

Toxicology and Biodistribution: The Clinical Value of Animal Biodistribution Studies

Beatriz Silva Lima^{1,2} and Mafalda Ascensão Videira¹

¹Universidade de Lisboa, Faculdade de Farmácia, iMED.Ulisboa, Lisboa 1649003, Portugal; ²NDA Advisory Board, Leatherhead, Surrey, UK

Since the human genome decoding, understanding and identification of genetic disturbances behind many diseases, including cancer, are intensively increasing. Scientific and technological advances in this area trigger the search for therapeutic (curative) approaches targeting the correction of gene disturbances. Gene therapy medicinal products (GTMPs) emerge in this context, bringing new challenges for their characterization. Compared to small molecules, biodistribution is fundamental to identifying target organs and anticipating safety and efficacy, may be integrated into safety and pharmacology studies, and may eventually be anticipated based on specificities of vectors and constructs. This review describes and discusses the requirements for nonclinical development and evaluation of GTMPs versus conventional ones and the needs and challenges of constructing nonclinical packages that assure GTMPs' human safety from early development, taking into consideration usefulness and/or limitations of many conventional, preclinical models. The experience gained in the European context is referenced.

The human administration of any medicinal product, either under experimental conditions in clinical trials or as established treatment after approval by regulatory authorities, requires an extensive body of information to be generated in anticipation of human administration to assure understanding of the benefits and the risks posed to humans, as well as to anticipate and estimate the potential benefit-to-risk ratio profile.

The required data are generated in studies performed in nonclinical models, *in vivo* and or *in vitro*. The composition of the preclinical or nonclinical development programs of medicines is relatively well established (pharmacodynamics, pharmacokinetics, and toxicology), but specific program and study adaptations are increasingly needed based on the type of products under development. Multiple guidance documents have been prepared by regulatory authorities, many of which have received global agreement under the remit of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) (<http://www.ich.org>) being largely and internationally applied.¹

Medicines are increasingly evolving into sophisticated formats, making the most standardized requirements for small molecules difficult or even impossible to apply in the development of many products. Recognizing the permanent scientific and technological evolutions, guidance documents are periodically revised according to the state

of the art, and new ones are produced according to identified needs. Case-based, product-dependent, scientifically based deviations from established guidelines (mostly produced for small molecules) are increasingly needed to address the nonclinical development of many innovative medicines or formulations. Such is the case with biopharmaceuticals, nanomedicines, or advanced therapy medicinal products (ATMPs).²

Gene therapy medicinal products (GTMPs) are among such innovative and complex medicines, and their development poses fascinating challenges to the scientific communities involved either in their experimental development or in their assessment, making it fundamental that close interaction between the basic and clinical scientists and the regulatory scientists is maintained along the full development process. Some of those challenges are addressed in this review, taking into consideration approved GTMPs.

The Preclinical Development of Medicinal Products: Objectives and Content

Irrespective of its origin, the development of any innovative product involves the generation of a set of data aimed at informing the human risks posed by its administration, from early-stage (first in human) into late-stage clinical trials and on to wider use after marketing. Understanding of product-related risks helps to determine the safe conditions of human use of the innovative product through the establishment of risk mitigation measures. With such objectives, the nonclinical development program of any innovative product includes three main areas: pharmacodynamics, pharmacokinetics, and toxicology. Pharmacodynamic studies are performed to characterize the mode of action and the primary and secondary targets that may be involved on wanted and unwanted biological effects (primary and secondary pharmacology and safety pharmacology). Pharmacokinetic studies are intended to characterize the profile of absorption, distribution, metabolism, and excretion (ADME) of the developing molecule. Toxicity studies address the toxicological properties in general terms (general toxicity, single-dose toxicity, or repeated-dose toxicity studies) and on specific aspects (reproductive toxicity, genotoxicity, carcinogenicity, phototoxicity, immunotoxicity, etc.).¹

<https://doi.org/10.1016/j.omtm.2018.01.003>

Correspondence: Beatriz Silva Lima, Universidade de Lisboa, Faculdade de Farmácia, iMED.Ulisboa, Lisboa 1649003, Portugal.

E-mail: beatrizlima@netcabo.pt





The nonclinical studies are conducted in several systems, *in vitro* and *in vivo*, which include animal models of disease, healthy animals, diverse animal, and human cell systems. Because the objective of these studies is to produce information assuring safe administration of the investigational product to humans, the test systems, either *in vivo* or *in vitro*, need to be relevant to the human situation, and strategies for human translation of the studies' outcomes are fundamental. The choice of human-relevant test systems is therefore a fundamental piece for safe and effective development of any molecule. The relevance of the test systems, either *in vitro* or *in vivo*, is based on the similarity of the pharmacology, pharmacodynamics, physiology, biochemistry, target biology, cellular cascades, kinetics, tissue responses, etc. When animal models are used, it is fundamental to understand how those aspects apply to humans. The use of irrelevant test systems, including animal models, healthy or diseased, might be as deleterious as their nonuse, because they could lead to misinterpretation of study outcomes and thus human risk overestimation or underestimation, which could lead to either exclusion of useful clinical candidates or triggering of unidentified, severe, or even potentially fatal reactions in humans.

Given the increasing human target specificity of innovative medicines under development, the search for human-relevant test systems becomes increasingly challenging, and rigorous human translation approaches are needed to avoid the emergence of unpredicted adverse reactions.

GTMPs and Their Preclinical Development

GTMPs are within the medicines for which, because of the human specificity of their activity, the search of human-relevant test systems to support the preclinical development is challenging yet highly needed. A GTMP consists of several components that, individually or together, determine the quality attributes and potential pharmacological, pharmacokinetic, and toxicological activity of the GTMP.^{3,4,5}

In general terms, the objective of the nonclinical characterization of a GTMP is the same as that for a small molecule: it should provide evidence of the mode of action, wanted and unwanted pharmacological effects, pharmacokinetics, and toxicity. However, contrarily to small molecules, which in general are able to interact with human and nonhuman targets, the GTMP has to be conceived (1) to be competent when infecting human cells with the selected vector, (2) to provide close-to-optimal gene product expression, and (3) to be as selective as possible for the intended target cells and tissues or organs. Because the nonclinical studies are intended to mimic the human effects of the developing products, the human specificity of the GTMP creates difficulties in finding the most appropriate models that respond to the product similarly to the response of human systems. In addition, compared to small molecules, GTMPs are intended to be administered once or a few times in life, with the objective of inducing a lifelong persistent effect. The nonclinical studies needed to support a first in human administration that might be the "last in human administration" are therefore more extensive.³

Relevance of In Vitro and In Vivo Models

As highlighted earlier, the relevance of the models used for the nonclinical testing of a GTMP is fundamental for accurate human translation of the findings generated in those models. Models for addressing the biological properties of any GTMP include *in vitro* and *in vivo* systems:

- (1) *In vitro* systems correspond to animal and (preferably) human cell systems that may allow the GTMP to be studied in terms of cellular tropism and upload, transfection, and gene product expression. Human cell systems, particularly those deriving from patients, may be of high relevance, because they may include the human components of the disease and better address the intended mode of action.
- (2) *In vivo* systems are animal models desirably reflecting the human disease condition, including genetically manipulated animals expressing a human-like condition and immunocompromised animals allowing the testing of the human GTMP and overcoming the immunogenicity displayed by the medicinal product or any of its components (e.g., the vector and the human gene product). Wild-type animals are also used in many situations. For any type of nonclinical test system, the fundamental condition is the model relevance, which needs to be known and well justified, because irrelevant models will compromise the full development and success of the GTMP. According to GTMP regulatory guidelines,⁴ when one animal model misses fulfilling the requirements for relevance, it may be necessary to test the GTMP in more than one species or model.

Factors of Relevance for the Nonclinical Test Systems of GTMPs. GTMPs integrate several molecular components that, individually or together, may affect the cellular responsiveness and response type and have to be considered in determining the human relevance of any test system. Therefore, model relevance depends on factors associated with the viral construct and the gene product.^{4,5}

Viral- or Vector-Dependent Factors of Model Relevance. Any relevant model, *in vitro* or *in vivo*, has to be sensitive to the viral or vector infection and transduction, as well as to its replication in the case of replicative viruses or vectors. Several factors should be addressed, including the following. First, the tissue vector tropism in animals and humans should be comparable. Second, the efficiency of cellular or tissue vector uptake of the GTMP depends on the expression and density of cellular receptors for the virus or bacteria in the (animal) model, which determine the tissue sequestration of the vector. Relevant models should therefore present similar cellular distribution of the receptors compared to humans. Alternatively, any differences need to be known and considered. Third, after having been distributed to and taken up by the target tissues and cells, the expression efficiency has to be compared in human and animal model cells as factors for model relevance, which determines the exposure level to the gene product and ultimately its activity.

Increasing knowledge is being gained with regard to species infectivity, tissue tropism, and cellular infectivity of different vectors.^{6,7} This knowledge is fundamental for the design of a gene therapy



product, vector selection, and the animal or *in vitro* model to be used for the preclinical testing of the GTMP.

