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Survey Report

Evaluation of genotoxicity testing of FDA approved large molecule therapeutics

Satin G. Sawant *, Mark R. Fielden, Kurt A. Black

Comparative Biology and Safety Sciences, Amgen Inc., Thousand Oaks, CA 91320, United States

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ABSTRACT

Large molecule therapeutics (MW > 1000 daltons) are not expected to enter the cell and thus have reduced potential to interact directly with DNA or related physiological processes. Genotoxicity studies are therefore not relevant and typically not required for large molecule therapeutic candidates. Regulatory guidance supports this approach; however there are examples of marketed large molecule therapeutics where sponsors have conducted genotoxicity studies. A retrospective analysis was performed on genotoxicity studies of United States FDA approved large molecule therapeutics since 1998 identified through the Drugs@FDA website. This information was used to provide a data-driven rationale for genotoxicity evaluations of large molecule therapeutics. Fifty-three of the 99 therapeutics identified were tested for genotoxic potential. None of the therapeutics tested showed a positive outcome in any study except the peptide glucagon (GlucaGen®) showing equivocal *in vitro* results, as stated in the product labeling. Scientific rationale and data from this review indicate that testing of a majority of large molecule modalities do not add value to risk assessment and support current regulatory guidance. Similarly, the data do not support testing of peptides containing only natural amino acids. Peptides containing non-natural amino acids and small molecules in conjugated products may need to be tested.

1. Introduction

Drug substances can be divided into two groups – small molecules and large molecules, with molecular weights typically less than 500 daltons and greater than 1000 daltons, respectively. Small molecule drugs can freely enter the cell and nucleus because of their small size and are therefore evaluated for their potential to cause DNA damage as recommended by the regulatory guidance (ICH S2(R1) (2011)). Small molecule drugs make up the majority of the therapeutic market today. However, due to the potential for high target specificity, fewer side effects, longer half-life, and reduced administration frequency, use of large molecule therapeutics is increasing, especially in the treatment of diseases, such as rheumatoid arthritis, multiple sclerosis, and cancer. Large molecule therapeutics, a class of protein- and peptide-based drugs, are manufactured mainly in genetically engineered cells such as microorganisms, and plant or animal cells. However, some large molecules, such as peptides, can be synthesized chemically and may contain non-natural amino acids or other chemical modifications.

Exposure of patients to therapeutics with potential to cause genetic damage is a public health concern as DNA damage can lead to adverse health consequences. Some mutations in somatic cells have been associated with cancer and other diseases, such as accelerated aging, cardiovascular and neurodegenerative diseases, and immune dysfunction (Altieri et al., 2008; Slatter and Gennery, 2010; Scott et al., 2012). Damage to germ cell DNA can cause infertility, spontaneous abortions, and heritable changes in subsequent generations (Aitken and De Iuliis, 2010). Therefore evaluation of novel pharmaceuticals for potential to induce genetic damage is an important component of preclinical safety studies.

Damage to DNA can manifest in various forms, such as point mutations, strand breaks, formation of adducts, and recombination events. As no single assay is able to detect all types of DNA damage, a test battery has been adopted to evaluate the genotoxic liability of pharmaceutical agents. Genetic toxicology testing is essential for safety evaluation of novel pharmaceuticals (ICH M3(R2), 2009) and the ICH S2(R1) guidance document outlines appropriate *in vitro* and *in vivo* tests. The guidance provides two options for the standard genetic toxicology battery that includes an assessment of mutagenicity in a bacterial reverse mutation assay and

* Corresponding author. Address: One Amgen Center Drive, Mail stop: 29-2-A, Comparative Biology and Safety Sciences, Amgen Inc., Thousand Oaks, CA 91320, United States. Fax: +1 805 499 2936.
E-mail address: ssawant@amgen.com (S.G. Sawant).

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0273-2300/

Table 1
ICH-compliant genetic toxicology test battery.

ICH S2(R1) options	Mutation	*In vitro* mammalian cell assay	*In vivo* test	
			First endpoint	Second endpoint
Option 1	•Bacterial reverse mutation test (471)	•Micronucleus test (487) or •Chromosome aberration test (473) or •Gene mutation test (476[a])	•Erythrocyte micronucleus test (474) or •Bone marrow chromosome aberration test (475)	•Not conducted
Option 2	•Bacterial reverse mutation test (471)	•Not conducted	•Erythrocyte micronucleus test (474)	•Comet assay or •Transgenic rodent somatic and germ cell gene mutation assays (488) or •DNA adduct assays or •Unscheduled DNA synthesis test with mammalian liver cells (486)

Titles of the OECD guidance (1997) documents (with the test number in parenthesis) are given for tests recommended in ICH S2(R1) options. The comet assay guideline was approved recently, but no OECD guideline is available for the DNA adducts endpoint.

[a] Multiple gene mutation assays (MLA, HPRT, gpt) from OECD 476 guideline are included in the ICH S2B guidance (1997); however, only mouse lymphoma assay is included in the ICH S2(R1) guidance document.

genotoxicity evaluation in mammalian cells using *in vitro* and/or *in vivo* tests (Table 1). Depending on the therapeutic indication, a positive outcome in any test can stop the drug development program and additional tests may be required to ensure safety of clinical trial participants.

Large molecule therapeutics are not expected to access the cytoplasm or nucleus like small molecule drugs (Torchilin, 2006) and therefore, genotoxicity studies are not applicable to large molecule therapeutics. The regulatory guidance on preclinical safety evaluation of biotechnology-derived pharmaceutical products (ICH S6, 1997) supports this approach. However, there are many examples of marketed large molecule therapeutics where sponsors have conducted tests for genotoxicity either following the standard ICH test battery or using *in vitro* assays. A survey of genetic toxicity studies conducted on large molecule therapeutic candidates collected from various sponsors was published in 1999 by Gocke et al. We conducted a retrospective evaluation of currently marketed large molecule therapeutics approved by US FDA since 1998 and the information was used to derive a data-driven rationale for the current regulatory recommendations and to identify considerations where genotoxicity assessment may be warranted for large molecule therapeutics.

