Production of therapeutic proteins

Therapeutic proteins: biopharmaceuticals

- Proteins as drugs
- Proteins as antigens (vaccines)

The industrial production of therapeutic proteins requires the use of recombinant DNA technology to obtain the necessary high amounts of proteins

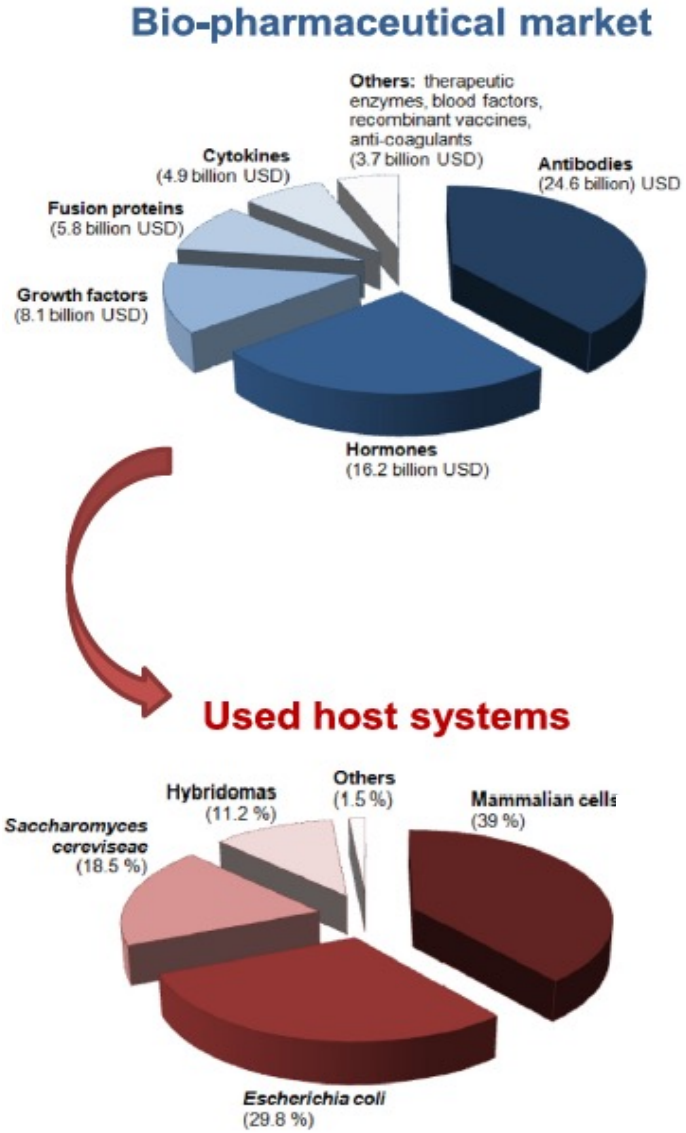


Table 1

Comparison of expression systems used for biopharmaceutical production [4,6,7]

	Higher eukaryotes		Yeast		*Escherichia coli*
Ease of genetic modifications	Moderate		Simple		Simple
Cultivation	Slow growth rates, expensive complex (or synthetic) media required		Fast and robust growth, defined minimal media		Fastest growth, defined minimal media
Contaminations	Risk of viral contaminations, viral clearance required		Little risks of endotoxins or viral DNAs		Endotoxins presence requires thorough purification, possible phage infections
Post translational modifications (PTMs)	Closely resembling human PTMs; usually mixtures of several glycoform variants		Most human PTMs achievable, but natural glycosylation patterns differ from humans, hypermannosylation, engineered strains can achieve human glycoforms and high uniformity		Limited set of PTMs, some human PTMs (e.g. glycosylation) difficult to achieve
Protein yields and secretory capacities	High yields, highly efficient secretion, high specific productivity		High yields, secretory capacities depending on the species		High expression capacities, secretion mostly inefficient, extensive purification and downstream processing required
Most commonly used species	Mammalian cells	Insect cells	*Pichia pastoris*	*Saccharomyces cerevisiae*	
Recently approved biopharmaceuticals[a]	32	2	2[b]	4	17
Additional information and specific differences between host species of the same class	Commonly used cell lines: CHO (Chinese Hamster Ovary), BHK (baby hamster kidney), murine-myeloma-derived NS0, SP2/0 cell lines [2] and HEK293	Baculo virus based systems most commonly used for transfection Easy scale up Contaminations less problematic Mammalianized glycosylation [5]	Efficient and selective secretion, often higher protein titers than *S. cerevisiae*, for example, [8**] Crabtree negative, high cell density cultivations GRAS status Hypermannosylation is less pronounced in *P. pastoris* and critical terminal α-1,3-mannose linkages were not observed [19], engineered strains providing fully humanized glycosylation not available for *S. cerevisiae*	Important eukaryotic model organism, high molecular- and cell biological knowledge Crabtree positive, leading to ethanol production	Fastest efficient expression system Inexpensive Well established processes suitable for mass production Folding problems may lead to the formation of inclusion bodies and require expensive refolding (yet, inclusion bodies provide a valuable strategy to achieve high protein yields and simple purification) Inefficient acetate metabolism may hamper high cell density cultivation of some strains

[a] Data from Walsh [1], time period: January 2006–June 2010, in total 58 biopharmaceuticals have been approved, two biopharmaceuticals produced in transgenic animals were not listed.
[b] In this number Jetrea by ThromboGenics is included (approved in 2012 and not listed by Walsh [1]).

Table 1 Examples of biopharmaceuticals produced in *E. coli*, yeast and mammalian cells (from [126])

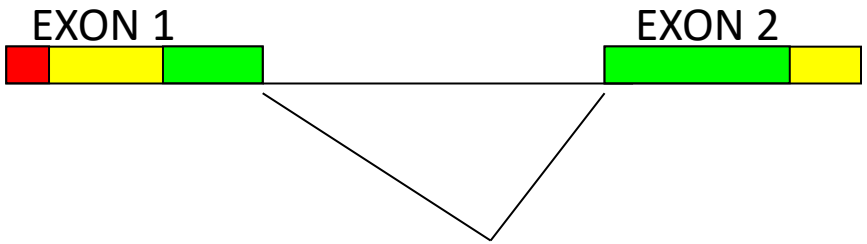
Therapeutic group	Recombinant protein	Host	Protein properties	
			Molecular weight (kDa)	Post-translational modifications
Blood factors, thrombolytics, anticoagulants	Factor VIII	Mammalian cells	267.0	Disulphide bonds, glycosylation, sulphation
	Tissue plasminogen activator	Mammalian cells, *E. coli* (fragment)	62.9	Proteolytic cleavage, disulphide bonds, glycosylation
	Hirudin	*S. cerevisiae*	7.0	Disulphide bonds, glycosylation, sulphation
Hormones	Insulin	*E. coli*, *S. cerevisiae*	12.0	Proteolytic cleavage, disulphide bonds
	Human growth hormone	*E. coli*, *S. cerevisiae*	24.8	Disulphide bonds, phosphoprotein
	Follicle-stimulating hormone	Mammalian cells	14.7 (subunit beta)	Disulphide bonds, glycosylation
	Glucagon	*E. coli*, *S. cerevisiae*	20.1	Amidation, proteolytic cleavage
Growth factors	Erythropoietin	Mammalian cells	21.3	Disulphide bonds, glycosylation
	Granulocyte-colony stimulating factor	*E. coli*, mammalian cells	22.3	Disulphide bonds, glycosylation
	Granulocyte–macrophage colony stimulating factor	*E. coli*	16.3	Disulphide bonds, glycosylation
Cytokines	Interferon-alpha	*E. coli*	21.5	Disulphide bonds, glycosylation
	Interferon-beta	*E. coli*	22.3	Disulphide bonds, glycosylation, phosphoprotein
Monoclonal antibodies	Infliximab	Mammalian cells	144.2	Disulphide bonds, glycosylation
Enzymes	Alpha-galactosidase	Mammalian cells	48.8	Disulphide bonds, glycosylation
	Deoxyribonuclease	Mammalian cells	31.4	Disulphide bonds glycosylation
	Uricase	*S. cerevisiae*	34.2	Acetylation

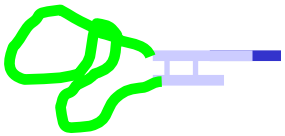
Protein properties that influence host selection include, among others, molecular weight and post-translational modifications, and were obtained from UniProt (http://www.uniprot.org/) and DrugBank (http://www.drugbank.ca/)

Biosynthesis of insulin in pancreas β cells

Chain A ⇒ 21 aa
Chain B ⇒ 30 aa

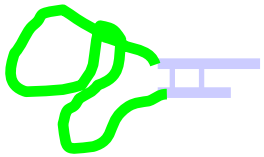
Linked by two S-S bridges





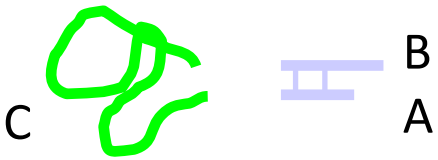
PREPROINSULIN

Signal peptide



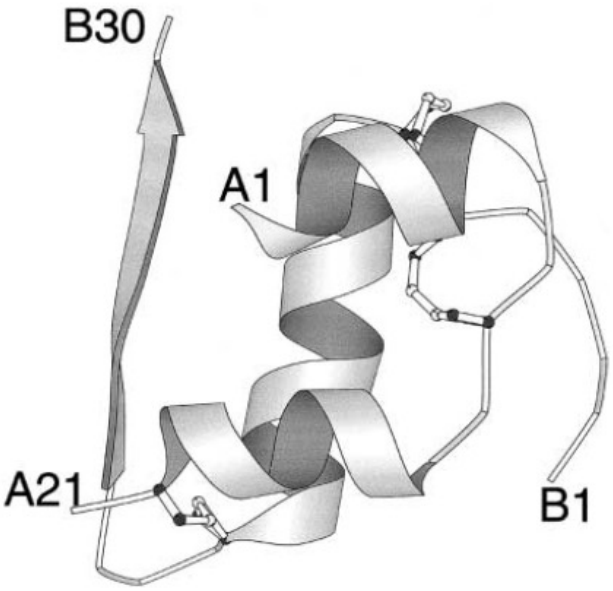
PROINSULIN

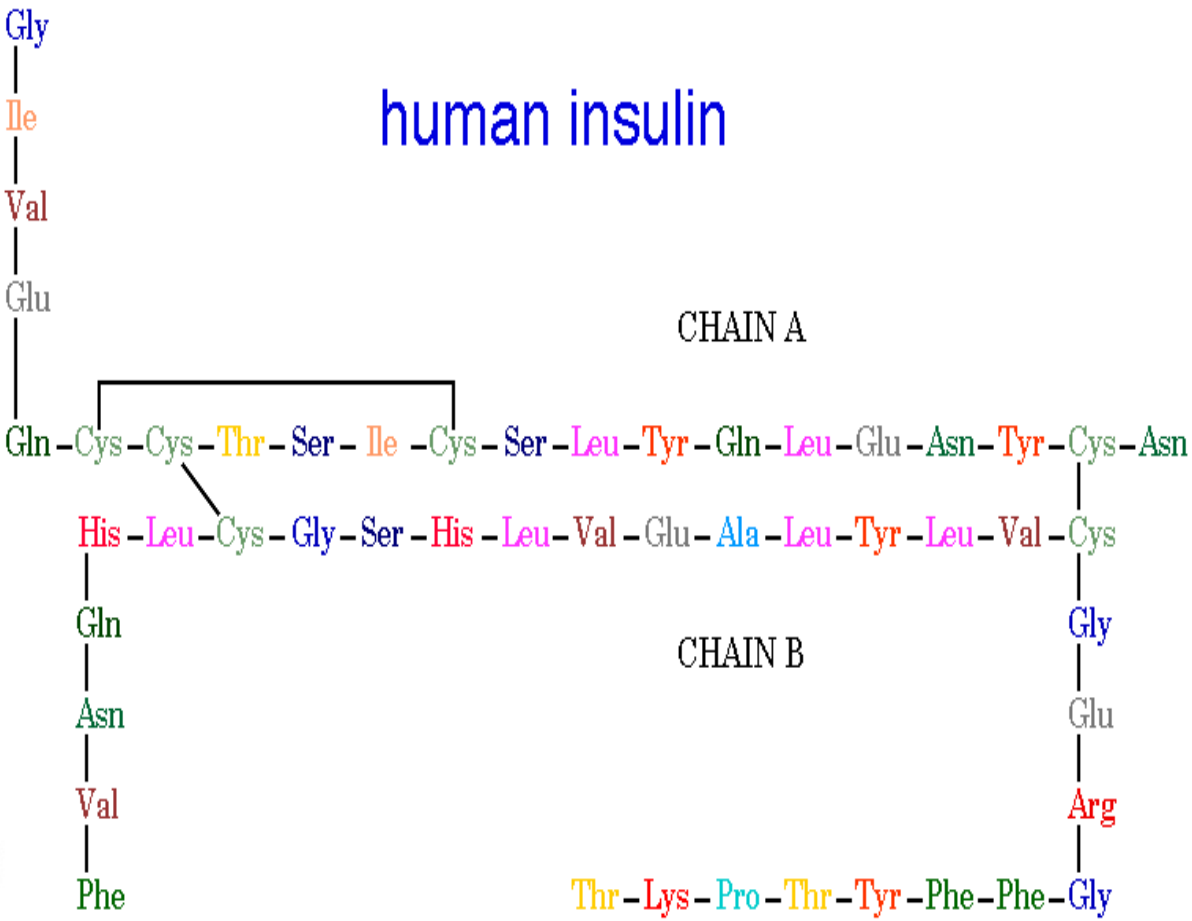
In the Golgi an enzyme removes 33 aa that constitute the C peptide



