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The Science of Biologics and Biosimilars: Why Proteins Are Different

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Introduction

The Food and Drug Administration (''FDA'') distinguishes between two broad classes of pharmaceutical drugs, the more common ''small molecules'' and the less common ''biologics.'' Small molecules and biologics have different properties and present different manufacturing challenges. Because of these and other differences, small molecules require approval of a New Drug Application (''NDA''), and biologics require approval of a Biologics License Application (''BLA''). The legal framework and procedures for approval of generic versions of small molecule drugs is provided by the Drug Price Competition and Patent Term Restoration Act of 1984, commonly referred to as ''Hatch-Waxman Act.'' The Biologics Price Competition and Innovation Act of 2009 (''BPCI''), a part of the Patient Protection and Affordable Care Act, for the first time provides a legal framework for the approval of biosimilars, which are products similar to, or interchangeable with, corresponding BLA-approved biologics. Because Congress created this separate regulatory framework for biologics predicated on different physiochemical (and thus, pharmacological) properties associated with these two classes of drugs, it is important to understand the basic science behind biologics and how it drives the different regulatory regimes.

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Definition of Biologics, Biosimilars, and Bioequivalents

''Biologics'' are defined as products produced in or by biological systems:

> The term ''biological product'' means a virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein (except any chemically synthesized polypeptide), or analogous product . . . applicable to the prevention, treatment, or cure of a disease or condition of human beings.[1]

''Biosimilar'' or ''biosimilarity'' means:

> [T]hat the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components; and
> that there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product.[2]

''Interchangeable'' or ''interchangeability,'' when referring to a biological product, means:

> [T]hat the biological product may be substituted for the reference product without the intervention of the health care provider who prescribed the reference product.[3]

[1] 42 U.S.C. § 262(i)(1). Prior to enactment of the BPCI, the definition of ''biologic'' in 42 U.S.C. § 262 did not include the term ''protein.'' Despite this, BLAs had been filed for numerous protein pharmaceuticals.

[2] 42 U.S.C. § 262(i)(2).

[3] 42 U.S.C. § 262(i)(3).

The FDA is responsible for establishing the regulatory criteria for defining biosimilarity and interchangeability, and must also decide whether a given drug meets the requirements to be approved as biosimilar to (or interchangeable with) a reference drug.[4] As of May 2014, the FDA has issued five ''Draft Guidance'' documents which provide the criteria for establishing biosimilarity and/or interchangeability.[5]

Of the various categories of biologics (and thus biosimilars), the FDA's primary focus is proteins:

> Although the 351(k) [abbreviated licensure] pathway applies generally to biological products, this guidance ***focuses on therapeutic protein*** products and gives an overview of important scientific considerations for demonstrating biosimilarity.[6]

There are currently dozens of FDA-approved biologic protein pharmaceuticals, with indications in a wide variety of therapeutic areas.[7]

The Complex Structure of Proteins

All molecules exist in three dimensions. However, the three-dimensional structures of proteins, commonly referred to as their ''conformations,'' are considerably more complex and variable than those of smaller molecules because of proteins' greater size.[8] A protein's conformation is critically important to its biological functions. A protein's amino acid sequence is the main determinant of protein conformation. Conformation is also influenced by various chemical modifications/alterations to which proteins are susceptible.[9]

In general, small molecules are synthesized using traditional, highly reproducible methods of chemical synthesis yielding individual molecules that are usually identical to one another[10]; generic versions of a drug under proper manufacturing conditions are typically, similarly identical to the name brand drug. In contrast, biologic proteins are generally produced in cells which, for reasons to be described below, results in considerable intra-molecular variation. Moreover, evaluation of such variations, which can influence the protein's conformation and potentially its pharmacological properties, is difficult and time consuming. For these reasons the Hatch-Waxman Act is inadequate for the approval of biosimilars. In the following sections we provide an overview of proteins' three-dimensional structure, and some of their more common chemical alterations that can occur within the cell in which the protein is synthesized or afterwards.

3-D Protein Structure

Four levels — or orders — of structure contribute to a protein's conformation. The *primary structure* of a protein is its sequence of amino acids, which are held together by peptide bonds. The *secondary structure* represents the three-dimensional twist and folds of a single chain of amino acids which are determined by hydrogen bonds between amino acids within the chain.

