Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmaceutics and Biopharmaceutics 58 (2004) 185–196
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Review article

Second-generation biopharmaceuticals

Gary Walsh*

Department of Chemical and Environmental Sciences, Industrial Biochemistry Program, University of Limerick, Limerick, Ireland

Received 5 February 2004; accepted in revised form 1 March 2004

Available online 7 May 2004

Abstract

The majority of first generation biopharmaceuticals are unengineered murine monoclonal antibodies or simple replacement proteins displaying an identical amino acid sequence to a native human protein. While some such products continue to be approved, an increasing number of modern biopharmaceuticals are engineered, second-generation products. Engineering can entail alteration of amino acid sequence, alteration of the glycocomponent of a glycosylated protein, or the covalent attachment of chemical moieties such as polyethylene glycol. Engineering has been applied in order to alter a protein's immunological or pharmacokinetic profile, or in order to generate novel fusion products. Better understanding of the links between protein structure and function will underpin the development of an increasing number of engineered biopharmaceuticals in the future.

Keywords: Biopharmaceutical; Protein engineering; PEGylation

1. Introduction

Ongoing elucidation of the molecular principles underlining both health and disease continues to reveal many regulatory polypeptides with obvious medical potential. The majority of such polypeptides are produced naturally in minute quantities. This initially rendered impractical their recovery by direct extraction in amounts sufficient to meet clinical demand. The development in the 1970s of the twin techniques of genetic engineering and hybridoma technology [1–3] finally overcame this technical hurdle and therapeutic proteins produced via such methods have been coined biopharmaceuticals [4].

The first biopharmaceutical to gain marketing approval was that of 'humulin' (recombinant human insulin developed and marketed by Genentech and Eli Lilly), initially approved in the United States in 1982 [5]. In the intervening years, the biopharmaceutical industry has grown rapidly. By 2003 some 140 biopharmaceuticals had gained marketing approval in some world region at least [6], commanding an estimated global market of some $30 billion [7]. Approximately 250 million patients had been administered these products and currently in the region of 1 in 4 new molecular entities approved for medical use are biopharmaceuticals. An estimated 500 candidates are in clinical trials, ensuring continued approval of new biopharmaceuticals into the future.

The majority of initially approved biopharmaceuticals may be classified as 'simple replacement proteins', i.e. proteins displaying an identical amino acid sequence to a native human protein and administered in order to replace or augment levels of that protein. Examples include recombinant forms of human insulin, growth hormone and blood factors. A more complete listing of such early first generation biopharmaceuticals is provided in Table 1. This excludes first generation (unmodified) murine monoclonal antibodies (Mabs), which are discussed separately later. It also excludes non-human therapeutic proteins such as subunit vaccines, details of which are included in Ref. [6]. Although recombinant forms of simple replacement proteins continue to be approved in latter years (Table 2), an increasing proportion of modern biopharmaceuticals have been engineered in some way in order to tailor their therapeutic properties. Such second-generation products display either an altered amino acid sequence (achieved via protein engineering or site directed mutagenesis), an altered glycocomponent (in the case of some glycosylated biopharmaceuticals) or a covalently attached chemical moiety such as polyethylene glycol (Table 3).

* Department of Chemical and Environmental Sciences, Industrial Biochemistry Program, University of Limerick, Castletroy, Limerick, Ireland. Tel.: +353-61-202664; fax: +353-61-202568.
E-mail address: gary.walsh@ul.ie

0939-6411/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2004.03.012

Table 1
First generation biopharmaceuticals ('simple replacement proteins') approved for general medical use in one or more world regions in the 1980s and early 1990s

Product	Company	Therapeutic indication	Approved
Bioclate (rh-Factor VIII produced in CHO cells)	Centeon	Haemophilia A	1993
Kogenate (rh-Factor VIII produced in BHK cells. Also sold as Helixate by Centeon)	Bayer	Haemophilia A	1993
Recombinate (rh-Factor VIII produced in an animal cell line)	Baxter Healthcare/ Genetics Institute	Haemophilia A	1992
Activase (Alteplase, rh-tPA produced in CHO cells)	Genentech	Acute myocardial infarction	1987
Humulin (rh-Insulin produced in *E. coli*)	Eli Lilly	Diabetes Mellitus	1982
Novolin (rh-Insulin)	Novo Nordisk	Diabetes Mellitus	1991
Protropin (rh-GH, produced in *E. coli*)	Genentech	hGH deficiency in children	1985
Humatrope (rh-GH produced in *E. coli*)	Eli Lilly	hGH deficiency in children	1987
Nutropin (rh-GH produced in *E. coli*)	Genentech	hGH deficiency in children	1994
BioTropin (rh-GH)	Biotechnology General	hGH deficiency in children	1995
Genotropin (rh-GH produced in *E. coli*)	Pharmacia and Upjohn	hGH deficiency in children	1995
Norditropin (rh-GH)	Novo Nordisk	hGH deficiency in children	1995
Gonal F (rh-FSH produced in CHO cells)	Serono	Anovulation and superovulation	1995
Epogen (rh-EPO produced in a mammalian cell line)	Amgen	Treatment of anaemia	1989
Procrit (rh-EPO produced in a mammalian cell line)	Ortho Biotech	Treatment of anaemia	1990
Neupogen (filgrastim, rG-CSF produced in *E. coli*)	Amgen	Chemotherapy-induced neutropenia	1991
Intron A (rIFN-α-2b produced in *E. coli*)	Schering Plough	Cancer, genital warts, Hepatitis	1986
Roferon A (rh-IFN-α-2a, produced in *E. coli*)	Hoffman-La-Roche	Hairy cell leukaemia	1986
Actimmune (rh-IFN-α-1b produced in *E. coli*)	Genentech	Chronic granulomatous disease	1990

Non-human therapeutic proteins such as subunit vaccines are excluded, although information regarding such products may be found in Ref. [6]. rh, recombinant human; hGH, human growth hormone; EPO, erythropoietin; IFN, interferon; G-CSF, granulocyte colony stimulating factor; tPA, tissue plasminogen activator; FSH, follicle stimulating hormone; CHO, Chinese hamster ovary; BHK, baby hamster kidney.

2. Protein engineering

Protein engineering generally refers to the controlled alteration of the nucleotide sequence of a gene/cDNA coding for a polypeptide, such that specific pre-determined changes in amino acid sequence are introduced [8]. Changes can include the substitution of a specific amino acid residue with an alternative amino acid, the insertion/deletion of one or more stretches of consecutive amino acid residues or sometimes the fusion of two polypeptides together. Protein engineering continues to provide valuable insights into the relationship between a protein's amino acid sequence, its three-dimensional structure and its function [9]. As such, the approach will also contribute to the still distant objective of de novo protein design. At a biotechnological level, protein engineering is used to tailor structural or functional attributes of commercially important proteins. Amongst its earlier applications was the generation of oxidation-resistant detergent enzymes. The bleach constituent of detergents can promote enzyme inactivation by oxidising sensitive surface amino acid residues such as methionine or cysteine. Replacement of these residues with non-oxidizable residues can overcome this problem [10].

Within the biopharmaceutical sector protein engineering has been applied to achieve a number of objectives, generally one of the following: (a) generation of faster/slower acting product; (b) alteration of a protein's biological half-life; (c) alteration of product immunogenicity; (d) generation of novel fused (hybrid) therapeutic proteins.