INSULIN

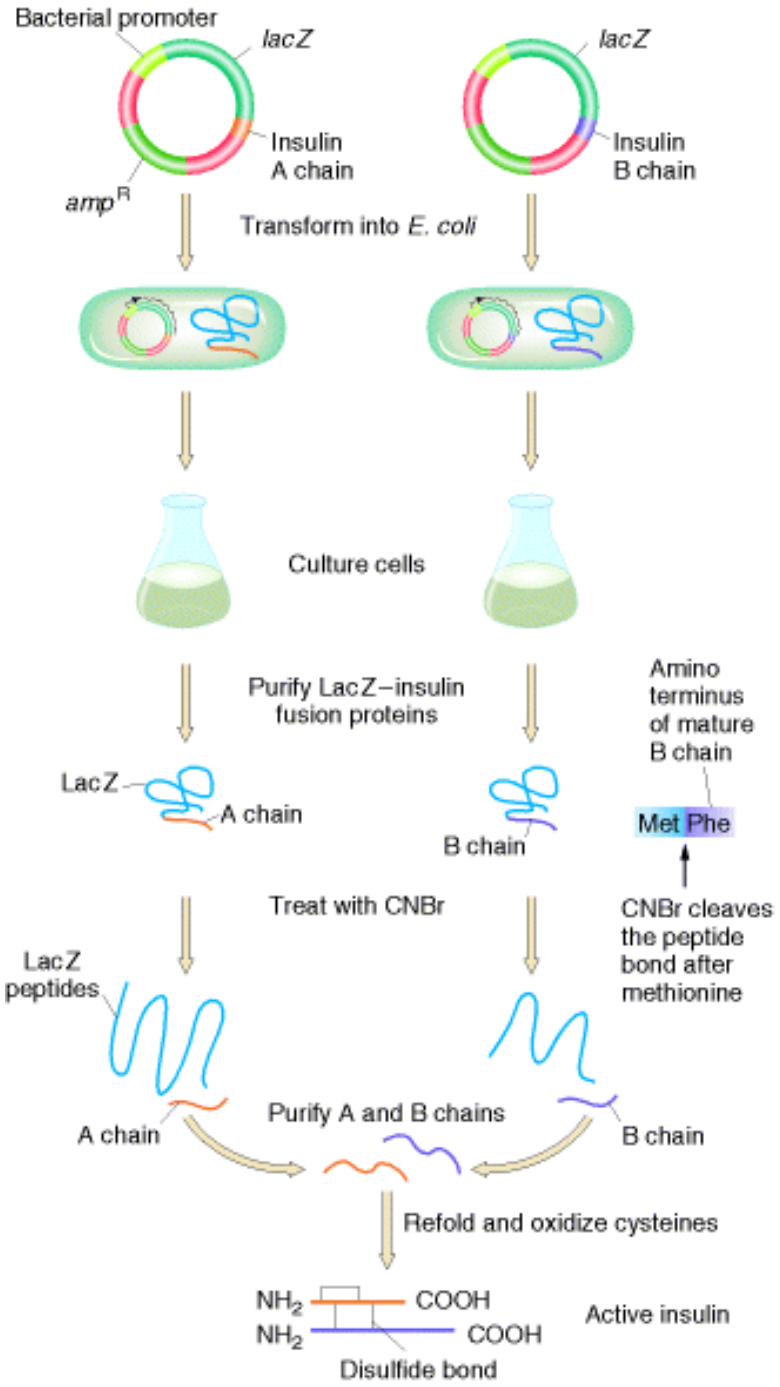
Insulin forms hexamers, stabilized by zinc, that modulate its solubility and bioavailability





Production of recombinant insulin in bacteria

- Separate plasmids encode A and B chains in separate E. coli strains
- A and B chains are produced under control of the *lac* promoter as fusion proteins with the N-terminal region of LacZ
- The LacZ portion is removed by treatment with cyanogen bromide (cleaves Met-X)
- Purified A and B chains are mixed in oxidizing conditions, disulfide bridges S-S form and the native form is purified



Production of recombinant insulin in the yeast Saccharomyces cerevisiae (proinsulin pathway)

Pro-insulin constituted by chain B1-29 (lacking Thr30) and chain A1-21 fused to α-factor signal peptide to produce secreted insulin (mini-peptide C).

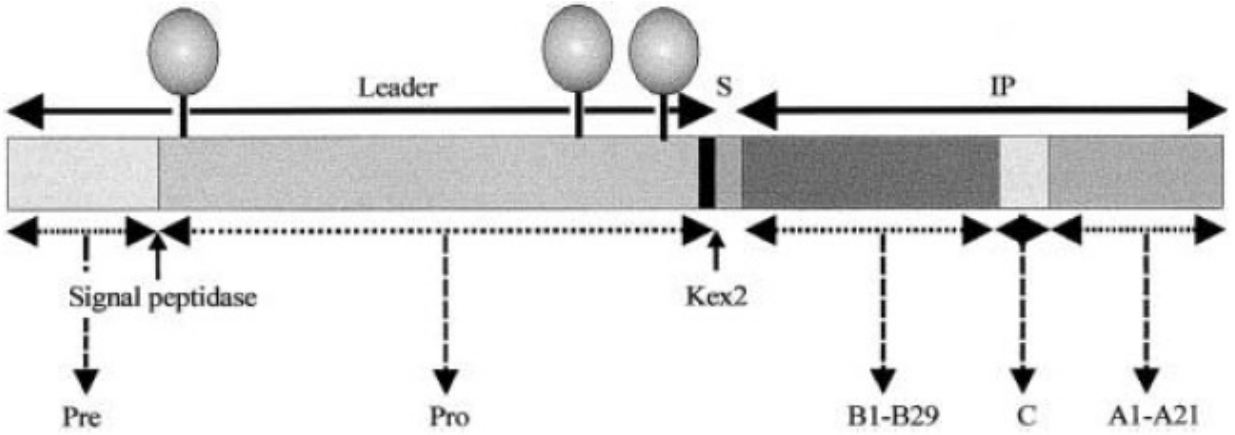


Fig. 2 Schematic representation of the leader-insulin precursor fusion protein expressed in *Saccharomyces cerevisiae*. A leader consists of a pre-peptide and a pro-peptide. Here the *S. cerevisiae* α-factor leader is indicated (*leader*). *Pre* indicates the pre-peptide (signal peptide) and *Pro* indicates the pro-peptide. The dibasic Kex2 endoprotease processing site, localised at the pro-peptides C-terminus, is shown in *black*. The single-chain insulin precursor (*IP*), comprising the first 29 amino acids of the human insulin B-chain (*B1–B29*) is joined to the 21 amino acids of the human insulin A-chain (*A1–A21*) by the mini C-peptide AAK (*C*) connecting lysine^{B29} and glycine^{A1}. Certain fusion proteins also feature a spacer peptide (*S*) with the amino acid sequence (EEAEAEAEPK, or derivatives of this sequence). Sites for attaching N-linked carbohydrate chains, for processing by the signal peptidase and for processing by the Kex2 endoprotease are indicated

Constitutive expression under control of TPI (triose-phosphate isomerase) promoter, selection by complementation with S. pombe TPI in S. cerevisiae tpi::LEU2 strain

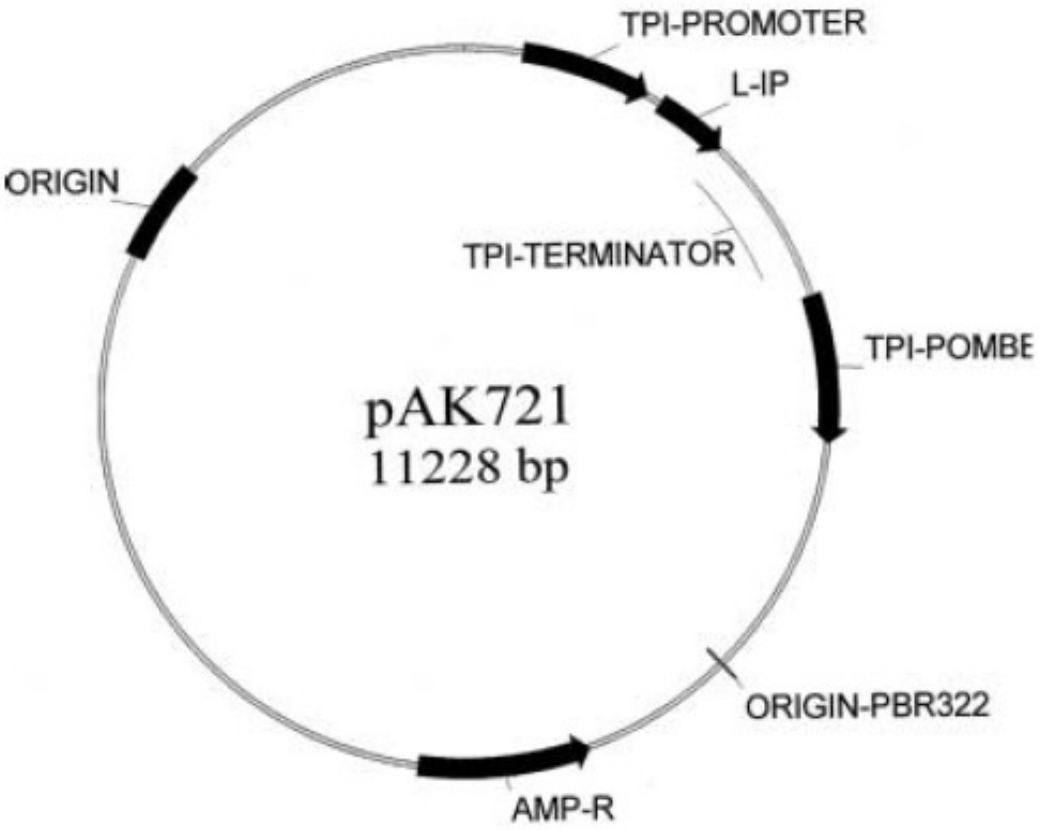


Fig. 4 The pAK721 *S. cerevisiae* expression plasmid expressing the LA19 leader-EEAEAEAEPK-insulin precursor fusion protein. The pAK721 expression plasmid was based on the *S. cerevisiae–Escherichia coli* shuttle *POT* plasmid (MacKay et al. 1990; Kawasaki and Bell 1999). *L-IP* indicates the fusion protein expression cassette, *TPI-PROMOTER* the *S. cerevisiae TPI1* promoter and *TPI-TERMINATOR* the *S. cerevisiae TPI1* terminator. *TPI-POMBE* indicates the *S. pombe POT* gene used for selection in *S. cerevisiae*; *ORIGIN* indicates a *S. cerevisiae* origin of replication derived from the 2-μm plasmid. *AMP-R* indicates the *β*-lactamase gene conferring resistance toward ampicillin and facilitating selection in *E. coli*. *ORIGIN-PBR322* indicates an *E. coli* origin of replication

Evaluation of efficiency of pro-peptide and *spacer* sequences in the production of recombinant insulin in yeast

Table 1 Fermentation yield of insulin precursor expressed in *Saccharomyces cerevisiae* by different leaders. Insulin precursors comprise the first 29 amino acids of the human insulin B-chain, joined to the 21 amino acids of the human insulin A-chain by a "mini C-peptide", AAK. The insulin precursor was expressed in the *S. cerevisiae* strain MT663[d] (a gift from Mogens Trier Hansen, Novo Nordisk; *MATa/MATα pep4-3/pep4-3 HIS4/his4 tpi::LEU2/tpi::LEU2* Cir$^+$) using the *POT* expression plasmid (Fig. 4). Each leader consists of a pre-peptide (the α-factor leader signal peptide or the Yap3 endoprotease signal peptide) and a pro-peptide, but only the pro-peptide and spacer peptides are shown here, see Fig. 2. Fermentation was at 30 °C for 72 h in YPD. Yield was determined by RP-HPLC of the culture supernatant, and is expressed as mean ± standard deviation (SD) of the values obtained in four individual fermentations. Where leaders lack the Kex2 endoprotease site, fermentation yield was determined as des(B30) insulin after maturation by *A. lyticus* lysyl specific endoprotease (EC 3.4.21.50). Consensus N-linked glycosylation sites [NX(T/S)] as well as mutated non-functional glycosylation sites (QTT) are underlined

S. cerevisiae strain	Pro-peptide	Pro-peptide amino acid sequence	Spacer peptide	Yield (mg/l)
MT748[d]	α	APVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFI NTTIASIAAKEEGVSLDKR	–	16.5 ± 0.2
yAK708	α[a]	APVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFI NTTIASIAAKEEGVSMAKR	EEAEAEAEPK	41.1 ± 0.9
yAK721	LA19	QPIDDTESNTTSVNLMADDTESRFATNTTLALDVVNLISMAKR	EEAEAEAEPK	50.3 ± 3.8
yAK775	LA34	––––––––QTT––––––––––––––––QTT–––––––––––– ΔΔ	EEAEAEAEPK	25.6[c] ± 3.9
yAK817	TA39	––––––––NTT––––––––––––––––NTT–GGLDVVNLISMAKR	EEGEPK	59.5 ± 6.7
yAK855	TA57	––––––––QTT––––––––––––A–––QTNSGGLDVVGLISMAKR	EEGEPK	79.2 ± 3.7

[a] An α-factor leader in which the C-terminus has been modified from "SLDKR" to "SMAKR". The LA19 pro-peptide amino acid sequence is shown; for the next three pro-peptides the sequence is given only at positions where it differs from the LA19 pro-peptide amino acid sequence

Scheme of the industrial production process of recombinant insulin by the proinsulin pathway.