There are two main types of secondary structure:

> (1) An *á-helix* is a coil, in which the backbone N-H group of one amino acid is bonded (via hydrogen bonds) to the C=O group of the amino acid four residues before it in the chain;
>
> (2) a *â-sheet* (also referred to as a â-pleated sheet) results from a folding of the chain back onto itself, such that one strand runs in one direction, the other strand in the other direction (the two strands being

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[4] In June 2003, the FDA transferred to the Center for Drug Evaluation and Research (CDER) some of the biological products that had previously been regulated by the Center for Biologics Evaluation and Research (CBER). The therapeutic biological products now under CDER's review include: (1) monoclonal antibodies for in-vivo use; (2) cytokines, growth factors, enzymes, immunomodulators; and thrombolytics; (3) proteins intended for therapeutic use that are extracted from animals or microorganisms, including recombinant versions of these products (except clotting factors); (4) other non-vaccine therapeutic immunotherapies.

[5] Guidance for Industry. Scientific Considerations in Demonstrating Biosimilarity to a Reference Product. February 2012.

Guidance for Industry. Quality Considerations in Demonstrating Biosimilarity to a Reference Protein Product. February 2012.

Guidance for Industry. Biosimilars: Questions and Answers Regarding Implementation of the Biologics Price Competition and Innovation Act of 2009. February 2012. (10 PLIR 173, 2/10/12)

Guidance for Industry. Formal Meetings Between the FDA and Biosimilar Biological Product Sponsors or Applicants. March 2013.(11 PLIR 437, 4/5/13)

Guidance for Industry: Clinical Pharmacology Data to Support a Demonstration of Biosimilarity to a Reference Product. May 2014. (12 PLIR 703, 5/16/14)

[6] Guidance for Industry. Scientific Considerations in Demonstrating Biosimilarity to a Reference product. February 2012, p. 1. (emphasis added).

Prior to enactment of the BPCI, BLAs had been filed for numerous protein pharmaceuticals. As such, the significance of the addition of the term ''protein'' is not clear.

[7] Therapeutic areas include pulmonary; rheumatology; bacterial and other infectious disease; ophthalmology; cancer; graft-vs-host disease; dermatology; diagnostic imaging; gastrointestinal and genetic metabolic disorders; peripheral neuropathy; renal, cardiac, and vascular disorders; viral infections; immunodeficiency disorders.

[8] The molecular weight of small molecules is typically in the range of 300-500, whereas proteins are considerably larger. The FDA defines a protein as an amino acid polymer greater than 40 amino acids in length. Guidance for Industry. Biosimilars: Questions and Answers Regarding Implementation of the Biologics Price Competition and Innovation Act of 2009. February 2012. pg 13. Because the molecular weight of amino acids is, on average, about 100, a protein (per the FDA criteria) will have a molecular weight greater than 4,000. Many proteins have molecular weights around 40,000. Thus, on average, biologics (and their biosimilars) are 10- to 100-fold larger than typical small molecules.

[9] The FDA recognizes the importance of such modifications:

> Unlike small molecule drugs, whose structure can usually be completely defined and entirely reproduced, proteins are typically more complex and are unlikely to be shown to be structurally identical to a reference product. . . . Because even minor structural differences (including certain changes in glycosylation patterns) can significantly affect a protein's safety, purity, and/or potency, it is important to evaluate these differences.

Guidance for Industry. Scientific Considerations in Demonstrating Biosimilarity to a Reference product. February 2012, p. 4.

[10] Many drugs have one or more asymmetric carbons, and can therefore exist in mirror image forms referred to as enantiomers. Most commonly, approved drugs are racemates, which are 50:50 mixtures of the enantiomers. However, some drugs are a single enantiomer.

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connected by a ''loop''). A given protein typically contains multiple á-helices and multiple â-sheets.

The *tertiary structure* results from folding of larger sections of the protein. The sections, each of which contains primary and secondary structures, are held together by any of a variety of chemical bonds (including hydrogen bonds, disulfide bonds, salt bridges, and hydrophobic bonds) between the amino acid side chains.