With regard to adeno-associated virus (AAV) tissue tropism, accumulated scientific information has been gathered by the regulatory authorities and published in the format of guidelines, such as the one addressing the specific tissue tropism of different serotypes of AAV.⁸ AAV1, AAV6, and AAV7 are effective at transducing muscle cells, AAV9 preferentially transduces the myocardium, and AAV5 is suggested to be more tropic to the airway epithelium and the CNS (at least in the mouse model). Preferential distribution does not mean exclusive distribution, and the specificity for the animal model studied needs to be understood. For instance, AAV5 is neurotropic in the mouse and binds and transduces in the airway epithelium more efficiently than AAV2, whereas AAV1 is more efficient in the cat brain.^{6,7} These aspects need to be taken into consideration when choosing the AAV serotype in relation to the species selection for efficacy and safety studies, as well as for the intended target tissue for efficacy in animals and humans. In addition, these aspects must be carefully considered when translating nonclinical data to humans.

Specific guidance on tissue tropism is provided in the “Reflection Paper on Quality, Non-clinical and Clinical Issues Related to the Development of Recombinant Adeno-Associated Viral Vectors”⁸ and in “ICH Considerations: Oncolytic Viruses.”⁹ When selecting the animal model for any vector-based GTMP, the comparability of tissue tropism in the selected animal model and humans should therefore be discussed and justified.

Gene Product-Related Factors for Animal Relevance. The activity of regulatory elements and their control to drive tissue-specific expression and the expression level of the transgene needs to be characterized both in human systems and in (animal) models, and the extension of similarity needs to be determined. Furthermore, the cross reactivity or binding of the gene product with the animal and the human targets needs to be known, qualitatively characterized (e.g., binding versus stimulation or inhibition of target-mediated cascades), and quantitatively evaluated. Ideally, the gene product should present the same level of activity in humans and in animals. However, given the human specificity of the gene products (e.g., specific enzymes, proteins, and neurotransmitters), species differences in the response to the human gene product are expected, at least in quantitative terms, and these need to be investigated and established. The condition for species relevance for GTMP testing includes the pharmacological responsiveness of the species to the gene product. This implies that the modulated genes should be similar in the species and humans, as well as the gene products. If the human gene product depends on different genes in humans and animals, this may lead to the adaptation of the GTMP for testing in animals (e.g., homologous GTMP) to obtain pharmacological responses similar to those in humans.

Immunogenicity of the GTMP or of the Gene Product

When infecting cells, whether human or from other species, the viral vector may trigger immunogenic responses. The previous exposure of

patients to the vector or viral component may therefore compromise the efficacy of the GTMP. Along same lines, if immunogenicity against the transgene or the gene product is developed in the testing species or model, this may compromise the species responses and their value for prediction of human efficacy or safety. Species immunogenicity of the GTMP in the experimental model, developed before or developing during the test period, will need to be considered when addressing the species or model relevance for predicting human safety and efficacy.

The main aspects determining the relevance of experimental models, animal or *in vitro*, for the preclinical study of any GTMP are addressed in specific guidelines, e.g., by the European Medicines Agency (EMA) and the Committee for Advanced Therapies (CAT).^{4,10}

Planning the Preclinical Development Plan for a GTMP

As stated earlier, for any other medicinal product, the preclinical studies with GTMP are performed or conceived to characterize the mode of action, the kinetics, and the potential toxicity, which will justify (in terms of potential benefit) and support (in terms of potential risks) human administration during clinical trials, for marketing authorization, and in the post-marketing period. For conventional products (e.g., small molecules and even biopharmaceuticals), the extension of the preclinical program is designed based on the duration of exposure to the product in clinical studies, e.g., single- or multiple-dose administration for short or long periods, the phase of the clinical development, and the extension of the target patient populations, following the ICH M3(R2) guideline.¹

For GTMPs, a single or a few administrations are performed, but the effects are intended to be long lasting and ideally should lead to a life-long effect. Therefore, supportive preclinical studies have to take into consideration the long-lasting exposure of patients and that healthy volunteers are usually not involved in even phase I clinical studies.³

Pharmacology Studies

The pharmacology studies will need to provide evidence on the mode of action of the GTMP at the cellular level and *in vivo*. The multiple factors associated with the activity of the GTMP will have to be studied and characterized, using relevant *in vitro* and/or *in vivo* systems:

- (1) *In vitro* pharmacology studies will inform on the infectivity of target cell systems of human and animal origin and on GTMP incorporation at the cytoplasmic or DNA level, as well as gene product expression or suppression as applicable.
- (2) For *in vivo* pharmacology studies, the GTMP effect will preferably be studied in animal models of disease when available to address the dose-response relationship and the therapeutic benefit of the GTMP. An animal model or models may include wild-type, immunocompromised, knockout, humanized, or transgenic animals, as well as disease models or homologous models (e.g., mouse cells analyzed in mice). For oncolytic viruses, which are classified as GTMPs, tumor-bearing xenograft models in immune-deficient or immunocompromised animals or a



syngeneic animal tumor model may be relevant to assessing the effects of viral replication in tumor cells in the nonclinical studies. This case is well illustrated with the recently approved GTMP under the trade name Imlygic, which is discussed later in the case examples section of this review.

When both *in vitro* and *in vivo* systems are used, which is often the case, it will be advisable that the GTMP be studied in (1) human cell systems, preferably from patients, and (2) animal cell systems, preferably originating from the same species used for the *in vivo* proof-of-concept and/or safety studies. This approach will allow the establishment of *in vitro* and *in vivo* correlations for the animal systems and the prediction or estimation of *in vivo* human responses based on observations collected in the human *in vitro* systems.

Altogether, *in vitro* and *in vivo* studies will provide information on the concentration/activity in human and animal cells and the *in vivo* dose response in the animal model of the disease. The *in vitro/in vivo* correlation in nonhuman systems may be helpful for the estimation of potential human reactivity of the GTMP, based on the *in vitro* results in human cell systems. As noted earlier, because clinical trials of GTMPs are generally conducted in patients and healthy volunteers are not involved, it might be appropriate, when feasible, to use animal models of disease for the safety evaluation of GTMPs, because these will be a better representation of the conditions of use from the start of human research than healthy animals, which by default are commonly used for the toxicity studies, particularly of small molecules or biopharmaceuticals. Therefore, when in the *in vivo* pharmacology studies animal models of disease are used, whenever possible, it may be of high value to incorporate safety endpoints, like the monitoring of biochemical and hematological parameters and terminal histopathology. In addition, it will be of value to collect from those models information on the tissues exposure to the GTMP, the GTMP components, and the gene products whenever appropriate. The possibility of the formation of aberrant gene products will need to be addressed, and when occurring, the biological activity, safety, and biodistribution will have to be studied and characterized. *In vitro* systems may be useful for this purpose, but *in vivo* studies in relevant species might allow a more integrated prediction in the whole body.

Pharmacokinetics

Conventional medicinal products once administered will be absorbed, will distribute throughout the body, and then will be metabolized and excreted. All these aspects of ADME need to be known in humans and animals and are important for the human translation of the nonclinical findings. GTMPs, once administered, are intended to access the target cells and be incorporated in the cytoplasm (plasmids) or in the genetic material of the cell, followed by expression of the gene product or gene suppression, depending on the mode of action. Therefore, the most relevant aspects of the kinetics of a GTMP are its distribution into target and nontarget cells, the concentrations reached, and the subsequent concentrations of the gene product reached within the sites where distribution is expected, is intended,

or has been observed. In this context, as earlier stated, biodistribution studies in the animal model of disease, when possible, may best reflect the human situation.

Appropriate qualitative and quantitative assay methods need to be established and be sensitive enough to detect the presence of the GTMP and related components in cells and tissues (e.g., through imaging techniques), as well as to quantify the GTMP and the gene products when applicable. The human value of animal biodistribution studies depends on comparative vector infectivity, cell tropism, viral receptor distribution, and gene expression in the animal and human cells. All these factors need to be known when selecting the species for the preclinical characterization of the GTMP.

The use of small and/or large animals for biodistribution characterization will depend on the model's predictivity and feasibility. Small animals, if relevant, can be used in larger numbers, and given the smaller organ volumes, the GTMP or related components may be easier to accumulate at high doses, identify, and track than in large animals. Large animals would require higher amounts of the GTMP compared to small animals and would be used in smaller numbers, which compromises the statistical and detection power of the studies.

Increasing knowledge is being gained on the characteristics and tropism of types of vectors, many of which are being developed with specific purposes in terms of cell targeting, e.g., AAV strains for liver cells, brain, or CNS. When vector platforms are built and characterized with regard to their cell tropism in human cells and species cells (*in vitro*) and organs (*in vivo*), the accumulated knowledge (e.g., on biodistribution, persistency, and vector safety, including integration) may be used for anticipating the biodistribution and the nonclinical biological profile of the GTMP built under such a platform. This will be of outstanding value to accelerate the development and better establish the preclinical programs to be adopted in support of the clinical trials, marketing authorization, and post-marketing therapeutic use. One good example of a platform concept is the one behind the development of the ATMP Heparesec, which was refused granting of the marketing authorization (MA) by the EMA CHMP (Committee for Medicinal Products for Human Use of the EMA) in June 2015.¹¹

If the constructs are not infectious in animals, or if the human gene product differs substantially in preclinical species, leading to species responses that are of lower magnitude or qualitatively different from human responses or even to no response, then the GTMP may have to be adjusted to become responsive in animals, e.g., by using a species-sensitive vector or construct and/or a different species gene product. In these situations, the clinical candidate may have to be tested only in human cells for the anticipation of distribution profiles, and the results obtained in animal studies with the homologous GTMP are only of qualitative, not quantitative, value. In such a situation, *in vitro* studies using different human cells to check for the ability of the GTMP to penetrate the cells and be incorporated and transduced will help to overcome the insufficiency of the animal



Table 1. Recommended Durations of Repeated-Dose Toxicity Studies to Support Clinical Trials According to the Approved Guideline¹

Maximum Duration of CT	Recommended Minimum Duration of Repeated-Dose Toxicity Studies to Support CTs	
	Rodents	Nonrodents
Up to 2 weeks	2 weeks	2 weeks
Between 2 weeks and 6 months	same as CT	same as CT
>6 months	6 months	9 months

models and might be of higher value for human efficacy and safety-related translation. Biodistribution components in relevant species or models are therefore among the most fundamental preclinical information on the GTMP to be collected, because they will inform on the potential target organs and tissues and will allow anticipation of which on-target or off-target effects might be expected. For instance, if the GTMP does not distribute in the gonads, the requirement for reproductive toxicity studies may need to be discussed only based on the potential effects of the gene product systemically, and a potential for a waiving or simplification of those studies may be considered if applicable. In the same perspective, if no biodistribution is seen in the gonads, gene transfer studies may not be needed.