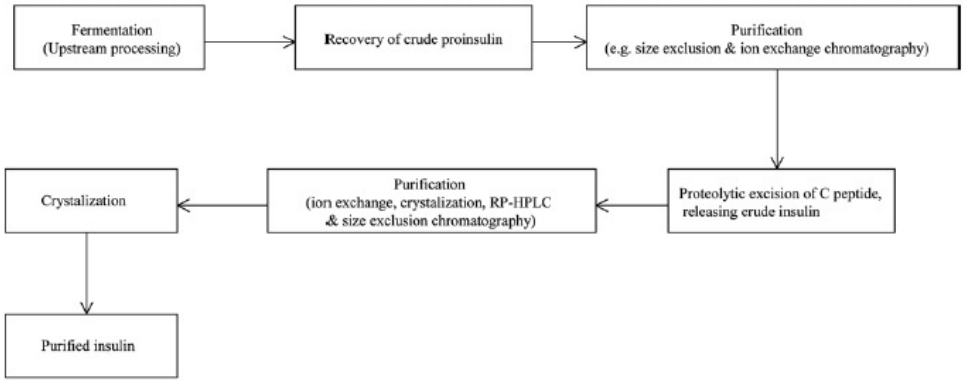


Fig. 1 A generalized overview of the industrial-scale production of modern high-purity recombinant insulins via the "proinsulin route" (**a**). An industrial-scale preparative HPLC column (photograph courtesy of NovaSep, France) (**b**)

Insulin analogues approved for medical use

Table 1 Recombinant human insulins/engineered insulins currently approved for general medical use in the USA and/or EU

Product	Description	Structure	Company	Approved
Humulin	Recombinant human insulin produced in *Escherichia coli*	Identical to native human insulin	Eli Lilly	1982 (USA)
Novolin	Recombinant human insulin produced in *Saccharomyces cerevisiae*	Identical to native human insulin	Novo Nordisk	1991 (USA)
Insuman	Recombinant human insulin produced in *E. coli*	Identical to native human insulin	Hoechst	1997 (EU)
Actrapid/Velosulin/ Monotard/Insulatard/ Protaphane/Mixtard/ Actraphane/Ultratard	All contain recombinant human insulin produced in *S. cerevisiae* formulated as short/intermediate/ long-acting product	Identical to native human insulin	Novo Nordisk	2002 (EU)
Humalog (insulin Lispro)	Recombinant short-acting human insulin analogue produced in *E. coli*	Engineered: inversion of native B28–B29 proline–lysine sequence	Eli Lilly	1996 (USA and EU)
Liprolog (insulin Lispro)	Recombinant short-acting human insulin analogue produced in *E. coli*	Engineered: inversion of native B28–B29 proline–lysine sequence	Eli Lilly	1997 (EU)
NovoRapid (insulin Aspart)	Recombinant short-acting human insulin analogue produced in *S. cerevisiae*	Engineered: B28 proline replaced by aspartic acid	Novo Nordisk	1999 (EU)
Novolog (insulin Aspart)	Recombinant short-acting human insulin analogue produced in *S. cerevisiae*	Engineered: B28 proline replaced by aspartic acid	Novo Nordisk	2001 (USA)
Levemir (insulin Detemir)	Recombinant long-acting human insulin analogue produced in *S. cerevisiae*	Engineered: devoid of B30 threonine and a C14 fatty acid is covalently attached to B29 lysine	Novo Nordisk	2004 (EU)
Apidra (insulin Glulisine)	Recombinant rapid-acting insulin analogue produced in *E. coli*	Engineered: B3 asparagine is replaced by a lysine and B29 lysine is replaced by glutamic acid	Aventis Pharmaceuticals	2004 (USA)
Lantus (insulin Glargine; Optisulin)	Recombinant long-acting human insulin analogue produced in *E. coli*	Engineered: A 21 asparagine replaced by glycine and B chain elongated by two arginines	Aventis Pharmaceuticals	2000 (USA and EU)

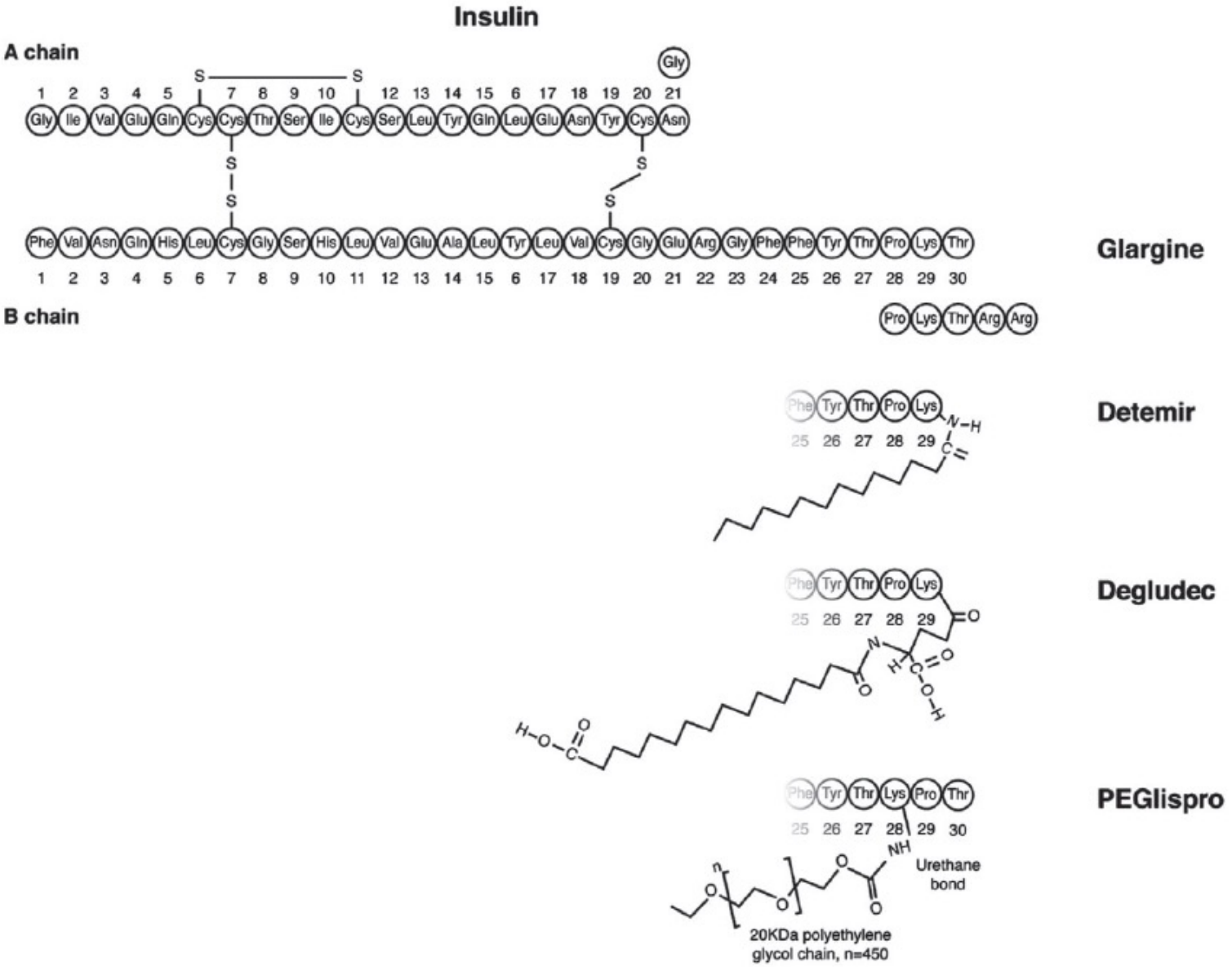


FIGURE 2 Molecular structure of insulin analogues. Molecular modifications made to the human insulin molecule in order to protract action are shown. The isoelectric point of IGlar U100 was raised by substituting glycine 21 on the A chain (A21) of human insulin for asparagine, and adding 2 asparagine molecules to the amino terminal of the B chain. IDet is an analogue in which threonine B30 has been removed and lysine B29 is acylated with a 14-carbon myristoyl fatty acid. Threonine B30 is also removed in IDeg but lysine B29 is attached to a 16-carbon fatty diacid via a glutamic acid spacer. In PEGlispro, the order of proline and lysine is reversed such that proline 29 follows lysine 28, which is attached to a polyethylene glycol chain via a urethane bond.

Pharmacokinetic properties of insulin analogues

Table 2 General pharmacokinetic characteristics of representative short-, intermediate- and long-acting native and engineered insulins

Category	Onset (time after administration)	Peak activity (time after administration)	Duration
Native insulin; short-acting formulation	30 min–1 h	2–5 h	6–8 h
Native insulin; intermediate-action formulation	2 h	4–12 h	Up to 24 h
Native insulin; long-acting formulation	4 h	10–20 h	Up to 36 h
Humalog (engineered, rapid acting)	20 min	30–90 min	3–5 h
Novorapid (engineered, rapid acting)	15 min	30–120 min	3–5 h
Lantus (engineered, long acting)	1 h	–	24 h

Therapeutic glycoproteins

Table 2 Approved glycosylated therapeutic proteins (listed by trade name)

Product category	Specific products (by trade name)
Blood factors, anticoagulants and thrombolytics	Activase, Advate, Benefix, Bioclate, Helixate/Kogenate, Metalyse/TNKase, Novoseven, Recombinate, Refacto, Xigiris
Antibodies	Avastin, Bexxar, Erbitux, Herceptin, Humaspect, Humira, Mabcampath/Campath-H1, Mabthera/Rituxan, Mylotarg, Neutrospec, Oncoscint, Orthoclone OKT-3, Prostascint, Raptiva, Remicade, Simulect, Synagis, Xolair, Zenapax, Zevalin, Tysabri
Hormones	Gonal F, Luveris, Ovitrelle/Ovidrel, Puregon/Follistim, Thyrogen
EPO and granulocyte-macrophage colony-stimulating factors	Epogen/Procrit, Leukine, Neorecormon, Nespo/Aranesp
Interferons	Avonex, Rebif
Additional	Aldurazyme, Amevive, Cerezyme, Enbrel, Fabrazyme, Inductos, Infuse, Osigraft/OP-1 implant, Pulmozyme, Regranex, Replagal, Myozyme, Naglazyme

Erythropoietin

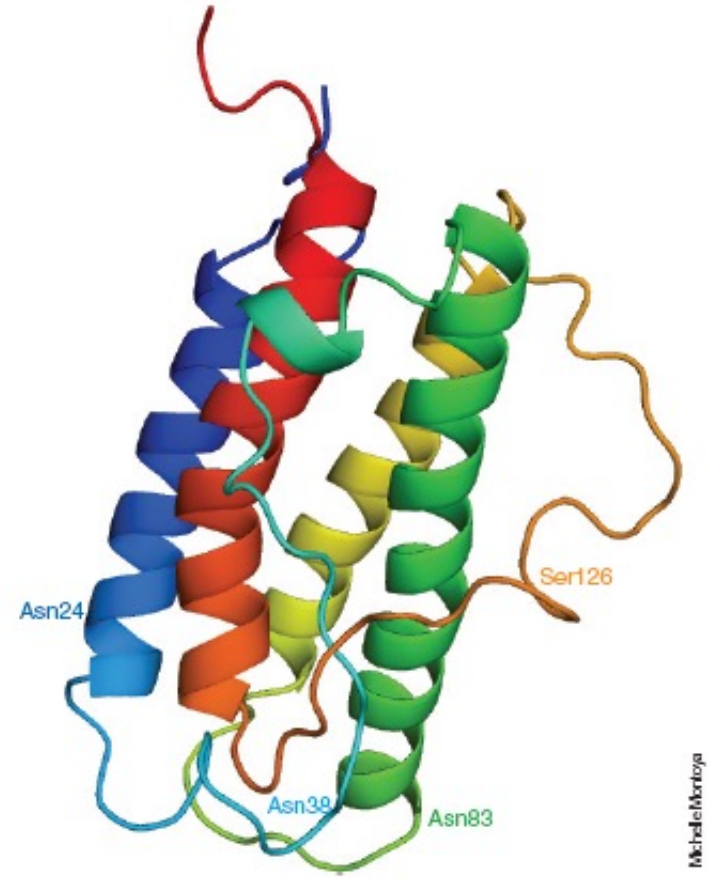


Figure 2 Schematic of human EPO indicating the positions of the three asparagine residues and single serine residue that are sites of N- and O-linked glycosylation respectively.

Erythropoietin (EPO) is a hormone that stimulates erythrocyte production. It is a glycoprotein of 165 aa with 4 oligosaccharide chains (3 N- and 1 O-glycosylation).
N-glycosylation is necessary for secretion and stability of EPO in plasma. O-glycosylation appears not to influence the biological activity of EPO.