Many proteins in their functional state are complexes consisting of multiple polypeptide chains, each with its own primary, secondary, and tertiary structure. Each polypeptide chain is referred to as a subunit. *Quaternary structure* refers to the interactions between these subunits. If the subunits are identical, the entire protein is referred to as homodimer (two subunits) or homotrimer (three subunits), etc. If the subunits are different, the protein is then referred to as a heterodimer, heterotrimer, etc.

Cellular-Mediated Modifications of the Protein Chain

Many proteins are chemically modified during (''co-translational'') or shortly after (''post-translational'') synthesis of the polypeptide chain.[11] As noted above, and by the FDA,[12] such changes can influence the protein's biological activity. Because many of these potential modifications occur in eukaryotic cells but not in prokaryotic cells[13], the choice of host cells can dramatically influence the structure of the therapeutic protein. Some of the more important modifications are shown in the following table, which is followed by a more detailed description of these modifications.

[11] Because post-translational modifications occur shortly after translation, such modifications, along with co-translational modifications, will be present on most therapeutic proteins isolated from the cells in which the proteins were synthesized.

[12] The FDA stated:

Unlike small molecule drugs, whose structure can usually be completely defined and entirely reproduced, proteins are typically more complex and are unlikely to be shown to be structurally identical to a reference product. . . . Because even minor structural differences (including certain changes in glycosylation patterns) can significantly affect a protein's safety, purity, and/or potency, it is important to evaluate these differences.

Guidance for Industry. Scientific Considerations in Demonstrating Biosimilarity to a Reference product. February 2012, p. 4. *See also* Guidance for Industry. Quality Considerations in Demonstrating Biosimilarity to a Reference Protein Product. February 2012, p. 7.

[13] Prokaryote cells are those without a nucleus; the most common example is bacteria. Eukaryotes are cells with a nucleus; these include plant cells, animal cells, as well as yeast and fungi.

CATEGORY	MODIFICATION	DESCRIPTION	FUNCTION
Glycosylation		Addition of sugar residues, typically to the extracellular domain of cell surface proteins.	Aids in cell-cell and cell-ligand recognition.
Phosphorylation		Addition of a phosphate group, to either a tyrosine residue, or a serine or threonine residue.	Alters enzymatic activity.
Carboxylation		Conversion of glutamate residues to ã-carboxyglutamate	Typically found in proteins of the blood-clotting cascade.
Membrane Anchoring: Lipidation	GPI Anchor	Addition of a GPI moiety, which serves a bridge between the protein and the cell membrane.	Allows attachment of the protein to the cell membrane.
	Myristoylation	Addition of myristoyl groups to a protein.	
	Palmitoylation	Addition of palmitoyl group to a protein.	
	Prenylation - farnesylation - geranylgeranlyation	Addition of prenyl groups to a protein.	
Sulfation		Addition of a sulfate group to tyrosine residues.	Strengthens protein-protein interactions.
Amidation		Modification of the	Commonly found in

CATEGORY	MODIFICATION	DESCRIPTION	FUNCTION
		C-terminal amino acid of a protein.	certain neuropeptides and hormones.
Investigator-Mediated Alterations	PEGylation	Attachment of chains of polyethylene glycol moieties to a protein.	Increases the *in vivo* half-life of the protein.
Environmentally-Induced Modifications	Denaturation	Unfolding of the protein's secondary and/or tertiary structure.	Typically impairs protein's biological activity.
	Oxidation	Chemical bonding of oxygen to the protein.	Typically impairs protein's biological activity.
	Aggregation	Formation of ''clumps''	Typically impairs protein's biological activity.

Glycosylation

One of the most common alterations, especially of proteins destined for secretion or insertion in the plasma membrane, is glycosylation, which refers to the process in which sugar residues are attached to proteins. Because the sugar residues are usually complex, they are referred to as ''oligosaccharides.'' There are two general categories of glycosylation. In ''O-linked'' glycosylation, oligosaccharides are covalently bonded to the *O*xygen in the R group[14] of the amino acids serine and/or threonine, whereas in ''N-linked'' glycosylation, the oligosaccharides are covalently attached to the *N*itrogen in the R group of the amino acid asparagine. The glycosylation process is, in part, stochastic. As a result, heterogeneity can and usually does exist between otherwise identical proteins, even when synthesized in the same cell. Moreover, different cell types display different glycosylation patterns, the most extreme case being prokaryotes, in which glycosylation does not occur. As a result, the choice of host cell can have a profound influence on the conformation of the expressed protein and can account for a difference between a biosimilar and its reference biologic drug.

