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REVIEW ARTICLE

Preclinical *in vitro* and *in vivo* models for the assessment of biological activity in biosimilarity studies

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Abstract

A drug that contains a recombinant protein as an active principle is called a biotechnological drug or biopharmaceutical. There are currently over 300 biopharmaceuticals worldwide. Many of these contains a similar active principle (biosimilar drug) as other previously registered (innovator drug). It has suggested that due to the complex implications in a formulation containing a protein, the manufacturing process is a key factor for efficacy and safety requirements. In fact, certain variability has been detected of the protein properties in different lots (or batches) of the same manufacturer, which produce changes at a clinical level. For this reason, the evaluation of biosimilar drugs has acquired great relevance, being the preclinical level of one of the more important stages of the development due to its lower cost (with respect to the clinical level) and its high capacity to detect formulation-manufacture problems. However, the demonstration of comparability at physicochemical, preclinical, and clinical levels is required in order to achieve market registration. In this review the in vitro and in vivo models used for the assessment of proposed biosimilars will be discussed. (Gac Med Mex. 2015;151:351-60)

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Introduction

Currently, biotechnological drugs (also known as biopharmaceuticals) account for an important part of the world's pharmaceutical market. The value of this market was calculated to be 109 billion US dollars in 2012, with 300 commercially available products worldwide[1]. In fact, 7 of the 15 best-selling drugs in the world in 2012 were biopharmaceuticals (Table 1)[2].

The increase in biotechnological drugs sales is mainly owing to their higher efficacy in the treatment of chronic degenerative conditions such as arthritis, cancer or diabetes[3,4]. A logical consequence of the rapid growth of this huge market is that in the past few years, several pharmaceutical companies have focused their efforts on formulating products with active ingredients similar to those of innovator drugs and marketing them after the expiration of innovator drugs' patents.

Since biotechnological drugs have a large-sized recombinant protein with a complex chemical structure as the active ingredient instead of a "small" molecule as in chemically synthesized drugs, some research groups have proposed that demonstrating that a biological drug is equally efficacious and safe than other with the same active ingredient is a complex task, since the synthesis-manufacture process has been detected to substantially affect the final product[5,6].

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Table 1. The 15 best selling drugs in 2012 world-wide

Place in sales	Estimated sales in 2012 (billions of dollars)	Drug	Laboratory	Active ingredient	Indication
1	9.48	Humira*	Abbot Laboratories/ Eisai	Adalimumab	Rheumatoid arthritis, Crohn's disease
2	8.37	Enbrel*	Amgen/Pfizer/Takeda	Etanercept	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis
3	8.00	Advair/Seretide	Glaxo SmithKline	Fluticasone/ salmeterol	Asthma, chronic obstructive pulmonary disease
4	7.67	Remicade*	Johnson & Johnson/ Merck/Mitsubishi Tanabe Pharma	Infliximab	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn's disease
5	6.94	Rituxan*	Roche	Rituximab	Chronic lymphocytic leukemia, non-Hodgkin lymphoma
6	6.65	Crestor	AstraZeneca/ Shionogi	Rosuvastatin	Hypercholesterolemia
7	6.12	Lantus*	Sanofi	Insulin glargine	Diabetes
8	6.08	Herceptin*	Roche	Trastuzumab	Breast cancer
9	5.98	Avastin*	Roche	Bevacizumab	Colorectal cancer, breast cancer, non-small cell lung cancer, renal cell cancer, ovarian cancer, malignant glioma
10	5.55	Lipitor	Pfizer/Astellas Pharma/ Jeil Pharmaceutical	Atorvastatin	Hypercholesterolemia
11	5.38	Abilify	Bristol-Myers Squibb/ Otsuka Holdings	Aripiprazole	Schizophrenia
12	5.11	Plavix	Sanofi/Bristol-Myers Squibb	Clopidogrel	Recent myocardial infarction, recent stroke, established peripheral artery disease, acute coronary syndrome
13	5.01	Cymbalta	Eli Lilly/Shionogi	Duloxetine	Depression, anxiety, chronic pain
14	4.72	Gleevec	Novartis	Imatinib mesilate	Leukemia
15	4.49	Spiriva	Boehringer Ingelheim	Tiotropium bromide	Chronic obstructive pulmonary disease

*Biotechnological drug.