The characterization of the biodistribution pattern *in vivo* may be conducted in dedicated studies or may rather be integrated in non-clinical safety and/or pharmacology studies appropriately designed to maximize the information to be extracted from those studies. For the sake of the 3Rs (refinement, reduction, replacement), the optimization of *in vivo* studies allowing extraction of multiple components (pharmacodynamics/biodisposition/toxicity [PD/BD/TOX]) is highly recommended; it is also appreciated by regulatory authorities.

Toxicology

The toxicological profile of any GTMP will depend on the attributes of its multiple components and of the gene product. GTMPs are constructed with specific purposes in terms of the biodistribution, cell or tissue specificity and tropism, and gene product expression. The risks associated with these products will therefore depend on the components (viral or vector, promoters, etc.) and the gene product. In principle, most safety aspects of GTMP might be anticipated when combining knowledge on the components to the biodistribution profile *in vivo* and at the cellular level where appropriate. The toxicological evaluation of the GTMP will therefore consist mostly of the search for anticipated or predicted toxicities, their characterization, and when possible, their quantification (dose-response relationship) to define the safe conditions of human therapeutic use. For ATMPs, a risk-based approach has been proposed by regulatory authorities, which consists of the design of preclinical packages based on the anticipated risks to be identified, confirmed, and characterized for that GTMP and the expected effects and target organs. Science-based, case-based protocols and programs may therefore be defined.¹⁰

Table 2. Recommended Durations of Repeated-Dose Toxicity Studies to Support Marketing Authorization¹

Duration of Treatment	Recommended Duration of Repeated-Dose Toxicity Studies to Support Marketing	
	Rodents	Nonrodents
Up to 2 weeks	1 month	1 month
>2 weeks to 1 month	3 months	3 month
>1 to 3 months	6 months	6 months
>3 months	6 months	9 months

Increased knowledge and experience collected with the preclinical and clinical studies of each GTMP will contribute to reinforcing the prediction of the product-associated attributes and effects related to the vector, the full construct, or the gene product. Because toxicity studies will have to represent the intended human situation of use of the GTMP, the most common approach for toxicology evaluation corresponds to single-dose administrations in relevant species, with subsequent long-term follow-up (e.g., up to 6 or 9 months), depending on the anticipated persistence of the gene and the gene product, to cover the potential effects of the gene product to which the patient will be chronically exposed after the single dose of the GTMP. If repeated administrations in humans are anticipated, the animal studies will also be of the repeated-dose type such that the administration schedule mimics the human one, and the duration of the effect (biodistribution, vector persistence, and gene product persistence) will be in line with that intended in humans. The durations of repeated-dose studies, established in the ICH M3(R2) guideline¹ with regard to the duration of human exposure, are given in Tables 1 and 2, which may be used as guides to decide on the duration of the study with the GTMP (with regard to the number of administrations or the timings for animal observation and terminal sacrifice).

Preclinical Safety Programs. While the toxicological concerns with GTMP might be similar to those for conventional medicinal products, e.g., toxicity after long-term exposure (which is equivalent to repeated dose toxicity), reproductive toxicity, carcinogenicity, genotoxicity, and immunotoxicity, they have to be addressed differently in accordance with the specificities of the GTMP. For instance, the evaluation of the reproductive toxicity or the carcinogenicity or tumorigenicity cannot be studied through repeated administration studies, because this will not mimic the therapeutic use of the GTMP. However, study protocols may need to be adjusted and, if available, combined with existing knowledge and the weight of evidence from the literature with regard to the potential effects of the vector, the gene product on the reproductive organs, or the reproductive function. This may require answers to questions such as “Does the gene product cross the placenta and reach the embryo or fetus?” If yes, what would be the consequences? In addition, the feasibility of studies may be limited by the availability of relevant species. If the only relevant species is a non-human primate (NHP), the design of the reproductive toxicity studies will take into consideration not only the particular aspects of

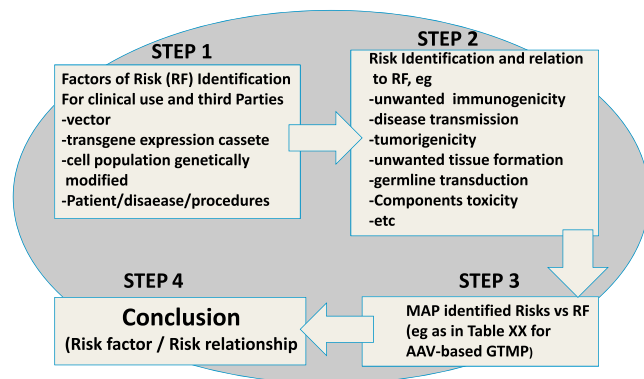


Figure 1. Risk Profiling for ATMPs following the Risk-Based Approach

the GTMP, e.g., concerning administration routes and scheduling, but also the limitations inherent to the species, as outlined in ICH S6(R1) guideline.¹² In addition, the use of rodents (mice or rats) for carcinogenicity or tumorigenicity testing is not meaningful, and such studies are therefore not advised. The difficulties or impossibility of getting the relevant models to cover more specific toxicities (other than general toxicity) may justify waiver of the studies and their replacement with risk anticipation based on existing knowledge and literature, complemented by risk minimization measures and implementation of registries for patient follow-up for certain periods.

Species Selection. The species used in toxicology studies need to be representative of the human situation. They need to be relevant, to be infected by the vector, to present similar cell tropism for the GTMP and its vector, and to present a similar cellular response in terms of gene product expression and subsequently in terms of the biological effects of the gene product.

In vitro experiments in multiple types of human and animal cells may constitute early and fundamental steps of any GTMP testing, including the search for relevant animal species to be used in subsequent *in vivo* tests and for subsequent pharmacodynamic, pharmacokinetic, or biodistribution and toxicity testing. Because different viral vectors and strains present different cell and species tropism, the increasing knowledge that is being gained in this aspect allows *a priori* selection of the vector to cope with its cell and species tropism, which contributes for the application of the risk-based approach concept in the planning of the nonclinical program.

When appropriately designed and conducted in a sufficient number of animals of a relevant species, *in vivo* studies may be of value in helping to characterize the safety of a GTMP, provided that all aspects of relevance described earlier and similar biodistribution in animal and human tissues are expected or anticipated based on the known characteristics of the vector and/or the gene product. When rodents are not considered relevant species, nonrodent species may have to be used, which in general limits the number of animals used, increases

the amount of test product needed, and makes it more difficult to obtain significant results. In these cases, the use and usefulness of *in vitro* human cell systems should be maximized to overcome the limitations placed by the use of larger animals.

When no relevant species exist, then animal models might not be considered meaningful, and the most human-relevant information may have to be generated in human cell systems. In this case, the tropism, integration, and expression of the GTMP are studied, including the tropism for multiple types of cells that may inform on the potential biodistribution and GTMP targeting effects.

Multiple human cell types obtained, e.g., from induced pluripotent stem cells (iPSCs), are increasingly available, making it possible to perform GTMP testing *in vitro*, to study the ability of the GTMP to penetrate the cell, and to express the gene products, which can be analyzed and characterized. The advances in iPSC technology are making possible the use of patient-derived multiple cell types, which can be used for pharmacology and for safety assessment studies. As described earlier, when human and species *in vitro* cell systems are available, studies in both systems are valuable assets for species-to-human translational approaches of the activity of the GTMP and should be used, together with *in vivo* studies in the relevant species, for appropriate human predictions.

The Risk-Based Approach

The risk-based approach consists of a strategy aiming (1) to determine the extent of quality nonclinical and clinical data to be included in the marketing authorization application (MAA), in accordance with the requirements of scientific guidelines, and (2) to justify any deviation from those requirements.^{4,10} The concept proposes an ongoing process for data collection before the submission of the MAA, starting at the beginning of product development and maturing over time as knowledge of the product and its characteristics increases (Figure 1). The methodology for the risk-based approach would lead to risk profiling, which is defined in four steps: (1) identification of risk factors for clinical use, (2) risk identification, (3) risk versus factors of risk correlation, and (4) risk profiling.