3. Second-generation insulins

At physiological concentrations insulin molecules exist in monomeric form. However, when stored at commercial therapeutic dose concentrations, individual insulin molecules interact with one another, predominantly forming dimers and hexamers [11]. Upon administration of the commercial product (via SC or IM injection), an insulin depot is formed, and individual insulin molecules must disassociate from each other before entering the bloodstream. This delays entry into the blood and, as a consequence, peak plasma insulin levels are typically witnessed 90–120 min post-injection. This necessitates administration of insulin to diabetics up to 1 h or more before eating, and the patients should not subsequently alter their planned mealtime.

The contact points between individual insulin molecules in oligomers mainly reside towards the C terminus of the molecule's B chain. It was therefore theorized that alteration of one or more amino acid residues in this region might reduce the propensity of individual insulin molecules to self-associate, thereby generating a fast acting insulin analogue which would enter the circulation more rapidly from the site of injection [12]. Initial protein engineering experiments mainly centred upon systematically removing/replacing amino acid residues in this region. None of these early analogues, however, proved therapeutically useful [13,14].

The generation of a usable fast acting insulin analogue eventually stemmed from parallel studies on a related

Table 2
Simple replacement protein-based biopharmaceuticals approved since the mid-1990s in one or more world regions

Product	Company	Therapeutic indication	Approved
Benefix (rh-Factor IX produced in CHO cells)	Genetics Institute	Haemophilia B	1997
Actrapid/Velosulin/Monotard/Insulatard/ Protaphane/Mixtard/Actraphane/Ultratard (all contain rh-Insulin produced in *S. cerevisiae* formulated as short/intermediate/long acting product)	Novo Nordisk	Diabetes Mellitus	2002
Insuman (rh-Insulin produced in *E. coli*)	Hoechst AG	Diabetes Mellitus	1997
Glucagen (rh-Glucagon produced in *S. cerevisiae*)	Novo Nordisk	Hypoglycemia	1998
Thyrogen (Thyrotrophin-α, rh-TSH produced in CHO cells)	Genzyme	Detection/treatment of thyroid cancer	1998
Nutropin AQ (rh-GH produced in *E coli*)	Schwartz Pharma AG	Growth failure, Turner's syndrome	2001
Serostim (rh-GH)	Serono Laboratories	Treatement of AIDS associated catabolism/wasting	1996
Saizen (rh-GH)	Serono Laboratories	hGH deficiency in children	1996
Puregon (rh-FSH produced in CHO cells)	N.V. Organon	Anovulation and superovulation	1996
Follistim (rh-FSH produced in CHO cells)	Organon	Some forms of infertility	1997
Luveris (rh-LH produced in CHO cells)	Ares-Serono	Some forms of infertility	2000
Ovitrelle also termed Ovidrelle (rh-CG produced in CHO cells)	Serono	Used in selected assisted reproductive techniques	2000
Neorecormon (rh-EPO produced in CHO cells)	Boehringer-Mannheim	Treatment of anaemia	1997
Viraferon (rIFN-α-2b produced in *E. coli*)	Schering Plough	Chronic Hepatitis B & C	2000
Rebif (rh IFN-β-1a, produced in CHO cells)	Ares Serono	Relapsing/remitting multiple sclerosis	1998
Alfatronol (rh-IFN-α-2b produced in *E. coli*)	Schering Plough	Hepatitis B, C, and various cancers	2000
Virtron (rh-IFN-α-2b produced in *E. coli*)	Schering Plough	Hepatitis B & C	2000
Avonex (rh-IFN-β-1a, produced in CHO cells)	Biogen	Relapsing multiple sclerosis	1996
Beromun (rh-TNF-α, produced in *E. coli*)	Boehringer-Ingelheim	Adjunct to surgery for subsequent tumour removal, to prevent or delay amputation	1999
Regranex (rh-PDGF produced in *S. cerevisiae*)	Ortho-McNeil Pharmaceuticals	Lower extremity diabetic neuropathic ulcers	1997
Fabrazyme (rh α-galactosidase produced in CHO cells)	Genzyme	Fabry disease (α-galactosidase A deficiency)	2001
Replagal (rh α-galactosidase produced in a continuous human cell line)	TKT Europe	Fabry disease (α-galactosidase A deficiency)	2001
Osteogenic protein 1 (rh-Osteogenic protein-1:BMP-7, produced in CHO cells)	Stryker/Howmedica	Treatment of non-union of tibia	2001
Infuse (rh Bone morphogenic protein-2, produced in CHO cells)	Medtronic Sofamor Danek	Promotes fusion of lower spine vertebrae	2002
Inductos (dibotermin alfa; rBone morphogenic protein-2 produced in CHO cells)	Genetics institute BV	Treatment of acute tibia fractures	2002
Xigris (drotrecogin-α; rh activated protein C produced in a mammalial (human) cell line)	Eli Lilly	Severe sepsis	2001

Non-human therapeutic proteins such as subunit vaccines are excluded, although information regarding such products may be found in Ref. [6]. r, recombinant; rh, recombinant human; CHO, Chinese hamster ovary; BHK, baby hamster kidney; tPA, tissue plasminogen activator; hGH, human growth hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; TSH, thyroid stimulating hormone; EPO, erythropoietin; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN, interferon; IL, interleukin; PDGF, platelet derived growth factor; TNF, tumour necrosis factor.

protein, insulin like growth factor-1 (IGF-1) [15]. IGF-1 displays a structural similarity to insulin and some 50% of the amino acid residues present in the IGF-1 A and B domains are identical to those present at the same position in the insulin A and B chain. IGF-1, however, self-associates to a much lesser extent than does insulin. The proline–lysine sequence found at positions 28 and 29 of the insulin B chain is reversed in IGF-1, prompting investigators to focus upon altering these two specific residues in native insulin [16–19]. Investigations revealed that simply reversing the B^{28} proline–B^{29} lysine sequence characteristic of insulin to B^{28} lysine–B^{29} proline generated an insulin analogue with identical hypoglycaemic potency to regular insulin [20], but which displayed a rapid onset of action in both pig and dog models [20–24]. Subsequent studies on humans confirmed its rapid acting effects and efficacy in controlling blood glucose levels [25–28]. Analytical ultracentrifugation studies illustrates that this insulin analogue displays a significantly reduced tendency to self-associate [14,17] and its dimerization constant is calculated to be 300-fold less than that of regular human insulin [29]. It is believed that movement of the proline residue in particular eliminates the formation of hydrophobic interactions between individual insulin molecules critical for self-association [25]. The product, produced by recombinant means in an engineered *Escherichia coli* K12 strain, is known as 'insulin lispro', and its three-dimentional structure had been elucidated (Fig. 1). It has been approved for general medical use under the trade names 'humalog' and 'liprolog' (Table 3). Insulin lispro may be administered either

Table 3
Second-generation (engineered) biopharmaceuticals now approved in one or more world regions