2. Materials and methods

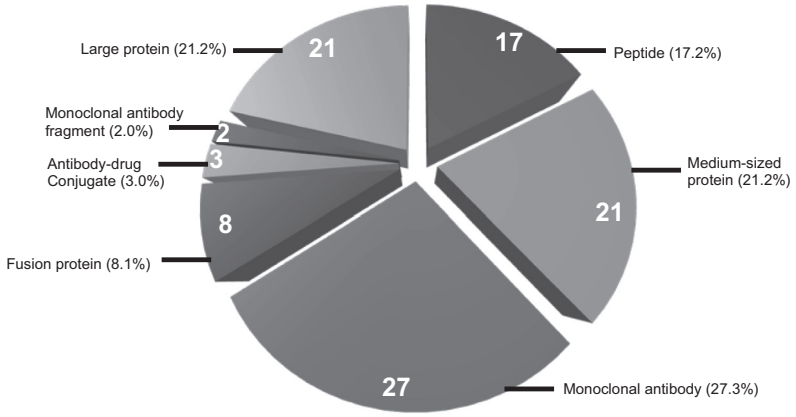
Novel large molecule therapeutics approved for human use by US FDA since 1998 were identified through Drugs@FDA website. The FDA database provides information on approved products regulated by FDA's Center for Drug Evaluation and Research including product labels and links to pharmacology, medical, chemistry, and summary reviews. However, no information is made available for drug candidates that were submitted for regulatory approval but were either not approved by the agency or the sponsor withdrew the application during the approval process. For the purpose of this review a large molecule therapeutic is defined as a protein- or peptide-based therapeutic administered parenterally and that interacts with a target present on or outside the cell. Based on the size and composition, large molecule therapeutics were divided into subgroups including monoclonal antibody (mAb), fusion protein, antibody drug conjugate (ADC), monoclonal antibody fragment, peptide with fewer than 40 amino acids, medium-sized protein with 40 to 200 amino acids, and large protein with more than 200 amino acids such as enzymes and toxins that are not included in any other groups. Gene therapy or antisense products are not covered in this review as they have different composition (DNA or RNA) and target molecules inside the cell.

The Drugs@FDA website was searched by 'Drug approval reports by month' from January 1998 to October 2013 for relevant large molecule therapeutics approved under a Biological License Application (BLA) or New Drug Application (NDA). Regulatory review documents provide summaries of submitted toxicology studies including reviews on genetic toxicology studies at various levels of detail. These documents were reviewed for information on genetic toxicology studies conducted.

3. Results

A retrospective evaluation was conducted to understand the scope of genetic toxicology studies conducted on US FDA approved large molecule therapeutics. A total of 99 novel large molecule therapeutics approved by US FDA for human use since 1998 were identified. Monoclonal antibodies, peptides, medium and large proteins made up 87% of the 99 approved therapeutics, whereas mAb fragments, ADCs, and fusion proteins made up the remaining 13% (Fig. 1).

Interestingly, more than half (53 of 99) of large molecule therapeutics approved have been tested in at least one genotoxicity assay (Table 2). Of the 53 compounds tested, 32 were evaluated in the standard ICH test battery, 12 were tested in *in vitro* assays only, eight were tested in one *in vitro* and one *in vivo* assay, and one was tested in one *in vivo* study. Number and types of genotoxicity studies conducted on these molecules include: 47 bacterial reverse mutation assays, 39 *in vitro* chromosome aberration assays, 19 mammalian cell gene mutation assays, four *ex vivo* rat liver UDS studies, three *in vivo* chromosome aberration tests, and 40 *in vivo* bone marrow micronucleus tests (Table 2). All *in vivo* studies were conducted by clinically relevant routes for the therapeutic molecules, except for oral administration of DM1, the cytotoxic agent in ado-trastuzumab emtansine, in a rodent micronucleus study. Not surprisingly, none of the large molecule therapeutics tested in the genotoxicity assays showed a positive outcome in any study, except glucagon, a 29 amino acid peptide with molecular weight of 3485 daltons. It was reported to be weakly positive in Ames and chromosome aberration assays. However, its mutagenicity in bacteria was attributed to a feeding effect (false positive due to increase in revertants from histidine and tryptophan released from the test protein) and the borderline increases in the *in vitro* chromosome aberration assay were not reproduced in a repeat



Number of US FDA approved large molecule therapeutics since 1998 is shown inside the pie diagram, Percentage of large molecule types is shown next to the corresponding label, Peptides: ≤ 40 amino acid residues, Medium-sized proteins: 41-200 amino acid residues, Large proteins: > 200 amino acid residues.

Fig. 1. Summary of US FDA approved large molecule therapeutic modalities.

Table 2
Genotoxicity studies conducted on FDA-approved large molecule therapeutics.

Large molecule modality	Number of large molecule therapeutics approved	Number of large molecule therapeutics evaluated for genotoxicity	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN	Total tests
Peptide[a]	17	16	16	12	8	1	1	14	52
Medium-sized peptide[b]	21	17	15	14	6	2	2	13	52
Monoclonal antibody	27	8	7	5	1	0	0	4	17
Fusion protein	8	4	3	4	2	0	0	2	11
Antibody-drug conjugate[c]	3	0	0	0	0	0	0	0	0
Whole molecule		0	0	0	0	0	0	0	0
Small molecule		3	2	0	1	0	0	3	6
Linker		0	0	0	0	0	0	0	0
Monoclonal antibody fragment (Fab)	2	1	1	1	0	0	0	1	3
Large protein[d]	21	4	3	3	1	1	0	3	11
Total	99	53	47	39	19	4	3	40	152

CA: chromosome aberration, UDS: unscheduled DNA synthesis, MN: micronucleus.
[a] 40 amino acids or less.
[b] 41–200 amino acids.
[c] Small molecule warheads of all antibody drug conjugates were evaluated for genotoxicity, linkers not tested for genotoxicity.
[d] Proteins (such as enzymes, toxins) containing more than 200 amino acids.

