

Second-generation protein drugs *children of the revolution*

The first biotech revolution was catalysed in the late 1970s with the development of recombinant DNA technology. This revolution generated numerous billion-dollar protein drugs such as erythropoietin and the interferons. Now that the patents on these drugs are expiring, will second-generation proteins, the children of the biotech revolution, dominate the protein drug market?

By Dr Jeffrey C. Way

The world of protein pharmaceuticals has seen two revolutions. The first began in the mid-1970s with the simultaneous development of recombinant DNA and monoclonal antibody technologies. The second revolution began with genomics, the sequencing of the human genome and the formation of hundreds of biotech companies. Genomics promises to have a profound impact on the world of marketed drugs, but this impact may be spread over a long timespan. Outside the genomics spotlight, a number of biotech companies are pursuing second-generation protein drugs. By analogy with second-generation small molecule drugs, this approach may be less risky and more conducive to predictable timelines, with better risk/reward ratios than genomic approaches to finding novel drugs. This article will focus on technologies and companies that enhance existing protein drugs, primarily through the improvement of protein pharmacokinetics and biodistribution.

Like small molecule drugs, proteins can be improved. However, unlike small drugs, strategies for improving proteins can often be generalised to a large number of proteins. As a result, numerous biotech companies have been formed around gen-

eral, patentable strategies for enhancing protein pharmacokinetics and biodistribution.

Unlike small molecules, proteins are the products of natural selection and their metabolism is often quite well understood. Hence, naturally occurring proteins are usually already optimised for binding to their targets, and improvement of protein pharmacokinetics and distribution is largely a matter of rational drug design.

Increasing serum half-life

Many protein drugs rapidly disappear from the body. Proteins with a molecular weight of less than about 50,000 Daltons are cleared through the kidneys and excreted in the urine. When low-molecular weight cytokines such as interleukin-2 (IL-2), interferon- α (IFN α) and interferon- β (IFN β) are injected intravenously, these proteins have a serum half-life of about five hours or less. Therapeutic proteins may also be degraded by proteases, either before or after internalisation by cells.

Chemical modification with large, inert groups and fusion to a second protein are two methods to extend a protein's serum half-life. These modifications increase the effective molecular weight of a therapeutic protein over the renal threshold of