Product name and company	Indication	Change introduced	Rationale for change
Altered amino acid sequence			
Humalog & Liprolog (Insulin analogue; Eli Lilly)	Diabetes	Inversion of the natural proline–lysine sequence on the B chain at positions 28 and 29 of native human insulin	Generation of a faster acting insulin
NovoRapid/Novolog (Insulin analogue, Novo nordisk)	Diabetes	Proline residue found at position 28 of the human insulin B chain has been replaced by aspartic acid	Generation of a rapid acting insulin
Lantus/Optisulin (Insulin analogue; Aventis)	Diabetes	Asparagine (A 21) is replaced by a glycine residue and two arginine residues have been added at the end of the B chain C-terminus	Generation of a long acting insulin
Retavase (tPA analogue; Boehringer-Manheim/ Centocor), also Ecokinase (Galenus Mannheim) and Rapilysin (Boheringer-Manheim)	Thrombolytic agent	Removal of three of the four domains characteristic of native tPA (the amino terminal finger domain, the EGF domain and the Kringle 1 domain)	Generation of thrombolytic agent with longer circulatory half-life
TNKase (tPA analogue; Genentech/Schering plough)	Thrombolytic agent	Amino acid substitutions introduced in native tPA's P and K_1 domains	Generation of thrombolytic agent with longer circulatory half-life
Simulect (Engineered Mab; Novartis)	Prevention of acute kidney transplant rejection	Chimaeric monoclonal antibody	Reduction of antibody immunogenicity
Remicade (Engineered Mab; Centocor)	Treatment of Crohn's disease	Chimaeric monoclonal antibody	Reduction of antibody immunogenicity
Mabthera (Engineered Mab; Hoffmann-La Roche)	Treatment of non-Hodgkin's lymphoma	Chimaeric monoclonal antibody	Reduction of antibody immunogenicity
Rituxan (Engineered Mab; Genentech and IDEC)	Treatment of non-Hodgkin's lymphoma	Chimaeric monoclonal antibody	Reduction of antibody immunogenicity
ReoPro (Engineered Mab; Centocor)	Prevention of blood clots	Chimaeric monoclonal antibody	Reduction of antibody immunogenicity
Zenapax (Engineered Mab; Roche)	Prevention of acute kidney transplant rejection	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Synagis (Engineered Mab; Abbott)	Prophylaxis of lower respiratory disease caused by respiratory syncytial virus	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Herceptin (Engineered Mab; Roche)	Treatment of some forms of breast cancer	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Xolair (Engineered Mab; Genentech and Novartis)	Treatment of moderate to severe persistent asthma	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Mabcampath/Campath (Engineered Mab; ILEX, Millennium and Berlex)	Chronic lymphocytic leukaemia	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Mylotarg (Engineered Mab; Wyeth)	Acute myeloid leukaemia	Humanized monoclonal antibody	Reduction/elimination of antibody immunogenicity
Infergen (IFN analogue; Amgen)	Treatment of chronic hepatitis C	Synthetic type 1 interferon, containing the most frequently observed amino acids in each corresponding position of several naturally occurring human IFN α subtypes	On a mass basis, displays higher anti-viral, anti-proliferative and NK cell activating activity as compared to several IFN α subtypes
ReFacto (Blood factor VIII analogue; Genetics Institute)	Haemophilia A	Differs from human factor VIII in that its B domain has been deleted	Production of a lower molecular mass blood factor which retains biological activity
Ontak (Fusion product; Seragen/Ligand)	Treatment of cutaneous T cell lymphoma	Fusion protein; diphtheria toxin linked to interleukin 2	Targets toxin to cells expressing an interleukin 2 receptor
Enbrel (Fusion product; Immunex)	Rheumatoid arthritis	Fusion protein consisting of the extracellular ligand-binding portion of the human TNF receptor linked to the F_c portion of human IgG	Inhibits activity of TNF by binding it
Amevive (Fusion product; Biogen)	Moderate to severe chronic plaque psoriasis	Dimeric fusion protein consisting of the extracellular CD2 binding portion of the human leukocyte function antigen 3 (LFA-3), linked to the F_c region of a human IgG	Targets lymphocytes predominantly involved in psoriatic lesions, while F_c immunoglobulin portion facilitated cellular destruction by attracting natural killer and other cytotoxic cells

Table 3 (*continued*)

Product name and company	Indication	Change introduced	Rationale for change
Altered carbohydrate component			
Cerezyme (Glucocerebrosidase enzyme; Genzyme)	Gaucher's disease	Sialic acid caps enzymatically removed from sugar side chains	Removal of sialic acid residues expose mannose residues underneath. This promotes macrophage-selective product uptake
Nespo/Aranesp (EPO; Amgen)	Anemia	Contains two additional sugar side chains when compared to native human EPO	Additional sugar side chains increases product's plasma half-life, facilitating reduced frequency of administration
Covalently attached polyethylene glycol			
Pegasys (IFN; Hoffman La Roche)	Hepatitis C	PEGylated	PEGylation increases product's plasma half-life, facilitating reduced frequency of administration
Viraferon Peg/PegIntron (IFN; Schering plough)	Hepatitis C	PEGylated	PEGylation increases product's plasma half-life, facilitating reduced frequency of administration
Somavert (hGH analogue; Pharmacia)	Acromegaly	PEGylated	PEGylation increases product's plasma half-life, facilitating reduced frequency of administration
Neulasta (G-CSF; Amgen)	Neutropenia	PEGylated	PEGylation increases product's plasma half-life, facilitating reduced frequency of administration

tPA, tissue plasminogen activator; IFN, interferon; EPO, erythropoietin; hGH, human growth hormone G; CSF, granulocyte colony stimulating factor; Mab, monoclonal antibody; TNF, tumour necrosis factor.

Fig. 1. Still image of the three-dimensional structure of Lys (B28) Pro (B29) human insulin (in the presence of phenol and a chloride ion). The structure was determined by X-ray diffraction at a resolution of 2.30 Å. Photo courtesy of the protein data bank (http://www.rcsb.org.pdb/).

15 min before a mealtime or immediately after a meal. This provides a much greater flexibility for the user in terms of forward mealtime planning.

'Novorapid' and 'Novolog' [30,31] are trade names given to yet another engineered fast acting insulin now approved for general medical use (Table 3). This product, also known as 'insulin aspart' differs from native human insulin by a single amino acid substitution; the proline residue at position 28 of the B chain has been replaced by an aspartic acid residue. As in the case of 'insulin lispro', the replacement of the proline likely eliminates the formation of inter-molecular hydrophobic interactions which would otherwise drive self-association. This analogue, produced in *Saccharomyces cerevisiae*, displays similar hypoglycaemic potency to that of native insulin, but may be administered immediately before consuming a meal [31].

Diabetes therapy often entails the administration of a slow or long acting insulin preparation, in an attempt to mimic normal physiological insulin baseline levels characteristic of healthy individuals. Slow acting insulin products are traditionally generated by formulation with zinc and/or protamines. Protein engineering has also been utilized to generate a long acting insulin analogue. 'Insulin glargine' (trade names 'Optisulin' and 'Lantus'; Table 3) differs from native insulin in that (a) the C terminal of the B chain has been elongated by two arginine residues and (b) the C terminal residue of the A chain (aspargine) has been replaced by a glycine. Collectively, these modifications increase the isoelectric point (pI; the pH at which the molecule displays a net zero charge) of insulin from 5.4 to more neutral values. Proteins generally are least soluble at their pI and some precipitate out of solution at this pH [32,33].

Insulin glargine is expressed in engineered *E. coli* cells. The finished product is formulated at pH 4.0, a pH at which

it is fully soluble. Upon SC administration, the product experiences an increase of pH towards 7.0, promoting the apparent formation of insulin microprecipitates at the injection site. Individual insulin molecules appear to enter circulation only upon their re-solubilization, a slow process. This supports product administration by just a once daily regime.

4. Second-generation thrombolytics

Tissue plasminogen activator (tPA) was amongst the first biopharmaceutical products to be subjected to protein engineering. Native human tPA is a 527 amino acid serine protease produced by vascular endothelial cells [34,35]. It triggers activation of the fibrinolytic (clot degrading) system by proteolytically activating plasminogen, forming plasmin. Plasmin in turn proteolytically degrades the fibrin strands of the blood clot (Fig. 2). Unmodified tPA displays five structural domains, each of which has a specific function (Table 4). The native molecule is also gylcosylated and the sugar component plays a role in facilitating hepatic uptake of the tPA and hence its clearance from plasma. tPA has proven effective in the emergency treatment of myocardial infarction. The first recombinant form of the molecule to be approved was Activase (also known as 'alteplase', Genentech, 1987) [34]. The enzyme is produced by recombinant means in a Chinese hamster ovary (CHO) cell line.