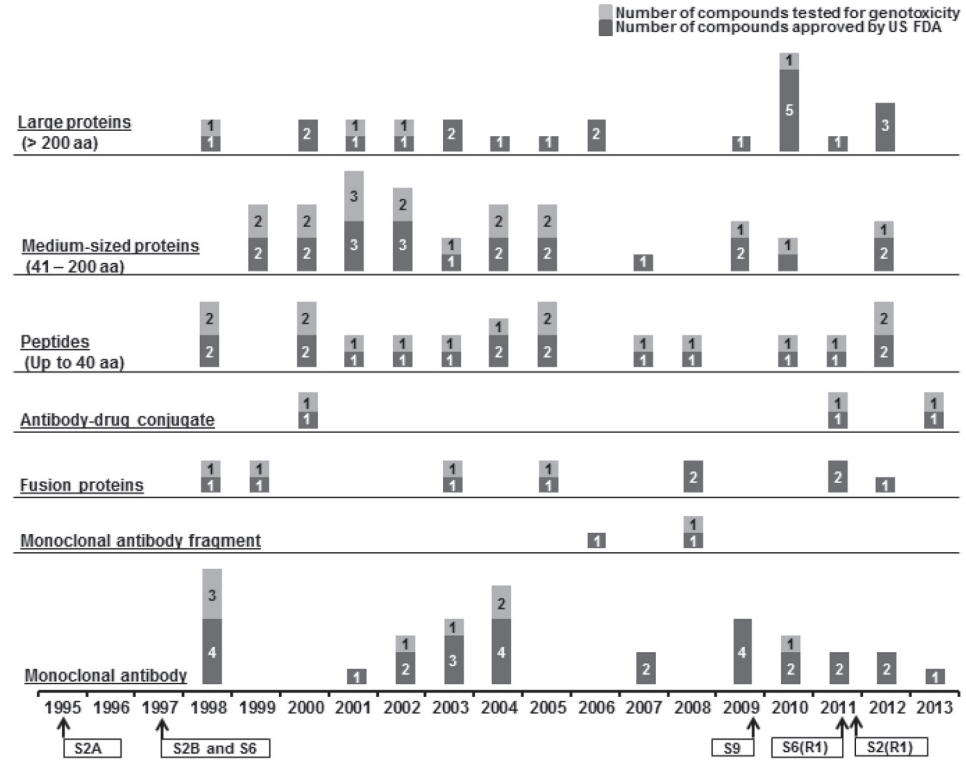
study (Gocke et al., 1999). The US FDA approval timeline for large molecule therapeutics in relation to the implementation of related ICH guideline documents is shown in Fig. 2.

3.1. Monoclonal antibody

A typical antibody is composed of two heavy chains and two light chains and has a molecular weight of about 150 kDa. They are produced by recombinant DNA technology in a single clone of cells or a cell line and consist of identical antibody molecules that bind to a specific protein outside the cell (cell surface receptor or soluble protein). Eight (29.6%) of the 27 mAb therapeutics approved by FDA since 1998 have been evaluated in genotoxicity tests, including bacterial reverse mutation, *in vitro* chromosomal aberration, gene mutation assays, and *in vivo* rodent bone marrow micronucleus tests (Table 3). Two mAb therapeutics (infliximab and trastuzumab) were tested in the standard ICH genetic toxicology test battery, four (basiliximab, omalizumab, natalizumab, and tocilizumab) in *in vitro* assays, and two (adalimumab and cetuximab) in one *in vitro* and one *in vivo* test. None of the mAb therapeutic molecules were positive in any of the genetic toxicology tests. Fourteen of the 27 mAb therapeutics were approved prior to 2004 and seven (infliximab, trastuzumab, basiliximab, omalizumab, natalizumab, adalimumab and cetuximab) were tested for genotoxicity; whereas, from the 13 mAb therapeutic molecules approved after 2004, only two were evaluated for genotoxic potential (Fig. 2). The likely reason for the initial high testing rate could be attributed to initiation of genotoxicity studies prior to 1997; the year ICH S6 guidance (1997) was implemented. Tocilizumab (Actemra®) is the only mAb therapeutic approved after 2004 that was evaluated for genotoxicity. It was tested in two *in vitro* assays and was found to be negative in both tests.

3.2. Monoclonal antibody fragment – Fab domain

The Fab domain (antigen-binding fragment) is a region of antibody that binds to antigen, whereas the Fc domain (fragment crystallization) interacts with various effector cells (macrophages,



ICH Guidance documents:
S2A: Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals (July 1995)
S2B: Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals (July 1997)
S6: Preclinical safety evaluation of biotechnology-derived pharmaceuticals (July 1997)
S9: Nonclinical evaluation for anticancer pharmaceuticals (Oct 2009)
S6(R1): Preclinical safety evaluation of biotechnology-derived pharmaceuticals (June 2011)
S2(R1): Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use (Nov 2011)

Fig. 2. Genotoxicity testing of large molecule therapeutics relative to ICH guidance implementation.

dendritic cells, neutrophils, NK cells, and B cells) and activates the complement cascade. Use of a Fab fragment as a therapeutic molecule instead of a monoclonal antibody avoids binding of the Fc fragment to effector cells and can permit more efficient tissue penetration due to its smaller size (Holliger and Hudson, 2005; Lawson, 2012). Fab fragments can be produced either as recombinant proteins in a variety of expression systems such as microorganisms, mammalian cells, or by enzymatic digestion of antibodies using papain, pepsin, or ficin (Coulter and Harris, 1983; Rousseaux et al., 1983; Backovic et al., 2010). Two Fab fragments have been approved by US FDA since 1998; ranibizumab (Lucentis®) and certolizumab pegol (Cimzia®). Both Fab fragment therapeutics are produced in an *Escherichia coli* expression system. Only certolizumab pegol was evaluated for its potential to cause genetic damage in the standard ICH test battery and was found to be negative in all three tests (Table 3).

3.3. Fusion protein

Proteins with a molecular weight less than 70 kDa are typically eliminated rapidly by renal filtration and have plasma half-lives in the range of minutes to a few hours. Fc fusion proteins are composed of an Fc region of immunoglobulin linked to another protein or peptide (Carter, 2011). The Fc region can confer the IgG-like property of long plasma half-life to proteins and peptides, thus reducing their dosing frequency (Kontermann, 2009). Fusion proteins are created by the joining of two or more genes coding different proteins with the resultant gene producing a single polypeptide exhibiting functional properties of both proteins (Uhlén et al., 1992; Czajkowsky and Shao, 2012). Seven Fc-based and one non-Fc-based fusion protein therapeutics have been approved since 1998. Six of the Fc-fusion proteins, etanercept (Enbrel®), alefacept (Amevive®), abatacept (Orencia®), rilonacept (Arcalyst®), belatacept (Nulojix®), and aflibercept (Eylea®), were created by joining the human IgG1 Fc portion to proteins of therapeutic utility, whereas Romiplostim (Nplate®) is the human Fc-fusion to a peptide therapeutic (Cines et al., 2008). Denileukin diftitox (Ontak®) is the only non-Fc fusion protein wherein an interleukin-2 (IL-2) fusion protein is created by replacing the receptor-binding domain of diphtheria toxin with IL-2 (Eklund and Kuzel, 2005).

Of the eight fusion protein therapeutics approved by the FDA, four (etanercept, denileukin diftitox, alefacept, and abatacept) were evaluated for their potential to cause genetic damage. However, none of the tests yielded a positive result (Table 4). All four fusion proteins tested in genetic toxicology studies were approved prior to 2005 and the genetic toxicology studies may have been initiated and/or completed before the implementation ICH S6 guidance in 1997 (Fig. 2). The sponsor of abatacept did acknowledge that "genotoxicity testing is usually not required for biotechnology-derived pharmaceuticals for it is not expected that these molecules interact directly with DNA or other chromosomal material", but did evaluate the product in *in vitro* genotoxicity tests (Abatacept, 2005). The remaining four fusion protein therapeutics, rilonacept, romiplostim, belatacept, and aflibercept were approved after 2007 and were not evaluated in genotoxicity studies.