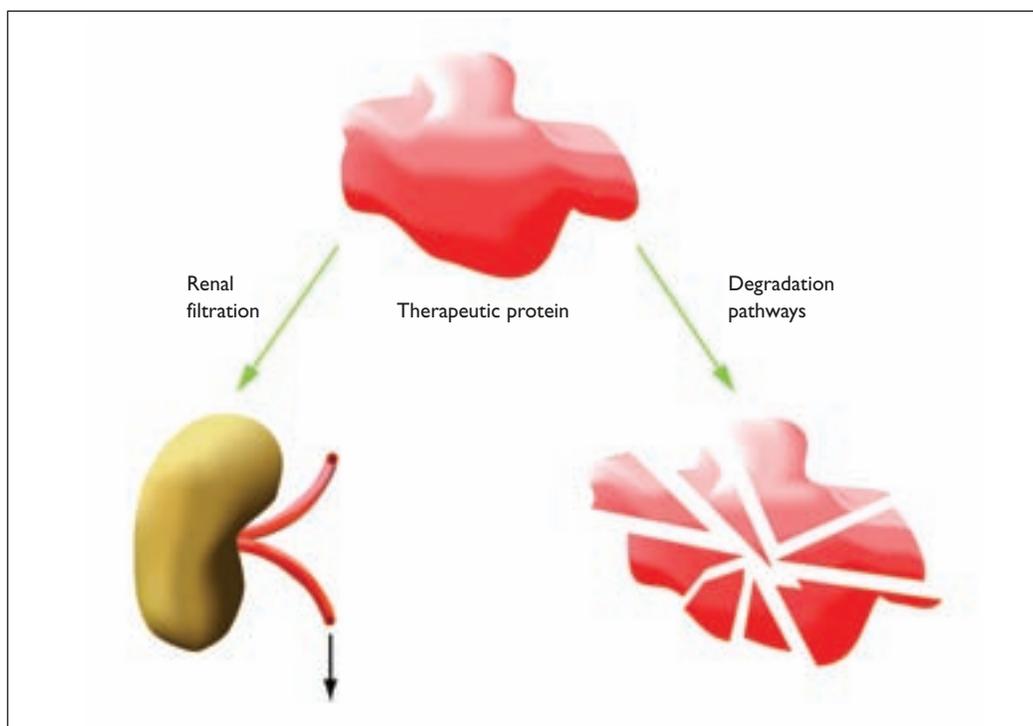


Figure 1
Therapeutic proteins *in vivo* are usually either excreted through the kidney into the urine or are degraded by proteases

50,000 Daltons. In some genetically engineered fusion proteins, the fusion partner does not simply increase mass, but also actively prevents degradation by proteases.

PEGylation is a well-established strategy for extending the serum half-life of proteins. Polyethylene glycols (PEG) are non-reactive polymers that can be chemically attached to therapeutic proteins. Enzon, Shearwater and Valentis are three companies whose success has been based on PEGylation technology.

Enzon, Inc was one of the first companies to exploit PEGylation technology, developing two niche products: a PEGylated adenosine deaminase (Adagen) for treating the genetic deficiency of adenosine deaminase; and Oncospar, a PEGylated L-asparaginase for treatment of acute lymphoblastic leukemia. These products demonstrated the utility of basic PEGylation technology, leading to Enzon's relationship with Schering-Plough and the development of PEG-Intron, a PEGylated interferon- α (IFN α).

PEG-Intron has a serum half-life about five times longer than IFN α . This extended serum half-life has enabled Schering-Plough to develop a weekly dosing schedule, in contrast to the three-times-per-week schedule for non-PEGylated IFN α .

Similarly, Shearwater generated Pegasys, using a branched derivative of polyethylene glycol attached to IFN α , and has formed a partnership

with Roche to sell this product. PEG-Intron was approved in January 2001, while Pegasys was approved in October 2002 and consequently Schering-Plough has a larger share of the hepatitis C market. The race between the Schering-Plough and Roche products illustrates how improvements in a protein drug are useful to carve out large shares of significant markets.

Products from the first phase of the recombinant DNA/biotech revolution are now going off-patent, so a number of similar confrontations are brewing. For example, the interferon- β for the multiple sclerosis market currently has three competitors: Betaseron from Berlex, Avonex from Biogen and Rebif from Serono. Betaseron is a form of IFN β made from bacteria, but the Avonex and Rebif products are both made from mammalian cells and are essentially identical. Biogen has investigated a PEGylated version of IFN β ¹.

Fusion proteins that extend serum half-life

An alternative strategy to PEGylation is to genetically link a therapeutic protein to a fusion partner to increase its mass beyond the renal threshold. This approach has been used extensively with two fusion partners: serum albumin and the Fc region of immunoglobulin G.

Human serum albumin fusion proteins² were developed by Principia Pharmaceuticals, a transient

biotech company that was spun out of Centeon and subsequently acquired by Human Genome Sciences (HGS) in a stock swap valued at \$120 million. This was a logical move for HGS, which has presumably identified thousands of secreted proteins in the human genome and can now construct albumin fusion proteins. Interestingly, the most advanced product that HGS has generated with this strategy is a fusion between albumin and IFN α ('Albuferon'). This speaks to the relative ease of marketing an improved version of an established protein drug, rather than identifying and developing a novel drug through genomics research.

The antibody Fc region has been used to construct fusion proteins with improved properties. Genentech³ generated X-Fc proteins, in which a therapeutic protein 'X' replaces the Fab portion of an antibody, while Lo et al⁴ generated Fc-X fusion proteins. Both classes of fusion proteins show extended serum half-lives, but differ in other ways. For example, X-Fc proteins have antibody-like effector properties that can be used to kill cells to which 'X' binds⁵, but Fc-X proteins do not. Fc-X proteins ('X' at the C-terminus of the Fc) are generally produced in mammalian cells at much higher levels than the corresponding X-Fc proteins or X alone.