Retevase (rapilysin) is an engineered form of tPA first approved in the EU in 1996 (Table 3). The molecule consists of only two native tPA domains, the catalytic (P) domain and the K_2 domain. It is produced in *E. coli* and hence is also unglycosylated. Lack of glycosylation as well as the absence of the EGF and the K_1 domains (Table 4) confers upon retevase a significantly extended half-life, allowing its administration by single iv injection. Native tPA displays a half-life of only 3 min, and hence has to be infused into patients, usually over a period of up to 90 min. Absence of the F1 domain also reduces the molecule's fibrin binding affinity, allowing it to diffuse more extensively into the interior of the clot. This potentially facilitates more rapid clot lysis [36–38].

TNKase (Tenecteplase) is yet another engineered tPA displaying an extended half-life (Table 3). Manufactured in a CHO cell line by Genentech, TNKase differs from native tPA by displaying amino acid substitutions at three positions. The effects of these substitutions are (a) to increase specificity for fibrin binding, which has a knock on effect of decreasing plasma clearance and (b) enhancing resistance to PAI-1 (plasminogen activator inhibitor-1, a natural tPA inhibitor) [39].

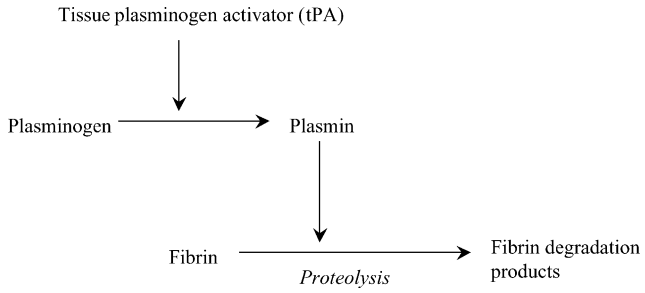

Fig. 2. The fibrinolytic system. Refer to text for details.

Table 4
The domain structure of human tissue plasminogen activator (tPA)

Domain name	Domain function
Finger domain (F)	Promotes high affinity binding to fibrin
Protease domain (P)	Displays plasminogen-specific proteolytic activity
Epidermal growth factor domain (EGF)	Mediates hepatic clearance from blood by binding to specific hepatic receptors
Kringle-1 domain (K_1)	Associated with hepatic binding
Kringle-2 domain (K_2)	Facilitates fibrin-based stimulation of proteolytic activity

Reproduced with permission from Ref. [74].

5. Second-generation antibodies

The development of hybridoma technology in the 1970s [3] facilitated the large scale production of monospecific antibody preparations generated against virtually any antigen of choice. Initially many Mabs were used primarily for in vitro diagnostic purposes, but they also find use for in vivo diagnostic and therapeutic purposes [40,41].

Earlier Mab based therapeutic products were invariably murine (mouse derived) antibodies, produced by classical hybridoma technology (Table 5). Some were used intact while other products consisted of antigen-binding fragments (F_{ab} or F_{ab2} fragments), generated by proteolytic cleavage of the intact antibody at the hinge region, with subsequent chromatographic removal of the F_c fragment, (Fig. 3).

Therapeutically these first generation murine Mabs suffered from a number of drawbacks [42–44]. They were themselves highly immunogenic when administered to man, due to their murine origin. A single injection of a murine Mab elicits an immune response in 50–80% of human patients. Human anti-mouse antibodies (HAMAs) are usually detected within 2 weeks of Mab administration and repeat administration will increase the HAMA response significantly. In consequence, the therapeutic efficacy of such products is often limited to the first and, at most, the second dose administered.

First generation monoclonals also displayed a relatively short half-life (typically 30–40 h), when administered to humans. Moreover, they proved to be poor triggers of human effector functions, such as activation of complement.

An obvious potential strategy for overcoming such limitations associated with murine monoclonals would be the generation and use of human Mabs. However, a number

Table 5
First generation monoclonal antibody-based biopharmaceuticals approved in one or more world regions

Product	Company	Indication	Approved
Orthoclone OKT3 (Muromomab CD3, murine Mab directed against the T-lymphocyte surface antigen CD3)	Ortho Biotech	Reversal of acute kidney transplant rejection	1986
OncoScint CR/OV (Satumomab Pendetide, murine Mab directed against TAG-72, a high molecular weight tumour associated glycoprotein)	Cytogen	Detection/staging/follow up of colorectal and ovarian cancers	1992
MyoScint (Imiciromab-Pentetate, murine Mab fragment directed against human cardiac myosin)	Centocor	Myocardial infarction imaging agent	1996
ProstaScint (Capromab Pentetate, murine Mab directed against the tumour surface antigen PSMA)	Cytogen	Detection/staging/follow-up of prostate adenocarcinoma	1996
Verluma (Nofetumomab murine Mab fragments (F_{ab}) directed against carcinoma associated antigen)	Boehringer-Ingelheim/NeoRx	Detection of small cell lung cancer	1996
CEA-scan (Arcitumomab, murine Mab fragment (F_{ab}), directed against human carcinoembryonic antigen, CEA)	Immunomedics	Detection of recurrent/metastatic colorectal cancer	1996
Indimacis 125 (Igovomab, Murine Mab fragment (F_{ab2}) directed against the tumour associated antigen CA 125)	CIS Bio	Diagnosis of ovarian adenocarcimona	1996
Tecnemab KI (murine Mab fragments (F_{ab}/F_{ab2} mix) directed against HMW-MAA, i.e. high molecular weight meloma associated antigen)	Sorin	Diagnosis of cutaneous melanoma lesions	1996
LeukoScan (Sulesomab, murine Mab fragment (F_{ab}) directed against NCA 90, a surface granulocyte non-specific cross reacting antigen)	Immunomedics	Diagnostic imaging for infection/inflammation in bone of patients with osteomyelitis	1997
Zevalin (Ibritumomab, murine monoclonal antibody directed against CD 20 antigen)	IDEC pahrmaceuticals	Treatment of non-Hodgkin lymphoma	2002
Bexxar (tositumomab, murine monoclonal antibody directed against CD 20 antigen)	Corixa/GlaxoSmithKline	Treatment of non-Hodgkin lymphoma	2003

of technical obstacles continue to render such an approach difficult to achieve in practice. Difficulties include the generation and sourcing of human lymphocytes producing antibody of appropriate specificity, reliable methodologies for human lymphocyte immortalization, as well as the poor stability and disappointing antibody production levels often associated with transformed human lymphocytes [42].

Genetic engineering provides a way of largely overcoming such problems via protein engineering and the majority of antibody-based products approved in latter years are engineered, second-generation products (Table 3). These may be classified as either ‘chimaeric’ or (more extensively engineered) ‘humanized’ antibodies.

Chimaeric antibodies are hybrid antibodies generated by splicing the gene sequence coding for the mouse-derived variable region (containing the antigen binding sites—CDR’s—which recognize the antigen of interest) to a nucleotide sequence coding for the constant regions of a human antibody (Fig. 4). The resultant antibody is, predictably, less immunogenic when administered to humans. Furthermore, it is capable of activating human immune system effector functions (which are associated with the antibody F_c region) and it displays an extended circulatory half-life (circa. 250 h as compared to circa. 40 h in the case of intact murine antibodies).