When the risk-based approach is used to support a development plan, this will be justified, namely, in the omission of some studies (e.g., reproductive toxicity, carcinogenicity, or tumorigenicity) based on the accumulated data or knowledge about the product and its associated risks (Table 3).¹⁰

Some Examples of the Nonclinical Development Plan of Approved GTMPs and the Use of the Risk-Based Approach

Despite intensive research in the area of GTMP, only three medications had received regulatory approval up to April 2016: Glybera, Imlygic, and Strimvelis. In the current section, the most relevant aspects of the nonclinical development programs of Glybera and Imlygic are addressed as examples, and the application of the risk-based approach is discussed.

Table 3. Example of Mapping of Risk versus Risk Factors for GTMP from the CAT Point of View

Risk Factor	Tumor Formation	Unwanted Immunogenicity	Treatment Failure	Toxicity Resulting from Unintended Alteration of Therapeutic Gene Expression
Recombination or mobilization	Recombination may lead to replicating AAV. Tumor formation depends on the level of AAV genome integration into the host genome. Addressed in CTD 3.2.P.5 (Control of DP) and CTD 4.2.3 (Toxicology) toxicology and integration studies.	Recombination or mobilization may lead to increased immunogenicity due to a higher number of vector or RCV particles. Addressed in CTD 3.2.P.5 (Control of DP) and CTD 4.2.3 (Toxicology).	Recombination during manufacture might lead to loss of the transgene and consequently loss of function. Addressed in CTD 3.2.P.5 (Control of DP).	Mobilization (with wild-type [WT] and helper coinfection) might result in higher levels of therapeutic gene expression. Toxic effects other than immunogenicity due to overexpression are considered low. Addressed in CTD 4.2.1 (Pharmacology) and CTD 4.2.3 (Toxicology) studies and justified by the literature.
Integration	AAV vectors are able to integrate into the genome, albeit at low levels. Integration studies are performed in CTD 4.2.3 (Toxicology). See also risk factor biodistribution in CTD 4.2.2 (Pharmacokinetics).	-	-	-
Type of transgene and transgene expression levels	-	The therapeutic gene is of human origin, and the respective endogenous gene product in patients is present but defective. This might cause unwanted immunogenicity. Expression of therapeutic protein is addressed and justified in CTD 5.3.5 (Reports of Efficacy and Safety Studies).	Impaired transgene expression might lead to treatment failure. Addressed in CTD 3.2.P.5 (Control of DP) and 4.2.1 (Pharmacology) transgene expression and potency studies and <i>in vivo</i> proof-of-concept studies.	Overexpression of the transgene in target cells is not considered to be of concern. Toxic effects other than immunogenicity due to overexpression are considered to be low. Addressed in CTD 4.2.1 (Pharmacology) and CTD 4.2.3 (Toxicology) toxicity studies and justified by literature data.
Vector type	AAV is not known to be tumorigenic per se. A low potential of AAV for insertional mutagenesis exists (see RF integration). Addressed in CTD 4.2.3 (Toxicology) integration studies. Justification of lack of tumorigenicity studies is based on respective integration data.	AAV is known to be immunogenic. Addressed in CTD 4.2.3 (Toxicology) immunogenicity and toxicity studies and CTD 5.3.5 (Reports of Efficacy and Safety Studies) clinical safety studies.	Pre-existing immunity to the vector might impair efficiency of treatment. Repeated administration may increase immunological responses against the vector that might also impair efficiency of treatment. Addressed in CTD 4.2.1 (Pharmacology) and CTD 5.3.5 (Reports of Efficacy and Safety Studies).	-
Impurities	Impurities might contribute to tumor formation. Full information and documentation on starting materials are given. Control of cellular and viral impurities are addressed in CTD 3.2.S.4 (Control of Critical Steps and Intermediates) release testing and CTD 3.2.P.5 (Control of DP).	AAV can be difficult to purify. The amount and type of impurities may lead to immunogenic reactions. Addressed in CTD 3.2.S.2 (Manufacture), CTD 3.2.S.4 (Control of DS), CTD 4.2.3 (Toxicology), and CTD 5.3.5 (Reports of Efficacy and Safety Studies).	Impurities can negatively influence the efficacy of treatment. Drug substance control is addressed in CTD 3.2.S.4 (Control of DS).	-
Biodistribution	Biodistribution of the vector contributes to the risk of tumor formation via vector persistence	Biodistribution of the vector to nontarget, immunogenic sites. Addressed in CTD 4.2.2 (Pharmacokinetics)	Treatment failure might be induced by unwanted immunogenicity due to biodistribution to nontarget,	Toxicity as a result of transgene-overexpression in nontarget cells is considered low. Evaluation of

(Continued on next page)



Table 3. Continued

Risk Factor	Tumor Formation	Unwanted Immunogenicity	Treatment Failure	Toxicity Resulting from Unintended Alteration of Therapeutic Gene Expression
	and integration events (see risk factor on integration). Inclusion of transduced nontarget organs in studies on episomal or integrated vector status. Addressed in CTD 4.2.2 (Pharmacokinetics) biodistribution and CTD 4.2.3 (Toxicology) integration studies.	biodistribution, CTD 4.2.3 (Toxicology) immunogenicity, and CTD 5.3.5 (Reports of Efficacy and Safety Studies) clinical safety studies.	immunogenic sites. Addressed in CTD 4.2.1 (Pharmacology) and CTD 4.2.2 (Pharmacokinetics) biodistribution and long-term transgene expression studies.	toxicity and transgene expression levels in nontarget tissues and cells. Addressed in CTD 4.2.2 (Pharmacokinetics) biodistribution and CTD 4.2.3 (Toxicology) toxicity studies.
Relevance of animal model	–	The animal model is not predictive for immunogenicity in patients due to differences in immune responses. An additional animal model to address immunogenicity was used. Addressed in CTD 4.2.3 (Toxicology) immunogenicity and CTD 5.3.5 (Reports of Efficacy and Safety Studies) clinical studies.	The animal model may not be predictive for treatment failure due to differences in the immune status of animals and patients. Immune status of the animal model has been matched to the patient's situation (e.g., pretreatment with the vector to induce seroconversion in animals). Addressed in CTD 4.2.1 (Pharmacology) and CTD 4.2.3 (Toxicology).	–
Patient related	–	Immune reaction might be triggered depending on the immune status of the patient. Addressed in CTD 4.2.3 (Toxicology) nonclinical studies using vector-pretreated animals and CTD 5.3.5 (Reports of Efficacy and Safety Studies) clinical safety studies.	Immune status, e.g., pre-existing immunity to the vector, of the patient might influence the efficiency of therapy. Addressed in CTD 4.2.1 (Pharmacology) nonclinical and CTD 5.3.5 (Reports of Efficacy and Safety Studies) clinical studies.	–
Disease related	The underlying disease might be linked to a higher incidence of cancer. This might bias the safety data. Addressed in CTD 5.3.5 (Reports of Efficacy and Safety Studies).	Variable levels of dysfunctional protein may be expressed in the patients, resulting in immune reactions to the therapeutic protein. Addressed in CTD 5.3.5 (Reports of Efficacy and Safety Studies).	Immune response against the transgene might compromise treatment efficacy. Addressed in CTD 4.2.1 (Pharmacology) nonclinical pharmacology and CTD 4.2.3 (Toxicology) toxicology studies and in CTD 5.3.5 (Reports of Efficacy and Safety Studies).	–
Medical procedure related	Concomitantly administered immune suppressants might lead to tumor formation. Addressed in CTD 5.3.5 (Reports of Efficacy and Safety Studies).	A high local dose administered i.m. might cause local inflammatory response due to immunoreaction to a vector component or the expressed therapeutic protein. Addressed in CTD 4.2.3 (Toxicology) and CTD 5.3.5 (Reports of Efficacy and Safety Studies).	Difficult administration of multiple injections i.m. might result in incomplete dosing. Addressed in CTD 5.3.5 (Reports of Efficacy and Safety Studies) and SmPC.	–

The AAV vector expressing the human ficcionase enzyme (FE) was administered i.m. CTD, common technical document; DP, drug product; RCV, replication-competent virus; SmPC, summary of product characteristics. Adapted from EMA/CAT/CPWP/686637/2011 (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139748.pdf).



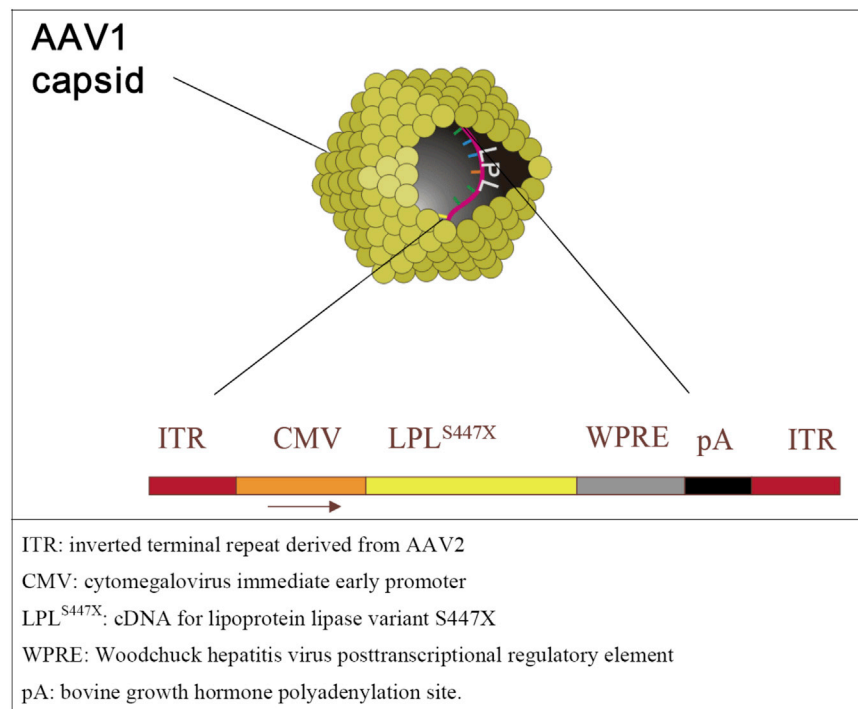


Figure 2. AAV1 Capsid Diagram Structure of Alipogene Tiparvovec

Extracted from European Public Assessment Report: Glybera (alipogene tiparvovec). EMEA/H/C/002145 (2012).¹⁴

The Preclinical Development Program of Glybera. The preclinical development of alipogene tiparvovec included pharmacodynamic studies, single-dose toxicity studies with extended observation times, biodistribution, carcinogenicity evaluation, and reproductive toxicity. This nonclinical package appears having been designed in line with the special attributes of a GTMP that is intended to be administered once (or a few times) and to induce an effect (LPL expression) that is as long lasting as possible, which means a single administration with a chronic effect.