Enbrel: an X-Fc protein

Tumour necrosis factor (TNF) is a potent inflammatory protein that plays a key role in many inflammatory and autoimmune disorders, such as rheumatoid arthritis and Crohn's disease. The protein drug Enbrel is an X-Fc fusion between the

extracellular domain of the TNF receptor (sTNF-R) and an Fc region (Figure 3). In this case, the Fc region serves two functions: prolonging the sTNF-R half-life and also dimerising the sTNF-R. TNF normally binds to at least two TNF receptors, so the dimeric sTNF-R in Enbrel has a higher affinity than a monomeric form. Enbrel is comparable to the anti-TNF antibody Remicade in treating TNF-mediated diseases.

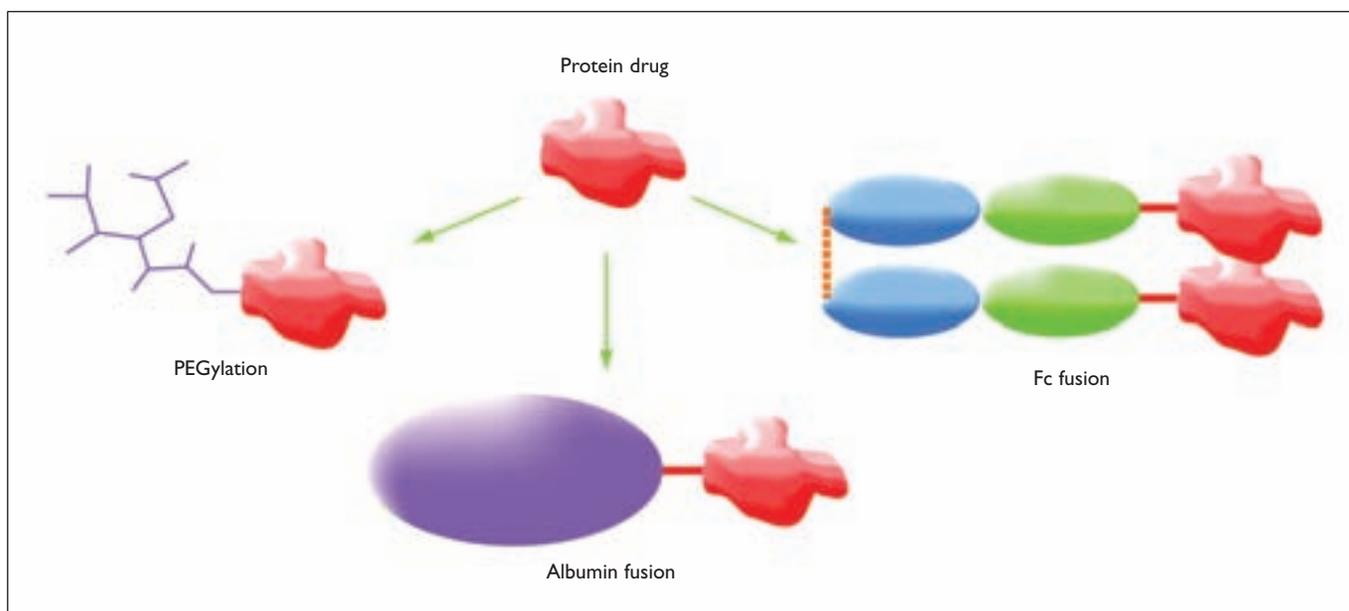
Recycling systems for albumin and the antibody Fc region

Albumin and the Fc portion of IgG have the added advantage that they are recognised by specific protein recycling systems that pump proteins out of cells. This is useful because a major site of protein degradation is within cells. For example, when signalling proteins such as IFN α , IFN β , erythropoietin and interleukin-2 bind to their cell-surface receptors, they are internalised by the cell and degraded. This degradation can be a major elimination pathway for a therapeutic protein. However, when such proteins are fused to albumin or Fc, these fusion proteins have a significant chance of being released intact from cells. These processes further lengthen the pharmacokinetic half-life of albumin and Fc fusion proteins beyond the effects of increased size.