Recombinant EPO is produced in CHO cells and in yeast

Physiology of EPO

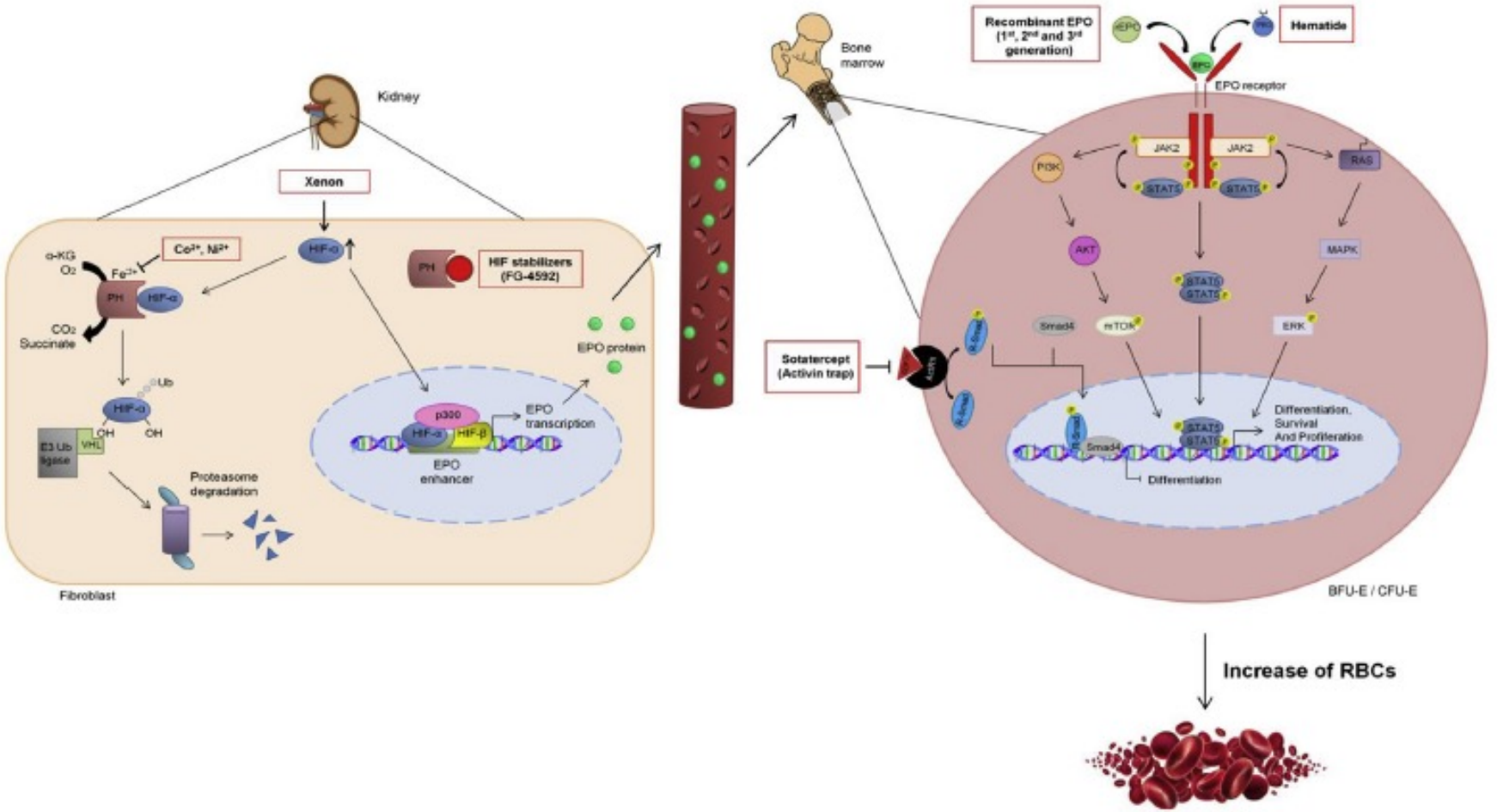


Fig. 2. Schematic model of the control of red blood cell production and the sites of action of erythropoiesis-stimulating agents. EPO is produced in the kidney under the control of HIFs which can be modulated by different substances. As a circulating endogenous hormone, EPO enters the bone marrow and activates the EPO receptor in BFU-E and CFU-E cells. Signal transduction through the JAK2/STAT5 pathway induces the differentiation, survival, and proliferation of red blood cell precursors. Recombinant human EPO and EPO biomimetic peptides also activate EPOR. Sotatercept prevents the binding of GDF-11 to its receptor and blocks the inhibition of differentiation of erythroblasts (Shenoy et al., 2014).

Production of human erythropoietin in glycoengineered *Pichia pastoris*

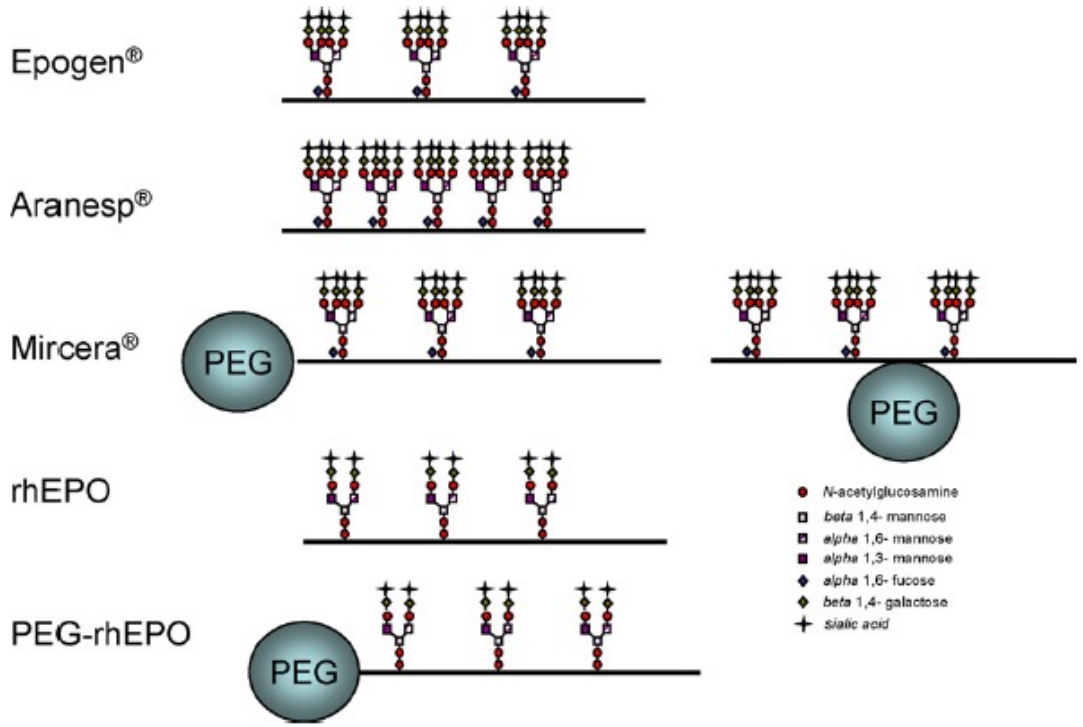
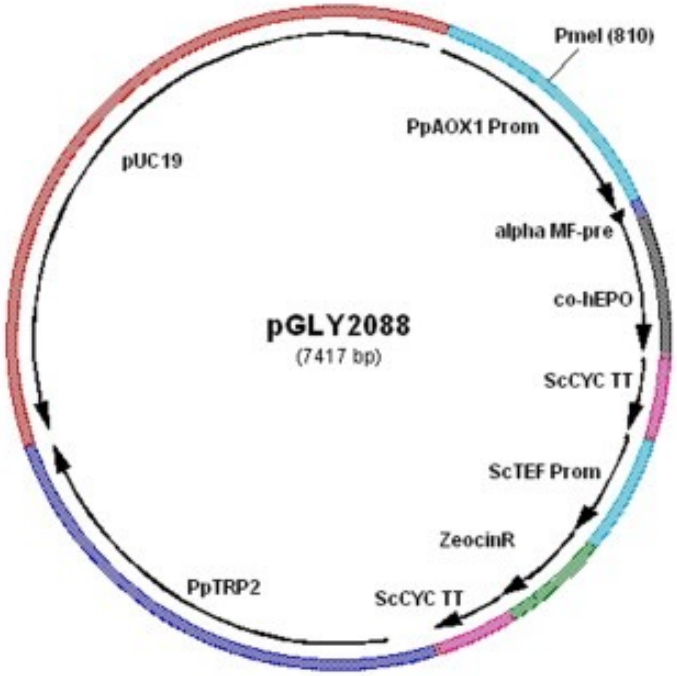


Fig. 1. Schematic structure of the three currently marketed erythropoiesis stimulating agents Epogen®, Aranesp®, Mircera®, and the PEGylated and non-PEGylated forms of rhEPO produced in glycoengineered *P. pastoris*.

Production of human erythropoietin in glycoengineered Pichia pastoris



Expression under control of the AOX1 promoter, α-factor secretion signal, induction for 40 hours, yield 50 mg/L (12-13 copies of the vector integrated in the genome)

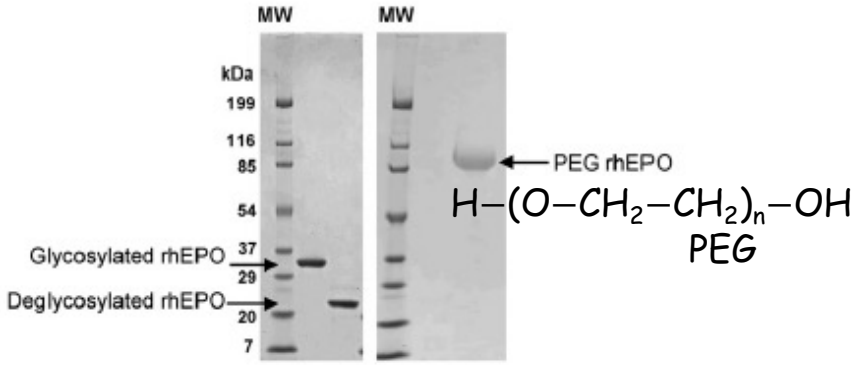
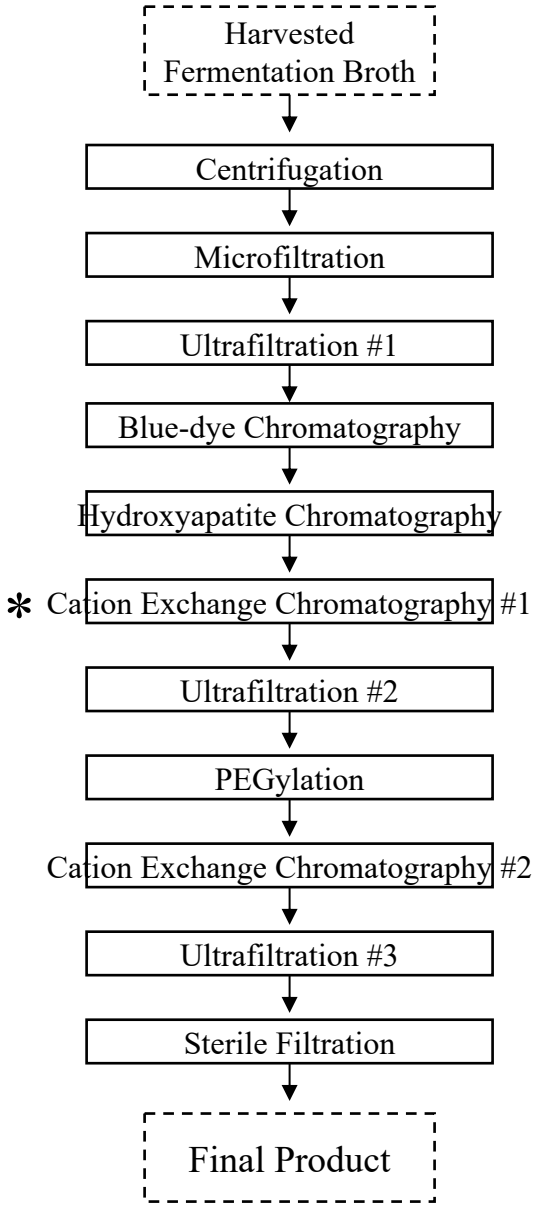


Table 1
Glycosylation differences between rhEPO produced in CHO and glycoengineered *P. pastoris*.

	CHO	Glycoengineered *P. pastoris*	Human
Antennae	2–4	2	2–3 (mainly 2)
Polylactosamine	Yes (heterogeneous)	No	No
Sialic acid linkage	α 2–3[a]	α 2–6[a]	α 2–3 and α 2–6[a]
Core fucose	Yes (heterogeneous)	No	Yes
NGNA (non-human)	Yes	No	No
O-glycosylation	*O*-GalNAc[b] (heterogeneous)	*O*-mannose (below detection limit of HPAEC-PAD)	*O*-GalNac, partially occupied

[a] Human contains both α 2–3 and α 2–6, predominant is α 2–6 linkage.
[b] A substantial fraction of human serum EPO lacks *O*-glycosylation at Ser-126 while the rhEPO produced in CHO is fully occupied (Skibeli et al., 2001).

Production of human erythropoietin in Pichia pastoris



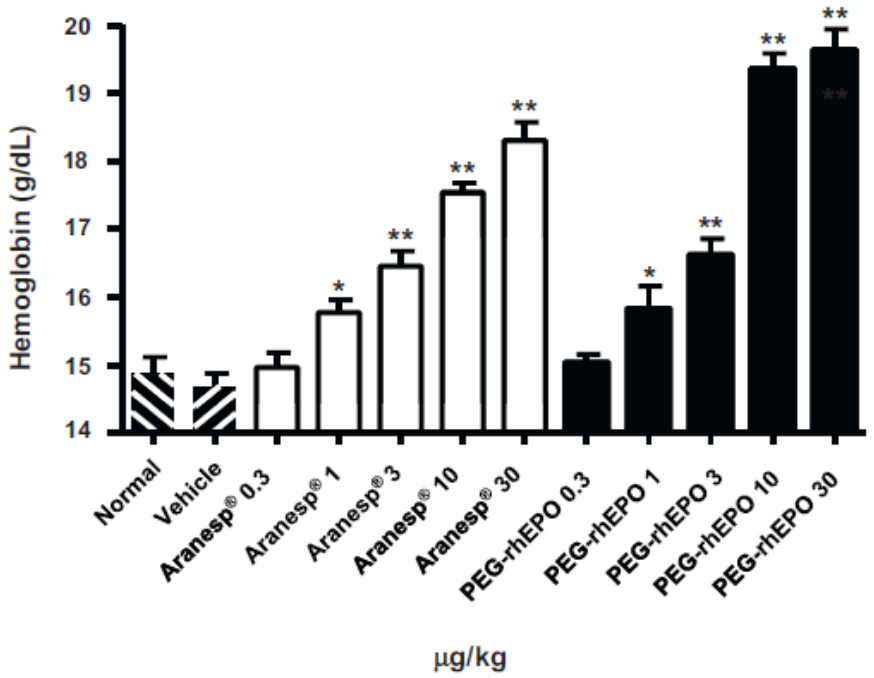


Fig. 6. Pharmacodynamic activity of PEG-rhEPO in mice is similar to Aranesp®. Groups ($n = 10$/group) of CD-1 mice were injected with various amounts of PEG-rhEPO or Aranesp® (between 0.3 and 30 µg/kg, molar equivalent based on protein mass) via IP route and hemoglobin levels were measured after 7 days. The data shown are group means of hemoglobin ± SEM. Statistical analyses were performed in GraphPad Prism. Significant difference from vehicle at $^{*}p < 0.05$, $^{**}p < 0.01$.

