Contribution of animal studies to evaluate the similarity of biosimilars to reference products

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The European Union (EU) was the first region to establish a regulatory framework for biosimilars, in which animal studies are required to confirm similarity to a reference product. However, animal studies described in European public assessment reports (EPARs) or marketing authorization applications (MAAs) did not identify clinically or toxicologically relevant differences despite differences in quality, suggesting that animal studies lack the sensitivity to confirm biosimilarity. Scientific advice provided learning opportunities to evolve existing guidance. Altogether, the data support a step-wise approach to develop biosimilars that focuses on quality and clinical efficacy of biosimilar. This approach might be more effective and does not necessarily require animal studies, which is also reflected in new EU draft guidance.

Introduction

When small-molecule drugs go off-patent, generic copies are allowed on the market after these copies are shown to be pharmaceutically equivalent to the originator product and their bioavailabilities lie within acceptable predefined limits. For small molecules, pharmaceutical equivalence is established through physicochemical characterization and demonstration of comparable bioavailability, usually in randomized, crossover studies in a limited number of subjects, without the need for animal studies [1]. Medicinal products of recombinant biotechnology are relatively large and complex proteins that are difficult to characterize fully [2]. In addition, biopharmaceuticals are mixtures of closely related molecules, the exact composition of which is dependent on the manufacturing process for the product, resulting in differences in, for example, protein aggregation and glycosylation patterns [3,4]. These differences can influence the pharmacodynamics (PD), pharmacokinetics (PK), or toxicity parameters of the drug [5–8]. Therefore, tailored regulatory requirements have been developed for the authorization of competing versions of biopharmaceuticals, so-called 'biosimilars'. Compared with small-molecule drugs, more studies are required to establish the similarity of the biosimilar in terms of quality, safety, and efficacy, compared with a reference product to obtain marketing authorization [9].

The EU was the first region to adopt legislation that allows the registration of biosimilars based on an abbreviated marketing authorization application (MAA) [10]. Overarching guidelines have been released that lay down quality, nonclinical, and clinical issues for biosimilars [11–13]. In addition, product-specific guidelines have been released [14–22]. In the current overarching guideline on nonclinical and clinical issues of biosimilars, emphasis is placed on performing comparative nonclinical studies that are sensitive enough to detect differences between the biosimilar and the reference product. This case-by-case approach limits the number of animal studies needed compared with a full application (Table 1) [12]. In June 2013, draft guidances relating to the revised biosimilar guidelines were released for consultation in which a risk-based, step-wise approach is suggested, opting for fewer or perhaps even no animal studies [23,24]. However, it remains unclear what the contribution is of animal studies in establishing biosimilarity and how biosimilar guidance has influenced nonclinical studies. Therefore,

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TABLE 1

Animal studies recommended to demonstrate biosimilarity in the current overarching guideline on non-clinical and clinical issues[a]

Type of study	Type of investigation	Necessity of study
PD	PD effect or activity relevant to clinical application	Recommended
Repeat-dose toxicity	Toxicity profile including toxicokinetics and determination of antibody titers, cross-reactivity, and neutralizing capacity	Recommended
Specific concerns	If relevant (e.g. local tolerance), addressed in the same repeat-dose toxicity	Recommended
Routine toxicological studies	Safety pharmacology, reproduction toxicology, mutagenicity, and carcinogenicity	Not recommended unless indicated by results of repeat-dose studies

[a] From [12].

we assessed nonclinical animal study programs of the MAA of all biosimilar products registered in the EU or submitted for authorization until 1 October 2013.

Review inclusion criteria and analysis

EPARs of all biosimilars up to 1 October 2013 were obtained from the website of the European Medicines Agency (EMA, http://www.ema.europa.eu). Data from the EPARs were supplemented with data from MAAs and scientific advice for registered biosimilar products. MAAs and scientific advice were obtained from the database of the Medicines Evaluation Board (CBG-MEB, Utrecht, The Netherlands). Nonclinical studies were tabulated and categorized by type of study and inclusion of a reference group. Categories for study type were ordered by studies recommended in the biosimilar guideline, including PD, repeat-dose toxicity, and local tolerance or studies that were not recommended in the biosimilar guidelines, such as single-dose toxicity, safety pharmacology, repeat-dose PK or toxicokinetics, developmental and reproductive toxicity studies, including embryo–fetal development and peri- and postnatal development, immunogenicity studies, and special toxicity studies. Only scientific advice pertaining to the nonclinical development or animal studies was included. The manufacturer's questions and the responses of the Committee for Medicinal Products for Human Use (CHMP) were first filtered for nonclinical questions. Nonclinical questions were categorized as either pertaining to the sufficiency of the nonclinical program, its design, or other regulatory requirements. The answers of the company were compared with the response of the scientific advice working party.