Pharmacodynamics. Animal models of disease were used in the proof-of-concept studies: LPL-deficient (LPL^{-/-}) mice and cats, treated with the test product designated

Glybera

Glybera (alipogene tiparvovec) is a GTMP intended for the treatment of lipoprotein lipase deficiency (LPLD), a rare autosomal recessive inherited condition caused by mutations in the enzyme lipoprotein lipase (LPL) gene under homozygosity or heterozygosity.^{13,14} Glybera aims to transduce LPL in skeletal muscles, to control or abolish symptoms of LPLD, and to prevent complications in adult patients clinically diagnosed with LPLD. The intended level of enzyme transduction should suffice to hydrolyze the triglyceride-rich lipoproteins, influence lipid homeostasis, and thus lead to clinical improvement or stabilization (Figure 2).^{13,14}

The original manufacturing process (AMT-010) used a plasmid-based system. The plasmids were then transfected into HEK293 to rescue the recombinant AAV. Subsequently, this process has been changed into a baculovirus production system (AMT-011), which has been introduced for commercial production.

LPLD patients present chylomicronemia and extremely high levels of circulating triglyceride-rich lipoproteins, resulting in several complications, with pancreatitis being the most severe. Pancreatitis attacks are responsible for multiple hospitalizations and, in severe cases, progress into chronicity, ultimately resulting in potentially fatal endocrine and exocrine pancreatic insufficiency. Until Glybera was approved, the only approach for the control of LPLD was a reduction in dietary fat by more than 80%. Even if accomplished, this proved to be ineffective in many cases, leading patients to remain at increased risk for potentially lethal pancreatitis. Glybera has not yet been commercialized because of pricing issues.

by AMT-010. Both animal models appear to be relatively well characterized.

LPL^{-/-} mice present sustained hypertriglyceridemia, increased total cholesterol (TC), and low levels of high-density lipoprotein cholesterol (HDL-C), but no acute pancreatitis develops in the model. This may be seen as a limitation, because this is the most severe complication observed in humans suffering from LPLD. LPL^{-/-} mice do not survive beyond an age of 24 hr, probably in association with starting to suckle, with resulting hypertriglyceridemia. LPL^{-/-} mice were treated shortly after birth with an LPL-expressing adenoviral vector, which made them not naive for LPL protein. This is similar to LPLD patients who are exposed to mutated, and then ineffective, LPL enzyme. The treatment of LPL^{-/-} mice (which are not naive to LPL protein) with adenovirus, resulting in expression of human LPL, enabled mice to survive well beyond the normal post-natal 24 hr.

LPL^{-/-} cats are a naturally occurring LPLD strain that, comparable to in humans, develops lactescent plasma, xanthomata, lipaemia retinalis, abnormally high hypertriglyceridemia, and controversially discussed pancreatitis. The survival rate of LPL^{-/-} cats is similar to that in humans and longer than for the mice model. The main findings reported in these two LPLD animal models are described in the next sections.

Study Outcomes in LPL^{-/-} Mice.

- (1) Transgenic animals responded with an increase in plasma triglyceride concentrations; in AMT-010-treated mice, recovery



- from this increase in plasma triglycerides was improved compared to untreated mice, with a reduction up to 99.2%.
- (2) Dose-related, quantifiable human LPL activity and protein content in plasma were observed up to 52 weeks post-administration, with a loss of activity over time, but were associated with complete and persistent resolution of visible lipemia over a year.
 - (3) Similar to natural LPL muscle expression, the injected muscle tissue expressed quantifiable human LPL activity in the outer surface.
 - (4) For a fixed dose, the transgene expression was independent of the number of injections (3 or 36), suggesting that, at least in mice, sufficient transgene to clear the circulating pool of triglyceride-rich lipoprotein was achieved with a fairly limited degree of muscle tissue.
 - (5) A single dose was able to induce long-lasting expression of transgene human LPL, associated with a clear effect to reduce raised triglycerides markedly.
 - (6) The activity was demonstrated in the dose range 1×10^{11} – 1×10^{13} gc/kg compared to the proposed human dose of 1×10^{12} gc/kg.
 - (7) When given an intravenous lipid challenge, on readministration, the second dose was not able to elicit a response, an effect attributable to formation of neutralizing antibodies to the AAV1 capsid.
 - (8) Immunosuppression did not ameliorate transgene expression. This is relevant because the clinical dosing strategy uses immunosuppression to inhibit antibodies to AAV infection, and it is fairly common for humans to have antibodies to AAV.
 - (9) Toxic effects were observed in the muscle at levels of 11- to 24-fold LPL overexpression.

Study Outcomes in LPL^{-/-} Cats.

- (1) In the cat model, AMT-010 corrected severe triglyceridemia and lipemia, acting within 3–4 days, and it was concluded that a dose needed for this effect was potentially achievable in humans.
- (2) Immune responses, possibly from trans-species reactions, resulted in loss of this effect.
- (3) Immunosuppression could delay the loss of response.

Altogether, the outcomes of the studies in the two LPLD animal models support the usefulness of the animal models to address the mode of action of alipogene tiparvovec, the ability to induce LPL expression in muscle, the efficiency of the enzyme to reduce hypertriglyceridemia, the value of this effect for the correction of the LPLD, and the prolongation of health status (cats) or lifespan (mice) of the LPLD-deficient animals. Furthermore, the animal models were helpful for the estimation of the human dose for efficacy and for safety. One important aspect highlighted in the mouse model refers to the possible lack of usefulness of immunosuppressive therapy to reduce antibody formation against LPL, which is an issue deserving further discussion and confirmation. The use of animal models also hints at the effects of excessive expression of LPL, so the LPL^{-/-} mouse model was useful for toxicological evaluation, which has been poorly explored. Clinically, LPL expression and activity were measured in muscle biopsies of treated patients. The outcomes were

not considered highly conclusive, meaning that the supportive proof-of-concept application might have relied on the outcome of the animal studies. It is however not clear on which extension, quantitatively, the studies in animal models contributed to the clinical use, because the proof of efficacy of Glybera has been difficult to accept by the regulatory authorities. Muscle lesions were generated by intramuscular (i.m.) injection of GTMP in animals and humans. These effects and the enzyme expression might have been fully characterized in the animal models of disease used in the pharmacology studies, which would have overcome further study repetitions for assessing toxicity. The integrated planning of the preclinical program to optimize and maximize the information to be generated with animal studies is therefore fundamental, and in the case of Glybera, it might have accelerated development, saving animal use and making it more cost effective.

Pharmacokinetics. The absorption of Glybera has not been studied, because it was presumed that the ATMP would stick in the muscle and be expressed there, and this has been shown in the pharmacodynamic studies. Biodistribution and persistence of the vector were studied in cats, mice, and rabbits. Vector DNA was mainly detected in the injected muscle, liver, spleen, and inguinal lymph nodes. In cats, it was also detected in the testis, epididymis, and motile sperm fraction, indicating some dissemination of the vector to their gonads. In rabbits, vector DNA presence was also observed in the semen. The presence of the vector in semen and reproductive organs has driven the conduction of reproductive toxicity studies with Glybera. In mice, a time course for loss of expression was evident, and longer expression was evident with a higher dose, but complete clearance was not confirmed over an observation period of up to 180 days. Injected muscle and, to a lesser extent, the inguinal lymph nodes retained expression. Regarding excretion, the only studies performed referred to shedding, which was studied in clinical samples, but not in preclinical studies.

Toxicology. General toxicity evaluation of alipogene tiparvovec has been performed using only one species, wild-type mice, which is acceptable according to the preclinical guidelines for GTMPs. The reproductive toxicity was also studied in mice. For general toxicity, single administrations were performed, but the post-dosing observation times were from 90, 105, and 180 days, corresponding to the adaptation of the concepts behind the duration of repeated-dose toxicity studies (for chronic use) in the ICH M3(R2) guideline¹ and the expected persistence time of the vector and gene product expression.