*This step was only applied at scales larger than 40 liters

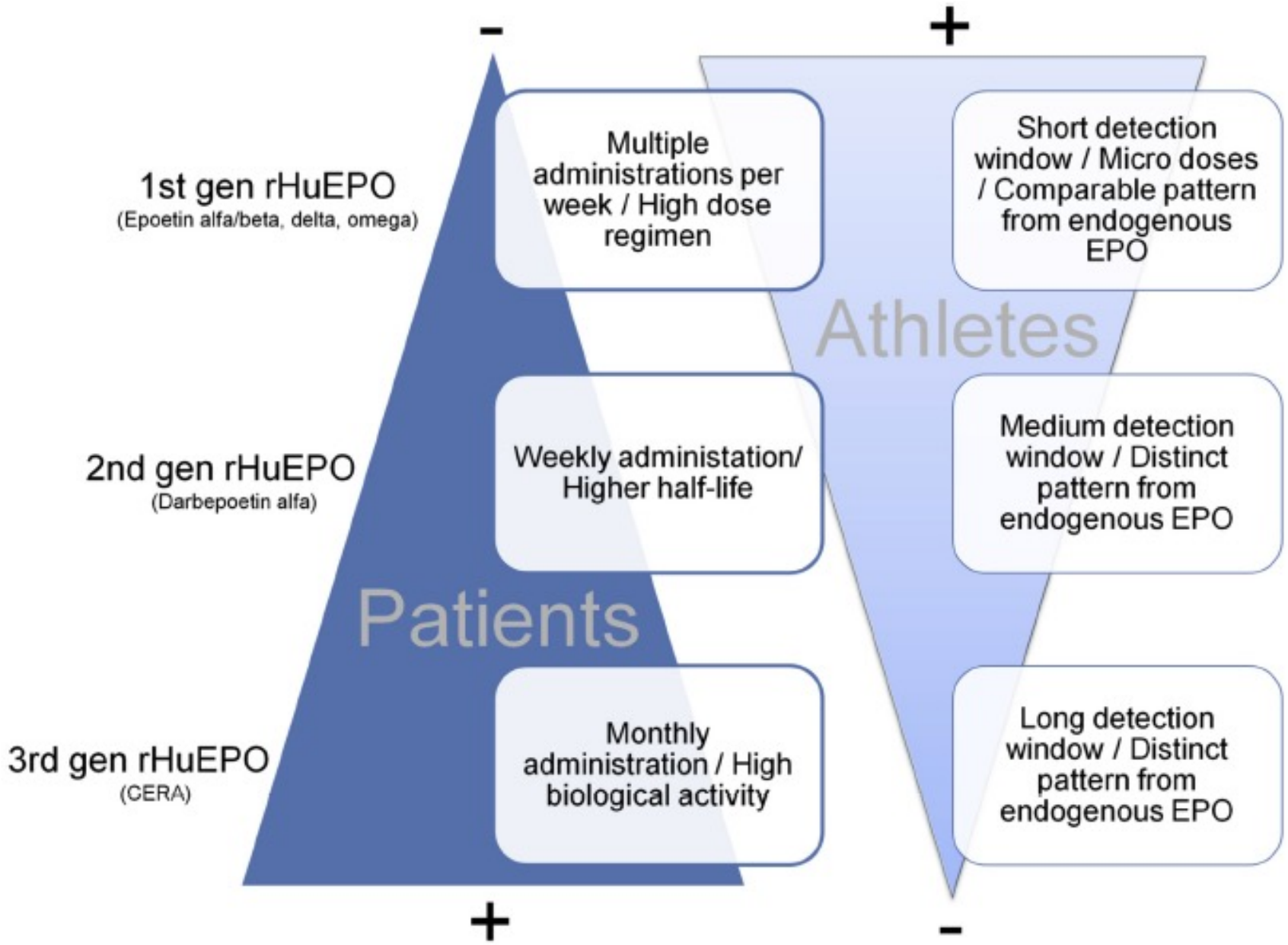


Fig. 1. Advantages and disadvantages of the three different generations of recombinant human EPO for athletes and patients. The benefits are inversely correlated between patients suffering from chronic anemia and cheating athletes for the different generations of rHuEPOs. The first generation is advantageous for athletes, because of its short detection window and its similarity to endogenous EPO. However, high doses are needed to treat anemia, and it requires multiple uncomfortable injections per week. The benefits are reversed with longer half-life rHuEPOs.

Direct analysis of recombinant EPO

Iso-Electro-Focusing at pH 2-6 identifies variants with different charge due to oligosaccharide chains

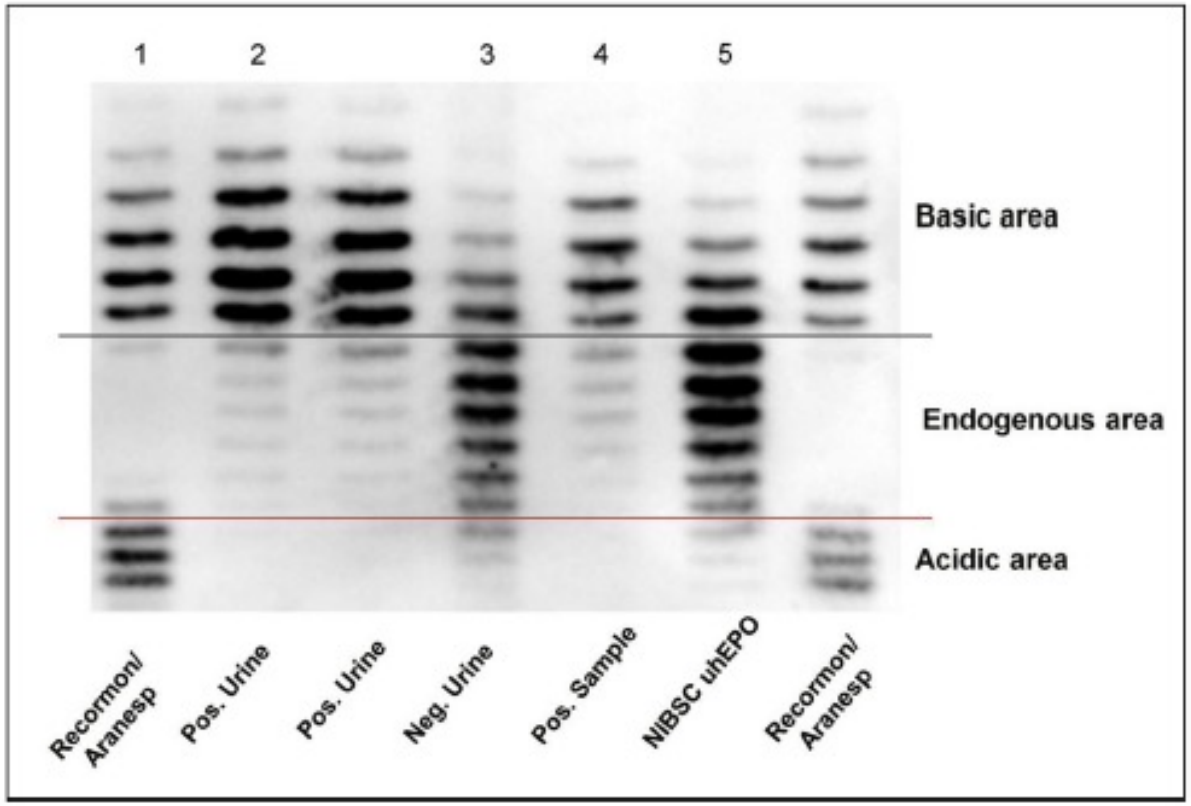
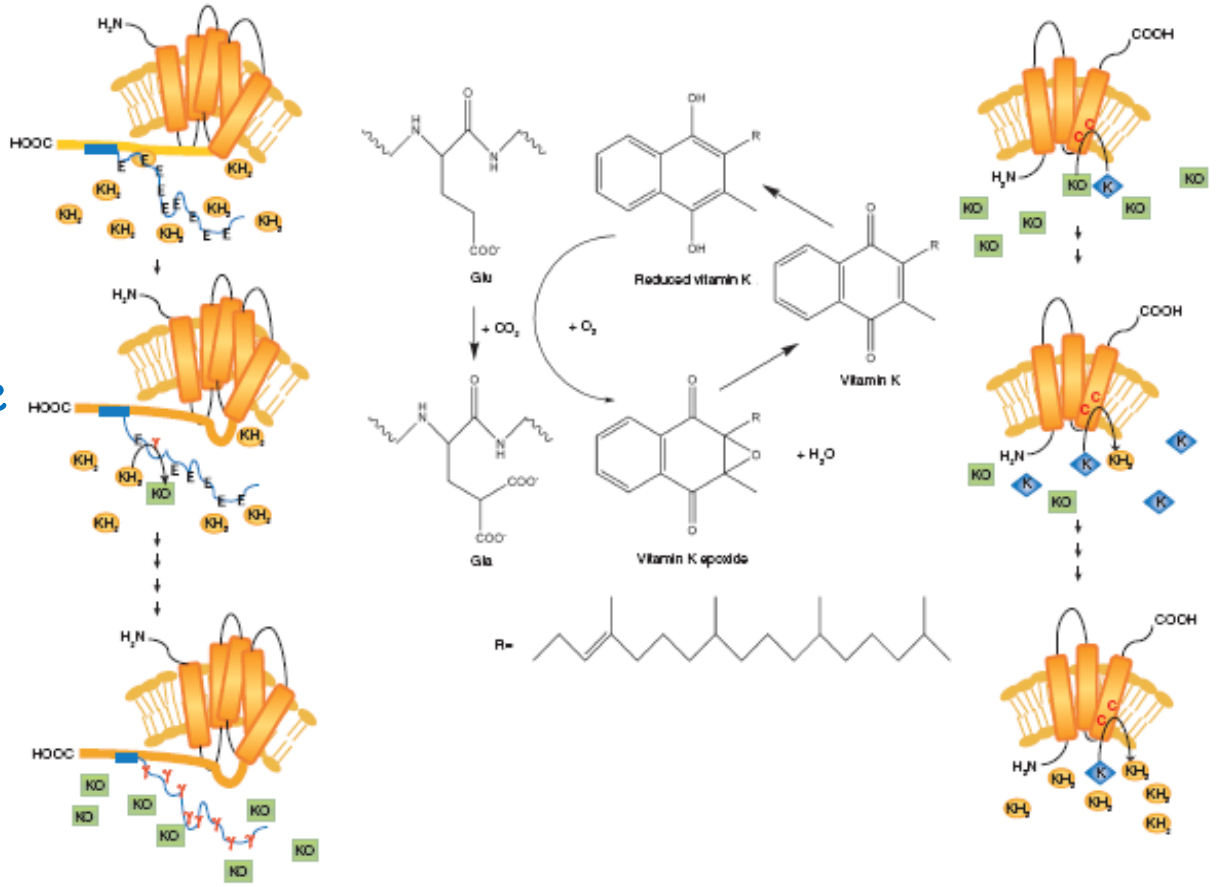


Fig. 3. Example of isoelectric focusing analysis of a sample positive for rhEPO. Lane 1: Reference marker corresponding to a mix of epoetin beta (Recormon) and darbepoetin alfa (Aranesp). Lane 2: human urine spiked with epoetin beta. Lane 3: endogenous urinary EPO. Lane 4: sample positive for epoetin alfa/beta. Lane 5: urinary EPO standard (National Institute for Biological Standard and Control; NIBSC).

An unusual post-translational modification: γ-carboxylation of glutamate

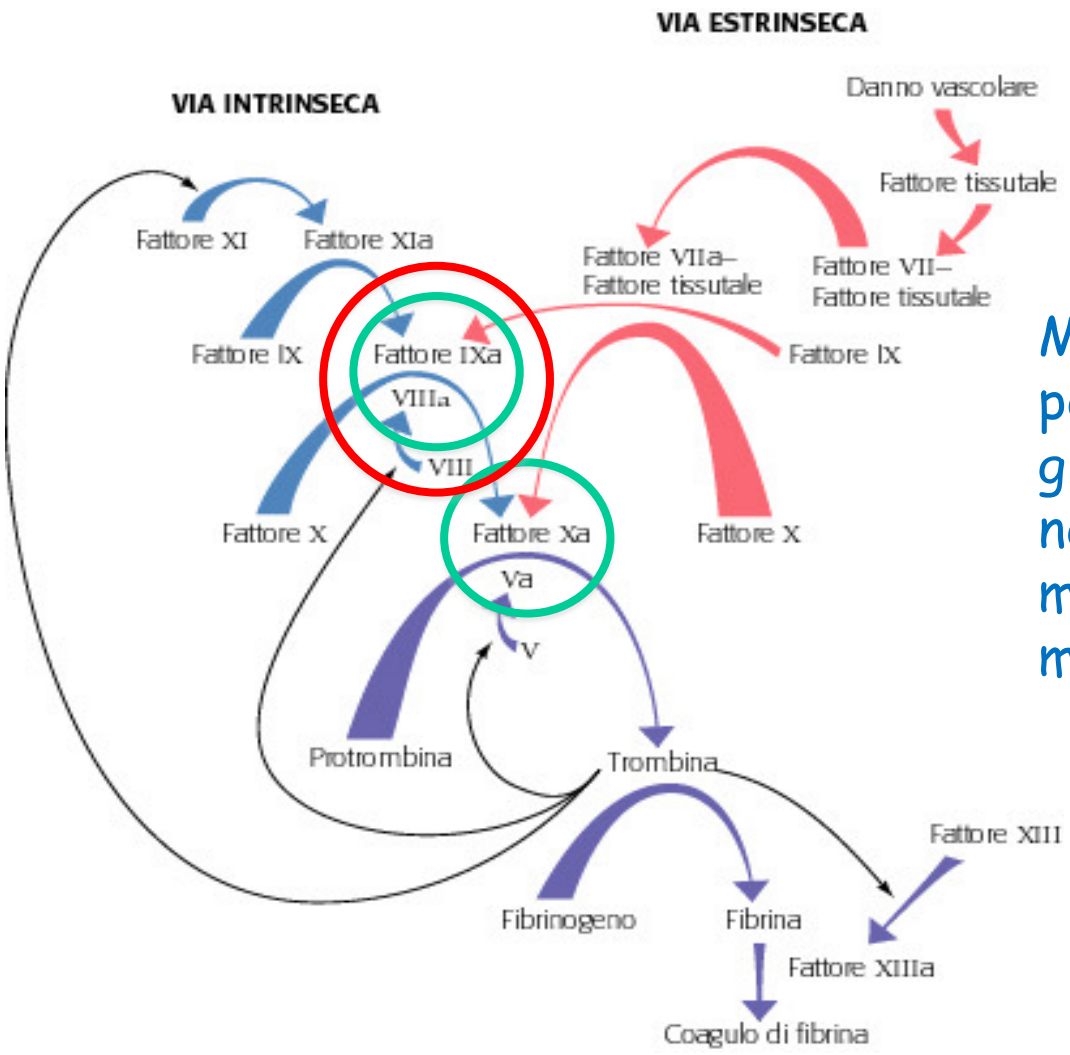
Vitamin K-dependent carboxylase



Vitamin K epoxide reductase

Figure 4 Protein carboxylation. Vitamin K-dependent carboxylase (left) converts glutamate (E) to carboxyglutamate (γ) by the addition of CO_2 to glutamate in a reaction requiring oxygen and reduced vitamin K. Carboxylation substrates (blue line) contain a conserved propeptide region, which binds to the carboxylase, acting as a docking site that localizes the glutamate residues near the carboxylase active site. Multiple carboxylations are accomplished via 'tethered processivity,' in which the propeptide remains bound throughout the reaction. Vitamin K epoxide reductase (right) reduces vitamin K after it has been oxidized in the carboxylation of glutamate. KH_2, reduced vitamin K; KO, vitamin K epoxide; K, vitamin K; Glu, glutamate; Gla, carboxyglutamate. (Source: Darrel Stafford, University of North Carolina at Chapel Hill, NC, USA.)