Table 3
Genotoxicity studies conducted on mAb and Fab fragment therapeutics.

Large molecule therapeutics	US FDA approval date	Target	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN
Monoclonal antibody therapeutics								
Basiliximab (Simulect)	5/12/98	CD25	Neg	Neg	–	–	–	–
Palivizumab (Synagis)	6/19/98	Respiratory syncytial virus F protein	–	–	–	–	–	–
Infliximab (Remicade)	8/24/98	TNF α	Neg	Neg	–	–	–	Neg
Trastuzumab (Herceptin)	9/25/98	HER2	Neg	Neg	–	–	–	Neg
Alemtuzumab (Campath)	5/7/01	CD52	–	–	–	–	–	–
Ibritumomab tiuxetan (Zevalin)	2/19/02	CD20	–	–	–	–	–	–
Adalimumab (Humira)	12/31/02	TNF α	Neg	–	–	–	–	Neg
Omalizumab (Xolair)	6/20/03	FcεRI	Neg	–	–	–	–	–
Tositumomab, Iodine I 131 Tositumomab (Bexxar)	6/27/03	CD20	–	–	–	–	–	–
Efalizumab (Raptiva)	10/27/03	CD11a	–	–	–	–	–	–
Cetuximab (Erbitux)	2/12/04	EGFR	Neg	–	–	–	–	Neg
Bevacizumab (Avastin)	2/26/04	VEGF	–	–	–	–	–	–
Fanolesomab (Neutrospec)	7/2/04	CD15	–	–	–	–	–	–
Natalizumab (Tysabri)	11/23/04	Integrin α4 subunit	–	Neg	Neg	–	–	–
Panitumumab (Vectibix)	9/27/07	EGFR	–	–	–	–	–	–
Eculizumab (Soliris)	3/16/07	Terminal complement component 5	–	–	–	–	–	–
Golimumab (Simponi)	4/24/09	TNF α	–	–	–	–	–	–
Canakinumab (Ilaris)	6/17/09	IL-1β	–	–	–	–	–	–
Ustekinumab (Stelara)	9/25/09	IL12/23 p40 subunit	–	–	–	–	–	–
Ofatumumab (Arzerra)	10/26/09	CD20	–	–	–	–	–	–
Tocilizumab (Actemra)	1/8/10	IL-6	Neg	Neg	–	–	–	–
Denosumab (Prolia, Xgeva)	6/1/10	RANKL	–	–	–	–	–	–
Belimumab (Benlysta)	3/9/11	B cell activating factor	–	–	–	–	–	–
Ipilimumab (Yervoy)	3/25/11	CTLA-4	–	–	–	–	–	–
Pertuzumab (Perjeta)	6/8/12	HER2	–	–	–	–	–	–
Raxibacumab (Raxibacumab)	12/14/12	Protective antigen	–	–	–	–	–	–
Gazyva (Obinutuzumab)	11/1/13	CD20	–	–	–	–	–	–
Fab fragment therapeutics								
Ranibizumab (Lucentis)	6/30/06	VEGF	–	–	–	–	–	–
Certolizumab pegol (Cimzia)	4/22/08	TNF α	Neg	Neg	–	–	–	Neg

–: No study conducted, Neg: study conducted with negative outcome.

Table 4
Genotoxicity studies conducted on fusion protein therapeutics.

Conjugated protein therapeutics	US FDA approval date	Composition	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN
Etanercept (Enbrel)	11/2/98	Human TNF receptor linked to human IgG1 Fc	Neg	Neg	Neg	–	–	Neg
Denileukin Diftitox (Ontak)	2/5/99	Diphtheria toxin fragments A and B linked to IL-2	Neg	Neg	–	–	–	–
Alefacept (Amevive)	1/30/03	Human leukocyte function antigen-3 linked to human IgG1 Fc	–	Neg	–	–	–	Neg
Abatacept (Orencia)	12/23/05	CTLA-4 homodimer linked to human IgG1 Fc	Neg	Neg	Neg	–	–	–
Rilonacept (Arcalyst)	2/27/08	Two IL-1 receptor domains fused to human IgG1 Fc	–	–	–	–	–	–
Romiplostim (Nplate)	8/22/08	Two thrombopoietin receptor-binding domains fused to human IgG1 Fc	–	–	–	–	–	–
Belatacept (Nulojix)	6/15/11	Human CTLA-4 fused to human IgG1 Fc	–	–	–	–	–	–
Aflibercept (Eylea)	11/18/11	Two domains VEGF receptors fused to human IgG1 Fc	–	–	–	–	–	–

–: No study conducted, Neg: study conducted with negative outcome.

3.4. Antibody-drug conjugate

Antibody-drug conjugates consist of an antibody linked, via an organic linker, to a highly potent cytotoxic agent. The antibody portion targets an antigen that is exclusively or preferentially expressed on cancer cells allowing the conjugate to bind and deliver the anticancer agent to the target cancer cell, thus minimizing its systemic exposure and untoward side effects on other non-antigen bearing cell types (Trail, 2013). Three such therapeutics have been approved by the US FDA since 1998 including gemtuzumab ozogamicin (Mylotarg®) in 2000 for leukemia, brentuximab vedotin (Adcetris®) in 2011 for Hodgkin lymphoma, and ado-trastuzumab emtansine (Kadcyla®) in 2013 for breast cancer. Gemtuzumab ozogamicin was subsequently withdrawn from US market in June 2010 for lack of efficacy (Jurcic, 2012). Calicheamicin, the cytotoxic agent used in gemtuzumab ozogamicin, was tested only in the rat micronucleus test and was found to be positive. The cytotoxic agents used in currently marketed ADCs are monomethyl auristatin E in brentuximab vedotin and a maytansine derivative (N^2-deacetyl-N^2-(3-mercapto-1-oxopropyl) maytansine, DM1) in ado-trastuzumab emtansine. Both cytotoxic agents inhibit tubulin polymerization leading to cell cycle arrest and subsequent apoptosis of the target cancer cells (Firer and Gellerman, 2012). The regulatory submission documents indicate that genotoxicity evaluation was conducted on both cytotoxic agents. Monomethyl auristatin E was tested in the bacterial reverse mutation test, mouse lymphoma cell gene mutation assay, and rodent bone marrow micronucleus tests. DM1 was evaluated for mutagenicity in the bacterial reverse mutation assay and genotoxicity in the rodent bone marrow micronucleus test. Both cytotoxic agents were positive in the rodent bone marrow micronucleus test and negative in the *in vitro* studies. Ado-trastuzumab emtansine (the whole conjugate, T-DM1) was also evaluated for genotoxicity in a non-human primate toxicity study. Bone marrow samples from a repeat dose cynomolgus monkey study with T-DM1 were collected seven days after the final dose (day 155) and evaluated for induction of micronucleated erythrocytes. Though the overall numbers of micronucleated cells were small; the numbers were increased in all drug-related groups compared to the vehicle only control. However, the study was considered inconclusive because exposure of bone marrow to T-DM1 was questionable due to low bone marrow cytotoxicity observed histologically. Also the time lag of seven days between the last dose and tissue collection exceeded the routine exposure time of 24–48 h in *in vivo* micronucleus studies, and the study lacked a positive control group.