Phosphorylation

Proteins, in particular enzymes, carry out myriad functions. For proper cellular functioning, it is necessary to regulate the proteins' activities; one key means by which this is accomplished is via the attachment and detachment of phosphate groups to the protein. Certain enzymes, termed kinases, add phosphate groups to substrates including proteins,[15] while other enzymes, termed phosphorylases, remove phosphate groups. There are two broad classes of protein kinases: (1) Tyrosine kinases which, as the name implies, add phosphate groups to particular tyrosines in a protein, and (2) serine/threonine kinases, which add phosphate groups to serine and/or threonine residues within the protein. Protein phosphorylation is a tightly regulated process, and a number of factors regulate the extent to which a protein, including heterologously expressed proteins, is phosphorylated; these include: (1) the protein itself, (2) the host cell in which it is expressed, and (3) the physiologic state of the host cell.

Carboxylation

Carboxylation is a process in which glutamate residues are converted, post-translationally, to ã-carboxyglutamate. The proteins most commonly carboxylated are those involved in the clotting cascades. Such proteins, which can now be produced in heterologous expression systems, are of considerable therapeutic utility in patients with bleeding disorders. As such, differences in carboxylation between a biologic therapeutic and its biosimilar are potentially quite important.

Membrane Anchoring: Lipidation

Many proteins that are destined to be inserted into the cell-surface plasma membrane (or secreted), contain a ''signal sequence,'' which causes the protein to be inserted into a membrane-bound organelle — the endoplasmic reticulum — during translation. Through a successive process of budding and fusion, membrane-bound vesicles containing the newly synthesized protein traverse various organelles, ultimately reaching the plasma membrane to which it fuses.

However, certain protein destined for the plasma membrane lack a signal sequence, and thus do not enter the endoplasmic reticulum during translation. Instead, co- or post-translationally, a lipid-containing moiety is attached to the nascent protein, a process referred to as ''lipidation.'' The lipid portion of said moiety attaches to the bi-lipid plasma membrane, thereby anchoring the protein to it. Attachment to the plasma membrane dramatically alters the protein's function. There are a number of different lipid moieties that can become attached to proteins and, thereby, influence protein function.

GPI Anchor

Certain proteins become attached post-translationally to glycosylphosphatidylinositol (''GPI'') which also binds to the cell membrane. The GPI thereby

[14] The R group of an amino acid is its side chain. Each amino acid has a distinctive side chain.

[15] The activity of kinases themselves is often regulated by phosphorylation.

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serves as a bridge between the protein and the cell membrane.

Myristoylation

In this process, a protein's N-terminal methionine is removed, then a moiety from myristic acid (which contains a 14 carbon chain) is covalently attached (via an amide bond) to a glycine.

Palmitoylation

Palmitic acid, a 16 carbon containing molecule, typically attaches to a cysteine residue, and less commonly to a serine or threonine.

Prenylation

Prenylation (also called isoprenylation), is a process in which a farnesyl molecule or a geranyl-geranyl molecule is attached to the C-terminus of a protein. Farneysl and geranyl-geranyl are both comprised of repeating prenyl groups, and differ in the length of the carbon chain (15 and 20 carbons, respectively). Prenyl groups are hydrophobic, and thereby facilitate attachment of the prenylated protein to the cell membrane.

Sulfation

Sulfation is a process in which a sulfate group is added to tyrosine residues of a protein; this is thought to strengthen protein-protein interactions. Sulfation occurs in eukaryotes but not prokaryotes.

Amidation

Amidation is a two-step process that modifies the C-terminus of certain proteins, wherein the penultimate C-terminal amino acid is glycine. In the first step, the glycine is oxidized to á-hydroxy-glycine. In the second step, the á-hydroxy-glycine is cleaved into the C-terminally amidated peptide. Proteins in which C-terminal amidation is important include certain neuropeptides and hormones.

