The protein science of biosimilars

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Abstract
A sea change is occurring in the off-patent drug manufacturing industry with a first wave of biotechnologically derived products reaching the end of their patent lives. However, recombinant proteins are in a different league from their chemical predecessors in terms of molecular complexity. Small differences in manufacturing processes can affect the efficacy and safety of the recombinant proteins in a manner which is not always measurable using analytical or in vitro techniques. Thus, comparable clinical profiles do not automatically follow from physicochemical likeness and can only be demonstrated through clinical studies. It is essential for patient safety that both innovator and biosimilar manufacturers ensure consistency in their production, by performing rigorous purity and activity profiling between batches, and implement tailored pharmacovigilance plans.

Keywords: assays; biopharmaceuticals; biosimilars; glycosylation; manufacturing; pharmacovigilance; physicochemistry

Requirements for generic drug manufacturing
A generic drug must meet several criteria to be approved for market release. Firstly, it has to contain the same active ingredient as the innovator drug and be administered by the same route. It must also have the same formulation, potency and conditions of use [1].

In the past, medicines have been almost exclusively low molecular weight compounds. Such chemical compounds (such as statins, angiotensin-converting enzyme inhibitors) are relatively easy to synthesize and precise copies of defined quality can be produced in a consistent manner. This reliability of synthesis methods is reflected in the regulatory requirements for generics manufacturers. They only need to demonstrate that their compound is physicochemically identical to the original drug and ‘bioequivalent’ (has the same pharmacokinetic profile) in a limited clinical study for the relevant indication.

A number of biotechnology products are currently marketed including insulin, growth hormone, interferon-β and factor VIII. These recombinant proteins are large molecules, produced from genetically modified cell lines, and extracted through complex and lengthy purification procedures. Because of the variability inherent in such processes, identical copies of original brands cannot be manufactured and, therefore, ‘biogenerics’ cannot exist. Instead, the term ‘biosimilars’ has been coined to describe these off-patent copies of therapeutic proteins.

Gauging biosimilarity
To clarify the debate on off-patent manufacturing, a new vocabulary is emerging which dissects different levels of similarity. ‘Comparability’ expresses the impact of changes to a single, established production process and is measured in terms of physicochemical properties. However, the goal of manufacturers of off-patent biologics is to achieve ‘similarity’: two products from different manufacturers are deemed similar if—importantly—the same safety and efficacy can be established.

Biological agents are large and complex
Biological therapeutic agents are much larger than chemically-synthesized compounds folding after translation into an elaborate three-dimensional structure. This molecular structure determines their function. Table 1 shows the molecular weights for a representative selection of medicinal agents produced by biotechnology compared with more classical therapeutic compounds. It can be seen that biologics are on average ~100–1000-fold larger than their chemical counterparts.
Proteins can undergo complex modifications

Proteins may undergo a number of co- and post-translational modifications to 'fine-tune' their activity. These include enzymatic cleavage (which can lead to activation, for example, hormones), attachment of lipids (for localization of the protein to a cell membrane) or glycans (which can affect properties such as serum half-life). Figure 1 illustrates some common modifications in an idealized protein and their possible functional roles.

Post-translational modifications affect protein activity

Many important therapeutic proteins are glycosylated including epoetin (EPO), granulocyte macrophage-colony stimulating factor (GM-CSF) and tissue-plasminogen activator (t-PA). Glycosylation is important because it can influence the biological activity of a protein through different mechanisms. First of all, glycosylation can affect half-life by influencing the active clearance of a protein. Certain pituitary glycoprotein hormones such as lutropin are rapidly removed from circulation by the liver if they bear a sulphated GalNAc residue [2]. A second example is EPO, the serum half-life of which is dependent on four sialylated N-glycans (sialic acid is itself a glycan). Poorly glycosylated forms of EPO are rapidly cleared by filtration in the kidney. In addition, under-sialylated EPO is rapidly removed by hepatocytes and macrophages. Thus, increasing the degree of sialylation and glycosylation decreases the renal clearance rate [3], and can increase EPO in vivo activity.

Glycosylation can affect the activity of a recombinant protein more directly. Rituximab, for instance, is a monoclonal antibody (mAb) used to treat non-Hodgkin's lymphoma through targeting of B-cell CD20 antigens. Its activity can be assessed with the antibody-dependent cellular cytotoxicity (ADCC) test, which measures the ability of an engineered mAb to lyse target cells. The ADCC of two types of mAb (one of which was rituximab) was found to vary inversely according to the mAb’s glycosylation state [4]. Rituximab was produced from Chinese hamster ovary (CHO) cells with a relatively high level of glycosylation and was found to be several-fold less cytotoxic in vitro than another anti-CD20 mAb (KM3065), which was synthesized with fewer glycan residues from a rat cell line.

Heterogeneity in biological preparations

Recombinant protein manufacturing processes are highly elaborate and sophisticated not only due to the complexity of the molecules themselves, but also as a consequence of cell-based production. Furthermore, the therapeutic properties of recombinant proteins are highly dependent on the manufacturing process. Maintaining batch-to-batch consistency ('comparability') is an ongoing challenge and different production processes yield measurably different proteins.