The blood coagulation cascade



Many coagulation factors possess Gla (γ-carboxy-glutamate) residues necessary for correct Ca^{2+}-mediated interaction with membranes.

Genetic disorders of coagulation: Hemophilia A (factor VIII) and hemophilia B (factor IX)

- Sex-linked genetic diseases characterized by bleeding of spontaneous and traumatic origin.
- The World Federation of Haemophilia estimates 400.000 patients in the world.
- Production of recombinant clotting factors would allow to overcome viral contamination risks that make plasma-derived proteins unsafe.

Table 1
Currently marketed recombinant coagulation factors.

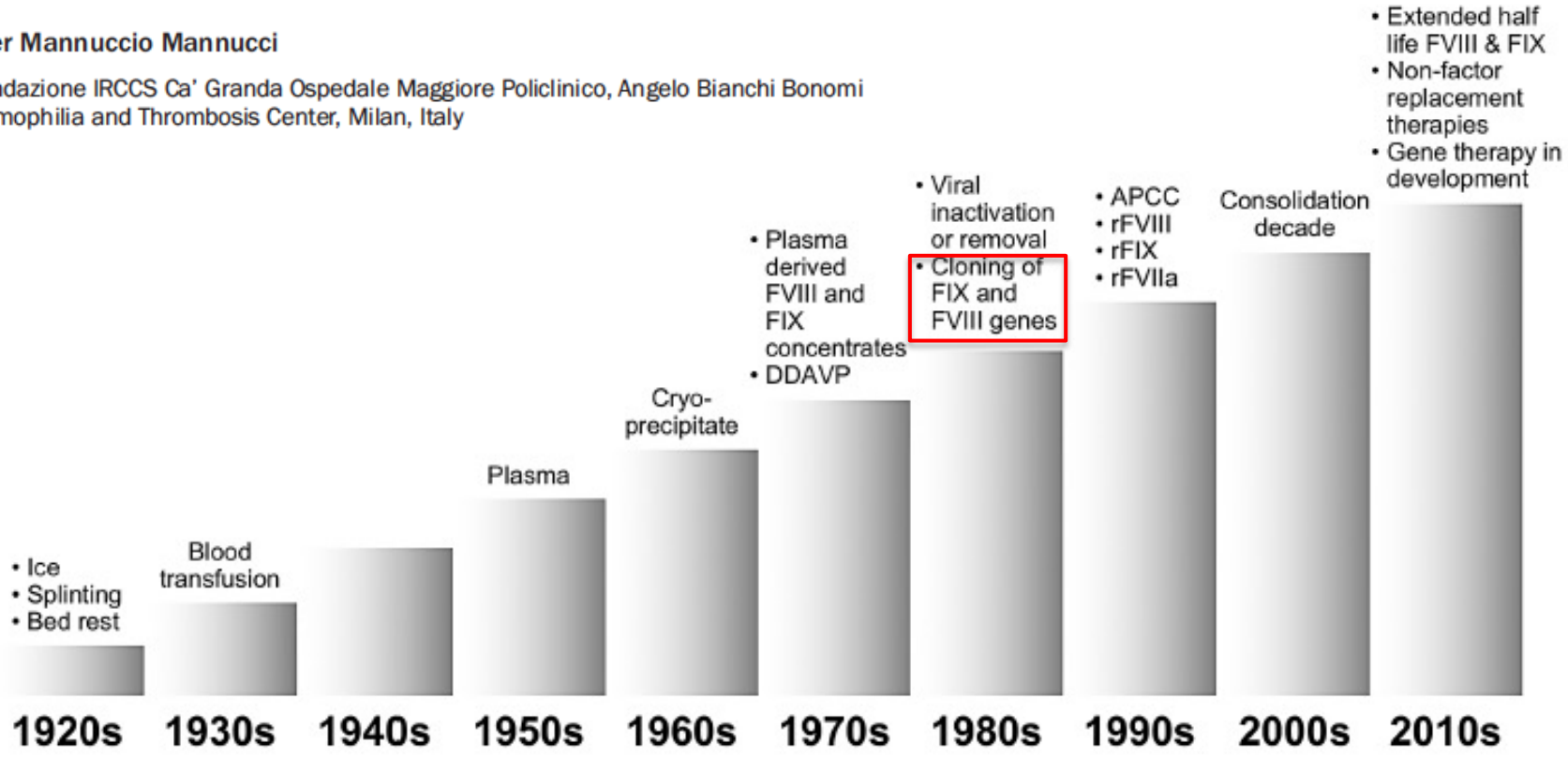
Segment	Manufacturer	Product	Molecule
Hemophilia A	Baxter	Recombinate®	rFVIII
		Advate®	rFVIII
	Bayer	Kogenate®	rFVIII
		Kogenate FS®	
	Wyeth	ReFacto®	BDD-rFVIII*
		Xyntha®	BDD-rFVIII
Hemophilia B	Wyeth	BeneFIX®	rFIX
Hemophilia A or B with inhibitors	Novo Nordisk	NovoSeven®	rFVIIa
		NovoSeven® RT	

*: B-domain deleted rFVIII.

Hemophilia therapy: the future has begun

Pier Mannuccio Mannucci

Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, Milan, Italy



rFVIII = recombinant FVIII; **rFIX** = recombinant FIX; **rFVIIa** = activated recombinant FVII; **APCC**= activated prothrombin complex concentrates.

Figure 1. Progress in hemophilia therapy. Each decade of the last and current century features the main weapons available at the time for the treatment of patients with hemophilia. Each column represents a decade.

Factor VIII

Factor VIII is a 2332 aa protein with a domain structure A1-A2-B-A3-C1-C2. the B domain is initially removed and in plasma factor VIII is associated and stabilized by von Willebrandt factor (vWF). The C2 domain is involved in binding vWF and membranes.

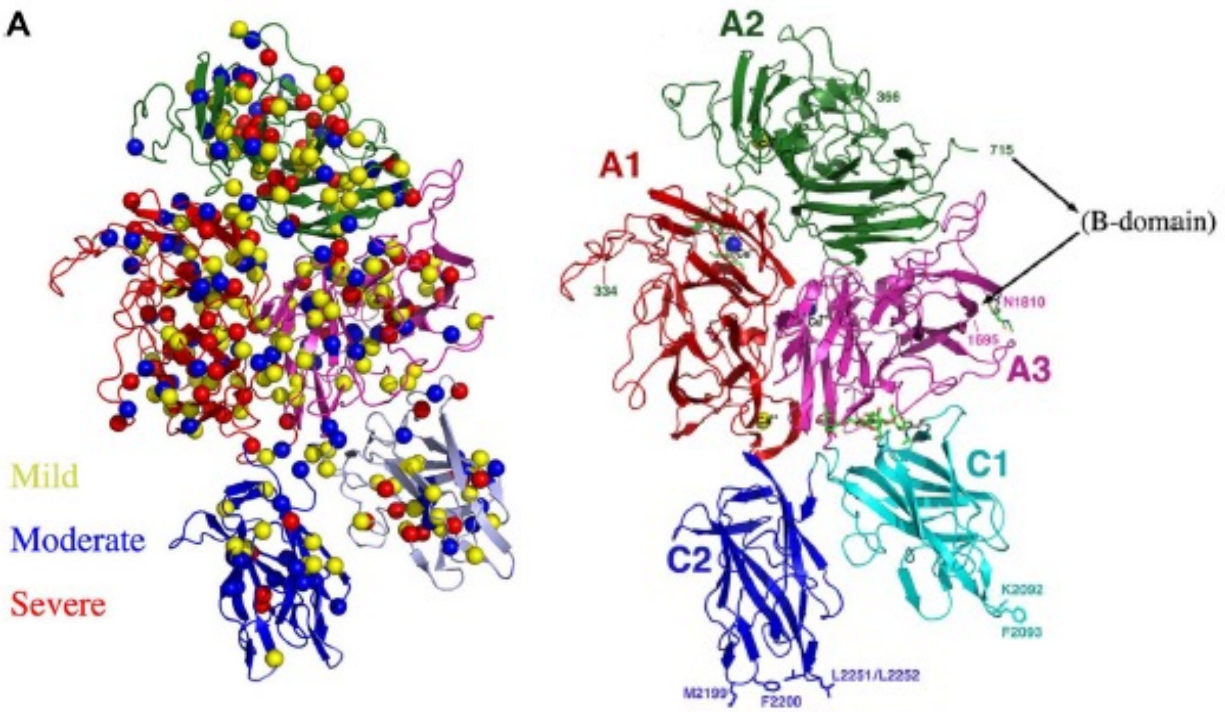


Figure 5. Location of hemophilia A–associated missense mutations across the r-fVIII structure. (A) The disease phenotype/symptom designations of "mild," "moderate," and "severe" are taken from the HamSters database[48] and correspond to plasma fVIIIa activity levels of 6% to 30%, 1% to 5% and less than 1%, respectively. (B) The presence of mutations in the interfaces of the C domains with each other and with the A3 domain.

Factor VIII: structural organization and post-translational modifications

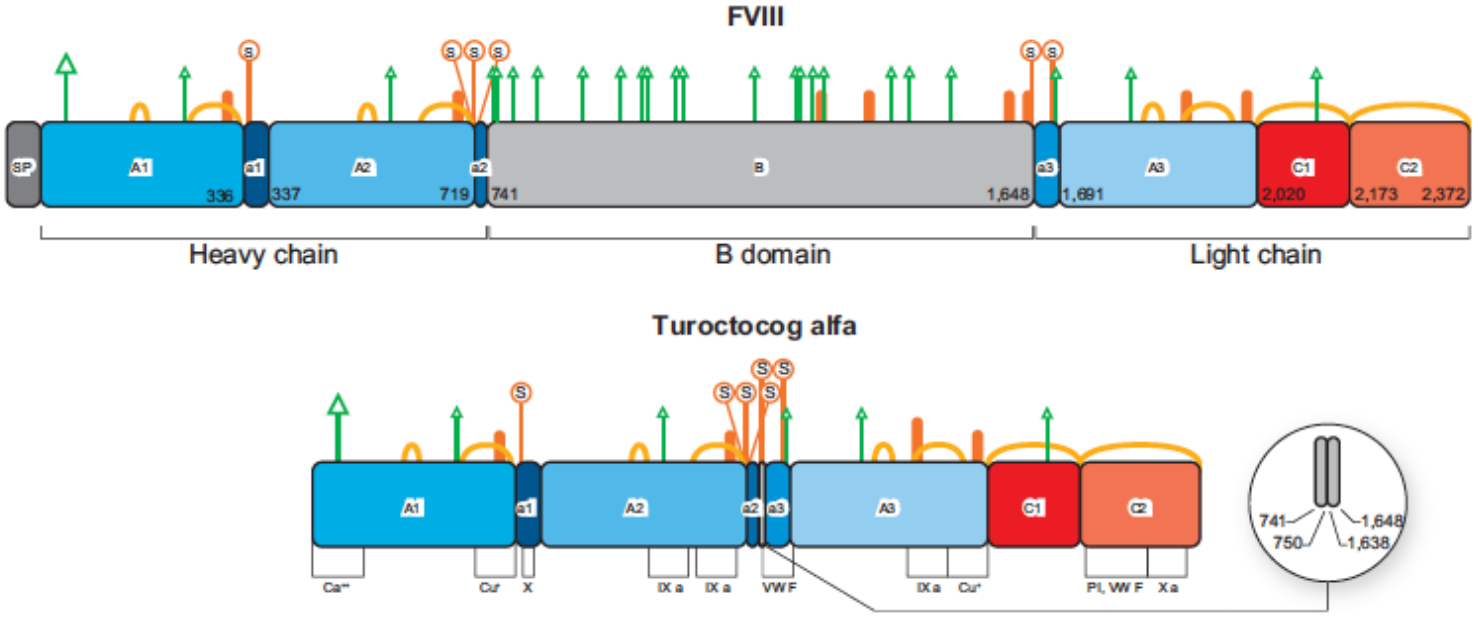


Figure 1 Protein structures and post-translational modifications reported for factor VIII and turoctocog alfa, respectively.
Notes: Both are characterized by the same heavy chain and light chain and differ in the B-domain. Domains are indicated with capital letters and subdomains with lower case letters. Glycosylation sites are denoted by triangles, disulfide bonds are denoted by arches, reduced cysteine residues are denoted by orange vertical lines, and "S" inside a circle indicates sulfated tyrosine residues. Brackets mark the areas of interaction with corresponding clotting factors, phospholipids (PI), von Willebrand factor (VWF), calcium (Ca^{2+}), and copper (Cu^{+}) ions.
Abbreviation: SP, signal peptide.