In disagreement with the scheme of bioequivalent drugs, different regulatory entities have started issuing guidelines to assess the comparability of biotechnological drugs, known as follow-on biologics in the European Union[7] and "biocomparable drugs" in Mexico[8] and the USA[9]. In fact, the International Conference on Harmonization (ICH) has updated its guidelines on biotechnological drugs manufacture and evaluation to include biocomparable drugs[10], and the World Health Organization (WHO) made consultations with different drug manufacturers and associations in 2006[11], which ended up with the issue of a guideline in 2009[12]. Other countries, such as India, China, Canada and some Latin American countries have also issued their assessment guidelines for this type of drugs[13]. After the October 2011 reform was implemented[8], Mexico positioned itself as a leader in biotechnological drugs regulation[14], and the first official standard to regulate the granting of registries was issued in 2012[12]. However, this standard was cancelled in 2013, since the standard that regulates bioequivalence (NOM-177-SSA1-2013) now includes biotechnological drugs and biocomparable candidates[16].

In broad terms, we could say that a biocomparable drug is a drug with comparable safety and efficacy to those of the innovator with the same active ingredient. This implies that these drugs have to be assessed prior to their marketing authorization approval. In fact, guidelines issued by the aforementioned regulatory entities contemplate this; therefore, these products must demonstrate their comparability at three levels: physicochemical-structural, pre-clinical and clinical. Nevertheless, given that one of the primary goals of biocomparable drugs development is to reduce the cost of the final product, the first stages (physicochemical-structural and pre-clinical) are the most important because they allow to save resources destined to clinical trials, although they don't substitute them[17,18]. As a matter of fact, clinical trials that have to be conducted to demonstrate the comparability of biocomparable candidates include: pharmacokinetics, pharmacodynamics, immunogenicity, safety, efficacy and drug surveillance studies (according to the molecule in question and the therapeutic indication the registry is required for)[2,7,9,12,13,16].

Physicochemical-structural evaluation is focused on distinguishing differences in physicochemical (isoelectric point [p/], presence of disulfide bonds or molecular weight) and structural properties (such as the peptide sequence, secondary and/or tertiary structure and post-translational modifications) of the protein contained in a biocomparable drug with regard to the innovator[18,19].

On the other hand, preclinical assessment of biocomparable formulations allow for their biological activity (effect) to be measured and compared with the biological activity of the innovator drug. This can be carried out *in vitro* (in cell lines) or *in vivo* (in animal models). At this point, two important considerations have to be taken into account: during the *in vitro* assays, only the effect of the protein contained in the formulations will be observed, whereas in the *in vivo* assays, the effect of the formulation as such will be observed, since the pharmaceutical vehicle contributes to the release-solubilization of the protein and, consequently, to its biological action. The purpose of this review is to address the different preclinical models that, to our knowledge, have been used in the assessment of drugs proposed as biocomparable formulations, in order to visualize their advantages or disadvantages in the face of the development of this type of drugs. Table 2 shows a summary of the preclinical models discussed in the present review.

Selection of articles

Articles published in international indexed journals included in PubMed and/or Google Scholar whose purposes would have been to assess one or more new products and/or biocomparable candidates versus a reference (or innovator) drug were searched and selected. Only *in vitro* and *in vivo* models to determine biological activity or efficacy profiles (pharmacodynamics) were included in the review. Regulatory agencies' guidelines and procedures described in pharmacopoeias were excluded from this review.

Preclinical models in vitro

Recombinant human erythropoietin (EPO)

Park et al.[20] looked into the physical and chemical properties of 12 recombinant EPO formulations marketed in Asia (Korea, China and India), and compared them with those of an innovative formulation produced by Amgen: Epogen®. One of the assays used was intended to determine the *in vitro* potency of the formulations. The procedure consisted in using an EPO-dependent human megakaryocytic leukemia cell line (in-house developed by Amgen); these cells were incubated at 37 °C for 4 h in presence of the analyzed samples and then were treated with a detergent for lysate and added luciferin as a substrate and luciferase enzyme (a reaction where luminescence is produced by the reaction between luciferin, ATP and Mg^{2+}, catalyzed with luciferase). The luminescence resulting from the reaction was measured with a luminometer and the obtained values were used for the determination of relative potency with regard to the innovator formulation.