An alternative engineering strategy entails the production of humanized monoclonals, and this approach has now become the favoured approach to therapeutic antibody engineering [45–49]. Humanization involves production of a murine monoclonal against the antigen of interest, isolating the nucleotide sequence coding for the antigen binding regions (CDR regions) and using these sequences to

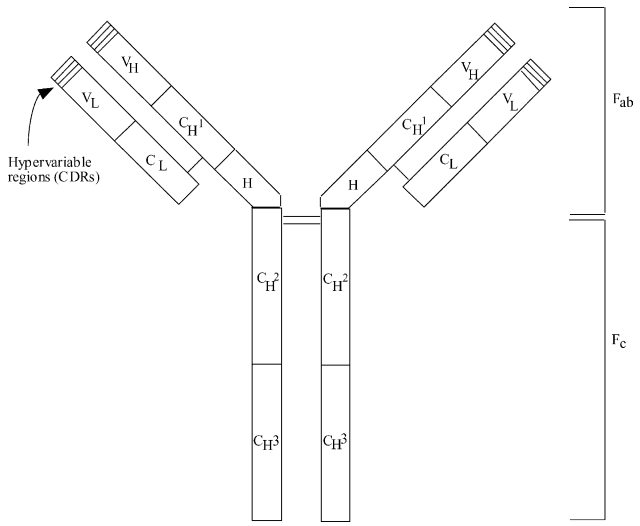

Fig. 3. Antibody (immunoglobulin G, IgG) structure. The molecule consists of four polypeptide chains, two identical light (L) chains and two identical heavy (H) chains, arranged in a Y shaped structure. Each chain houses discrete domains, as shown. Variable (V) domains of monoclonal antibodies displaying different binding specificities vary in amino acid sequence, whereas the sequence of their constant (C) domains do not. The actual antigen binding regions (the hypervariable regions or CDR’s) are located at the tips of the Y shaped structure as shown. Reproduced with permission from Ref. [71].

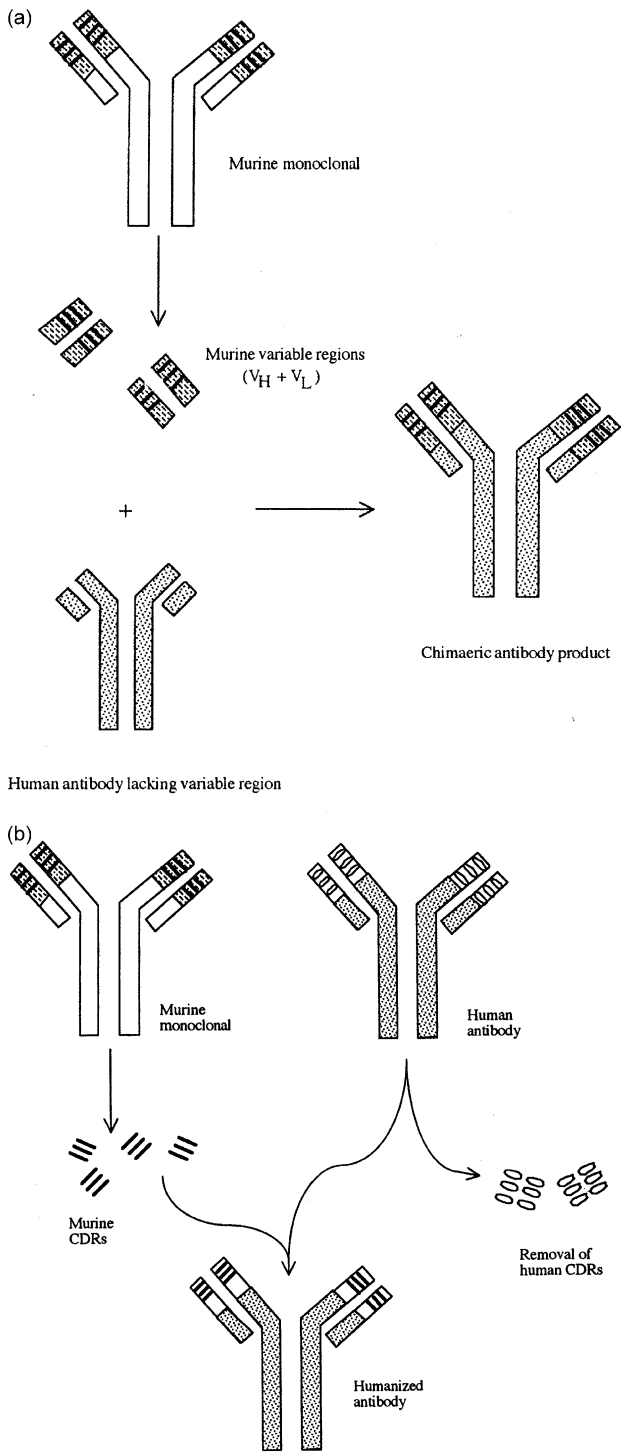

Fig. 4. Production of chimaeric (a) and humanized (b) antibodies (via recombinant DNA technology). Chimaeric antibodies consist of murine monoclonal V_H and V_L domains grafted onto the F_c region of a human antibody. Humanized antibody consists of murine CDR regions grafted into a human antibody. Reproduced with permission from Ref. [42].

replace the CDR sequences of the human antibody gene. The resultant antibody is entirely human in nature apart from its antigen-binding region. Predictably it is significantly less immunogenic even when compared to chimaerics, and it displays a half-life approximating to fully native human antibodies.

6. Fusion products

Protein engineering also facilitates the generation of novel 'fusion' or hybrid biopharmaceuticals. Such products consist of two (or potentially more) naturally occurring polypeptides (or, more usually, fragments thereof) linked directly or via a short linker sequence. In general one section serves a molecular recognition function while the second serves an effector function (Fig. 5). Three such fusion products have gained general marketing approval to date, 'Ontak', 'Enbrel' and 'Amevive' (Table 3).

Ontak, also known as denileukin diftitox, is indicated for the treatment of adult patients with cutaneous T cell lymphoma (a form of non-Hodgkin's lymphoma), whose malignant cells express the CD25 component of the interleukin 2 (IL-2) receptor on their surface [50–52]. It is a 58 kDa fusion protein expressed in an engineered *E. coli* cell line. It consists of a modified form of the diphtheria toxin fused to human IL-2. More specifically it consists of amino acid residues 1–386 and 484–485 from the diphtheria toxin (encompassing the toxin's cytotoxic and translocation domains), and residues 2–133 of IL-2.

Upon administration, Ontak binds to the lymphoma cells via their surface IL-2 receptor. Binding induces internalization of the receptor–ligand complex and it appears that a proportion of the now internalized toxin-containing product evades intracellular destruction. Cell death is induced by toxin-mediated inhibition of protein synthesis. The product can also target additional cells expressing the IL-2 receptor (usually activated B and T lymphocytes and macrophages), likely mediating the increased incidence of infections observed as a side effect of product administration in some patients.

Enbrel (Etanercept) is a recombinant fusion protein consisting of the extracellular, ligand binding portion of the human tumour necrosis factor (TNF) receptor (p75), linked to the F_c portion of a human IgG antibody. The product is expressed in an engineered CHO cell line, from which it is released as a 150 kDa dimeric soluble protein. It is indicated for the treatment of rheumatoid and some related forms of arthritis [53,54]. TNF is a major pro-inflammatory cytokine.