The uptake of albumin, antibodies and therapeutic proteins are all examples of 'receptor-mediated endocytosis'. In this process, internalised proteins are sent to a subcellular compartment termed the endosome. Unless a recycling receptor inter-

Figure 2

Modification of protein drugs to extend serum half-life. The purpose of PEGylation or fusion to albumin or Fc is to increase the effective molecular weight of a therapeutic protein to above 50,000 D, which is the cutoff for renal filtration



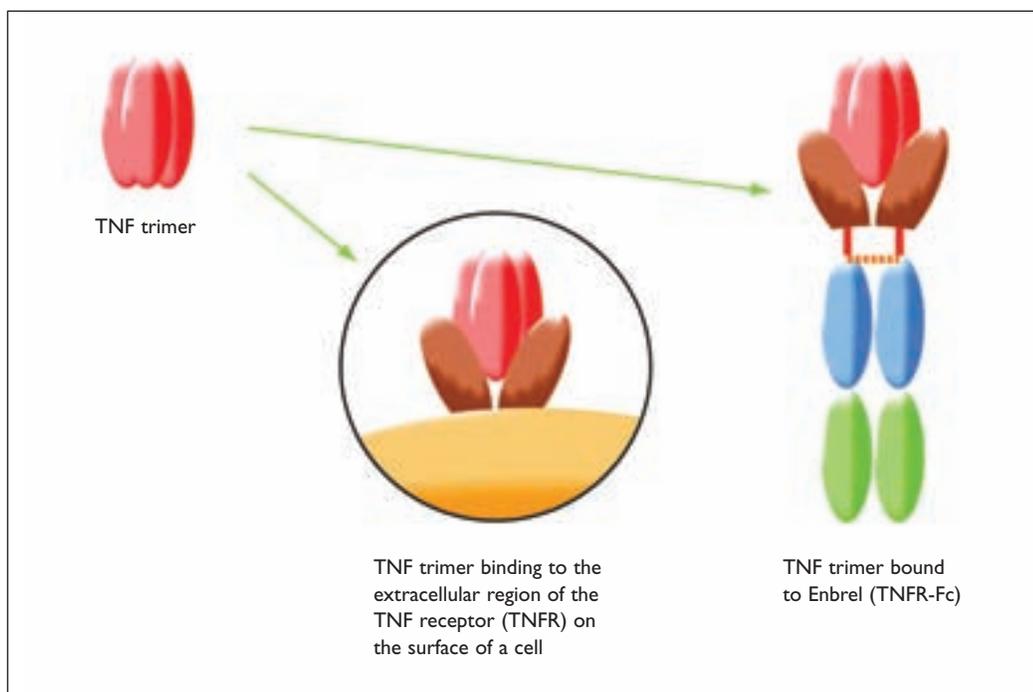


Figure 3
Enbrel, a fusion of the extracellular region of the TNF receptor to an Fc region. Enbrel binds to TNF (a pro-inflammatory protein; red trimer) and blocks TNF binding to TNF receptors (brown) on cell surfaces

venues, proteins in the endosome are then sent to the lysosome and degraded.

Antigen-antibody complexes form when an antibody binds to a second molecule (the antigen). The complexes are then taken up by cells such as macrophages, which digest the antigen but recycle the antibody to work again. The recycling process is mediated by the Fc ‘protection’ receptor (FcRp), which specifically identifies antibodies within cells and transports them out (Figure 4). This ‘protection’ receptor turns out to be identical to the neonatal Fc receptor, which is expressed in the intestine. In this context, FcRp transports antibodies in breast milk across the wall of the intestine and into an infant’s circulation.

Aranesp: less is more

Amgen’s second-generation erythropoietin, Aranesp, uses a surprising trick to enhance the lifetime of the drug in the body. A major elimination route of erythropoietin (Epo) from the body is through binding to Epo receptors on the numerous red blood cell precursors in the bone marrow. Aranesp is a derivatised Epo with a reduced affinity for the Epo receptor. Paradoxically, Aranesp is more active *in vivo*. Because Aranesp binds to its receptor more slowly, it disappears more slowly and has improved pharmacokinetic properties⁶.