The obtained results allowed for a high biological potency heterogenity to be elucidated between all different evaluated drugs, which for the most part differed from Amgen's innovator drug.

Tumor necrosis factor (TNF) receptor 2-Fc fusion protein (etanercept)

Maity et al.[21] reported the clonation, the development of an expression vector and the *in vitro* and *in vivo* physicochemical assesment of a non-innovator and an innovator formulation (Enbrel®) of etanercept. In this study, the *in vitro* assessment consisted in conducting a cytotoxicity-neutralization assay using a TNF-sensitive cell line (L929). The L929 cells were incubated

Table 2. Reported preclinical models for biological activity assessment in biocomparability studies

Drug	Type of evaluation	Assay	Subjects	Response variable	Analytical method used	References
EPO	*In vitro*	Cell stimulation	EPO-dependent megakaryocytic leukemia cell line*	Luciferin-luciferase reaction luminescence	Luminometry	Park, et al.[20]
	In vivo	Reticulocyte-production stimulation	Normocythemic mice	Total reticulocyte count	Flow cytometry	Ramos, et al.[31]
Etanercept	*In vitro*	Cytotoxicity neutralization	TNF-sensitive cell line (L929)	MTS conversion into formazan	530-590 nm/490-630 nm spectrophotometry	Maity, et al.[21] Tan, et al.[22]
	In vivo	Articular histopathological level comparison	Heterozygous mice originating from transgenic humanized mice with TNF over-expression	Joint-damage score obtained	Optical microscopy	Maity, et al.[21]
Filgrastim	*In vitro*	Receptor-binding	N/A	Liquid scintillation	Liquid scintillation counter	Skrlin, et al.[23]
		Cell proliferation	Myeloid leukemia murine cell line (NFS-60)	MTS conversion into formazan	570 and 620 nm spectrophotometry	Skrlin, et al.[23], Sörgel, et al.[24]
	In vivo	Neutrophil production	Balb/C mice with ifosfamide-induced neutropenia	Neutrophil count	Flow cytometry	Vanz, et al.[33]
Gonadotropin	*In vitro*	Progesterone production	Leydig tumor cell line (CRL-2065)	Progesterone concentration	Immunoassay	Seo, et al.[25]
	In vivo	Ovulation rhythm	Gonadotropin-imprinted immature rats	Egg count	Optical microscopy	Seo, et al.[25]
		Ovulation rhythm	FSH-imprinted immature rats	Egg count	Optical microscopy	Seo, et al.[25]
		Ovulation rhythm	Mice with androgenic sterilization	Egg count	Optical microscopy	Seo, et al.[25]
Interferon β	*In vitro*	Cytopathic effect reduction	Human glioblastona cell line (2D9)/human adenocarcinoma cell line (A549)	Live cell count	620 nm spectrophotometry	Meager, et al.[26]
	In vivo	Neopterin-production stimulation	Rhesus Monkeys *(Macaca mulatta)*	Neopterin temporary profile	Immunoassay	Hu, et al.[27]
Rituximab	*In vitro*	Protein-binding	Lymphoma cell lines: Ramos, daudi, Raji and K562	Count of cells with drug bound	Flow cytometry	Dorvignit, et al.[28], Visser, et al.[29], Da Silva, et al.[30]
		Competition between test and reference	Ramos lymphoma cell line	Biotinylated rituximab displacement	Flow cytometry	Dorvignit, et al.[28]
		Cytotoxicity	Ramos lymphoma cell line	Live cell count	Flow cytometry	Dorvignit, et al.[28], Visser, et al.[29], Da Silva, et al.[30]
	In vivo	Anti-tumor effect	Mice with SU-DHL-4 and Jeko-1 cell lines xenografts	Tumor size reduction	N/A	Da Silva, et al.[30]
		Effect on B-lymphocyte counts	Cynomolgus monkeys *(Macaca fascicularis)*	B-lymphocyte counts reduction	Flow cytometry	Da Silva, et al.[30]

*In-house-developed cell line, not commercially available.
N/A: not applicable

overnight in 96-well tissue culture plates at 37° C; then, the formulations' protein was added in serial dilutions and the cells were incubated again for 20 h at 37° C. Subsequently, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate (MTS/PMS), a pair of compounds contained in a reagent for cell-viability measurement that only reacts with active mitochondrial reductases and yields a colorful compound known as formazan, were added, and the cells were incubated again for 4 h. Absorbance readings of the wells where the reaction took place ranging from 530 and 590 nm were recorded for the formazan quantification. The formazan concentration is directly proportional to the number of cells alive. The presence of etenercept in the mixture creates a protecting effect against TNF-mediated cytotoxicity. The authors reported a comparable profile between the assessed formulations.