Overview of biosimilar applications

Seventeen biosimilars were registered in the EU up to 1 October 2013. With the exception of Remsima™ and Inflectra™, these were all copies of endogenous proteins. Several biosimilars are registered under different trade names and, effectively, nine biosimilar applications have been developed (Table 2). Alpheon® (interferon α-2a) was refused market entry and the MAA for Insulin Human Rapid Marvel® (insulin) was withdrawn by the manufacturer [25]. The manufacturer of Epostim® (epoetin) withdrew its biosimilar application following a request from CHMP for additional data [26]. Filgrastim Ratiopharm® (filgrastim) and Valtropin® (somatropin) received marketing authorization, but were withdrawn from the market for commercial reasons in April 2011 and May 2012, respectively. Neither of these products was marketed in any EU country [27,28].

The submission of the MAA of XM-02 was intended as a full application, but was registered in the EU as a biosimilar. For the MAA of LBD-009, the manufacturer referred to the biosimilar guideline for the development of their product [29]. However, the submitted nonclinical package was a full application containing a safety pharmacology study in nonhuman primates, developmental and reproductive toxicity studies, single- and repeat-dose PK and toxicity studies (in a rodent and a nonrodent animal model), and genotoxicity and antigenicity studies. Extensive direct comparisons between Valtropin and its reference product were not done on the basis of comparable pharmacological activity in parallel PD studies. LBD-009 and XM-02 were registered in the USA via a new drug application, which could explain the full package of nonclinical studies. Valtropin was also marketed in Korea. Omnitrope® (somatropin) was registered in the USA via a biological license application. In a pre-investigational new drug (IND) meeting with the US Food and Drug Administration (FDA), an abbreviated *in vivo* package was justified, which was also submitted to support approval for this drug as a biosimilar in the EU. Infliximab and insulin glargine have both been submitted as a biosimilar in the USA [30,31].

In vitro comparability

Most study programs included both *in vivo* and *in vitro* characterization of the biosimilar to support marketing authorization. In addition to the thorough physicochemical characterization of the biosimilar protein required to evaluate the quality of the product, *in vitro* receptor-binding assays and biological activity assays were performed for HX-575, SB309, EP2006, PLD-108, XM-02, and CT-P13 (Table 3). Similarity of the biosimilar to the reference product of both receptor-binding affinity and *in vitro* potency measured by various assays was established for all these products. For the development of the biosimilar CT-P13, extensive *in vitro* testing to establish PD biosimilarity was done exclusively *in vitro*.

Use of animals

In total, 7590 animals were used in 72 studies for nine distinct nonclinical biosimilarity exercises, including studies required according to the European or United States Pharmacopoeia monographs. Pharmacopoeial assays to determine potency or PD were often extensive, including multiple batches over multiple dose ranges and controls or reference standards requiring the use of a large amount of animals. A median of four (interquartile range, IQR 4–9) animal studies was done to support demonstration of

TABLE 2

Approved, refused, and withdrawn biosimilar applications in the EU up to 1 October 2013 by trade name, active substance, and development code