Table 2 Function specificity of sulfated tyrosine residues in factor VIII

Tyrosine residues	Significance and activity for FVIII
346 and 1664	Increase of affinity for thrombin interaction and thereby the rate of FVIII activation by thrombin
718, 719, and 723	Increase of specific procoagulant activity of FVIIIa in the complex with FIXa and FX bound to the phospholipid membrane
1680	Prerequisite for complex formation with VWF, influencing half-life of FVIII in the circulation

Proteolytic processing of Factor VIII

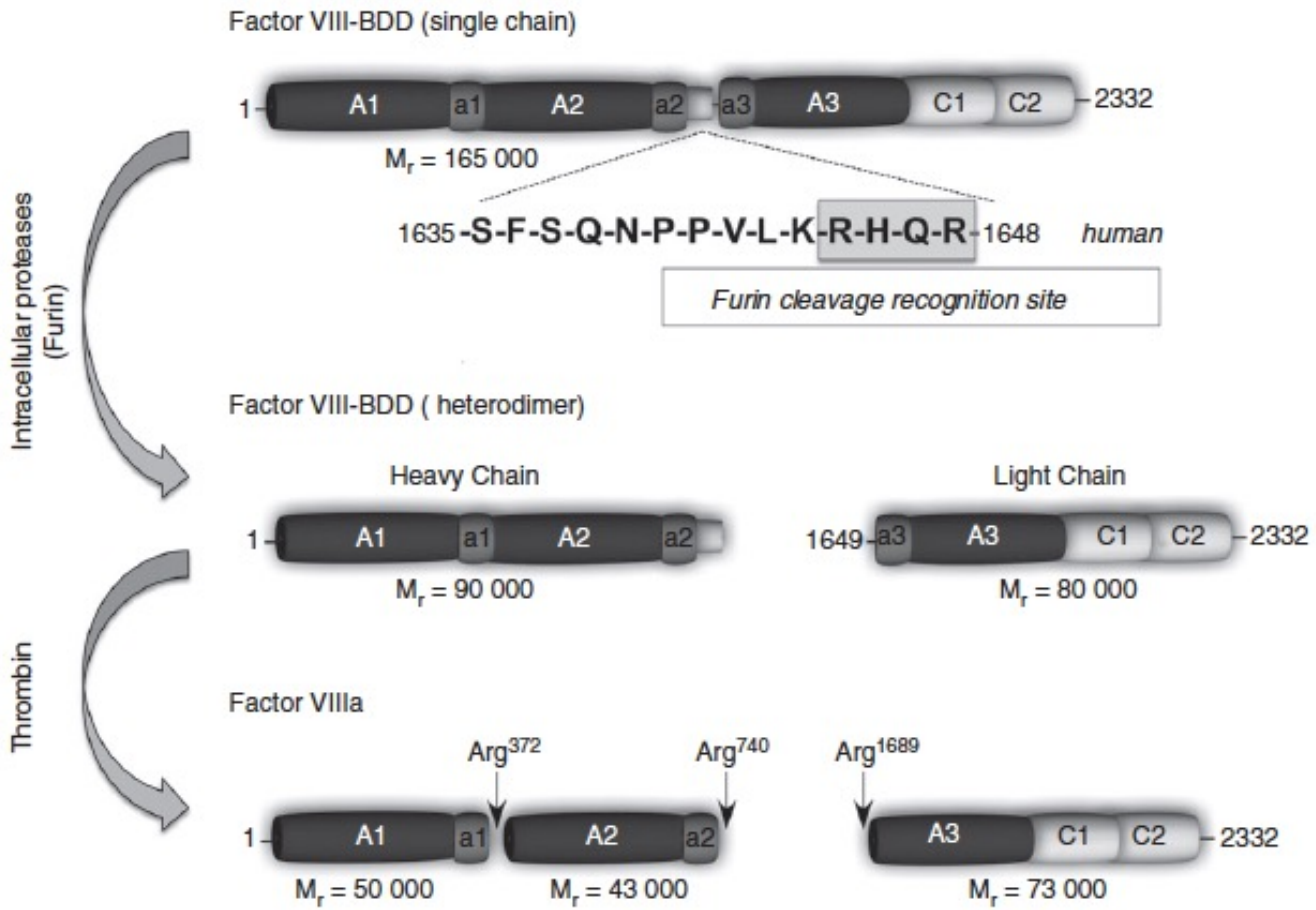


Fig. 1. Factor (F) VIII processing. FVIII has a domain structure that includes the A1-A2-B-A3-C1-C2 domains. The B domain is not required for procoagulant activity. In the B-domain-deleted form of FVIII (FVIII-BDD) there are 14 residual amino acids and within that region is the furin recognition motif (R-X-X-R). Furin cleaves after the arginine residue (R1648) to give rise to two polypeptide chains, the heavy chain (HC) and the light chain (LC). The HC and LC form a heterodimer that is the predominantly secreted form of FVIII in humans. Cleavage by thrombin (IIa) generates a heterotrimer, the activated form of the protein.

Recombinant Factor VIII

- Expressed at low levels in mammalian BHK and CHO cells: inefficient secretion and very high costs (over 100.000 $/year per patient)
- Production of hybrid human/porcine factor VIII (sequences of porcine FVIII in domains A1 and A3 improve expression levels by increasing secretion rate) with a lentiviral system under control of EF-1α promoter in BHK cells
- Expression levels: 9 pg/cell/day about 7.3 mg in 2.5 lt collected from day 3 to day 7 of culture
- Purification by cation exchange chromatography on SulfoPropyl-Sepharose: 4.9 mg of factor VIII with high purity

Recombinant Factor VIII

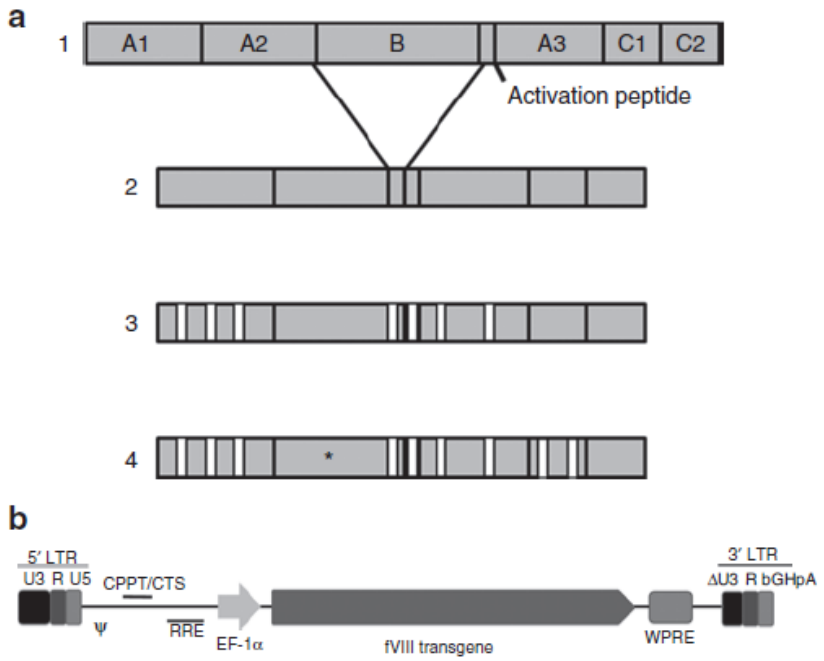


Figure 1 Design of ET-801i and the lentiviral production platform. (**a**) Design of ET-801i. The percent amino acid identities to B-domain-deleted recombinant human fVIII (h-fVIII) are 100, 90, and 88, respectively for constructs 2, 3, and 4. FVIII domain boundaries were defined using the human fVIII amino acid sequence numbering[21] as follows: residues 1–372 (A1), 373–740 (A2), 1,649–1,689 (*ap*), 1,690–2,019 (A3), 2,020–2,172 (C1), and 2,173–2,332 (C2). (**b**) Schematic representation of the ET-801i transgene in the LentiMax expression vector. ET-801i was cloned into the LentiMax expression vector and recombinant lentivirus vector encoding the ET-801i transgene under the control of the elongation factor 1-α (EF-1α) promoter was generated using the LentiMax production system. cPPT, central polypurine tract; CTS, central termination sequence; LTR, long-terminal repeat; RRE, Rev-responsive element; WPRE, woodchuck hepatitis post-transcriptional regulatory element.

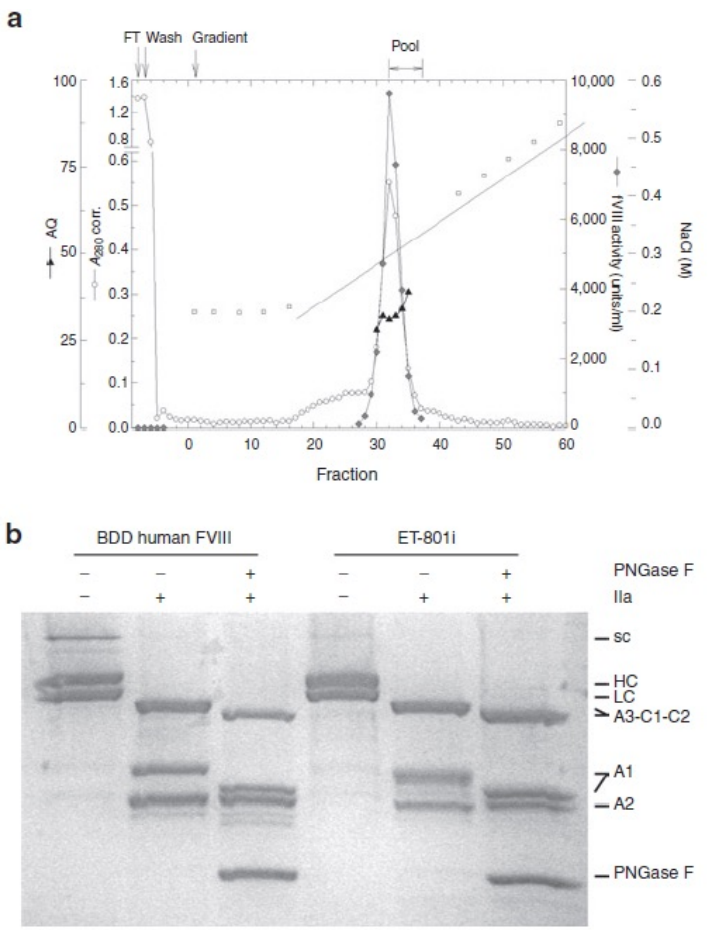


Figure 5 Analysis of purified ET-801i. (**a**) FVIII-containing cell culture supernatant was loaded onto an SP-Sepharose Fast Flow column and ET-801i was eluted using a linear 0.18–0.7 mol/l NaCl gradient. Fractions collected were assayed for A_{280}, fVIII activity, AQ, and conductivity. (**b**) Two µg of human fVIII and ET-801i were subjected to 4–15% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions and visualized by silver staining. Where indicated, samples were treated with 100 nmol/l porcine thrombin ± PNGase F endoglycosidase for 5 minutes before analysis to demonstrate proteolytic activation and the presence of N-linked glycan modifications, respectively. BDD, B-domain-deleted; HC, heavy chain; LC, light chain; SC, single chain.

Recombinant Factor VIII: optimization of expression system and purification

Table 1. Comparison of BAY 81-8973 and rFVIII-FS manufacturing processes.

	BAY 81-8973	rFVIII-FS
Cell line/bank	• rBHK-21-HSP70	• rBHK-21
FVIII	• Full-length, unmodified human FVIII • 2332 amino acids	• Full-length, unmodified human FVIII • 2332 amino acids
Glycosylation	• N- and O-linked glycans • High level of highly branched sialylated glycans • Higher level of sialylated glycans • No α-gal–linked glycans detected	• N- and O-linked glycans • Lower level of highly branched sialylated glycans • High level of sialylated glycans • Low level of α-gal–linked glycans
Cell culture	• Perfusion-based bioreactor • No HPPS in culture media	• Perfusion-based bioreactor • HPPS used in culture media
Purification	• Three chromatography columns • Addition of 2 membrane-based chromatography steps • Removal of gelatin-sepharose chromatography • Addition of 20-nm viral filter	• Six chromatography columns
Drug product	• Same process, presentation, excipients, and fill sizes	• Same process, presentation, excipients, and fill sizes

BHK, baby hamster kidney; FVIII, factor VIII; HPPS, human plasma protein solution; rBHK-21-HSP70, baby hamster kidney-21 clone expressing recombinant FVIII and transfected with human heat-shock protein 70; rFVIII-FS, recombinant factor VIII formulated with sucrose.

Recombinant Factor VIII: characteristics of different production and purification platforms

Table 2. Comparison of principal manufacturing steps for BAY 81-8973 and selected marketed rFVIII products.

	BAY 81-8973 (Kovaltry®) [17]	Kogenate® FS [9,26]	Advate® [27]	Xyntha® [28,29]	Novoeight® [30,31]	Nuwiq® [32,33]	Afstyla® [34–36]	Eloctate® [37,38]	Adynovate® [39]
Host cell	BHK	BHK	CHO	CHO	CHO	HEK	CHO	HEK	CHO
FVIII molecule	Full length	Full length	Full length	B-domain deleted	B-domain truncated	B-domain deleted	B-domain truncated; single chain	B-domain–deleted Fc fusion protein	Full length; PEGylated
Human- or animal-derived additives in cell culture, purification, or formulation processes	None	HPPS in cell culture medium	None	None	None	None	None	None	None
Purification	Rapid membrane adsorber capture Immunoaffinity chromatography using monoclonal antibody Metal chelate affinity Ion exchange chromatography Membrane adsorber Ultrafiltration	Ion exchange chromatography Immunoaffinity chromatography using monoclonal antibody Metal chelate affinity Ion exchange chromatography Ultrafiltration/ diafiltration	Series of chromatography steps Immunoaffinity chromatography using monoclonal antibody	Series of chromatography steps, including affinity chromatography using synthetic peptide affinity ligand	Ion exchange chromatography Immunoaffinity chromatography using monoclonal antibody Gel filtration	Multimodal cation chromatography Ion exchange chromatography Affinity chromatography Size exclusion chromatography	Multistep chromatography process	Affinity chromatography Ion exchange chromatography Ultrafiltration/ diafiltration	Series of chromatography steps, including immunoaffinity chromatography using monoclonal antibody
Nanofiltration	20 nm	None	None	35 nm	20 nm	20 nm	None	15 nm	None
Viral inactivation	Detergent	Solvent/detergent	Solvent/detergent	Solvent/detergent	Detergent	Solvent/detergent	2 dedicated viral inactivation and removal steps	Detergent	Solvent/detergent

BHK, baby hamster kidney; CHO, Chinese hamster ovary; FVIII, factor VIII; HEK, human embryonic kidney; HPPS, human plasma protein solution; rFVIII, recombinant factor VIII.