The main toxicity findings were observed at the injection sites, with consistently observed histopathological findings, including myodegenerative changes and subacute inflammation after i.m. administration of either 1×10^{12} or 1×10^{13} gc/kg AMT-010 and AMT-011. The incidence and severity of myodegeneration seemed to be treatment related and dose dependent, and these were also seen to be regressing in the 180-day follow-up study. The muscle lesions did not appear to affect muscle function, though a dedicated investigation on this aspect

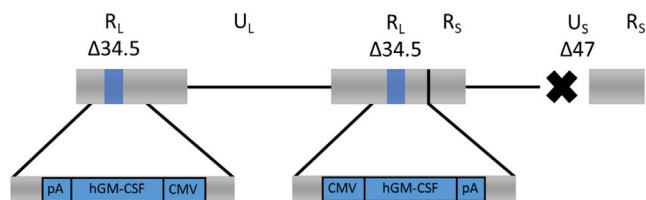


Figure 3. Talimogene Laherparepvec Has Been Modified to Replicate within Tumors and to Produce the Immune Stimulatory Protein Human GM-CSF

The schematic diagram illustrates the mechanism from which T-VEC was developed. Basically, herpes simplex 1 virus render the modified vector known as talimogene laherparepvec (T-VEC) by deletion of ICP34.5 and ICP47 and posterior insertion of the coding sequence GM-CSF. The genome is composed of a unique long (U_L region) flanked by long repeats (R_L) and a unique short region (U_S) flanked by short repeats (R_S). The site of the human GM-CSF cassette insertion is expanded to show the composition; the CMV (cytomegalovirus) promoter, human GM-CSF cDNA, and a (polyadenylation) pA signal. Adapted from Imlygic EPAR, EMA/734400/2015 (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002771/WC500201082.pdf).

had not been performed. No CD8⁺ T cells were detected, indicating that the cellular infiltrates that were detected do not represent cytotoxic T cells. Furthermore, in mice, no functional effect was identified in general toxicity studies, although no dedicated muscle function tests were included. The toxicological findings in animal studies were well matched clinically, because in patients, signs of local muscle degeneration and regeneration, with some seemingly dose-related cellular infiltration, were observed in injected muscles up to approximately half a year after drug administration during the study.

Carcinogenicity. Because Glybera is to be given just once in a lifetime (repeated dosing is in principle not possible due to immunogenicity) but the expression of LPL is expected or intended to be long-term persistent (ideally with lifelong expression), the possibility for carcinogenicity to occur would need to be addressed. However, conventional carcinogenicity studies do not apply and are not possible. Glybera contains two elements that may pose a tumorigenic hazard, these being the woodchuck post-transcriptional element and insertional mutagenesis. The woodchuck post-transcriptional regulatory element (WPRE) acts to amplify transgene expression and achieve sufficient levels of expression. Woodchuck post-transcriptional regulatory element (WFPRE) contains an element that promotes the woodchuck hepatitis virus (WHV) X protein, which is associated with the development of liver tumors in WHV-infected woodchucks, related partly to the association between hepatitis B virus X (HBx) protein. HBx appears to act as a tumor promoter, not an initiator.

To address the carcinogenic potential of Glybera, the risk-based or weight-of-evidence approach was used through analysis of the possible attributes affecting in this potential, together with evidence generated *in vitro* and *in vivo*: insertional mutagenesis was studied and not observed, and the WHV X protein was not detected in two cell lines after transfection. In toxicity studies with Glybera that lasted 105 and 180 days, no increase in tumor risk was identified in the liver or any other tissue (e.g., based on histopathology). Therefore, it has

been concluded by the applicant and accepted by CAT and CHMP that there is no evidence pointing toward a carcinogenic risk posed by Glybera.

Reproduction Toxicity. Persistent signals observed in the gonads of cats, mice, and rabbits triggered further testing in rabbits using cell fractionation methods to determine whether vector DNA would localize within sperm cells. Because vector was found in both seminal fluid and sperm cells, the need for breeding studies to investigate its possible transmission to the F1 generation was discussed. Clinical testing also indicated positive signals in semen, indicating the relevance of requiring further animal testing. As for carcinogenicity, conventional reproductive toxicity studies designed according to the ICH guideline on reproductive toxicity¹⁵ cannot be applied to GTMPs, and protocol adaptations were needed: (1) female mice were treated with AMT-011 (single dose) 4 weeks before mating, and the presence of vector DNA to fetuses, which could be indicative of germline transmission via the maternal line, was investigated and was not observed, and (2) a breeding study in male CD-1 mice was conducted and did not evidence paternal germline transmission of AMT-011. Nevertheless, warnings regarding the use of Glybera by women of potential childbearing age and during pregnancy were included in the product information.

It could be concluded that the animal studies (1) were supportive of the use of Glybera in LPLD patients for the expression of LPL and subsequent improvement of the highly adverse hyperlipidemia profile, (2) identified the local inflammatory effects after *i.m.* administration, and (3) led to the identification of potential reproductive toxicity concerns that were subsequently clarified. When analyzing the Glybera preclinical program, which has been long lasting and was developed well before dedicated guidelines for GTMPs were available, it becomes apparent that the pharmacology studies *in vivo* might well have been adapted to incorporate safety and kinetics or biodistribution. This option would have avoided the conduction of repeat studies just to evaluate the biodistribution and would have provided a better use of the animal models of disease. Diseased models could have been more relevant to mimic the patient's situation than healthy animals, because healthy volunteers would never be treated with Glybera or even in phase I clinical trials. A more restricted development plan, with fewer animal studies, could have been possible. This requires thorough planning of the preclinical studies, but if appropriately done, it may lead to reinforcement of the usefulness of animal models for preclinical testing and would eliminate irrelevant studies. Better translational value of animal studies and reduction of preclinical development testing in terms of extension and duration might be the most prominent outcomes of this well-planned strategy. Researchers in academia and in research institutes should keep these aspects in mind when planning their research projects.

Imlygic

Talimogene laherparepvec (T-VEC) is an oncolytic immunotherapy derived from herpes simplex virus 1 (HSV-1) (Figure 3), which has been approved as an antitumor medicine for the treatment of

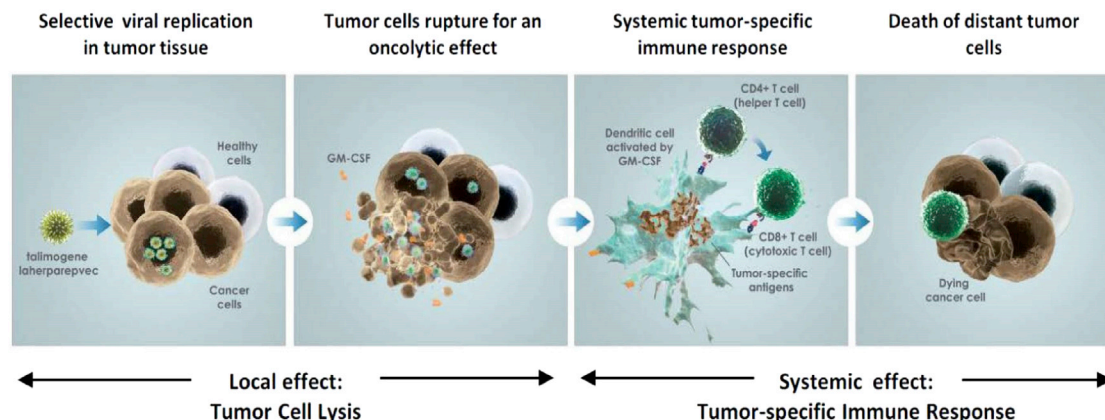


Figure 4. Proposed Mechanism of Action for Talimogene Laherparepvec

Extracted from Imlygic EPAR, EMA/734400/2015.

adults with unresectable melanoma that is regionally or distantly metastatic (stage IIIB, IIIC, and IVM1a) with no bone, brain, lung, or other visceral disease.¹⁶ T-VEC has been conceived to replicate within tumors and to produce the human immune stimulatory protein granulocyte-macrophage colony-stimulating factor (GM-CSF). T-VEC acts by causing the death of tumor cells and the release of tumor-derived antigens, which will promote a systemic antitumor immune response and an effector T cell response (Figure 4).¹⁶

The Preclinical Development Program of Imlygic. The nonclinical development program of Imlygic included pharmacology and toxicology studies designed to evaluate the mechanism of action, biodistribution and shedding, general safety of T-VEC following single and repeat administration, and effects on embryo-fetal development.

Pharmacodynamics. *In vitro*, *in vivo*, and *ex vivo* studies were performed to address the mode of action of T-VEC.

***In Vitro* Studies.** The lytic potential was studied in a range of human tumor cell lines (melanoma, colorectal, breast, brain, pharynx, prostate, and squamous cell carcinoma cell lines), while the dose and time dependence of the lytic and human GM-CSF secretion were studied in melanoma cell lines 24 and 48 hr post-infection.

***Ex Vivo* Studies.** The T cell immune response after OncoVEX has been studied and shown *ex vivo*, measuring the stimulation of interferon gamma (IFN γ) release by CT26-specific cytotoxic T lymphocytes (CTLs) obtained after intratumoral injection of a mouse surrogate of OncoVEX (described later). Mice bearing A20 tumors were treated with JS1/34.5⁻/47⁻ (OncoVEX backbone). The immune response was measured *ex vivo* by the T lymphocyte activation as a response to IFN γ release and by development of specific cytotoxic T cells using CTL assay. The IFN γ release was stimulated, most notably in splenocytes isolated from the surrogate-treated A20 tumor cells bearing mice.