Trade name	Active substance	Development code[f]	Status	Authorization date	Refs
Omnitrope®[a]	Somatropin	EP2000	Authorized	12 April 2006	[49]
Valtropin®[b,c]	Somatropin	LBD-009	Withdrawn	24 April 2006	[29]
Binocrit®	Epoetin alpha	HX-575	Authorized	28 August 2007	[50]
Abseamed®	Epoetin alpha	HX-575	Authorized	28 August 2007	[51]
Epoetin alfa Hexal®	Epoetin alpha	HX-575	Authorized	28 August 2007	[52]
Retacrit®	Epoetin zeta	SB309	Authorized	18 December 2007	[34]
Silapo®	Epoetin zeta	SB309	Authorized	18 December 2007	[53]
Ratiograstim®[d]	Filgrastim	XM-02	Authorized	15-9-2008	[32]
Biograstim®	Filgrastim	XM-02	Authorized	15-9-2008	[54]
Tevagrastim®	Filgrastim	XM-02	Authorized	15-9-2008	[55]
Filgrastim Ratiopharm®[c]	Filgrastim	XM-02	Withdrawn	15-9-2008	[56]
FilgrastimHexal®	Filgrastim	EP2006	Authorized	6-2-2009	[57]
Zarzio®	Filgrastim	EP2006	Authorized	6-2-2009	[58]
Nivestim®	Filgrastim	PLD-108	Authorized	8-6-2010	[59]
Remsima™[e]	Infliximab	CT-P13	Authorized	10-9-2013	[60]
Inflectra™[e]	Infliximab	CT-P13	Authorized	10-9-2013	[61]
Ovaleap®	Folitropin	XM-17	Authorized	27-09-2013	[62]
Alpheon®	Interferon	NA	Refused	Not approved	[63]
Insulin human long, rapid and 30/70 mix Marvel®	Human insulin	NA	Withdrawn	Not approved	[25]
Epostim®	Epoetin alpha	NA	Withdrawn	Not approved	[26]

[a] Marketed in the USA as Omnitrope® (somatropin) via a new drug application.
[b] Marketed in the USA as Valtropin® (somatropin) via a new drug application.
[c] Withdrawn in the EU for commercial reasons.
[d] Marketed in the USA as tbo-filgrastim (filgrastim) via a biological license application.
[e] Submitted to the FDA under the new biosimilar pathway.
[f] NA, not applicable.

TABLE 3

Results of an *in vitro* similarity exercise comparing approved biosimilar applications with for all registered biosimilar products[a]

Product (development code)	Similar?	Results
EP2000	NA	No *in vitro* potency assays were available, only *in vivo* bioassays
LBD-009	NA	No *in vitro* potency assays were available, only *in vivo* bioassays
HX-575	Yes	Characterization of receptor binding and signal transmission; dose response curves of reference and biosimilar were similar
SB309	Yes	Receptor binding, proliferation, and second messenger activation were evaluated; similarity at the level of receptor binding as well as in functional assays was demonstrated
XM-02	Yes	Relative receptor binding and biological activity showed similar binding affinities of the biosimilar and reference product and were equally effective in inducing cellular proliferation
EP2006	Yes	*In vitro* potency of the product samples was comparable to that of the reference product; comparative receptor binding studies showed similar affinity to the receptor
PLD-108	Yes	A bioassay and receptor binding assay was performed to evaluate PD; receptor-binding affinity and effects of the biosimilar and the reference product were similar
CT-P13	Yes	33 *in vitro* studies were done and included comparative tissue and species cross-reactivity studies, receptor-binding affinity assays, TNFα-neutralizing capacity assays, and assays to investigate FcγRI, FcγRII, and C1q complement binding; CDC, ADCC, and apoptosis activation were also investigated. In all studies, the biosimilar and its reference product were comparable. Binding affinity of the biosimilar to FcγRIIIa was not significantly comparable to its reference product but was not considered to have PD or functional significance
XM-17	Yes	Receptor activity and receptor affinity were similar between biosimilar and reference product

[a] *Abbreviations*: ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; FcγRI, Fc γ receptor I; FcγRII, Fc γ receptor II; FcγRIIIa, Fc γ receptor IIIa; NA, not applicable; TNFα, tumor necrosis factor α.

comparability, with a minimum of three and a maximum of 24. For all but one product, the nonclinical studies described in the dossier were also found in the EPARs. Most animals used for the *in vivo* studies were rodents (rat and mouse). Dog was predominantly used as a nonrodent species and, to a lesser extent, guinea pigs and rabbits were also used. For two products, six studies included nonhuman primates; 104 cynomolgus and 12 Rhesus monkeys.