Erythropoietin is a glycoprotein with three N-linked glycosylation sites and one O-linked glycosylation site. The N-linked glycosyl moieties are

essentially large trees of sugar molecules that end in a negatively-charged sialic acid. The association of Epo to its receptor is partly driven by the positively charged amino acids on the binding face of Epo, and the negative charges on the receptor. The negatively-charged sialic acids create a negative electrostatic field around Epo, reducing the on-rate of binding to the receptor because of electrostatic repulsion.

Amgen’s Aranesp is a genetically engineered form of Epo that has two additional N-linked glycosylation sites. Compared to normal Epo, the resulting protein is even more negatively charged, has an even slower rate of receptor binding, is less biochemically active and has a longer pharmacokinetic half-life. Aranesp can be administered once per week instead of three times per week for Epo, resulting in significant patient convenience.

Control of distribution in engineered proteins

The organ-specific distribution of small molecules is generally a matter of chance rather than choice. In contrast, engineered proteins can often be targeted to a specific tissue by fusion to antibody variable domains or by fusion to another tissue-specific protein. Tumour-specific antibodies have been used to target anti-tumour agents such as radioactive atoms, *Pseudomonas* exotoxin, RNase I, and cytokines (these approaches are reviewed in Carter⁷ and Huston and George⁸).

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Potency and side-effects are key issues in choosing a molecule that will be delivered by an antibody to a tumour site. The absolute number of antibody binding sites on a tumour cell surface simply is not adequate to deliver enough of a typical chemotherapy agent, so the focus has shifted to targeting extremely potent molecules that may have unacceptable side effects without targeting.

Radioactive antibody conjugates (Figure 6b): Rituxan is a monoclonal antibody used for treating B cell lymphoma. Zevalin and Bexxar are similar to Rituxan but have been chemically modified to contain 131I and 90Y, respectively. Once they bind to a tumour cell, nearby cells are specifically irradiated and killed through a bystander effect. The conjugated isotopes ideally have a short decay path length and a half-life comparable to the residence time of the antibody at the tumour site, so that most of the decay events irradiate the tumour.

Antibodies fused to cytotoxic proteins: On a molecule-by-molecule basis, proteins are generally much more potent than small molecules. For example, Pseudomonas exotoxin (PE) catalytically modifies and inactivates eukaryotic ribosomes. A few internalised PE molecules can kill a mammalian cell. The enzyme RNase digests RNA, so a small amount of RNase in the cell cytoplasm can also be lethal. Both of these proteins can kill nor-

mal as well as cancer cells and are most effective and specific when targeted.

Ira Pastan and his co-workers have pioneered the use of targeted PE (Figure 6c). In one of the most advanced versions, PE is fused to a single-chain Fv, which consists of the variable domains from an antibody heavy and light chain connected by an artificial peptide linker; this minimal antibody combining site is then fused to PE.

Similarly, Suzanna Rybak and her colleagues have investigated human eosinophil RNase as an anticancer agent (Figure 6d). This protein can be internalised by cells, somehow crossing the cell membrane and ending up in the cytoplasm, where it can degrade mRNAs, tRNAs and so on. Rybak and her co-workers have fused RNase to the Fab portion of a tumour-specific antibody, creating a molecule with an improved therapeutic index. Versions of the PE and RNase fusion proteins have been tested in small clinical trials.