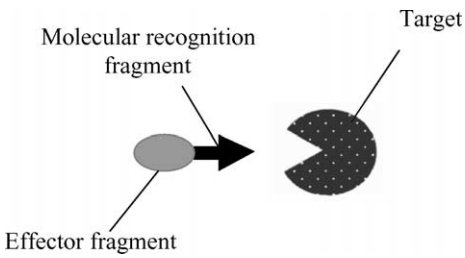

Fig. 5. Generalized schematic diagram of a fusion product. Refer to text for further details.

It is present at elevated concentrations in the joints of patients suffering from rheumatoid arthritis, where it promotes inflammation and tissue damage characteristic of this condition. Enbrel effectively acts as a competitive inhibitor of TNF by competing with true cellular TNF receptors for ligand binding. The antibody portion of the product appears to increase its half-life in the blood.

Amevive (Alefacept) is a more recently approved fusion product [55–57]. The 91.4 kDa protein consists of the extracellular CD 2 binding portion of the human leukocyte function antigen 3 (LFA-3) linked to the F_c region of a human IgG1 antibody. It is indicated for the treatment of moderate to severe chronic plaque psoriasis. Upon administration, amevive binds primarily to T lymphocytes exhibiting the CD 2 antigen on their surface. This prevents lymphocyte activation. It also promotes a reduction in overall lymphocyte counts, likely by bridging the gap between these cells and the F_c receptors found on the surface of cytotoxic cells such as natural killer (NK) cells. The ensuing immunosuppressive action blocks the immune system cells believed to play a significant role in the pathophysiology of the target condition.

7. Post-translational engineering

Several approved recombinant therapeutic products are engineered post-synthesis. This approach normally entails the covalent attachment of a chemical group to the polypeptide's backbone, or the alteration of a pre-existing post-translational modification, such as a glycosylation pattern.

The covalent attachment of one or more molecules of polyethylene glycol (PEG) to the polypeptide backbone represents a common form of such engineering [58]. PEGylation is relatively straightforward and generally increases the plasma half-life of the protein drug by reducing the rate of systemic clearance [59,60]. As a result, less frequent dosage regimes are necessitated, with consequent economic savings and (usually) improved patient compliance and convenience. Several PEGylated biopharmaceuticals have gained regulatory approval over the last few years (Table 3). The majority have been (PEGylated forms of) first generation interferon-based products [61–63]. The plasma half-life of native interferons is typically of the order of 3–5 h. In consequence, first generation interferon-based therapies often entailed product administration trice weekly. Covalent attachment of PEG, while not effecting the biological activity of such products, typically increases their plasma half-life to the order of 24 h, allowing their administration just once weekly.

Somavert is the trade name given to a particularly interesting PEGylated biopharmaceutical in that it also displays an altered amino acid sequence. The product consists of a PEGylated form of a human growth hormone (hGH) analogue, engineered to introduce a total of nine specific alterations in amino acid sequence. While retaining the ability to bind the hGH receptor, the analogue fails to trigger an intracellular response. It is indicated for the treatment of acromegaly, a rare endocrinological disorder characterized by elevated plasma hGH concentrations [64,65]. The product is PEGylated in order to increase its plasma half-life [66]. The PEGylation process results in the attachment of 4–6 PEG molecules to the product's polypeptide backbone.

Two approved biopharmaceuticals have been engineered by modification of their carbohydrate component (Table 3). Cerezyme (trade name) is a carbohydrate-modified form of glucocerebrosidase, a lysosomal enzyme involved in the catalytic degradation of glycolipids. Gaucher's disease is a genetic condition caused by lack of lysosomal glucocerebrosidase activity, with tissue-based macrophages being amongst the most severely effected cell type. The product, which is naturally glycosylated, is produced by recombinant means in a CHO cell line and downstream processing includes an enzyme-based processing step using an exoglycosidase. The exoglycosidase removes sialic acid sugar residues that cap the oligosaccharide side chains. This exposes mannose residues underneath, facilitating specific uptake by macrophages via macrophage cell surface mannose receptors. In this way the product is specifically targeted to the cell type most affected by the disease [67,68]. Unmodified glucocerebrosidase, if administered, is quickly removed from the bloodstream by the liver.

Nespo (known as Aranesp in the USA) is a recombinant 166 amino acid human erythropoietin (EPO) used to treat anaemia associated with chronic renal failure. Produced in a recombinant CHO cell line, it displays an increased overall carbohydrate content when compared to native EPO. The natural molecule harbours three N-linked carbohydrate side chains whereas the recombinant product displays five such side chains. The increased carbohydrate content extends the product's half-life, facilitating once weekly (and in some circumstances once every second week) administration [69,70].

8. Engineered veterinary products

While the majority of biopharmaceuticals approved to date are destined for human use, a number of veterinary medicines are now also produced via recombinant DNA technology. Several of these are engineered forms of single (pathogen derived) polypeptides or are engineered viruses and are used as animal vaccines (Table 6). Porcilis AR-T DF (trade name), for example, represents a multi-component vaccine containing a modified polypeptide toxin from *Pasteurella multocida*. It is produced by recombinant means in *E. coli*.

Ibraxion (trade name; Table 6) is an example of an engineered bovine herpes virus. The product is used to vaccinate cattle against infectious bovine rhinotracheitis,

Table 6
Recombinant/engineered veterinary products thus far approved in the European Union

Product	Company	Therapeutic indication	Approved
Porcilis Porcoli (combination vaccine containing r *E. coli* adhesins)	Intervet	Active immunization of sows against neonatal enterotoxicosis	1996
Fevaxyn Pentofel (combination vaccine containing r Feline leukaemia viral antigen as one component)	Fort Dodge Laboratories	Immunization of cats against various feline pathogens	1997
Neocolipor (vaccine containing four inactivated *E. coli* strains; two wild type strains expressing *E. coli* adhesins F6 and F41 and two recombinant strains, engineered to express F4 and F5 adhesins)	Merial	Reduction of neonatal enterotoxicosis of young piglets caused by *E. coli* strains expressing F4, F5, F6 or F41 adhesins	1998
Porcilis AR-T DF (combination vaccine containing a modified toxin from *Pasteurella multocida* expressed in *E. coli*)	Intervet	Reduction in clinical signs of progressive atrophic rhinitis in piglets: oral administration	2000
Porcilis pesti (vaccine containing r Classical swine fever virus E_2 subunit antigen produced in an insect cell baculovirus expression system)	Intervet	Immunization of pigs against classical swine fever	2000
Ibraxion (vaccine consisting of an inactivated, bovine herpes virus type 1 engineered by removal of the viral glycoprotein gE gene)	Merial	Active immunization of cattle against infectious bovine rhinotracheitis	2000
Bayovac CSF E2 (vaccine consisting of r Classical swine fever virus E2 subunit antigen produced using a baculovirus vector system)	Bayer	Immunization of pigs against classical swine fever virus	2001
Eurifel FELV (vaccine consisting of an engineered canarypox virus into which the *gag*, *env* and a partial *pol* gene of feline leukaemia virus have been inserted)	Merial	Immunization of cats against feline leukaemia virus (FeLV)	2000
Vibragen Omega (rFeline interferon omega)	Virbac	Reduce mortality/clinical signs of canine parvovirusis	2001
Eurifel RCPFEVL (multi-component vaccine containing as one component an engineered canarypox virus into which the *gag*, *env* and a partial *pol* gene of feline leukaemia virus have been inserted) (see Eurifel FELV above)	Merial	Active immunization of cats against viral pathogens, including feline leukaemia virus	2002
Gallivac HVT IBD (live multi-component vaccine containing as one component an engineered herpes virus of turkeys (HVT) housing a gene coding for the protective VP2 antigen of the infectious bursal disease virus (IBDV))	Merial	Active immunization of chickens against, amongst others, the viral causative agent of infectious Bursal disease	2002
ProteqFlu (vaccine containing two strains of engineered canarypox virus expressing haemagglutinin HA genes from two strains of influenza virus)	Merial	Active immunization against equine influenza	2003

a condition characterized by animal production losses and increased risk of abortion. The engineered virus differs from the wild type pathogen in that one structural gene (the gE gene) has been deleted. Administration of (inactivated) virus induces immunological protection in cattle. The serum of vaccinated animals, however, is devoid of anti-gE antibodies, which allows immunological differentiation between vaccinated and infected (and hence infectious) animals.