In a comparative study of two etanercept commercial formulations[22], a physicochemical characterization and a bioassay *in vitro* were conducted for the determination biological potency. The experiment was a bioassay similar to that of Maity et al.[21], with differences only in some incubation times (18-22 h instead of 20 h and 2-4 h instead of 4 h) and the wavelength at which the readings were recorded (490 and 630 nm instead of 530-590 nm). The results in this study pointed towards biological activity comparability, in spite of some differences found in the primary sequence and in post-translational modifications. According to the authors, comparability is maintained because the differences found are located in areas of the protein not affecting the interaction with its target.

Granulocyte Colony-stimulating factor (G-CSF) (filgrastim)

In 2010, Skrlin et al.[23] published a physicochemical and biological evaluation study of two filgrastim formulations, one manufactured by Hospira and the other by Amgen: Neupogen® (reference formulation). In this study, two assays *in vitro* are described: one for receptor binding and one for biological activity assessment. In the receptor binding assay, biotinylated G-CSF was added in plates with streptavidine. After several washes were performed and a blocking solution (Bovine Serum Albumin/Phosphate Buffer Saline [BSA/PBS]) was added, a solution was applied at different concentrations of the filgrastim formulation to immediately add G-CSF labeled with iodine-125 and incubate overnight. The plates were washed and a commercial scintillation cocktail was added for measurement in a liquid scintillation counter.

The biological activity assay was based on the stimulating effect of filgrastim for the proliferation of NFS-60 murine myeloid leukemia cell lines, comparing the proliferation obtained with the analyzed products with an international recombinant G-CSF standard produced in yeast to calculate the relative potency. Proliferation was measured spectrophotometrically with the formazan method (described in the etanercept section), but using another substrate (MTT) instead of MTS. The values obtained with the formulations were within the expected range and there were no differences between the analyzed batches.

In another study developed by Sörgel et al.[24], a bioassay similar to that previously reported by Skrlin et al.[23] is described, but without performing a receptor-binding assay. The obtained results allowed for biological activity comparability of the assessed formulations to be found.

Recombinant human chorionic gonadotropin

In this report published by Seo et al.[25], an evaluation was made of the purification process efficiency of a recombinant human chorionic gonadotropin (rhCG), as well as of *in vivo/in vitro* biological potency in comparison with the reference rhCG Ovidrel®. The biological potency *in vitro* assay involved the determination progesterone production in a mouse Leyden tumor cell line (CRL-2065). These cells were cultured in 6-well plates and incubated for 48 h; then, the culture medium was removed and the cells were washed with PBS. The rhCG samples were added to the plates together with the cells and incubated for 2 h; then, the supernatant was collected and the progesterone concentration was measured with a commecial immunoassay kit. The results allowed visualizing that there was no difference between the test and reference rhCG evaluated doses; however, the data obtained for an international rhCG standard were significantly lower.

Interferon-beta

Meager et al.[26] described a model *in vitro* for biological activity measurement of 16 drug batches (7 non-innovator batches and 9 innovator batches: Avonex® and Rebif®), where a cytopathic-effect reduction method

was applied using 2D9 human glioblastoma or A549 human adenocarcinoma cell lines. In this procedure, the cells are incubated in a culture medium adequate for each cell line, together with serial dilutions of the drug and addition of encephalomyocarditis virus (EMCV). After 24-h incubation at 37° C, living cells are counted by previously adding amido-blue black stain and performing spectrophotometric reading of the wells at 620 nm. With the obtained results, mean potency against an international standard and specific potency against the activity reported in the label of the innovator formulation were calculated.