Commercial manufacturing processes are complex

Biological manufacturing processes involve many steps from cloning the desired gene via selection of a suitable cell type, fermentation and purification to formulation of the end product (Figure 2). A variety of undesired chemical alterations to the drug product

| Table 1. |
|-----------------|-----------------|-----------------|-----------------|
| Chemical drug   | Molecular weight (Da) | Biological drug  | Molecular weight (Da) |
| Glucophage®     | 166             | Neupogen®       | 18 800          |
| Vioxx®          | 314             | Roferon-A®      | 19 625          |
| Prozac®         | 346             | Humatrope®      | 22 125          |
| Zantac®         | 351             | Avonex®         | 22 500          |
| Paxil®          | 375             | Epogen®         | 30 400          |
| Zocor®          | 419             | Pulmozyme®      | 37 000          |
| Augmentin®      | 420             | Enbrel®         | 75 000          |
| Crisivan®       | 712             | Zenapax®        | 144 000         |
| Taxol®          | 854             | Rituxan®/MabThera® | 145 000      |
|                 |                 | Factor VIII     | 264 000         |

Source: EuropaBio.
can occur during this time including oxidation or deamidation. A frequent problem is also the formation of protein aggregates.

Glycosylation is notably sensitive to cell growth conditions. Changes in culture pH, the availability of precursors and nutrients, and the presence or absence of various cytokines and hormones can each affect the extent of glycosylation. In addition, the presence of sialidases and other glycosidases, either secreted or released by dead cells, can cause degradation of a previously intact product.

Differences between EPO preparations

Biological preparations, whether commercial or from a laboratory can, and do, vary markedly from one another. One study revealed marked variation between two laboratory preparations of human urinary erythropoietin and four preparations of EPO [5]. The observed differences in isoform profiles were attributed to differences in glycosylation, as ascertained by a special binding assay developed by the investigators. In a second study, which compared
eight different laboratory EPO preparations by mass spectrometry, it was shown that each sample contained a distinct mixture of isoforms with at least 23 observed glycan structures [6]. Complementary \textit{in vivo} measurements in mice demonstrated that the exact N-glycan structure was a major determinant for EPO biological activity.

Scaled-up commercial production of biologics suffers from the same problems as laboratory preparation. Heterogeneity between 12 commercial EPO samples manufactured outside of the US and Europe was demonstrated by isoelectric focusing (IEF) [7]. The variations in isoform composition can be seen clearly in Figure 3. Another comparability study of biosimilar products manufactured outside the US and Europe revealed that products differed widely in composition, did not always meet self-declared specifications and exhibited batch-to-batch variation [8]. These studies were in turn echoed by a Brazilian study [9], which, in addition, found unacceptable bacterial endotoxin levels in three products.

\section*{Immunogenicity}

Immunogenicity is a critical issue in biotechnologically derived medicines. The risk of immune responses against recombinant proteins was made clear by cases of pure red cell aplasia (PRCA) in patients receiving a brand of EPO approved in Europe. These cases were traced to a minor change in the production process, the consequences of which eluded the rigorous controls in place at the time [10]. Affected patients experienced an antibody-mediated neutralization of endogenous EPO and a complete block in the differentiation of red blood cells. This incident has been instrumental in creating awareness of the importance of careful control of biotechnology drugs, particularly with regard to immune responses in patients.

Immune responses can be elicited by various qualities of recombinant protein preparations. For instance, deglycosylation has previously been associated with immunogenicity in the case of interferon-β by uncovering hydrophobic residues and reducing solubility [11] or in the case of GM-CSF by exposure of antigenic sites [12]. Also, misfolding or aggregation during manufacturing or storage can yield products which cause an immune response. Finally, immune responses can be triggered by impurities. Such impurities may include process-derived chemicals or antibiotics, or may result from microbial or viral contamination [13].

\section*{Can we manage biopharmaceutical heterogeneity?}

Maintaining patient safety and treatment efficacy with recombinant proteins represents a challenge especially with the advent of biosimilars. Therapeutic benefit is critically dependent on a finished drug product of a very high quality. Both biosimilar manufacturers and innovators must ensure consistency in their production. They should have a complete description of their manufacturing process, monitor batch variation through impurity profiling, physicochemical and functional analyses, and maintain a historic database of in-process control data to assess the impact of any manufacturing changes.

Currently, neither analytical methods nor \textit{in vitro} assays are guaranteed to fully characterize a given recombinant protein and there are no methods for predicting clinical efficacy. Animals transgenic for the human endogenous gene equivalent to a recombinant protein could provide useful quantitative models to gauge immunogenicity, but have not yet been validated [14]. Thus, in order to demonstrate unequivocally that a biosimilar has the same efficacy and safety as its branded predecessor, a similarity assessment should include pre-clinical and clinical data. Current drafts
of European guidelines state that the amount of clinical data required depends on the nature of the recombinant protein under consideration [15]. Proteins which have a unique endogenous counterpart with a critical function or which are particularly large and complex (such as mAbs) should undergo extensive clinical testing [16]. Because even phase III trials are typically not large enough to detect adverse events as rare as immune responses, tailored pharmacovigilance plans are also essential.

Conclusion

A change is occurring in the biotechnologically derived drug industry with the impending arrival of biosimilars on the market. Experience with brand products indicates that manufacturing of recombinant proteins must be supported by stringent controls. Recombinant proteins are much more complex molecules than traditional chemical drugs. They require highly elaborate and sophisticated manufacturing processes, and their properties are highly dependent on the process used. Different processes inevitably yield different products, which cannot always be fully characterized by analytical methods, yet may have different clinical safety and efficacy profiles. Current drafts of European guidelines, still under discussion, express a requirement that biosimilar manufacturers provide pre-clinical and clinical data to support a marketing application, and perform quality checks as extensively as innovator companies. For the same reasons, biosimilars should also be accompanied by a pharmacovigilance plan. Future experience with biosimilars will determine how these requirements should be defined.

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