9. Conclusions

The production of therapeutic proteins represents the first truly industrial application of recombinant DNA technology. Initially recombinant protein-based production was undertaken to produce proteins in sufficient quantities to meet medical demand and/or to produce product virtually free from risk of contamination with pathogens. Advances in protein science and molecular biology now also render possible the generation of proteins tailored to better achieve a pre-specified therapeutic goal. Engineered products have made a genuine impact upon the management of a range of medical conditions. Moreover, the design and development of engineered biopharmaceuticals will become more commonplace as protein scientists gain further insight into the molecular rules governing the relationship between protein structure and function [71–73].

References

[1] D.A. Jackson, R.H. Symons, P. Berg, Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV 40 DNA molecules containing lambda phage genes and the galactose operon of *Escherichia coli*, Proc. Natl Acad. Sci. USA 69 (1972) 2904–2909.
[2] P.E. Lobban, A.D. Kaiser, Enzymatic end to end joining of DNA molecules, J. Mol. Biol. 78 (1973) 453–471.
[3] G. Kohler, C. Milstein, Continuous culture of fused cells secreting antibody of pre-defined specificity, Nature 256 (1975) 495–497.
[4] G. Walsh, Biopharmaceuticals and biotechnology medicines: an issue of nomenclature, Eur. J. Pharm. Sci. 15 (2002) 135–138.
[5] I. Johnson, Human insulin from recombinant DNA technology, Science 219 (1983) 632–637.
[6] G. Walsh, Biopharmaceutical benchmarks—2003, Nat. Biotechnol. 21 (8) (2003) 865–870.
[7] K. Robinson, An industry comes of age, Biopharm. Int. 15 (11) (2002) 20–24.
[8] P. Carey, Protein Engineering and Design, Academic Press, London, 1996.
[9] J.A. Brannigan, A.J. Wilkinson, Protein engineering 20 years on, Nat. Rev. Mol. Cell Biol. 3 (12) (2002) 964–970.