In the same study, a reporter gene assay is also described, which measures alkaline phosphatase secreted to the medium by transfected cells when they are stimulated by the presence of interferon. In this assay, HEK 293 transfected cells harboring alkaline phosphatase deoxyribonucleic acid (cDNA) linked to the interferon-stimulated response promoter. The cells are cultured in 96-well microtiter assay plates together with serial dilutions of the analyzed formulations and incubated for 48 h. Then, aliquots of each mixture's supernatant are transferred to other microtiter plate in the same positions as the previous plate, and a *p*-nitrophenyl phosphate substrate is added; the plates are incubated 2-3 h and absorbance of wells with the supernatant is read at 405 nm. Biological activity of the evaluated batches was heterogeneous; some non-innovator formulations had activity above, others below the activity of the innovator drug batches.

The authors conclude that the values obtained with both methods are very similar, but the antiviral activity assay is extremely complex, laborious and involves many steps in the procedure and, therfore, from their perspective, the best assay is that of the reporter gene, since it is simple and quick.

Other study that included an *in vitro* assay similar to that by Meager et al.[26] was described by Hu, et al.[27]. In this study, an interferon-b-1a pegilated formulation was assessed and compared with a non-pegilated one. The experiment was carried out with an A549 human lung carcinoma cell line and the protecting effect of the formulations was determined in the presence of EMCV. Incubation of the cells with the virus and serial dilutions was for 30 h and, after this incubation, the plates were stained with 0.75% violet crystal in formaldrhide for fixation. The plates were visually inspected to determine the minimal concentration at which the study products were able to protect the cells against the ENCV. To determine the potency, the last positive dilution was multiplied by the limit of detection of the standard and the dilution factor. Specific activity was determined by dividing the potency by the concentration of the sample.

Anti-CD20 antibody (rituximab)

Dorvignit, et al.[28] describe the expression and physicochemical and biological evaluation of an anti-CD20 biosimilar candidate antibody compared with the reference, commercialized by Roche: Rituxan®/MabThera®. First, they measure the binding abilty between the antibody and the CD20 protein of 4 varieties of lymphoma cell lines: Ramos, daudi, Raji and K562. The method consists in incubating the cells together with the antibody, wash out the molecules not bound to cells and then incubate them with an anti-immunoglobulin G antibody (ab') conjugated with fluorescein in order to measure cell labeling with flow cytometry. Subsequently, they carried out a competition assay between rituximab and the test antibody; this assay consisted in incubating cells of the Ramos line together with biotinylated rituximab at constant concentration (5 µg/ml) and the test antibody or unlabeled rituximab at different concentrations; thus, the displacement of biotinylated rituximab was considered as the relative affinity by carrying out this measurement with flow cytometry.

Assays in Ramos cells for the determination of complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), apoptosis and caspase 3 activation testing, as well as decrease in B-lymphocyte counts in *Macaca fascicularis* monkeys (the latter will be described and discussed in the *in vivo* preclinical models section) were carried out to complement the already mentioned binding and competition assays. The authors conclude that this series of assays were adequate to verify the anti-CD20 antibodies biological activity, since by verifying that the binding affinity of both antibodies to cells expressing CD20, as well as to those expressing CDC and ADCC is comparable, the study focused on generating an *in vivo* model as an approach to its efficacy in patients.

In 2013, two other groups[29,30] reported a biological activity characterization based on similar assays to those reported by Dorvignit et al.[28], measuring the ability to bind to CD20, CDC and ADCC, as well as apoptosis (the latter was not measured in the study by Da Silva et al.[30]).

Preclinical models *in vivo*

Recombinant human EPO

Ramos, et al.[31] published on an optimization of the biological assay in normocythemic mice, as well as a comparison between visual methods and flow cytometry for reticulocyte count by evaluating 12 commercially available products, as well as an European recombinant human EPO standard. Randomized groups of male and female mice (n = 8) with a body weight of 27-32 g and an approximate age of 8 weeks were used in the assay. The assay was divided in two phases: one with a single administration and the other with multiple administrations. During the single administration phase, each mouse was subcutaneously (s.c.) given a single dose of the formulations at three different concentrations. Five days after the administration, a blood sample was drawn from the orbital venous sinus for reticulocyte count. In the multiple administration phase, three concentrations, the s.c. route and the same sample-taking method were used; however, administrations were carried out every 24 h each over 4 days and the blood sample was drawn on the fifth day. All samples were drawn between 9 and 11 a.m.

Reticulocyte counts were more accurate and reproducible in females than in males, as well as by using flow cytometry, although this is a more expensive method. The results demonstrated that this assay is adequate to assess and compare EPO formulations, since it enabled to find differences in biological potency for the assessed products.

