pursuit events.

Lazutina, who tied an Olympic record Sunday with her 10th medal when she won the women's 30K classic, will be forced to forfeit that medal. But she will be allowed to keep two medals she won earlier in these Olympics.
Danilova was disqualified from the 30K classic in which she finished eighth.

Muehlegg had become the toast of Spain, even fielding congratulations from King Juan Carlos following his success in Salt Lake City.

Since darbepoetin is relatively new, it is not on the IOC's list of banned substances. But it has similar properties to the banned hormone erythropoietin, or EPO.

Lazutina, 36, was disqualified from the women's relay Thursday for having high levels of performance-boosting hemoglobin, a blood molecule that helps carry oxygen to muscles. She took another blood test Sunday morning.

The heavily favored Russians had to withdraw from the relay. Russian officials claimed drug-testers were targeting their athletes, and the dispute led to widespread complaints of biased judging in other sports.
On Sunday, the Spanish team doctor acknowledged that Muehlegg had tested positive for darbepoetin, which is **stronger than EPO and stays in the body longer**. He and members of the Spanish delegation went before the IOC disciplinary committee to ask for a second test, but the results have not been released.

Spectators at Soldier Hollow were treated to thrilling performances over the 16 days of competition, but all anyone could talk about Sunday was doping, which also resulted in the disqualification of Russians Larissa Lazutina and Olga Danilova.

Frode Estil, a Norwegian cross-country skier who won two individual silvers and a relay gold at the games, said he was "very disappointed" in Muehlegg.

"It's very sad for the sport to have this scandal," Estil said. American skier Carl Swenson argued that **Muehlegg's testing was "good news" for the sport, since it showed that the system can detect use of darbepoetin.**

"The sport's bigger than any one doping scandal," Swenson said. "It's going to keep going, and the more they catch, the cleaner it's going to become, eventually. if someone's caught, it's that much cleaner."
Erythropoietin receptor:

• In neural cell lines (PC12, SK-N-MC cells)
• In hippocampal and cerebral cortical neurons

Erythropoietin:

• In *in vitro* cultured astrocytes and neurons
• In brain
Erythropoietin is neuroprotective against damage from:  
- Ischemia  
- Mechanical trauma  
- Excitotoxins  
- Neuroinflammation  
- An animal model of Parkinsonism.

Erythropoietin addition to in vitro cultured neuronal cells inhibits death caused by serum deprivation or kainic acid exposure.

Activation of neuronal erythropoietin receptor prevents death induced by nitric oxide and other toxic chemicals.

Similar to its role in erythroid progenitor cells, Epo prevents the death of certain nerve cells.