Pharmacological similarity and safety *in vivo* of approved, refused, and withdrawn biosimilar applications

Pharmacological biosimilarity was almost always demonstrated (Table 4). Only in the case of XM-02, one PD study in a rodent disease model did not show similar potency between the biosimilar and reference product. However, there was a tendency towards comparable potencies in terms of the *in vivo* biological activity [30]. In animals receiving low- and high-dose biosimilar filgrastim, male animals had statistically significant lower CD4+ counts at the end of the first period compared with males treated with the reference product. However, in repeat dose studies, the results were similar.

Biosimilar products were well tolerated. Local tolerance studies generally did not include a reference group, but for those studies that did, no differences were observed. For CT-P13, local tolerance parameters were incorporated in the repeat-dose toxicity studies. Safety pharmacology, although not recommended in the current guideline, had been performed for three products, in one case comparing the product with positive controls.

Safety profiles of biosimilars obtained from repeat-dose toxicity studies showed dose-dependent expected pharmacology-mediated

TABLE 4

Similarities and differences in PD and repeat-dose toxicity studies of approved biosimilars and their comparators[a]

Product (development code)	Study	Similarity demonstrated?	Note
EP2000	PD	Yes	No significant difference
EP2000	RDT	Yes	Comparable to other products on the market
LBD-009	PD	Yes	Potency of the biosimilar was within the established limits, although the overall potency was lower for the biosimilar, which was considered to be a result of the variability of the PD assay
LBD-009	RDT	No comparator	Primate studies had high intergroup variability, although no ADA was detected
HX-575	PD	Yes	No differences in PD
HX-575	RDT	Yes	Comparable PD effects, no unexpected findings or differences in safety profile; marked increase in LDH in male dogs given biosimilar at week 13
SB309	PD	Yes	Increase in PD marker without consistent differences in PD effects and within 80–125% of stated reference potency; marked within-group variability was observed in all drug treated groups
SB309	RDT	Yes	Substantial treatment-related mortality in the high-dose groups of both biosimilar and reference compound that was comparable; dosing had to be stopped before the end of the study; toxicology findings were comparable, but biosimilar caused slight reduction in urinary specific gravity and significant increase in urine volume in males after six weeks compared with the reference
XM-02	PD	Yes	In a PD animal model of disease, possible differences in potency were measured; statistical meta-analysis revealed inconsistencies; both biosimilar and reference product induced expected pharmacological effects to a similar extent in the animal disease model with a tendency towards comparable potencies; in healthy animals, low- and high-dose biosimilar-treated male animals had a statistical significant lower mean outcome for a PD marker at the end of the first period compared with males treated with the reference product
XM-02	RDT	No comparator	In a 28-day immunogenicity study with rats, a higher ADA response to the biosimilar was observed compared with the reference product in the lowest dose at the end of the study, but a lower ADA response in the highest dose was observed at both time points
EP2006	PD	Yes	No differences in PD
EP2006	RDT	Yes	Similar findings were obtained for the biosimilar and reference product
PLD-108	PD	Yes	No differences in PD
PLD-108	RDT	Yes	No differences in toxicology, and observed effects were similar between biosimilar and reference; no unexpected toxicology
CT-P13	RDT	Yes	Comparable kinetics, well tolerated without development of ADA for either the biosimilar or reference product; no test article-related effects or adverse effects on the clinical pathology were observed; nontoxicologically significant effects were observed for both biosimilar and reference products and were comparable
XM-17	PD	Yes	No differences in PD between the biosimilar and reference product, determined *in vivo* using a pharmacopoeial ovary weight gain assay and *in vitro* in a functional binding assay
XM-17	RDT	Yes	Observed effects were similar in quality between the biosimilar and reference product, but slight quantitative differences were seen, which were considered incidental; kinetics and local tolerance were comparable

[a] *Abbreviation*: RDT, repeat-dose toxicity study.

adverse effects (Table 4). The repeat-dose studies never resulted in unexpected or new toxicities for the biosimilar or the reference product. One manufacturer also examined safety via the oral route.

The safety profiles of biosimilars were generally considered similar in incidence and response to that of the reference product. Only in a few cases were minor differences observed. In male, but not female, dogs treated with HX-575, there was a marked increase in lactate dehydrogenase (LDH), a marker of hemolysis. Male, but not female, rats treated with SB309 had slight reductions in urinary specific gravity and significant increase in urine volume and there was a significant difference in values for reticulocytes and red blood cells between animals in the high-dose groups of the biosimilar and reference product after six weeks. These results were not seen in dog and it was concluded that it was likely the result of higher potency of the biosimilar product. Variability inherent to animal studies was also considered as a source of these differences, which were not considered to be toxicologically meaningful [32].