In other report, Brinks et al.[32] assess the quality of 4 EPO commercial formulations and compare them with an international standard, by using a method similar to that from Ramos et al., differing in that they only use female Balb/C mice; body weight was 17-21 g and n = 6.

TNF receptor 2-Fc fusion protein (etanercept)

In the previously described study by Maity et al.[21], an efficacy preclinical evaluation was also included, performed in 3-week old heterozygous mice originating from a cross of humanized transgenic mice with TNF over-expression (Tg197), which develop arthritis. Several groups were included; one (n = 4) was slaughtered at the beginning and three were twice-weekly intraperitoneally (i.p) administered for seven weeks: a positive control that received Enbrel® (n = 8), a placebo group that received a buffer of the test formulation (n = 8) and an experimental group that received the test formulation (n = 16). A serum sample was obtained by cardiac puncture after 7 weeks of treatment; ankle joints were collected, fixed in formalin, decalcified, embedded in paraffin blocks, stained with hematoxylin/eosin and microscopically analyzed to verify their histopathological status.

There were no significant differences in the score obtained for the experimental and positive control groups; in contrast, the placebo group and the slaughtered group had significantly higher values than the treated groups.

G-CSF (filgrastim)

Vanz et al.[33] published a characterization of a filgrastim formulation that was cloned, expressed and purified by themselves, comparing it with an international standard. In this characterization, one of the assessments was an *in vivo* biological assay in male Balb/C mice (n = 6) of 19-24 g with neutropenia induced with a single dose of ifosfamide on day 0 of the experiment. Subsequently, on day 1 to 4, they were i.p. administered three concentrations of each formulation. 6 h after the last administration, a blood sample was obtained from the venous orbital sinus to microscopically analyze the samples and manually carry out the neutrophil count. This model allowed for a dose-dependent response to be visualized for each formulation, which was not statistically different between all different concentrations of the analyzed formulations. The determinations between each concentration were statistically different and superior to the control.

Recombinant human chorionic gonadotropin

In the assessment by Seo et al.[25], three different multiple-dose *in vivo* assays were used (n = 8): ovulation rhythm in a model of immature rats imprinted with gonadotropin; ovulation rhythm in a model of immature rats imprinted with follicle-stimulating hormone (FSH); and ovulation rhythm in a model of mice with androgenic sterilization.

The first one of them was carried out in Sprague Dawley female rats that were s.c. injected pregnant mare serum gonadotropin for follicle maturation on day 0, and on day 3, the products were assessed for

ovulation induction. The animals were slaughtered on day 4, and the numbers of eggs from the dissected ovarian ducts were counted by microscopic analysis.

The second model was carried out with the same characteristics of the first one, only that in this case, follicle maturation induction of the rats was performed with four FSH injections (one every 12 h). Twelve hours after the last injection, the products were s.c. administered for ovulation induction and the animals were slaughtered 20 h later for egg counts.

For the third model, female mice were used, which were s.c. injected with testosterone proprionate for androgenic sterilization. At 8-9 weeks of the injection, an ovarian polycystic syndrome was induced. Subsequently, the same procedure than in the second model was followed by administering FSH and rhCG and counting the eggs by means of microscopic analysis.

The results obtained with all three models consistently showed the absence of differences between the tested formulation, the reference and the standard. The authors conclude that with all three addressed models, sufficient clinical evidence is obtained to demonstrate the biological activity of rhCG *in vivo*.

Interferon-beta

Hu et al.[27], during the assessment of a pegilated interferon β-1a formulation versus a non-pegilated reference formulation, in addition to the previously described *in vitro* assay, conducted a pharmacokinetics and pharmacodynamics assay in male Rhesus monkeys *(Macaca mulatta)* (n = 5), which received a single administration of the assessed interferon formulations by the s.c. or intramuscular (i.m.) routes. Eleven blood samples were obtained over the 168 h following the dose for neopterin serum determination as a biomarker of pharmacodynamical response. Neopterin was quantified by employing an immunoassay commercial kit. Neopterin serum concentration curves versus time allowed for a mean concentration increase after the dose to be observed, which confirmed the biological activity of the products. No significant differences were found between administration routes and the assessed formulations.