Antibody-cytokine fusions: Cytokines are potent proteins that can direct an immune response against a tumour. The cytokines interleukin-2 (IL-2) and interferon- α (IFN α) have been approved for treatment of certain uncommon cancers, but both molecules cause significant side-effects. IL-2, for example, causes fever and generally reproduces the sensation of fighting off a bad infection. Another cytokine, IL-12, has been tested in clinical trials

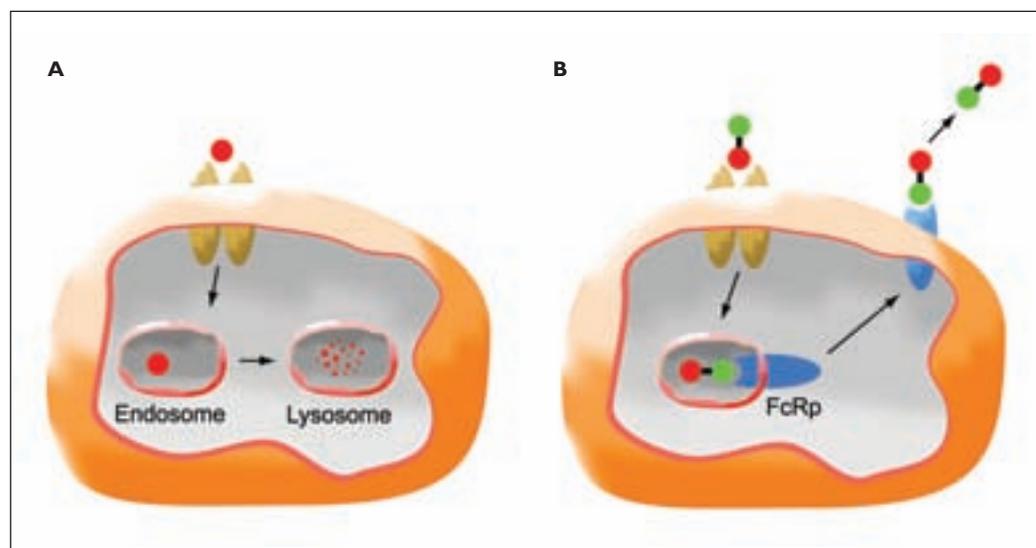


Figure 4

Receptor mediated endocytosis leads to destruction of protein drugs. Normally, a signalling protein (red) binds to a cell-surface receptor (yellow) and is internalised. The protein is sent to the endosome, then ultimately sent to the lysosome, where it is degraded. A fusion of a therapeutic protein to an Fc region (green) is similarly internalised. However, upon reaching the endosome, the Fc protection receptor (FcR α ; blue) binds to the Fc region and recycles the fusion protein out of the cell. Thus, an Fc fusion protein remains in the body longer and can be recycled numerous times. Albumin fusion proteins probably benefit from a similar mechanism

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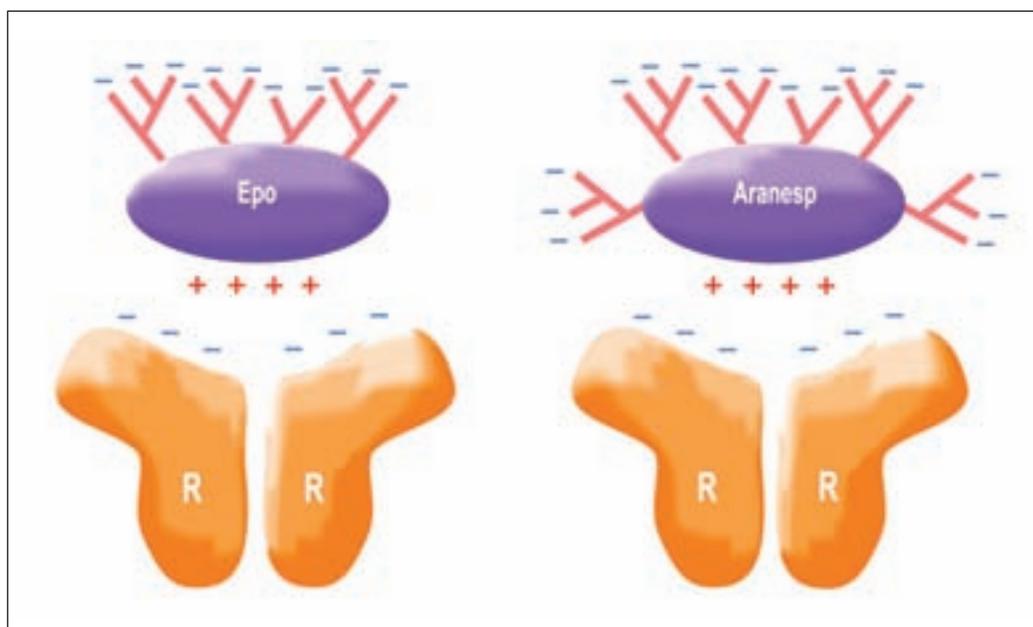