The assessment of antibody formation to the biosimilar during repeated administration was assessed and, in general, the incidence of (neutralizing) anti-drug antibody (ADA) formation was similar for the biosimilar and its reference product. In XM-02-treated animals, there was a higher ADA response in the lowest dose at the end of a repeat dose study compared with the reference product, but a lower ADA response in the highest dose compared with the reference products. It was thought that this was probably a reflection of normal interanimal variability, rather than being a real difference in immunogenicity between the two products. For XM-17, higher antibody titers in mid- and high-dose animals might have led to lower serum follicle-stimulating hormone (FSH) levels compared with the reference product at the end of a 28-day study. However, given that immunogenicity in animals is not considered predictive, the differences were not thought to be relevant for the evaluation of biosimilarity.

Except for CT-P13, every nonclinical program included at least one *in vivo* PD study with a reference compound. Repeat-dose studies with or without a reference compound were done for all biosimilar applications and a local tolerance study was conducted for all biosimilar applications except CT-P13 (Table 5). For three biosimilars, the local tolerance study did not include a reference compound group.

Three products were reviewed by CHMP and were either withdrawn by the applicant or refused by CHMP. Alpheon (interferon alfa 2a) was refused marketing access on the European market. The major objections to the claim of biosimilarity were based on the quality and clinical dossier. Nonclinical studies were also evaluated, but not considered to be a major objection. Animal data were not considered to enable a clear conclusion regarding similarity of the biosimilar and the reference product because of several deficiencies in the submitted four-week repeat-dose toxicity study in monkeys. Few, and only female, animals were used to populate the study groups, sampling times were far apart, and only one dose group for the comparator was included in the study. Therefore, differences in dose–response effects were difficult to evaluate. Nevertheless, various endpoints, such as toxicology, biological activity, PK, immunogenicity, safety pharmacology, and local tolerance, were included in the study design. Differences in safety profiles were observed and were related to food consumption being lower in the reference group, and some clinical hematology and chemistry values were changed only in the high-dose biosimilar group and the reference group, but not the lower-dose biosimilar group corresponding with the reference dose. In addition, circulating interferon levels were higher for the biosimilar than for the reference product [33].

TABLE 5

Types of animal study conducted to support the biosimilarity exercise

Type of study	Programs that included a study (%)	Number of studies (total)
Recommended[a]		
PD[b]	8 (88%)	16
Repeat-dose toxicity	9 (100%)	17
Local tolerance	8 (88%)	9
Not recommended[a]		
Single-dose toxicity	3 (25%)	9
Safety pharmacology	3 (25%)	6
Single-dose PK	2 (13%)	2
Repeat-dose PK	3 (38%)	5
Developmental and reproductive toxicity studies[c]	1 (13%)	4
Special toxicity	1 (13%)	1
Immunogenicity and/or antigenicity	2 (25%)	4

[a] Recommendations based on the current European guidelines on similar biological medicinal products containing biotechnology-derived proteins as active substance: nonclinical and clinical issues [12].
[b] Including PD studies according to the European or United States Pharmacopoeia.
[c] Including embryo–fetal development and peri-postnatal development.

Biosimilar insulin, produced by Marvel, was withdrawn by the manufacturer because there were major objections based on quality and clinical data. The nonclinical data package included extensive *in vitro* PD studies in isolated cell lines to evaluate binding activity, functional binding, and activation of downstream proteins. However, the justification for the selection of the assays, the description of the methods, and the interpretation of the results were insufficient to prove similarity. Animal studies comprised single- and repeat-dose toxicity studies in rodent and a local tolerance study in rabbit. Marvel insulin was well tolerated without unexpected or new toxicity, but no kinetics were performed, blood glucose was infrequently measured, and relative immunogenicity was not assessed. Taken together, additional justification, clarification, and/or data would be needed to support the claim of biosimilarity [25].