Anti-CD20 antibody (rituximab)

Da Silva et al.[30] describe the development of a proposed rituximab biocomparable formulation; they include some *in vitro* assays (discussed in the *in vitro* models section) and three *in vitro* assays: one with xenografted mice, a pharmacokinetics study and a pharmacodynamics study in cynomolgus monkeys *(Macaca fascicularis)*. The xenograft model was carried out in two differente types of severely compromised immunodeficient (SCID) mice grafted with SU-DHL-4 (n = 50) and Jeko-1 (n = 24-40) non-Hodgkin lymphoma cell lines. The mice were s.c. injected the cell lines on the flank, and those with tumor volumes of 100-150 mm^3 (SU-DHL-4) or 200 mm^3 (Jeko-1) 22 days after the injection were randomized to receive the treatments by the i.p. route, in two different concentrations for the mice with SU-DHL-4 and three for Jeko-1, once-weekly for 4 weeks. The tumors were measured twice-weekly.

The pharmacodynamics model in cynomolgus monkeys consisted in assessing the effect of the administration on total and relative counts of two B-cell populations found in the monkeys: $CD20^{high}CD20^{low}CD21^{-}$ and $CD20^{high}CD20^{low}CD21^{+}$. The treatments were administered by the intravenous (i.v.) route in two modalities: single dose at 10 weeks' follow-up (n = 7) or multiple doses (n = 8) at two different concentrations administered weekly for 4 weeks and with follow-up initially for 4 weeks and then for 6 months (only a subgroup of 4) free of dosing. Cell counts were obtained using an automated system with cell sub-populations immunostaining.

Both models showed results with no significant differences between treatments and with dose-dependent responses at the evaluated concentrations. However, the analysis of the SU-DHL-4 model presented higher model-related heterogeneity associated with the increased follow-up time.

Discussion and conclusions

The growing demand for more efficacious therapies in the treatment of diseases, as well as numerous drug's patent expirations, have prompted the need for accurate assessment models to be created for the development of formulations with high probability of possessing safety and efficacy comparable to those of innovator products, since most of these type of medications are directed towards complex therapeutical indications such as cancer.

The assessment models here described have proven to be able to act as comparison tools during the development of the evaluated drugs; however, it is important to point out that none of the models included in this review has scientifically validated capacity to

predict efficacy at the clinical level, due, in part, to the marked difference between human physiology and that of the used models. This situation can be appreciated by the fact that some of the mentioned products are in development or clinical evaluation phase, while others are already available in the market[34-36]. For example, the Binocrit® EPO formulation, whose physichochemical properties and biological activity *in vivo* are described in the previously discussed study bt Brinks et al.[32], was registered in 2007 by the European Medicines Agency (EMA)[34]. In a report by Brockmeyer et al.[37], a compilation of the physicochemical and preclinical assays conducted for the registry of Binocrit® is presented, as well as a list of clinical trials that were included to substantiate the safety and efficacy of this formulation. One of the Binocrit® clinical studies, published in 2009[38], was a multiple-dose comparative clinical trial in 80 healthy volunteers that assessed the pharmacokinetic and pharmacodynamic profile, with Eprex® as reference drug. Other of the studies, published in 2011[39], assessed the efficacy, therapeutic equivalence (with regard to the reference drug) and safety of the formulations in patients with chemotherapy-produced anemia.

This registration pathway for biocomparable biotechnological drugs has already been travelled many times by regulatory agencies such as the EMA in the European Union and the Food and Drug Administration (FDA) in the USA, and registration of new biocomparable drugs in our country is expected to be carried out this way[40].

In Mexico, regulation started in 2011[8] and culminated in 2013 with the issue of the NOM-177-SSA1-2013 standard[16], provides with a general pathway for assessment and registration of this type of drugs; however, specificity of the guidelines will depend on the experience that could be accumulated in the next few years as a consequence of the regularization of health registrations already in the national market, and registration granting to new drugs that are biocomparable to innovator drugs with gradually expiring patents. In Mexican regulations[16,40], as in international regulations[7,9,12,13], the three mentioned assessment levels are contemplated: physichochemical, preclinical and clinical.

This type of preclinical models allows for the pharmaceutical industry to make important decisions before advancing into clinical trials to demonstrate the efficacy in the treatment of the clinical indications the drug will be prescribed for, as well as its safety.

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