[10] J. Van Ee, Enzymes in Detergency, Marcel Dekker, New York, USA, 1997.
[11] A. Bristol, Recombinant DNA derived insulin analogues as potentially useful therapeutic agents, Trends Biotechnol. 11 (1993) 301–305.
[12] Z. Vajo, W. Duckworth, Genetically engineered insulin analogues: diabetes in the new millennium, Pharmacol. Rev. 52 (1) (2000) 1–9.
[13] R.E. Chance, B.H. Franks, J.M. Radziuk, R. Di Marchi, Discovery and development of insulin lispro, Drugs Today 34 (Suppl. C) (1998) 1–9.
[14] D.N. Breams, L.A. Alter, M.J. Beckage, Altering the association properties of insulin by amino acid replacement, Protein Eng. 5 (6) (1992) 527–533.
[15] W. Cohick, D. Clemmons, The insulin-like growth factors, Annu. Rev. Physiol. 55 (1993) 131–153.
[16] H.B. Long, Human insulin analogues with rapid onset and short duration of action, in: J.A. Smith, J.E. Rivier (Eds.), Peptides. Chemistry and biology, Proceedings of the Twelfth American Peptide Symposium, ESCOM, Leiden, 1992, pp. 88–90.
[17] B.H. Franks, Manipulation of the position of proline in the B chain produces monomeric insulins, Diabetes 40 (Suppl. 1) (1991) 423A.
[18] R.E. Chance, Insulin analogues modified at position 29 of the B chain, US Patent 5,514,646, 1996.
[19] L.J. Slieker, G.S. Brooke, R.D. Di Marchi, Modification in the B10 and B26–30 regions of the B chain of human insulin alter affinity for the human IGF-1 receptor more than for the insulin receptor, Diabetologia 40 (Suppl. 2) (1997) S54–S61.
[20] W.N. Shaw, K. Su, Biological aspects of a new human insulin analog: [Lys(B28), Pro(B29)]-human insulin, Diabetes 40 (Suppl. 1) (1991) 464A.
[21] J.A. Galloway, Human insulin and its modifications, in: M. Reidenberg (Ed.), The Clinical Pharmacology of Biotechnology Products, Elsevier, Amsterdam, 1991, pp. 23–34.
[22] K.S. Su, Using dog model for comparing time action of insulins after subcutaneous (s.c.) injection: prediction of rapid onset of a new insulin analogue [Lys (B28), Pro (B29)]-human insulin (KP), Pharm. Res. 11 (10) (1994) S357.
[23] D.L. Bakaysa, J. Radziuk, H.A. Havel, Physicochemical basis for the rapid time action of Lys^{B28} Pro^{B29}-insulin: dissociation of a protein–ligand complex, Protein Sci. 5 (12) (1996) 2521–2531.
[24] J. Radziuk, J.C. Davies, W.S. Pye, Bioavailability and bioeffectiveness of subcutaneous human insulin and two of its analogs—Lys^{B28} Pro^{B29}—human insulin and Asp^{B10} Lys^{B28} Pro^{B29}—human insulin—assessed in a conscious pig model, Diabetes 46 (1997) 548–556.
[25] R.E. Chance, N.B. Glazer, K.L. Wishner, Insulin Lispro (Humalog), in: G. Walsh, B. Murphy (Eds.), Biopharmaceuticals, An Industrial Perspective, Kluwer Academic Publications, Dordecht, The Netherlands, 1999, pp. 149–171.
[26] D.C. Howey, R.R. Bowser, R.L. Brunelle, [Lys (B28), Pro (B29)]-human insulin: effect of injection time on postprandial glycemia, Clin. Pharmacol. Ther. 58 (1995) 459–469.
[27] L. Heinemann, J. Woodworth, Pharmacokinetics and glucodynamics of insulin lispro, Drugs Today 32 (Suppl. C) (1998) 23–36.
[28] L. Heinemann, T. Heise, L.C. Wahl, Prandial glycaemia after a carbohydrate-rich meal in type 1 diabetic patients: using the rapid acting insulin analogue [Lys (B28), Pro (B29)] human insulin, Diabet. Med. 13 (1996) 625–629.
[29] B.H. Frank, J.C. Baker, D.L. Bakaysa, Lys^{B28} Pro^{B29}-human insulin (insulin lispro): solution properties of a rapid-acting insulin, Diabetologia 38 (Suppl. 1) (1995) A189.
[30] T.M. Chapman, S. Noble, K.L. Goa, Insulin aspart: a review of its use in the management of type 1 and 2 diabetes mellitus, Drugs 62 (2002) 1945–1981.
[31] S.M. Setter, C.F. Corbett, R.K. Campbell, Insulin aspart: a new rapid acting insulin analog, Ann. Pharmacother. 34 (2000) 1423–1431.
[32] D.R. Owens, S. Griffiths, Insulin glargine (Lantus), Int. J. Clin. Pract. 56 (2002) 460–466.
[33] K. McKeage, K.L. Goa, Insulin glargine: a review of its therapeutic use as a long acting agent for the management of type 1 and 2 diabetes mellitus, Drugs 61 (2001) 1599–1624.
[34] J. Gillis, A.J. Wagstaff, K.L. Goa, Alteplase: a reappraisal of its pharmacological properties and therapeutic uses in acute myocardial infarction, Drugs 50 (1995) 102–136.
[35] M. Verstacte, Third generation thrombolytic agents, Am. J. Med. 109 (2000) 52–58.
[36] M. Waller, U. Kohnert, Reteplase, a recombinant plasminogen activator, in: G. Walsh, B. Murphy (Eds.), Biopharmaceuticals, An Industrial Perspective, Kluwer Academic Publisher, Dordecht, The Netherlands, 1999, pp. 185–216.
[37] M.B. Wooster, A.B. Luzier, Reteplase: a new thrombolytic for the treatment of acute myocardial infarction, Ann. Pharmacother. 33 (1999) 318–324.
[38] S. Noble, D. McTavish, Reteplase. A review of its pharmacological properties and clinical efficacy in the management of acute myocardial infarction, Drugs 52 (1996) 589–605.
[39] L. Davydov, J.W. Cheng, Tenecteplase: a review, Clin. Ther. 23 (2001) 982–997.
[40] M. Berger, V. Shankar, A. Vafai, Therapeutic applications of monoclonal antibodies, Am. J. Med. Sci. 324 (2002) 14–30.
[41] F. Breedveld, Therapeutic monoclonal antibodies, Lancet 355 (2000) 735–740.
[42] G. Walsh, Biopharmaceuticals: Biochemistry and Biotechnology, second ed., Wiley, Chichester, UK, 2003, pp. 403–461.
[43] W. Harris, Antibody Therapeutics, CRC Press, Florida, USA, 1997.
[44] R. Kontermann, Antibody Engineering, Springer, Berlin, 2001.
[45] S. Nihira, Experiences in drug discovery and development of anti-erbB2 (HER 2) monoclonal antibody; Herceptin, J. Pharmacol. Sci. 91 (2003) 63P.
[46] G. Hendler, Her-2: the making of herceptin, a revolutionary treatment for breast cancer, Libr. J. 123 (15) (1998) 102.
[47] Anon, Palivizumab (Synagis)—monoclonal antibody for disease prevention, Am. J. Nurs. 99 (1) (1999) 52.
[48] J. Lacha, M. Simova, L. Noskova, V. Telpan, S. Vitko, Zenapax versus OKT-3 prophylaxis in immunologically high risk kidney transplant recipients, Transplant. Proc. 33 (3) (2001) 2273–2274.
[49] S. Emre, A. Vanin, E. Crevantes, D. Heidt, T. Fishbein, P. Sheiner, M. Schwartz, C. Miller, Use of daclizumab (Zenapax) as initial immunosuppression in liver transplant recepients with kidney failure, Transplantation 67 (9) (1999) 231.
[50] E. Olsen, M. Duvic, A. Frankel, Pivitol phase III trial of two dose levels of denileukin diftitox for the treatment of cutaneous T cell lymphoma, J. Clin. Oncol. 19 (2001) 376–388.
[51] F.M. Foss, Interleukin-2 fusion toxin: targeted therapy for cutaneous T cell lymphoma, Ann. N.Y. Acad. Sci. 941 (2001) 166–176.
[52] M. Duvic, T. Kuzel, E.A. Olsen, Quality of life improvements in cutaneous T cell lymphoma patients treated with denileukin diftitox (ontak), Clin. Lymphoma 2 (2002) 222–228.
[53] L.W. Moreland, Soluble tumor necrosis factor receptor (p75) fusion protein (Enbrel) as a therapy for rheumatoid arthritis, Rheum. Dis. Clin. North Am. 24 (1998) 579–591.
[54] A. Alldred, Etanercept in rheumatoid arthritis, Expert Opin. Pharmacother. 2 (2001) 1137–1148.
[55] C. Ellis, G.C. Krueger, Treatment of chronic plaque psoriasis by selective targeting of memory effector T lymphocytes, N. Engl. J. Med. 345 (2001) 248–255.
[56] J.E. Frampton, A.J. Wagstaff, Alfacept, Am. J. Clin. Dermatol. 4 (2003) 277–286.
[57] G.G. Krueger, K.P. Callis, Development and use of alefacept to treat psoriasis, J. Am. Acad. Dermatol. 49 (2003) 587–597.
[58] Z.Y. Jiang, S.W. Xu, Y.Q. Wang, Chemistry for Pegylation of protein and peptide molecules, Chin. J. Org. Chem. 23 (2003) 1340–1347.
[59] R.B. Greenwald, Y.H. Choe, J. McGuire, C.D. Conover, Effective drug delivery by PEGylated drug conjugates, Adv. Drug Deliv. Rev. 55 (2003) 217–250.

[60] J.M. Harris, N.E. Martin, M. Modi, Pegylation—a novel process for modifying pharmacokinetics, Clin. Pharmacokinet. 40 (2001) 539–551.
[61] Y. Wang, S. Youngster, M. Grace, Structural and biological characterization of PEGylated recombinant interferon α2B and its therapeutic implications, Adv. Drug Deliv. Rev. 54 (2002) 547–570.
[62] K. Patel, J. Hutchison, PEGylated interferon α-2b: a new approach to improving response in hepatitis C patients, Expert Opin. Pharmacother. 2 (2001) 1307–1315.
[63] S.J. Hadziyannis, G.V. Papatheodoridis, Peginterferon-alpha (2a) (40 kDa) for chronic hepatitis C, Expert Opin. Pharmacother. 4 (2003) 541–551.
[64] C. Parkinson, P.J. Trainer, The place of Pegvisomant in the management of acromegaly, Endocrinologist 13 (2003) 408–416.
[65] Anon, Pegvisomant–Trovert–Somavert—treatment of acromegaly, Drug Future 26 (2001) 911–913.
[66] R. Clark, K. Olson, G. Fiuh, Long acting growth hormones produced by conjugation with polyethylene glycol, J. Biol. Chem. 271 (1996) 21969–21977.
[67] H. Hoppe, Cerezyme—recombinant protein treatment for Gaucher's disease, J. Biotechnol. 76 (2000) 259–261.
[68] C. Niederau, First long term results of Imiglucerase therapy of type 1 Gaucher's disease, Eur. J. Med. Res. 3 (1998) 25–30.
[69] J.A. Glaspy, J.S. Jadeja, G. Justice, Darbepoietin alfa given every 1 or 2 weeks alleviates anaemia associated with cancer chemotherapy, Br. J. Cancer 87 (2002) 268–276.
[70] M.S. Joy, Darbepoetin alfa: a new erythropoiesis-stimulating protein, Ann. Pharmacother. 36 (2002) 1183–1192.
[71] C. Berg, R. Nassar, K. Pang, The evolution of biotech, Nat. Rev. Drug. Discov. 1 (2002) 845–846.
[72] M.J. Glennie, P.W.M. Johnson, Clinical trials of antibody therapy, Immunol. Today 21 (2000) 403–410.
[73] O.H. Brekki, I. Sandlie, Therapeutic antibodies for human disease at the dawn of the twenty-first century, Nat. Rev. Drug. Discov. 2 (2003) 52–62.
[74] G. Walsh, Biopharmaceuticals: first versus second generation, New Drugs 2 (2002) 26–37.