3.5. Peptide (up to 40 amino acids)

Peptides are composed of multiple amino acids linked by peptide bonds. They offer potential advantages over small molecule drugs by reducing the likelihood for off-target effects; and advantages over larger proteins with better tissue distribution due to their smaller size (Craik et al., 2013). Therapeutic peptides can be manufactured either by chemical synthesis or using recombinant DNA technology, depending on the number of amino acids it contains. Commercial production of peptides with up to 50 amino acids is now possible with chemical synthesis (Vlieghe et al., 2010), whereas recombinant DNA technology is more efficient for producing longer polypeptides. Chemical synthesis allows incorporation of non-natural amino acids, which have the potential to resist degradation by proteolysis in order to extend the plasma half-life (Werle and Bernkop-Schnurch, 2006; Thayer, 2011). FDA have considered polymers composed of 40 or fewer amino acids to be peptides and not proteins (Guidance for Industry: Biosimilars, 2012) and will typically regulate them as small molecule drugs under the Federal Food, Drug, and Cosmetic Act (FD&C Act). Similarly, all chemically synthesized peptides, regardless of length, are not considered as a "biological product" and are also regulated as small molecule drugs under the FD&C Act. For the purpose of this evaluation, we define peptides as polymers containing 40 or fewer amino acids.

A total of 17 peptides were approved by the US FDA since 1998. Sixteen of these peptides were studied in 52 genotoxicity tests with 14 evaluated in the standard ICH test battery and two (nesiritide recombinant and linaclotide) tested for their mutagenic potential in *in vitro* assays only (Table 5). None of the peptides tested in the genotoxicity assays showed a positive outcome in any study; except glucagon (GlucaGen®), which was reported to be borderline positive in *in vitro* assays. However, the increases in the number of revertant colonies in the bacterial reverse mutation assay seen with the recombinant form, as well as synthetically produced glucagon, were attributed to a feeding effect resulting from histidine and tryptophan release from the peptide (Gocke et al., 1999). The borderline increases reported in the *in vitro* chromosome aberration study were not reproduced in a repeat study (Gocke et al., 1999) and therefore GlucaGen® is considered to be equivocal in *in vitro* genotoxicity studies. The product label states that "the weight of evidence indicates that GlucaGen is not different from glucagon pancreatic origin and does not pose a genotoxic risk to humans" (GlucaGen®, 2012). ChiRhoStim®, a chemically produced human secretin peptide used in the diagnosis of pancreatic exocrine dysfunction, was the only peptide not tested for its genotoxic potential. The sponsor provided 'intended single use of the product' as the justification for not conducting any genotoxicity study (ChiRhoStim®, 2004).

Of the 17 peptides approved since 1998, 11 are composed of all natural amino acids and the remaining six (eptifibatide, cetrotide, bivalirudin, somatuline depot, degarelix, and icatibant acetate) contain at least one non-natural amino acid. All six non-natural amino acid-containing peptides were tested in the standard ICH test battery (a total of 24 genotoxicity tests). None of these peptide molecules showed a positive outcome in any genotoxicity test. These six marketed peptides contain a total of 20 unique non-natural amino acids, including mercaptopropionyl (des-amino cysteine) in eptifibatide; D-3-(2′-naphtyl)-alanine, D-4-chlorophenylalanine, D-3-(3′-pyridyl)-alanine, D-citrulline, and D-alanine in cetrotide; D-phenylalanine in bivalirudin; [cyclo S-S]-3-(2-naphthyl)-D-alanine in somatuline depot; D-2-naphthylalanine, D-4-chlorophenylalanine, D-3-pyridylalanine, (L-hydroorotyl)-4–4-aminophenylalanine, [4-(aminocarbonyl)amino]-D-phenylalanine, N^6-isopropyllysine, and D-alanine in degarelix; and D-arginine, L[(4R)-4-hydroxyprolyl]-glycine, L[3-(2-thienyl)]alanine, D-(1,2,3,4-tetrahydroisoquinolin-3-ylcarbonyl), and L[(3aS,7aS)-octahydroindol-2-ylcarbonyl] in icatibant acetate.

3.6. Medium-sized proteins (41–200 amino acids)

For the purpose of this review, medium-sized proteins are defined as polypeptides containing 41 to 200 amino acids. A total of 21 medium-sized protein therapeutic molecules were approved since 1998 and 17 molecules were tested in 52 genotoxicity tests. Only two medium-sized proteins, tesamorelin acetate (Egrifta®) with 44 amino acids and peginesatide (Omontys®) with 42 amino acids, were chemically synthesized. The remaining 19 were manufactured by recombinant DNA technology. All approved medium-sized protein therapeutics are composed of only natural amino acids. Both chemically synthesized medium-sized protein therapeutics, tesamorelin acetate and peginesatide, were tested in the standard ICH test battery. Of the other 19 medium-sized proteins, nine were tested in the standard ICH test battery, three in *in vitro* assays, and three in *in vitro* and *in vivo* tests. None of the medium-sized proteins showed a positive outcome in any study (Table 6).

Table 5
Genotoxicity studies conducted on peptide therapeutics.