The MAA of Epostim® was withdrawn by the manufacturer after major objections were raised by CHMP. The epoetin copy was not considered to be comparable to the reference product in terms of quality, and consistency of manufacturing was not demonstrated. In a pharmacopoeial normocythemic mouse study to measure potency, the reference potency was higher, although the specific activity was lower compared with Epostim®. Repeat-dose studies in rat and dog showed different PK parameters and potential formation of neutralizing ADA in one species, but this was not characterized. Overall, the studies did not reveal significant differences in PK. Safety profiles of the biosimilar and the reference products were similar but the mortality of animals in the biosimilar groups was higher. Major objections were the overall design

of the study, such as the incorporation of too few animals and the lack of an immunogenicity assessment, which is required according to the guideline. It was recommended not to request additional nonclinical clarification until the major quality issues were resolved.

Scientific advice of the EMA during biosimilar development

All but one of the nine products specified that scientific advice had been sought on nonclinical issues in their MAA or the EPAR. One manufacturer asked about the necessity for any preclinical studies because most endpoints could also be studied in humans [34]. The sufficiency of the nonclinical testing strategy was also discussed for all other programs that requested scientific advice and was generally considered adequate, but refinement of the development program was the result of scientific advice in several cases. Most nonclinical programs were considered sufficient by the regulatory authorities. In several cases, additional comments and suggestions to improve the studies were made. For instance, when one manufacturer suggested using only one dose group of the reference compound, CHMP considered it necessary to include more than one dose because the purpose of the repeated-dose toxicity study was also to compare PK, PD, and unexpected toxicity. Establishing a dose response was considered important for a proper comparison, although for PK a single-dose group would suffice. Nevertheless, the recommendation was to study the PD of the reference compound at three dose levels similar to the test compound. Another example concerns a manufacturer of a similar product who suggested using only two dose groups for their repeat-dose toxicity study, which was accepted by CHMP.

Manufacturers have also overestimated regulatory expectations. This is illustrated by a case where a 13-week repeat-dose toxicity study in dogs had been conducted and was considered adequate to fulfill the regulatory requirements. Despite this, the regulatory authorities commented that a four-week rat repeat-dose toxicity study would have also produced data of equal relevance.

Other questions in scientific advice dealt with more specific issues related to study design, regulatory requirements, or suggestions to reduce regulatory requirements. Additional *in vivo* studies were requested for four products and manufacturers were always compliant with these requests.

Discussion

The MAAs of all registered, refused, and withdrawn biosimilars were used for this study. Our results show that none of the animal studies submitted within biosimilar applications that were subsequently approved for marketing authorization showed relevant differences to the reference product. The clinical relevance of the differences that were detected is questionable, because no clinical correlates were observed. Differences also did not preclude clinical trials and, invariably, animal data supported the claim of similarity for marketed biosimilars. Previously, Schellekens and Moors have shown that, even in products with known quality differences between the reference and biosimilar product in terms of level of impurities, host cell type, formulation, or post-translational modification, animal studies supported biosimilarity whereas differences were observed in clinical PK profiles [35]. In addition, considerable differences in potency, content, and isoform profile have been reported for several marketed epoetins, including biosimilar epoetins [36]. Therefore, animal data contributed little to the overall biosimilarity evaluation. Animal studies are unlikely to be sensitive enough to detect subtle differences between products or to translate them into measurable endpoints because of the inherent variability of animal models and the variability of assays. When differences in quality between the reference product and biosimilar application are identified, these are not always reflected in subsequent animal and clinical studies. Quality issues are more likely to determine whether a product can be considered as a biosimilar. Therefore, focusing on, and refining methods to improve, physicochemical characterization of proteins might be an important determinant of success in establishing biosimilarity between products [37,38]. The importance of quality is illustrated by the refusal of Alpheon® and the withdrawals of Epostim® and Marvel insulin.