***In Vivo* Studies.** The antitumor activity of OncoVEX was studied in nude (xenograft studies) or immunocompetent (syngeneic studies) BALB/c mice. Mouse models of melanoma and of reticulum cell sarcoma were tested. The antitumoral effects of T-VEC (1) after local intratumoral injection or (2) occurring systemically after injection in the contralateral, tumor-free animal flank were studied.

Because the human GM-CSF does not bind to mouse GM-CSF receptor, animals had to be treated with a mouse surrogate HSV-1 vector encoding the murine GM-CSF (OncoVEXmGM-CSF; JS1/34.5⁻/47⁻/murine GM-CSF) instead of using the clinical candidate. The studies included the analysis of antitumor effect of OncoVEXmGM-CSF on mice previously exposed to wild-type HSV-1.

B16F10-muNectin1 Melanoma Syngeneic Tumor Model in Female C57BL/6 Mice. The mouse model using the B16F10 cell variants has high metastatic potential to distant visceral organs, most notably the lungs, and has been ideal for *in vivo* studies because of its swift growth pattern and high turnover, inducing death within 2 to 4 weeks after subcutaneous (s.c.) injection into mice. Tumors were initiated by s.c. implantation of 1×10^5 B16F10-mNectin-1 tumor cells into the right flank of C57BL/6 mice. One control group and two dose groups (n = 10 each) were studied. 10 days post-tumor cell injection, animals received three intratumoral doses of T-VEC (or other viral constructs or vehicle) on days 10, 13, and 16. One group of mice received only one dose of T-VEC at day 10 post-tumor implantation.

In mouse reticulum cell sarcoma (A20)-induced tumors in BALB/c mice, the direct antitumor effect (injected right flank tumors) was evaluated after intratumoral injection (p < 0.001) of T-VEC mouse surrogate (murine GM-CSF) administration in mouse reticulum cell sarcoma (A20) BALB/c mouse model. The systemic effect (uninjected left flank tumor) was studied after the injection of the GTMP in the contralateral (tumor free) flank (left flank). Positive effect has been observed in this condition with both T-VEC and the mouse surrogate vector, which had a similar but slightly more potent antitumor effect.



In immunosuppressed BALB/c mice bearing A20-induced tumors given cyclosporin (50 mg/kg) from 2 days before the first dose of T-VEC (or human GM-CSF-deficient T-VEC) throughout the study, the tumor diameters were significantly smaller in the T-VEC (or GM-CSF-deficient T-VEC) groups compared to the control at days 14 and 18. Significant levels of serum HSV-1 antibodies were seen in treated animals on day 27. Antibody levels were similar in the animals treated with either T-VEC or GM-CSF-deficient T-VEC. Because the study did not seem to include a group of T-VEC-treated, cyclosporine-nontreated animals, it is not clear from the report whether immunosuppression has or has not affected the antitumor effect of the GTMP.

Pharmacokinetics. Nonclinical pharmacokinetics evaluation included single-dose and repeated-dose studies addressing biodistribution, viral shedding, and replication of T-VEC. Contrary to Glybera, the repeated-dose studies were designed to simultaneously address biodistribution, viral shedding in excreta (urine and feces), shedding tissues (lacrimal glands, nasal mucosa, and salivary glands), and toxicology.

Regarding *in vivo* biodistribution and viral shedding, the distribution and persistence of T-VEC at the site of administration, as well as in blood and all other tissues, were evaluated in naive or tumor-bearing BALB/c mice following single or multiple subcutaneous, intravenous, and intratumoral dosing. The biodistribution was most extensive after intravenous dosing, followed by intratumoral and subcutaneous dosing. Highest contents of viral DNA were detected in the injection site (tumor or subcutaneous site in the flank of the animal), blood, organs, and tissues with high blood perfusion, such as heart, liver, lungs, kidneys, and spleen. Although rapid clearance was observed, possibly due to antibody formation, some viral DNA was detected in some tissues after a prolonged time, up to 84 days after the last dose, suggesting persistence and possible replication of the virus. Viral DNA was detected in a low number of samples in the brain, testes, ovaries, duodenum, liver, lung, lymph node, and spleen. The presence of viral DNA in the brain and in the trigeminal ganglia was only analyzed at early time points, which left a safety concern in the risk management plan. This insufficiency might have been better addressed at later time points, which could have eventually allowed relief of the clinical concern. This approach would be in line with the risk-based approach, because some concern that the mutated virus could still be present and replicating in nervous tissues persists based on some published findings.¹⁷ After injection of T-VEC in the dog's prostate, viral DNA was also detected in lumbar and cervical spinal cord. This suggested that despite ICP34.5 removal from the viral genome, which would eliminate the replication ability in neurons,^{18–21} the virus kept some affinity for nervous system tissue, which is in line with the findings from Lasner et al.¹⁷ A post-authorization prospective safety study of a cohort of patients treated with T-VEC in clinical practice has therefore been undertaken to characterize the risk of herpetic illness among patients, close contacts, and healthcare providers. After intratumoral administration in A20 tumor-bearing BALB/c mice (three doses were used), viral DNA was detected in 90% and 100% of tumor samples collected 24 hr post-dose in the

low- and high-dose groups, declined at subsequent sampling time points, and was not detectable at post-last dose days 50 and 70. However, a viral presence appeared again 84 days after the last dose (15% and 25% of tumor samples from the low- and high-dose groups, respectively), suggesting that some viral replication might have occurred.

Viral shedding was studied in animals and humans. In BALB/c mice, following multiple s.c. doses of T-VEC, viral assay was performed 24 hr to 4 weeks post-dose, while a 12-week post-dose time point was planned, but not studied, based on the negativity of earlier time points. Viral presence was detected in 2 of 10 urine samples 24 hr post-dose below the assay limit of quantification, and no presence was seen 4 weeks post-dose. Following three intratumoral injections of T-VEC in A20-bearing BALB/c mice, shedding tissues (lacrimal glands, nasal mucosa, and salivary glands) or excreta samples (urine and feces), which were collected at multiple post-last dose days (i.e., 24 hr, 7 days, and 84 days), were analyzed. The results did not raise concerns about risks of shedding and viral transmission to third parties.

Data from humans revealed low copy numbers of the viral DNA detected in blood (30% of subjects) and urine (20% of subjects) samples across the studies from 1 hr to 1 week after intralesional injection. Available samples from 2 weeks post-injection were negative. The longest time that T-VEC DNA was detected in the injection site swabs was 2 weeks post-injection.

Limited data were generated on shedding and biodistribution for T-VEC after the intratumoral injection with the highest dose. Preliminary data of a clinical study in patients with melanoma (n = 20 subjects) showed that T-VEC DNA was detected in 36% samples of blood and 2% samples of urine, and the proportion of positive blood or urine samples was highest during the second cycle. 17% of samples from occlusive dressing tested positive for T-VEC DNA, but none tested positive for the presence of infective virus. Among samples of oral mucosa, only one tested positive for T-VEC DNA but negative for infectious virus.

For human translation of animal shedding studies, despite the limited clinical information, it appears that the studies in mice, particularly after intratumoral administration, were predictive of viral shedding of T-VEC in humans in urine and blood, at least in qualitative terms.

Toxicology. Information on the tolerability of T-VEC was obtained from (1) nonpivotal repeated-dose studies through the intratumoral route administration under clinically relevant conditions, e.g., in tumor-bearing animals that allow viral replication as anticipated in patients, and (2) pivotal repeated-dose studies in tumor-free mice, after s.c. and intravenous (i.v.) routes of administration, to inform the safety of T-VEC under conditions that are similar to the planned clinical dosing route in a study unconfounded by the presence of a tumor. In two of the pivotal repeated-dose studies, a group of high-dose animals was used to assess biodistribution.



Pivotal Repeated-Dose Studies. In one study, both the mouse homolog and the clinical candidate were tested, and observation was performed up to 28 days post-dose. Reversible cellulitis at the site of injection and increased hematopoiesis in the splenic red pulp were the only safety findings registered. Two additional studies were performed in BALB/c mice. First, 5 subcutaneous doses of GSF-OncoVEX were given with a 3-day interval between doses, followed by observation periods of 1, 28, or 56 days, during which the high-dose group was also used for biodistribution evaluation. The reported adverse effects were cellulitis and increased hematopoiesis, as described earlier. Second, once a week for 12 weeks (or 5 weeks for biodistribution), a repeated-dose study was performed with up to 84 days of post-dose observation.

Genotoxicity. No specific studies were conducted, and literature review has been the basis for genotoxicity assessment. T-VEC is a genetically modified HSV-1, which is an enveloped, double-stranded DNA virus that forms stable, circular episomes and does not integrate with host DNA; therefore, no direct mutagenicity would be expected.²²

Carcinogenicity. Similar to genotoxicity, the carcinogenic potential of T-VEC was addressed through a thorough review of published studies investigating the epidemiology of HSV-1 infection and cancer risk in human populations as follows: (1) a case control study of 410 cases with oral cancer and 410 matched controls in Sweden, with exposure assessment based on patient recollection of symptoms; (2) a case control study of 260 oral carcinoma cases and 445 matched controls, with exposure assessment based on serological evidence; (3) a case control study of 131 oral carcinoma cases and 136 controls in the United States, with exposure assessment based on serological evidence, and (4) a case control study of 164 head and neck cancer patients with 295 matched controls in United States. Two of the studies concluded that a previous HSV-1 oral infection was a risk factor for oral carcinoma, but no evidence of a direct carcinogenic effect has been reported.