Peptide therapeutics	FDA approval date	Amino acid residues (molecular weight)	Production system	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN
Glucagon [rDNA origin] (GlucaGen)	6/22/98	29 Natural aa (3483 Da)	*Saccharomyces cerevisiae*	Pos[a]	Pos[a]	–	–	–	Neg[b]
Eptifibatide (Integrilin)	5/18/98	Heptapeptide with one non-natural aa (831 Da)	Chemical synthesis	Neg	Neg	Neg	–	–	Neg
Cetrotide (Cetrorelix)	8/11/00	Decapeptide with five non-natural aa (1200 Da)	Chemical synthesis	Neg	Neg	NC	–	Neg	Neg
Bivalirudin (Angiomax)	12/15/00	19 Natural and 1 non-natural aa (2180 Da)	Chemical synthesis	Neg	Neg	Neg	Neg	–	Neg
Nesiritide recombinant (Natrecor)	8/10/01	32 Natural aa (3464 Da)	*Escherichia coli*	Neg	–	–	–	–	–
Teriparatide (Forteo)	11/26/02	34 Natural aa (4100 Da)	*Escherichia coli*	Neg	Neg	Neg	–	–	Neg
Enfuvirtide (Fuzeon)	3/13/03	36 Natural aa (4500 Da)	Chemical synthesis	Neg	–	Neg	–	–	Neg
Secretin Synthetic Human (ChiRhoStim)	4/9/04	27 Natural aa (3039 Da)	Chemical synthesis	–	–	–	–	–	–
Prialt (Ziconotide)	12/28/04	25 Natural aa (2639 Da)	Chemical synthesis	Neg	–	Neg	–	–	Neg
Pramlintide (Symlin)	3/16/05	37 Natural aa (4000 Da)	Chemical synthesis	Neg	Neg	Neg	–	–	Neg
Exenatide (Byetta, Bydureon)	4/28/05	39 Natural aa (4200 Da)	Chemical synthesis	Neg	Neg	–	–	–	Neg
Somatuline Depot (Lanreotide)	8/30/07	Octapeptide with one non-natural aa (1100 Da)	Chemical synthesis	Neg	–	Neg	–	–	Neg
Degarelix (Firmagon)	12/24/08	Decapeptide with seven non-natural aa (1600 Da)	Chemical synthesis	Neg	Neg	Neg	–	–	Neg
Liraglutide (Victoza)	1/25/10	31 Natural aa lipidated peptide (3700 Da)	*Saccharomyces cerevisiae*	Neg	Neg	–	–	–	Neg
Icatibant Acetate (Firazyr)	8/25/11	Decapeptide with five non-natural aa (1300 Da)	Chemical synthesis	Neg	Neg	–	–	–	Neg
Linaclotide (Linzess)	12/30/12	14 Natural aa (1520 Da)	Chemical synthesis	Neg	Neg	–	–	–	–
Teduglutide (Gattex)	12/21/12	33 Natural aa (3750 Da)	*Escherichia coli*	Neg	Neg	–	–	–	Neg

–: No study conducted, Neg: study conducted with negative outcome, Pos: study conducted with positive outcome.
[a] Borderline positive.
[b] Higher incidence of micronuclei in mouse bone marrow study.

3.7. Large proteins

Large molecule therapeutics (such as enzymes, toxins) with more than 200 amino acids that are not included in any groups discussed above were classified as large proteins. Of the total 21 large protein therapeutics approved since 1998, only four were evaluated in 11 genotoxicity assays. Rasburicase (Elitek®) and collagenase (Xiaflex®) were tested in the standard ICH test battery, thyrotropin alfa (Thyrogen®) in bacterial mutagenicity assay, and drotrecogin alfa (Xigris®) in *in vitro* and *in vivo* tests. None of the genotoxicity tests showed a positive outcome for any large protein therapeutics (Table 7).

3.8. PEGylated proteins/peptides

Peptides are prone to enzymatic digestion and high renal clearance resulting in short plasma half-lives (minutes to hours). Conjugating peptides to carrier molecules, such as polyethylene glycol (PEG), is one way to extend the plasma half-life. PEGylation may also make the therapeutic protein invisible to the host's immune system, thus reducing immunogenicity (Werle and Bernkop-Schnurch, 2006).

A total of eight PEGylated large molecule therapeutics have been approved since 1998 (Table 8) and include PEG conjugation to a Fab fragment (certolizumab pegol), six medium-sized proteins (peginterferon α-2B, pegfilgrastim, peginterferon α-2A, somavert, methoxy polyethylene glycol-epoetin β, and peginesatide) and one large protein (pegloticase). Of the eight PEGylated large molecule therapeutics approved, five were tested in 13 genotoxicity studies – three (cetolizumab pegol, peginterferon alfa-2B, and peginesatide) were tested in the standard ICH test battery and two (peginterferon alfa-2A and somavert) in *in vitro* assays. Although mutagenicity studies were not performed with pegfilgrastim, the active peptide (recombinant human granulocyte-colony stimulating factor), was tested and found to be negative for genotoxicity in *in vitro* mammalian test systems. Three of four PEGylated products approved prior to 2003 and two of the remaining four products approved after 2007 were evaluated for genotoxic potential (Fig. 2). None of the PEGylated large molecule therapeutics were positive in any genotoxicity test.

Table 6
Genotoxicity studies conducted on medium-sized protein therapeutics.

Medium-sized protein therapeutics	FDA approval date	Amino acid residues	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN
Interferon gamma-1B (Actimmune)	2/25/99	140 natural aa	Neg	–	–	–	–	Neg
Epoetin alfa (Eprex)	2/25/99	165 natural aa	Neg	Neg	Neg	–	–	Neg
Insulin Glargine (Lantus)	4/20/00	53 natural aa	–	–	Neg	–	Neg	–
Insulin Aspart recombinant (NovoLog)	6/7/00	51 natural aa	Neg	Neg	Neg	Neg	–	Neg
Peginterferon alfa-2B (Pegintron, Sylatron)	1/19/01	153 natural aa	Neg	Neg	–	–	–	Neg
Darbepoetin alfa (Aranesp)	9/17/01	165 natural aa	Neg	–	Neg	–	–	Neg
Anakinra (Kineret)	11/14/01	153 natural aa	Neg	Neg	Neg	–	–	Neg
Pegfilgrastim (Neulasta)	1/31/02	175 natural aa	–	–	–	–	–	–
Interferon beta-1A (Rebif)	3/7/02	166 natural aa	Neg	Neg	–	Neg	Neg	Neg
Peginterferon alfa-2A (Pegasys)	10/16/02	165 natural aa	Neg	Neg	–	–	–	–
Somavert (Pegvisomant)	3/25/03	191 natural aa	Neg	Neg	–	–	–	–
Insulin glulisine recombinant (Apidra)	4/16/04	51 natural aa	Neg	Neg	–	–	–	Neg
Palifermin (Kepivance)	12/15/04	140 natural aa	Neg	Neg	Neg	–	–	Neg
Insulin detemir (Levemir)	6/16/05	51 natural aa	Neg	Neg	–	–	–	Neg
Mecasermin Recombinant (Increlex)	8/30/05	70 natural aa	–	Neg	–	–	–	Neg
Methoxy polyethylene glycol-epoetin beta (Mircera)	11/14/07	165 natural aa	–	–	–	–	–	–
Interferon beta-1B (Extavia)	8/14/09	166 natural aa	Neg	Neg	–	–	–	–
Ecallantide (Kalbitor)	11/27/09	60 natural aa	–	–	–	–	–	–
Tesamorelin acetate (Egrifta)	11/10/10	44 natural aa	Neg	Neg	–	–	–	Neg
Peginesatide (Omontys)	3/27/12	42 natural aa	Neg	Neg	–	–	–	Neg
Tbo-filgrastim (XM02, Neutroval)	8/29/12	175 natural aa	–	–	–	–	–	–

–: No study conducted, Neg: study conducted with negative outcome.