Expression of Factor IX in CHO cells

Factor IX (415 aa) expressed in CHO at 180 μg/ml (active form only 1.5 μg/ml). Expression levels increase if furin is co-expressed. Furin is a protease that removes a signal peptide required for γ-carboxylation. Approved with the name BeneFIX in 1998.

Table 3 Comparison of PTMs of plasma-derived and recombinant blood factor IX[a]

Structural character	Plasma-derived factor IX	Recombinant factor IX
Primary structure	Ala148/Thr148	Ala148
Secondary/tertiary structure	Indistinguishable by fluorescence, circular dichroism, or analytical ultracentrifugation	
γ-Carboxyglutamic acid (Gla)		
12 of 12 Gla residues	100%	60%
11 of 12 Gla residues	0%	35%
10 of 12 Gla residues	0%	5%
β-hydroxyaspartic acid (64)	37%	46%
Carbohydrate		
N-linked glycans		
Asn157	High heterogeneity	Low heterogeneity,
Asn167	Fully sialylated	Less fully sialylated
O-linked glycans		
Ser53	$(Xyl)_{1-2}$-Glc	$(Xyl)_2$-Glc
Ser61[1]	NeuAcGalGlcNAcFuc	NeuAcGalGlcNAcFuc
Thr159, Thr167, Thr172	Classical, partially filled	Classical, partially filled
Tyr155 sulfation	>90%	<15%
Ser158 phosphorylation	>90%	<1%

[a]Reproduced with permission from ref. 137.

Expression of Factor IX in HEK293 cells

Four-fold increase in expression levels of active FIX by co-expression of VKORC1 (vitamin K epoxide reductase)

TABLE 1 Barium citrate adsorption of recombinant human FIX. Factor IX antigen and activity was assessed in conditioned media prior to barium citrate adsorption ($-Ba^{2+}$) or after barium citrate adsorption ($+Ba^{2+}$) in the precipitate and supernatant. The data are the means of three individual experiments

		FIX –Ba2+		FIX $+Ba^{2+}$				
				Precipitate			Supernatant	
Cell type	Time (hr)	Concentration (ng/ml)	Activity (mU/ml)	Concentration (ng/ml)	Activity (mU/ml)	FIX recovery (%)	Concentration (ng/ml)	Activity (mU/ml)
HEK-hFIX								
	24	57	4	35	3	62	21	2
	48	102	15	58	9	58	41	6
	72	124	34	80	21	64	42	12
HEK-hFIX-VKORC1								
	24	169	20	138	16	82	27	3
	48	248	48	183	35	74	60	12
	72	305	109	233	82	76	64	23

Abbreviations: FIX, factor IX; HEK, human embryonic kidney.

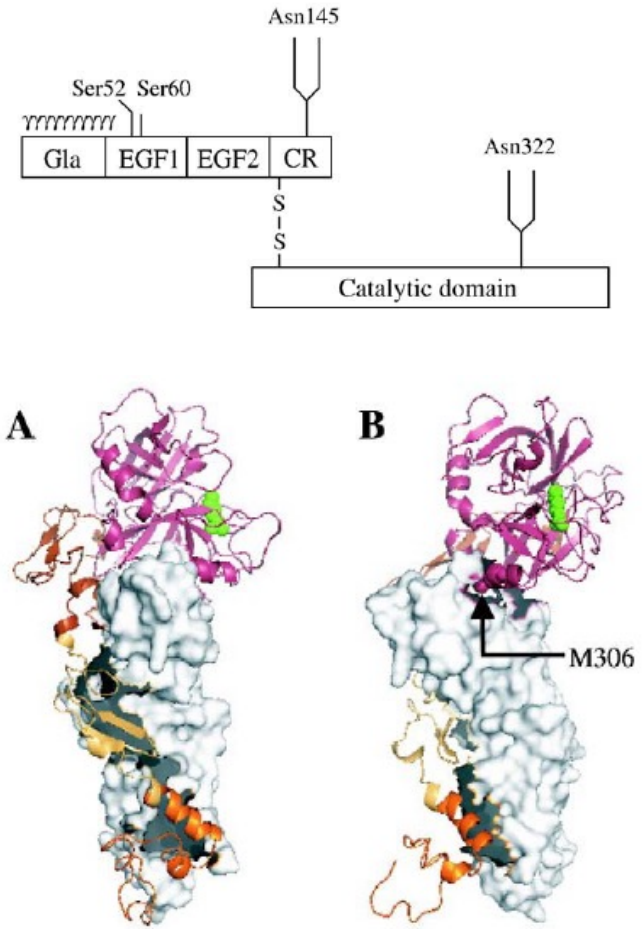


Fig. 2. Three-dimensional structure of the FVIIa-TF complex. The FVIIa molecule is shown in cartoon representation with the Gla domain in orange, the first and second EGF-like domains in yellow and brown, respectively, and the serine protease domain in raspberry. The TF molecule is shown in surface-rendered representation in gray. An arginine sidechain positioned in the S1 pocket of FVIIa is shown in green spheres. Panel A illustrates the extensive interface between the two proteins resulting in high affinity. Panel B is rotated along the vertical axis to show the location of methionine-306 of FVIIa. This residue is depicted as spheres at the bottom of the serine protease domain and buried in a TF surface pocket. An arrow points to the bottom of the surface pocket to assist in locating methionine-306. The figure was generated using the coordinates from PDB entry code 1.dan [10] and the PyMOL software version 1.1.

Recombinant Factor VIIa

Factor VII is expressed in BHK cells as single-chain protein. Proteolytic activation (proteolysis R152-I153) is spontaneous. Purification by ion exchange chromatography, affinity chromatography on a monoclonal antibody against the Gla domain and other two ion exchange chromatography steps.

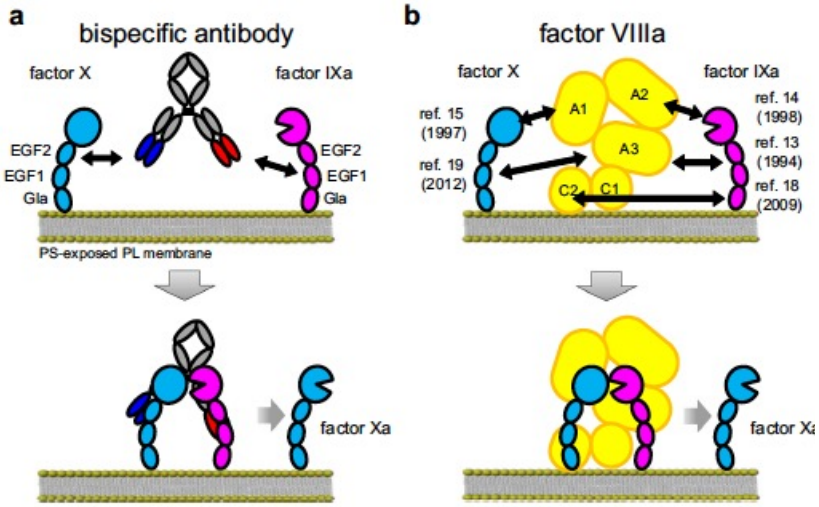
Factor VIIa is indicated in hemophilia A or B patients that develop inhibitory antibodies against factors VIII or IX.

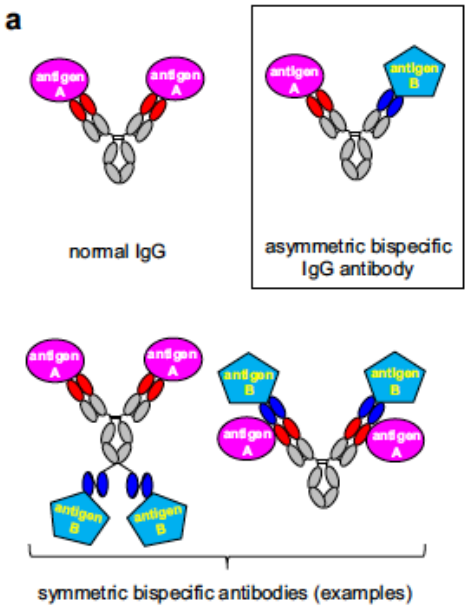
Novel approaches to increase the half-life of FVIII, FIX and FVIIa

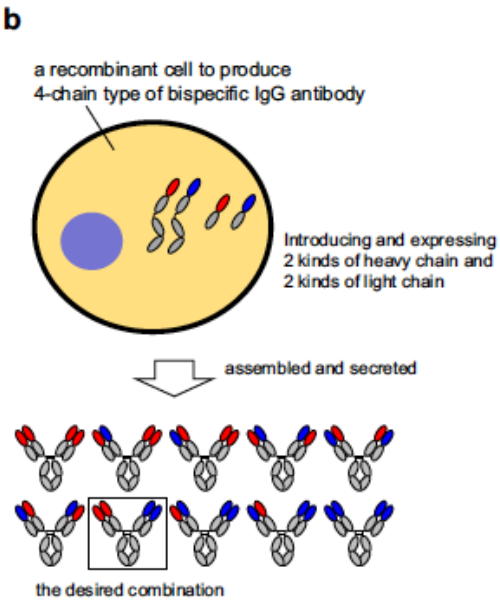
Table 1.

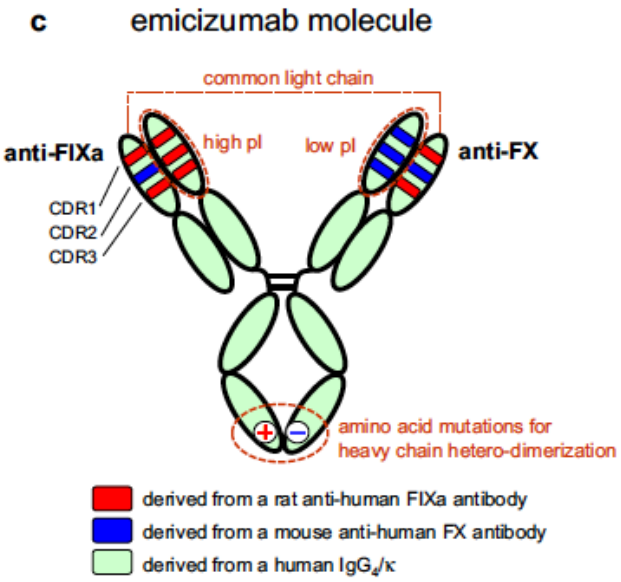
Protein	Name	Modification	Clinical status*
Protein replacement strategies			
FVIII	wt FVIII	Pegylated liposomes	No increase in $T_{1/2}$; phase 3 completed
	N8-GP	Single 40 kDa PEG attached to 21 amino acid B-domain	Well-tolerated; in phase 3
	BAX855	~ 2 mol PEG/full-length FVIII	~ 1.5× increased $T_{1/2}$; in phase 1
	BAY94–9027	60 kDa PEG attached to single site in A3-domain	In phase 3
	rFVIII-Fc	FcIg fusion to rFVIII	~ 1.5-2× increased $T_{1/2}$; phase 3 completed
	scFVIII	Single chain FVIII	~ 1.14× increased $T_{1/2}$
	rhFVIII h-cl	rFVIII manufactured in a human cell line	In clinical trials
FIX	N9-GP	40 kDa PEG attached to activation peptide	5× increased $T_{1/2}$; phase 3 completed
	Fc-FIX	FcIg fusion protein to FIX	~ 3-4× increased $T_{1/2}$; phase 3 finished
	rFIX-FP	rFIX fusion to albumin	~ 5× increased $T_{1/2}$; in phase 3
Bypassing strategies			
FVIIa	N7-GP	40 kDa attached to 1 glycan	Characterization stopped due to lack of effect
	rFVIIa-FP	hFVIIa fusion to albumin	~ 5× increased $T_{1/2}$ in clinical trials
mAb	hBS23 Xase mimetic	Bispecific mAb binds FIXa and FX	Preclinical

Emicizumab: a bispecific antibody for hemophilia treatment









Bispecific antibody for FIXa and FX mimics the role of FVIIIa

Gene Therapy (2013) **20,** 607–615

www.nature.com/gt

ORIGINAL ARTICLE

Generation of an optimized lentiviral vector encoding a high-expression factor VIII transgene for gene therapy of hemophilia A

JM Johnston[1,2], G Denning[3], CB Doering[1] and HT Spencer[1]

We previously compared the expression of several human factor VIII (fVIII) transgene variants and demonstrated the superior expression properties of B domain-deleted porcine fVIII. Subsequently, a hybrid human/porcine fVIII molecule (HP-fVIII) comprising 91% human amino-acid sequence was engineered to maintain the high-expression characteristics of porcine fVIII. The bioengineered construct then was used effectively to treat knockout mice with hemophilia A. In the current study, we focused on optimizing self-inactivating (SIN) lentiviral vector systems by analyzing the efficacy of various lentiviral components in terms of virus production, transduction efficiency and transgene expression. Specifically, three parameters were evaluated: (1) the woodchuck hepatitis post-transcriptional regulatory element (WPRE), (2) HIV versus SIV viral vector systems and (3) various internal promoters. The inclusion of a WPRE sequence had negligible effects on viral production and HP-fVIII expression. HIV and SIV vectors were compared and found to be similar with respect to transduction efficiency in both K562s and HEK-293T cells. However, there was an enhanced expression of HP-fVIII by the SIV system, which was evident in both K562 and BHK-M cell lines. To further compare expression of HP-fVIII from an SIV-based lentiviral system, we constructed expression vectors containing the high expression transgene and a human elongation factor-1 alpha, cytomegalovirus (CMV) or phosphoglycerate kinase promoter. Expression was significantly greater from the CMV promoter, which also yielded therapeutic levels of HP-fVIII in hemophilia A mice. Based on these studies, an optimized vector contains the HP-fVIII transgene driven by a CMV internal promoter within a SIV-based lentiviral backbone lacking a WPRE.