Investigator-Mediated Alterations

Certain biologic drugs are intentionally modified from their naturally occurring form, usually to improve their pharmacokinetic properties. One of the most common modifications is the covalent attachment of polyethylene glycol, a process referred to as ''PEGylation.''

A patient's immune system is less likely to recognize, and thereby inactivate, a PEGylated protein (as compared to the non-PEGylated protein). In addition, PEGylation reduces clearance of the protein by the kidney. Both processes serve to prolong the amount of time the therapeutic protein remains in the body.

PEG is a polymer, and its exact length varies from molecule to molecule. Moreover, the PEGylaton procedure is inherently variable with regard to which amino acid in the protein becomes PEGylated and the extent to which the protein is PEGylated. These factors result in intra-molecular variability in protein structure and conformation.

''Environmentally''-Induced Modifications

Proteins — both biologics and biosimilars — are susceptible to environmental conditions, including temperature, moisture, formulation, and impurities, amongst others.

Denaturation

The loss of a protein's secondary and/or tertiary structure is termed ''denaturation,'' which can be reversible or irreversible. A familiar example of permanent protein denaturation is that which occurs when an egg is either fried or hard-boiled, whereby heat causes the egg protein albumin to irreversibly unfold. Thus unfolding causes obvious and dramatic changes in the albumin's physical characteristics. Less dramatic changes can occur if a protein is exposed to moderate heat, as might occur during shipping or storage. Denaturation of a biologic medicine can essentially eliminate its intended biological activity.

Oxidation

Like denaturation, oxidation of a protein can alter its biologic activity. Oxidation can occur intracellularly (*e.g.*, during period of oxidative stress), or extracellularly, for example during purification or storage.

Aggregation

Aggregation is a modification whereby protein molecules adhere to one another, forming ''clumps.'' The clumps are commonly insoluble, and are associated with alterations in higher-order protein structure.

The Use of Structural and Functional Assays to Establish Biosimilarity

Because of the greater complexity and variability of candidate generic biologics compared to traditional small molecule generics, the FDA has developed additional guidelines for establishing biosimilarity between candidate generics and the branded biologics which require further testing.

In the case of small molecules, the requirements for demonstrating similarity between an approved drug and a putative generic are well-established. An Abbreviated New Drug Application (''ANDA'') seeking approval of a candidate small molecule generic must contain information to show that the generic is pharmaceutically equivalent (same activity, same strength, same route of administration and same dosage form) and ''bioequivalent to the listed drug.''[16]

The FDA describes the criteria for bioequivalence between a small molecule generic drug and the approved drug as requiring similar pharmacokinetic (''PK'') data (which measure the effect of the body on the drug). The FDA requires that the ''generic version must deliver the same amount of active ingredients into a patient's bloodstream in the same amount of time as the innovator drug.''[17] Typically, the FDA does not require pharmacodynamic (''PD'') studies (which measure the effect of the drug on the body at various concentrations) for small molecule generics).[18]

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[16] 21 U.S.C. § 355(j)(2).

[17] FDA, *Abbreviated New Drug Application (ANDA): Generics*, http://www.fda.gov/Drugs/DevelopmentApprovalProcess/HowDrugsareDevelopedandApproved/ApprovalApplications/AbbreviatedNewDrugApplicationANDAGenerics/default.htm (last updated May 20, 2014).

[18] ''[W]e do not recommend pharmacodynamic studies for drug products that are intended to be absorbed into the systemic circulation and for which a pharmacokinetic approach

However, for biosimilars, the FDA *does* require PD studies (in addition to structural and PK studies) because of (1) the numerous ways in which a proposed biosimilar may differ structurally from the approved biologic, (2) the difficulties in determining such structural differences,[19] and (3) the difficulty in predicting the biologic significance of such structural differences.[20] PD studies may comprise both clinical studies (*i.e.*, those in humans), as well as non-clinical *in vivo* animal studies and *in vitro* assays.