Table 7
Genotoxicity studies conducted on large protein therapeutics.

Large protein therapeutics	FDA approval date	Amino acid residues	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* MN
Thyrotropin alfa (Thyrogen)	11/13/98	210	Neg	–	–	–	–
Tenecteplase (Tnkase)	6/2/00	527	–	–	–	–	–
Botulinum toxin type B (Myobloc)	12/8/00	1280	–	–	–	–	–
Drotrecogin alfa (activated) (Xigris)	11/21/01	~500	–	Neg	–	–	Neg
Rasburicase (Elitek)	6/12/02	1204	Neg	Neg	Neg	Neg	Neg
Agalsidase beta (Fabrazyme)	4/24/03	796	–	–	–	–	–
Laronidase (Aldurazyme)	4/30/03	628	–	–	–	–	–
Hyaluronidase (Vitrase)	5/5/04	447	–	–	–	–	–
Galsulfase (Naglazyme)	5/31/05	495	–	–	–	–	–
Alglucosidase alfa (Myozyme)	4/28/06	896	–	–	–	–	–
Idursulfase (Elaprase)	7/24/06	525	–	–	–	–	–
AbobotulinumtoxinA (Dysport)	4/29/09	~1300	–	–	–	–	–
Collagenase (Xiaflex)	2/2/10	1000	Neg	Neg	–	–	Neg
Velaglucerase alfa (Vpriv)	2/26/10	497	–	–	–	–	–
OnabotulinumtoxinA (Botox)	9/3/10	1296	–	–	–	–	–
IncobotulinumtoxinA (Xeomin)	7/30/10	~1300	–	–	–	–	–
Pegloticase (Krystexxa)	11/14/10	300	–	–	–	–	–
Asparaginase Erwinia Chrysanthemi (Erwinaze)	11/18/11	~1200	–	–	–	–	–
Voraxaze (Glucarpidase)	1/17/12	390	–	–	–	–	–
Jetrea (Ocriplasmin)	4/16/12	249	–	–	–	–	–
Taliglucerase alfa (Elelyso)	5/1/12	506	–	–	–	–	–

–: No study conducted, Neg: study conducted with negative outcome.

4. Discussion

The data from the present review of the currently marketed FDA approved large molecule therapeutics indicate that genotoxicity testing has been performed on a substantial number of large molecule therapeutics despite being categorized as unwarranted by regulatory guidance documents. A total of 53 (53.5%) of the 99 approved large molecule therapeutics were evaluated for their potential to cause genetic damage in 152 tests. However, none of the large molecules tested in the genotoxicity assays showed a positive outcome in any study; except glucagon, the borderline positive result for which could be explained by artifact or weren't reproduced. The data from this review also shows that the frequency of genotoxicity testing is inversely proportional to the number of amino acid residues in the therapeutic molecules. Almost all (16/17) peptides, 81% (17/21) medium-sized proteins, and only 34% (21/61) of the remaining larger protein therapeutics were tested for genotoxicity. Thirty-three (69%) of the 48 large

Table 8
Genotoxicity studies conducted on PEGylated protein/peptide therapeutics.

PEGylated protein/peptide therapeutics	US FDA approval date	Parent protein/peptide	Ames	*In vitro* CA	*In vitro* mammalian cell gene mutation assay	*Ex vivo* rat liver UDS	*In vivo* CA	*In vivo* MN
Fab fragment								
Cetolizumab pegol (Cimzia)	4/22/08	Anti TNFα Fab fragment	Neg	Neg	–	–	–	Neg
Medium-sized protein (41 to 200 amino acids)								
Peginterferon alfa-2B (Pegintron, Sylatron)	1/19/01	Interferon α-2b	Neg	Neg	–	–	–	Neg
Pegfilgrastim (Neulasta)	1/31/02	Granulocyte-colony stimulating factor	–	–	–	–	–	–
Peginterferon alfa-2A (Pegasys)	10/16/02	Interferon α-2a	Neg	Neg	–	–	–	–
Somavert (Pegvisomant)	5/25/03	GH hormone analog	Neg	Neg	–	–	–	–
Methoxy polyethylene glycol-epoetin beta (Mircera)	11/14/07	Erythropoietin analog	–	–	–	–	–	–
Peginesatide (Omontys)	3/27/12	Erythropoietin analog	Neg	Neg	–	–	–	Neg
Large protein (more than 200 amino acids)								
Pegloticase (Krystexxa)	11/14/10	Uricase	–	–	–	–	–	–

–: No study conducted, Neg: study conducted with negative outcome.

molecule therapeutics approved prior to 2004 were evaluated for genotoxicity, whereas only 17 (33%) of the remaining 51 large molecule therapeutics approved after 2004 were tested for genotoxicity. It is likely that genotoxicity studies with large molecule therapeutic candidates approved earlier were initiated and/or completed prior to 1997, the year ICH S6 guidance was implemented.

The ICH S6 guidance and amended S6 (R1) guidance documents suggest that the range and type of genotoxicity studies routinely conducted for small molecule pharmaceuticals are not applicable to biotechnology-derived pharmaceuticals and therefore are not needed. A typical molecular weight of a peptide containing 40 amino acids is about 5 kDa and the largest protein therapeutic (mAb) is about 150 kDa. Therapeutic molecules larger than 5 kDa are not expected to enter the cell and thus have reduced potential to interact directly with DNA or other nucleic acid physiological processes. Therefore, genotoxicity studies should not be required for large molecule therapeutics with molecular weights greater than 5 kDa, such as medium-sized proteins, large proteins, monoclonal antibodies and their fragments, and fusion proteins. This recommendation is similar to the scientific rationale that supports the hERG channel testing requirement for small molecule drug candidates, but not protein-based therapeutics (Vargas et al., 2013). The present analyses also show that none of the protein therapeutics was positive in any genotoxicity study. An earlier survey of large molecule therapeutic candidates from various sponsors also concluded that genotoxicity testing is generally inappropriate and unnecessary for large molecule therapeutics (Gocke et al., 1999). Considerations for peptides less than 5 kDa are discussed below.