Figure 5
 Aranesp is a derivative of erythropoietin with additional negatively charged oligosaccharides. The extra negative charges reduce the binding to the erythropoietin receptor (R). Since erythropoietin is destroyed after binding to its receptor, the decrease binding rate of Aranesp to EpoR allows Aranesp to have a longer serum half-life

and has potential anticancer activity, but IL-12 doses are limited by its side-effects.

Several groups have constructed targeted versions of IL-2. Gillies and his colleagues at EMD Lexigen Pharmaceuticals have tested whole-antibody fusions to IL-2 and also IL-12 ('immunocytokines' **Figure 6e**) in a wide variety of animal models^{9,10}. Neri and co-workers have studied single-chain Fv fusions to IL-2, targeted to tumour-specific blood vessels (**Figure 6f**)¹¹. The Epstein group has pursued cytokine proteins using antibodies targeted to necrotic tumour-associated tissue¹². Morrison and her colleagues have focused on fusion proteins using IgG3, an antibody with a particularly long, flexible hinge¹³.

Cytokines are particularly attractive anti-tumour agents that benefit greatly from targeting. By their nature, cytokines act locally: typically, a cytokine will be produced at a site of infection as a 'cry for help' that stimulates immune cells in the immediate area. Fusion proteins that target cytokines to tumours significantly reduce cytokine-related side-effects, and may mimic the localised production of cytokines. Two different whole-antibody-IL2 fusion proteins have been studied in Phase I safety trials. These proteins show moderate side-effects that appear to derive entirely from the IL-2 portion of the molecule and do not show organ-specific effects that might result from an undesired concentration of IL-2 activity.

A key advantage of antibody-targeted radiation and cytokines is that they can act on neighbouring tumour cells. A major problem in cancer treatment

is that cancer cells have a high mutation rate and evolve rapidly in response to drug treatment. For example, treatment of a cancer with an antibody fused to Pseudomonas exotoxin or RNase would select cancer cells that no longer express the tumour-specific target protein. However, a short path-length radioactive decay will kill cells in a general area, even if they no longer have the tumour-specific surface marker. Similarly, cytokines bound to the surface of one tumour cell will stimulate the immune system to attack all of the tumour cells in the vicinity.

Future improvements in protein drugs

Protein drugs lend themselves to a novel type of 'rational drug design' in which a desired property can be added to a protein simply by fusing it to a second protein. This has been used to improve pharmacokinetics and biodistribution, but could also be used to address a fundamental problem with proteins: the need for injection. There are a number of specific protein transport systems in the human intestine. Two biotech companies, Arizeke Pharmaceuticals (San Diego, CA) and Syntonic Pharmaceuticals (Waltham, MA), are trying to engineer and formulate proteins to take advantage of these protein transport systems, so that proteins can be taken in pill form. Other key developments are the expression of protein drugs in plants such as corn and tobacco (which could reduce the cost of producing an antibody drug by 10-fold) and the systematic reduction of immunogenicity¹⁴. When combined with the ease of engineering proteins,

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these scientific advances may lead to a revolution in pharmaceutical development in which protein and small molecule drugs achieve parity in the marketplace.