Because it is not always possible, or practical, to measure the ultimate intended biological response to the biological product, the FDA describes the use of ''markers,'' which serve as a proxy for the biologic's intended action. The FDA states:

> The PD marker(s) used to measure response may be a single biomarker or a composite of markers that effectively demonstrate the characteristics of the. product's target effects.
>
> . . .
>
> When determining which markers should be used to measure response, it is important to consider the following:
>
> - The time of onset of the PD marker relative to dosing
> - The dynamic range of the PD marker over the exposure range to the biological product
> - The sensitivity of the PD marker to differences between the proposed biosimilar product and the reference product
> - The relevance of the PD marker to the mechanism of action of the drug
> - The relationship between changes in the PD marker and clinical outcomes.[21]

One example of a biomarker is an enzyme assay, applicable to those biological products which are enzymes.[22] The substrate used in the assay may be the endogenous substrate, or a substrate which has been chemically modified so as to make more readily detectable the action of the enzyme (for example, catalysis) on said substrate. Two useful metrics for enzymatic activity are K_M and V_{MAX}; if either or both differed significantly from those of the approved biologic, it would suggest the existence of important structural differences between the approved biologic molecule and its putative biosimilar.

Other functional assays are well known; the decision of which bioassay/biomarker to use would depend on the particular biological properties of the approved biologic molecule. The question of which studies are required to establish biosimilarity for particular candidate generic biologics, and how much difference is acceptable between the approved biologic and the biosimilar, are certain to be subjects of intense inquiry and much debate because of the nature of biologics' structural variability.

Summary and Conclusions

A considerable number of therapeutic proteins produced in heterologous expression systems have received FDA approval.[23] It is likely this number will increase dramatically in the coming years. The sheer size and complexity of proteins and their variable three-dimensional structures present different, and in most cases greater, challenges for regulatory approval than for small molecules. Moreover, though proteins are typically defined by their amino acid sequence (i.e., their primary structure), they are subject to a variety of chemical modifications which, while not changing the primary sequence, can often profoundly alter their three-dimensional structures (i.e., conformation) and thus their biological activity and other pharmacological properties. Adding to this complexity is the fact that the chemical changes in proteins often create variability within a population of protein molecules which may be difficult to detect and evaluate. For these reasons, the Hatch-Waxman Act's process for approving generic drugs proved inadequate for proteins, necessitating a new process for the approval of biosimilars.

As of June 2014, no biosimilars have been approved in the United States, although approximately 20 biosimilars have been approved in Europe.[24] It is unclear what degree of structural analyses will be required by the FDA for approval of a biosimilar and the extent of structural difference from the reference product that will be deemed acceptable. Because various structural alterations differentially impact a protein's biological activity, it is possible, and perhaps likely, that the FDA will be unable to establish hard and fast rules for all biosimilars resulting in a less predictable case-by-case approach. It seems clear that for the foreseeable future, those seeking approval for biosimilars will face considerable challenges and hurdles.

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can be used to establish [bioequivalence].'' Guidance for Industry. Bioequivalence Studies with Pharmacokinetic Endpoints for Drugs Submitted Under an ANDA. December 2013, p. 7.

[19] ''Because of the complex molecular structure of biological products, conventional analytical methods used for chemical drugs may not be suitable for biological products . . . .'' Guidance for Industry: Clinical Pharmacology Data to Support a Demonstration of Biosimilarity to a Reference Product. May 2014, p. 6.

[20] ''The objective of a well-designed clinical PK and PD study in a biosimilar development program is to evaluate the similarities and differences in the PK and PD profiles between the proposed biosimilar product and the reference product.'' Guidance for Industry: Clinical Pharmacology Data to Support a Demonstration of Biosimilarity to a Reference Product. May 2014, p. 3.

[21] Guidance for Industry. Clinical Pharmacology Data to Support a Demonstration of Biosimilarity to a Reference Product. May 2014, p. 3-4.

[22] One example is Fabrazyme (agalsidase beta), used to treat Fabray's Disease.

[23] *See*, FDA, *Biological Approvals by Year*, http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/BiologicalApprovalsbyYear/default.htm (last updated Feb. 22, 2010).

[24] Generics and Biosimilars Initiative, *Biosimilars Approved in Europe*, http://www.gabionline.net/Biosimilars/General/Biosimilars-approved-in-Europe (last updated Jan. 31, 2014).

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