Reproductive and Development Toxicity. Effects of T-VEC on embryo and fetal development were evaluated in dedicated studies in mice. While no relevant effects were observed, negligible amounts (<0.001% of maternal blood levels) of T-VEC DNA were found in fetal blood, suggesting transplacental passage, which led into a warning in the summary of product characteristics (SPC). No description of dedicated fertility studies is found in the product EPAR, but it is stated that no effects had been seen in reproductive organs. In biodistribution studies, no reference is found to the presence of T-VEC in the reproductive tissues.

In summary, the analysis of the preclinical development program of T-VEC shows a more integrated strategy compared to that of Glybera, which is understandable given T-VEC's more recent development. Repeated-dose studies were designed to incorporate biodistribution using healthy as well as animal models of tumors. The program illustrates how a well-planned development program could be simultaneously used for assessing proof of concept and screening for effective doses or exposures, biodistribution, and toxicity of a GTMP.

The relevance of biodistribution studies, which, according to the state of the art can only be comprehensively performed in animal models, is shown with either Imlygic or Glybera. The outcome of such studies will dictate the need for testing the toxicological consequences at the tissues or organs of biodistribution through general toxicity or dedicated studies. With Imlygic, concerns about nervous system, as well as fertility and reproductive, toxicity studies were among those needing to be addressed. Because no effects (or distribution) of T-VEC in the reproductive tissues were seen (possibly in repeated-dose studies), dedicated fertility studies were not conducted. Embryo-fetal studies could be justified not only by the need to address biodistribution and transplacental passage but also by the need to study the potential effects of the GTMP or the gene product on fetal development. For genotoxicity and carcinogenicity, the risk-based approach has been used, putting together the attributes of the viral vector, existing scientific knowledge related to the viral vector (on genome integration), and epidemiological information in patients exposed to the wild-type virus with regard to the susceptibility for developing cancer.

Imlygic versus Glybera Development

Since the implementation of the regulation on ATMPs, multiple guidelines have been generated to help researchers and manufacturers in the discovery and development of gene or cell therapies, producing the appropriate information and studies to support the efficacy and safety of such products. In the extensive period during which Glybera was tested, no dedicated guidelines were in place. This is apparent in the development program, which has somehow followed the general regulatory guidance developed for conventional medicinal products.

For Imlygic, the principles behind the guidelines produced for GTMPs by the CAT are already apparent, highlighted by aspects such as the impact of biodistribution on the design of other studies, the use of a risk-based approach for addressing genotoxicity and carcinogenicity, the integration of biodistribution studies in repeated-dose studies, maximizing animal usefulness, and reducing timings for such combined versus separate studies. In addition, the biodistribution pattern captured in relevant experimental *in vivo* models, like the models of disease and the use of homologous vector-based GTMP to assure the infectivity of the test species, has substantially contributed to human prediction of efficacy and multiple aspects of safety, including potential reproductive effects, viral vector shedding, and replication potential, as was the case for Imlygic. If appropriately designed using a science-based rationale, rather than a checkbox-based rationale, the few cases of approved GTMPs point toward a useful contribution of *in vivo* biodistribution studies in determining the full preclinical and clinical development strategy for this type of product.

Future Trends

The lack of a wide regulatory framework in the past led to divergent national approaches that hindered patients' access to products, hampered the growth of this emerging industry, and affected competitiveness in a key biotechnology area. In the European Union (EU), a regulation on advanced therapies² has been settled upon, in which the main elements intended to improve and accelerate the development



of advanced therapies (ATMPs) are (1) establishing a centralized marketing authorization procedure to benefit from the pooling of expertise at the European level and direct access to the EU market, (2) creating the CAT as a new and multidisciplinary expert committee within the EMA to assess advanced therapy products and follow scientific developments in the field, (3) establishing technical requirements adapted to the particular characteristics of these products, and (4) providing special incentives for small and medium-sized enterprises. Since the implementation of the CAT, 8 ATMPs have received a positive opinion in the EU as of June 2016.²³ However, difficulties are identified related to the cost effectiveness of such ATMPs, given the very high costs of fulfilling the quality requirements of the development process, which is affecting the access of patients to such medicines upon reaching the market and persisting there. For the future, one possible contribution for overcoming this problem may be related to a gain in knowledge associated with progress in the areas of cell therapy medicinal products (CTMPs) and GTMPs. For instance, with regard to GTMPs, the use of vector platforms incorporating the therapy-specific gene entities will facilitate gains in knowledge on the biodistribution and safety aspects related to those specific vector platforms, allowing the application of such knowledge to the characterization of the related GTMP and then reducing their preclinical, and eventually supportive clinical, programs. In addition, the creation of shared facilities where such products might be produced and characterized could be a strategy to reduce the production costs and accelerate development and access. Altogether, using and sharing acquired knowledge and practical experience (precompetitive components) will most likely help to make GTMPs accessible to patients and healthcare systems. Science-driven solutions are needed and will constitute the next challenge for approved GTMPs (and ATMPs in general) to become effectively usable by patients. For the sake of patient interests worldwide, globalized approaches based on international cooperation, knowledge sharing, and precompetitive alignments will be needed to achieve efficient and fair patient access to GTMPs as potentially disruptive therapies for many incurable diseases.

REFERENCES

- European Medicines Agency. (2009). ICH guideline M3(R2) on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002720.pdf.
- European Medicines Agency. (2007). Regulation on advanced therapy medicinal products. <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2007:324:0121:0137:en:PDF>.
- European Medicines Agency. (2007). Guideline on the nonclinical studies required before first clinical use of gene therapy medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003743.pdf.
- European Medicines Agency. (2015). Note for guidance on the quality, preclinical and clinical aspects of gene transfer medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2015/05/WC500187020.pdf.
- Food and Drug Administration. (2013). Guidance for industry preclinical assessment of investigational cellular and gene therapy products. <http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/default.htm>.
- Zabner, J., Seiler, M., Walters, R., Kotin, R.M., Fulgeras, W., Davidson, B.L., and Chiorini, J.A. (2000). Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J. Virol.* 74, 3852–3858.
- Vite, C.H., Passini, M.A., Haskins, M.E., and Wolfe, J.H. (2003). Adeno-associated virus vector-mediated transduction in the cat brain. *Gene Ther.* 10, 1874–1881.
- European Medicines Agency. (2010). Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/07/WC500094345.pdf.
- European Medicines Agency. (2009). ICH considerations: oncolytic viruses. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/07/WC500094345.pdf.
- European Medicines Agency. (2013). Guideline on the risk-based approach according to annex I, part IV of directive 2001/83/EC applied to advanced therapy medicinal products. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139748.pdf.
- European Medicines Agency. (2015). European Public Assessment Report of HEPARESC-human heterologous liver cells. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/003750/WC500204185.pdf.
- European Medicines Agency. (2011). ICH guideline S6(R1)—preclinical safety evaluation of biotechnology-derived pharmaceuticals. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002828.pdf.
- European Medicines Agency. (2012). European public assessment report: glybera (alipogene tiparvovec). http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002145/WC500135476.pdf.
- Schaefer, E.J., Tsunoda, F., Diffenderfer, M., Polisecki, E., Thai, N., and Asztalos, B. (2000). The measurement of lipids, lipoproteins, apolipoproteins, fatty acids, and sterols, and next generation sequencing for the diagnosis and treatment of lipid disorders. In Endotext, L.J. De Groot, G. Chrousos, K. Dungan, K.R. Feingold, A. Grossman, J.M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, et al., eds. (MDText.com). <https://www.ncbi.nlm.nih.gov/books/NBK355892/>.
- European Medicines Agency. (1995). Note for guidance on the detection of toxicity to reproduction for medicinal products & toxicity to male fertility. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002809.pdf.
- European Medicines Agency. (2015). EMA/734400/2015. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002771/WC500201082.pdf.
- Lasner, T.M., Tal-Singer, R., Kesari, S., Lee, V.M., Trojanowski, J.Q., and Fraser, N.W. (1998). Toxicity and neuronal infection of a HSV-1 ICP34.5 mutant in nude mice. *J. Neurovirol.* 4, 100–105.
- Brown, S.M., Harland, J., MacLean, A.R., Podlech, J., and Clements, J.B. (1994). Cell type and cell state determine differential in vitro growth of non-neurovirulent ICP34.5-negative herpes simplex virus types 1 and 2. *J. Gen. Virol.* 75, 2367–2377.
- Chou, J., Kern, E.R., Whitley, R.J., and Roizman, B. (1990). Mapping of herpes simplex virus-1 neurovirulence to gamma 134.5, a gene nonessential for growth in culture. *Science* 250, 1262–1266.
- Chou, J., Chen, J.J., Gross, M., and Roizman, B. (1995). Association of a M(r) 90,000 phosphoprotein with protein kinase PKR in cells exhibiting enhanced phosphorylation of translation initiation factor eIF-2 alpha and premature shutoff of protein synthesis after infection with gamma 134.5– mutants of herpes simplex virus 1. *Proc. Natl. Acad. Sci. USA* 92, 10516–10520.
- Chou, J., and Roizman, B. (1994). Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc. Natl. Acad. Sci. USA* 91, 5247–5251.
- Morissette, G., and Flamand, L. (2010). Herpesviruses and chromosomal integration. *J. Virol.* 84, 12100–12109.
- European Medicines Agency. (2016). EMA/CAT/358066/2016. http://www.ema.europa.eu/docs/en_GB/document_library/Committee_meeting_report/2016/07/WC500209627.pdf.