Antibody drug conjugates have typical molecular weight of about 150 kDa and are composed of three parts – the antibody, a small molecule warhead, and a linker. To date, all approved ADCs have been for advanced cancer indications and thus regulated under ICH S9 guidance. The antibody portion of the conjugate is expected to pose no genotoxicity risk to patients due to its large size. The small molecules used in currently marketed ADC therapeutics are potent cytotoxic compounds used for killing cancer cells. If the cytotoxic agent is a known genotoxicant, then additional genotoxicity studies will be of little value and should not be needed. When the cytotoxic agent has not been characterized in genotoxicity studies, such studies are recommended on the small molecule component of the conjugate. In oncology indications, additional *in vivo* genotoxicity tests may not be required if the small molecule is positive in the *in vitro* assays (ICH S9, 2010). The ICH S6 and S6(R1) guidance indicates that the presence of an organic linker in an antibody-drug conjugate is a cause for concern and therefore recommends genotoxicity testing on the linker. However it does not provide guidance on what studies may be appropriate and which molecule (e.g., free linker or the whole antibody drug conjugate) to test for linker's genotoxic potential. Organic linkers are small but reactive molecules and may not be appropriate to test in genotoxicity assays as they are not expected to be present in the free reactive form after administration of ADC to patients. Evaluation of the intact ADC molecule in genotoxicity studies is also not appropriate as the cells used in the *in vitro* studies and in normal animals may not express corresponding antigens to internalize the conjugate molecule needed for inducing DNA damage. For those ADC's with a genotoxic warhead, testing of the linker would not add significantly to the safety assessment of the drug product. For non-oncology ADCs with novel linkers, testing of the linker should be considered. Also, if unreacted linker is present in such products or a derivative of the linker is present as an impurity in the drug substance or product, consideration should be given to assessment of its genotoxic potential.

The FDA considers therapeutic large molecules with up to 40 amino acids to be peptides and regulates them as a small molecule drug under the FD&C Act (Guidance for Industry: Biosimilars, 2012). Therefore genotoxicity testing of peptide therapeutic candidate would be expected for regulatory approval similar to small molecule drugs. However, there is no clear scientific rationale to believe that peptide therapeutics composed purely of natural amino acids, the endogenous building blocks of proteins, can compromise DNA integrity. In fact such testing may be problematic as false or irrelevant positive results have been observed in the bacterial reverse mutation assay with certain amino acids and proteins. For instance L-cysteine, a natural amino acid, has been reported to be positive in bacterial reverse mutation assay (Glatt and Oesch, 1985) but does not induce gene mutations in mammalian cells (Glatt et al., 1990). The authors of these studies suggest that the lack of genotoxicity in mammalian system is due to the presence of effective protective mechanisms in mammalian cells compared to the bacterial system (Glatt et al., 1990). Increases in revertant colony number have been observed in proteins containing certain amino acids (such as, histidine and tryptophan) due to their ability to complement histidine/tryptophan auxotrophy, i.e., a "feeding effect" (Thompson et al., 2005). The data collected in this review strongly suggest that genotoxicity testing of peptides composed of purely natural amino acids, irrespective of their size and ability

Table 9
Recommendations for genetic toxicology assessment of large molecule therapeutics.

Modality	Description	Perform standard ICH test battery
Monoclonal antibody	Identical antibody molecules produced by a single clone of cells or a cell line	No
Monoclonal antibody fragment	Fab domain of monoclonal antibodies	No
Antibody drug conjugate	Monoclonal antibodies attached to biologically active small molecule via chemical linker	No[a,b]
Conjugated protein	Fusion proteins	No
	Protein attached to biologically active small molecule via chemical linker	No[a,b]
	PEGylated protein/peptide with natural amino acids	No
	PEGylated protein/peptide with non-natural amino acids	Yes[c]
Peptide	Peptides containing natural amino acids	No
	Peptides containing non-natural amino acids	Yes

[a] Consider need to test organic chemical linkers in conjugated therapeutics or as potential chemical impurities.
[b] The cytotoxic agent may need testing.
[c] Consider testing the non-natural amino acid containing protein or peptide.

to pass the cell membrane, is not appropriate and should not be required. As peptides containing only natural amino acids produced by recombinant or chemical technique are identical; testing of chemically synthesized peptides containing only natural amino acids is also not appropriate and should not be required. Although errors in peptide synthesis (such as deletions, insertions) may occur to a small degree in both methods, such errors would not be expected to produce peptides of a foreign nature that would have genotoxic potential given the natural amino acid substrates of synthesis. Non-peptide impurities in peptide therapeutic products should be assessed for genotoxicity similar to small molecule drugs, as outlined in ICH M7 guidance document.

Of the 17 peptide therapeutics approved since 1998, six peptides contained a total of 20 non-natural amino acids. All six non-natural amino acid containing peptides were tested in the standard ICH test battery and none of these peptide molecules showed a positive outcome in any genotoxicity study. Many more non-natural amino acids exist however (Hendrickson et al., 2004) and genotoxic activity of these non-natural amino acids may not be established. Therefore potential genotoxic liability of non-natural amino acids and their metabolites supports evaluation of non-natural amino acid containing peptides using the standard ICH genetic toxicity test battery.

Polyethylene glycol is a large molecule that does not cross cellular membranes and has established an acceptable safety profile in clinical use. Therefore, conjugation of PEG to a protein or peptide containing natural amino acids should pose no genotoxic risk to patients and therefore testing of PEGylated proteins/peptides is not appropriate. However, PEGylated large molecule therapeutics containing non-natural amino acids and/or linkers may warrant genotoxicity evaluation in the ICH test battery, for the reasons discussed above.

Based on the scientific rationale and data from FDA-approved large molecule therapeutics presented in this review, our recommendations for conducting genotoxicity evaluations on various large molecule modalities are summarized in Table 9. Genotoxicity testing of monoclonal antibodies, fusion proteins, antibody fragments, the intact antibody-drug conjugates, medium and large protein therapeutic candidates is not appropriate as they are not expected to enter cell and nucleus due to their high molecular weights (>5 kDa). If the genotoxic potential of the ADC warhead is unknown, then characterization of the warhead in genotoxicity studies is recommended. For those ADC's with a genotoxic warhead, testing of the chemical linker would not add significantly to the safety assessment of the drug product. For ADCs with novel linkers developed for non-oncology indications, testing of the linker should be considered. Testing of peptides containing natural amino acids is not warranted, as most of these peptides will not cross cellular membrane and upon degradation the peptides will release only natural amino acids that do not pose any genotoxic liability. Testing of peptide therapeutics containing non-natural amino acids should be considered for genotoxicity evaluation.

Conflict of interest

The authors are employed by Amgen Inc., a biopharmaceutical company.

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