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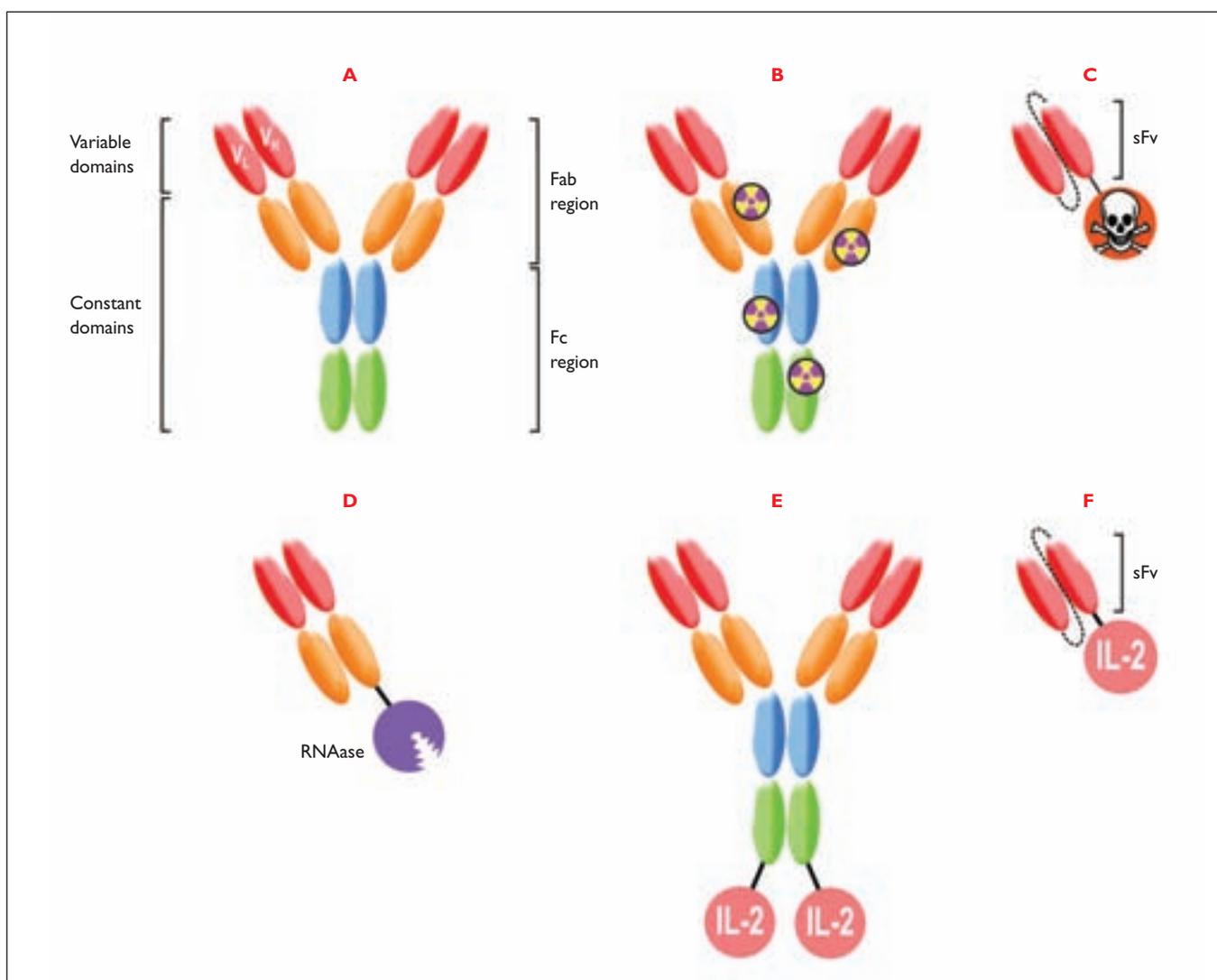


Figure 6

Targeted anti-tumour agents that have been clinically studied. In each fusion protein, antibody variable domains (V domains; red) are present to achieve targeting. **A** An intact antibody, showing the V domains that bind to an antigen and target the antibody, and the constant regions (orange, blue, green). **B** An antibody with radioactive atoms attached by chemical conjugation. **C** A 'single-chain Fv' (sFv), which contains the heavy and light V domains attached by a peptide linker (dotted line), fused to Pseudomonas exotoxin (skull and crossbones). **D** An antibody Fab region fused to the enzyme RNAase (purple). **E** An intact antibody fused to interleukin-2 (pink). **F** A sFv fused to IL-2