The first guidelines came into effect in the EU in 2004. In the early regulatory guidelines, relatively extensive animal studies were considered essential to determine similarity in potency, pharmacological effects, and safety of new biosimilars. However, most, if not all, companies started the development of their biosimilar before the first biosimilar guideline appeared. Nonclinical strategies focused on existing guidelines for biopharmaceuticals, such as ICH S6, common scientific sense, and scientific advice of regulatory authorities [39]. Scientific advice was important in increasing the regulatory knowledge on biosimilar applications, and is increasingly being sought for the development of biosimilar monoclonal antibodies [40]. Scientific advice was sought for almost all biosimilar products and the nonclinical program of the manufacturer was the starting point of these discussions. Questions predominantly focused on whether the nonclinical study package was sufficient. The responses of the regulatory authority were generally affirmative, although in some cases additional studies were requested. Manufacturers have proposed omitting certain animal studies, using a single species, route of administration, or fewer dose groups. In some cases, this resulted in reduced, scientifically justified, nonclinical development plans. Therefore, scientific advice enabled companies to align their development strategies with regulatory expectations. In addition, the regulatory dialog was driven by experience of the pharmaceutical industry in biosimilar development and has increased the knowledge of regulatory authorities. This has facilitated the revision of the current biosimilar guidelines.

In 2010, a draft guidance on nonclinical and clinical issues for the development of biosimilar monoclonal antibodies appeared, which was approved in 2012 [20]. The basis for this guidance is a step-wise approach, where the necessity for animal studies depends on the need for additional information. The use of nonhuman primates in repeat-dose toxicity studies is not recommended and neither are studies in nonrelevant species. However, most monoclonal antibodies are only pharmacologically responsive in humans or nonhuman primates. Therefore, in theory, most *in vivo* studies are no longer recommended. This is reflected in the development of CT-P13, where the selection of a relevant animal model was the main challenge. However, similar to other biosimilars, the development of this product was also started before the release of the new monoclonal antibody biosimilar guideline, and evaluation of potential off-target effects was evaluated in a

nonresponsive rat repeat-dose toxicity study. By contrast, nonhuman primates have been extensively used to assess the biosimilarity of some rituximab biosimilars [41,42]. In the EU, studies in nonhuman primates might no longer be required, given the new overarching draft guidance for biosimilar development, in which the limited relevance of animal studies is acknowledged [43]. Alternative strategies to evaluate the similarity of new biopharmaceutical copies have also been proposed [35]. Furthermore, it has been suggested that the regulatory demand for comparative clinical studies could be foregone [35]. Comparative clinical trials to demonstrate similarity preclude informative data because of practical consequences, such as undefined acceptance ranges for PK parameters. This further stresses the use of a step-wise, risk-based approach to demonstrate analytical comparability before performing any subsequent studies. Indeed, the regulatory thought process that helped shape the new draft guidance to favor a risk-based approach, where animal studies could be omitted, hinges on thorough physicochemical and biological characterization [44]. The continuing improvements of analytical and *in vitro* studies are likely to further reduce the need to perform animal studies [45].

Concluding remarks

Our data suggest that animal studies are not suited to detect subtle differences between biosimilars and reference products or to translate them into measurable endpoints. This is probably because of the inherent variability of animal models and of assays. The next wave of biosimilars comprises mainly nonhomologous proteins, including monoclonal antibodies. These are species-specific products and the nonhuman primate is often the only available relevant species [46]. However, the relevance of studies with biosimilar applications with nonhuman primates is limited and studies in these or irrelevant animal models are not recommended [44,47,48]. For monoclonal antibodies specifically, the effects that are seen in repeat-dose toxicity studies generally conform to the intended pharmacology. This can also be studied *in vitro*, including the evaluation of binding affinity to the target antigen and Fc receptors, Fab-arm function, Fc-function (ADCC/CDC), and the comparability of these outcomes between biosimilar and reference. Establishing comparability in terms of quality and clinical response are more relevant characteristics of biosimilarity, to which animal studies add little additional evidence. To support biosimilarity from a nonclinical perspective, thorough *in vitro* evaluation should, following a risk-based approach, enable reasoned justification to forego animal studies. Only when unforeseen differences emerge will further evaluation in relevant animal models be warranted. Most biosimilars were developed in the absence of detailed regulatory guidance. Thus, manufacturers faced unknown regulatory requirements before the establishment of detailed guidance on the development of biosimilars. Scientific advice has been an important regulatory tool to streamline nonclinical development programs and increase regulatory knowledge. This process, perhaps as a prime example of learning by doing, was also important in shaping regulatory thinking to improve the biosimilar guidelines. Therefore, early contact with regulatory authorities through scientific advice might improve the efficiency of not only future development strategies, but also regulatory guidelines.

Conflict of interest

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