EXPERT REVIEWS

Safety profile of recombinant adeno-associated viral vectors: focus on alipogene tiparvovec (Glybera)

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There has been great interest over the past two decades in developing gene therapies (GTs) to treat a variety of diseases; however, translating research findings into clinical treatments have proved to be a challenge. A major milestone in the development of GT has been achieved with the approval of alipogene tiparvovec (Glybera) in Europe for the treatment of familial lipoprotein lipase deficiency. At this important stage with the evolution of GT into the clinic, this review will examine the safety aspects GT with adeno-associated virus (AAV) vectors. The topics that will be covered include acute reactions, immunological reactions to the AAV capsid and expressed transgene, viral biodistribution and shedding, DNA integration and carcinogenicity. These safety aspects of GT will be discussed with a focus on alipogene tiparvovec, in addition to other AAV vector GT products currently in clinical development.

Keywords: adeno-associated viral vectors • alipogene tiparvovec • AAV1-LPLS^{447X} • gene therapy • lipoprotein lipase • lipoprotein lipase deficiency

For almost 20 years, the promises made about the utility of gene therapy (GT) have not been translated into the development of clinical treatments. However, the scientific community's focus on GT has been rekindled following several positive results with different approaches. In October 2012, alipogene tiparvovec (Glybera, uniQure, Amsterdam, The Netherlands) became the first GT to be approved in Europe [1]. It is indicated for the treatment of familial lipoprotein lipase deficiency (LPLD), a very rare inherited condition that leads to increased fasting plasma triglycerides and other complications, including potentially fatal pancreatitis [2]. Alipogene tiparvovec introduces a healthy lipoprotein lipase (LPL) gene into the body, which results in the production of functional LPL enzyme. Clinical study data indicated that alipogene tiparvovec was associated with sustained improvements in the postprandial metabolism of newly formed, large/buoyant chylomicrons, the major cause of acute pancreatitis in LPLD patients [3,4]. There was also a clinically relevant reduction in the frequency of acute pancreatitis, a debilitating complication associated with LPLD.

The transition from clinical investigational phase to a marketing authorization means moving under different legal boundaries and a higher regulatory scrutiny. Therefore, the approval of a completely new, first-in-class product in pharmaceutical history always raises many questions on safety aspects. Given that a GT had never been approved in the West, it is noteworthy that two of the most likely sources of concern for this type of product – safety issues related to viral vector-mediated gene transfer and the process of manufacturing and characterizing a viral vector – did not prove to be major obstacles to approval [5]. The expected theoretical concerns, including insertional mutagenesis, immunotoxicity or germ-line transmission were all resolved during the review procedure (available from the EMA website).

In the case of LPLD, many questions were also raised on how to prove clinical efficacy in ultrarare disease especially when there are also many unknowns about the disease and the

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etiology of the clinical manifestations. The approval of alipogene tiparvovec has been therefore a long procedure leading to many debates and the European Public Assessment Report is a good reflection of these debates (available from the EMA website). A LPLD registry has been set up upon authorization of Glybera in Europe aiming to collect even more information on the disease and the effects of alipogene tiparvovec in a higher number of patients. Nonetheless, the approval of alipogene tiparvovec has further confirmed that GT can provide real treatment options for patients with genetic diseases.

Among a number of different DNA delivery systems, significant progress has been made in the development of recombinant adeno-associated virus (rAAV) for therapeutic use, and over the past decade, a good clinical safety record has been established for rAAV. To date, over 60 clinical trials have used different adeno-associated virus (AAV) serotypes as a vector, across a range of indications including alpha1-antitrypsin deficiency, arthritis, Batten's disease, Canavan's disease, cystic fibrosis, hemophilia B, Leber's congenital amaurosis, Parkinson's disease, muscular dystrophy and LPLD. However, there have been some concerns regarding the safety of patients undergoing treatment with AAV vectors, although in some cases these safety concerns related to concomitant factors rather than the treatment with AAV vectors. For example, in 2007, a 36-year-old woman with rheumatoid arthritis died as a result of a serious fungal infection during a trial of GT using an AAV vector. Following a subsequent investigation by the FDA, however, the trial was allowed to restart after the findings indicated that the GT was unlikely to have caused the death [6]. As the first GT is now approved for clinical use in Europe, the aim of this review article is to examine the safety profile of GT approaches with AAV vectors, with a particular focus on alipogene tiparvovec.

Acute reactions following administration AAV-based GT

Local injection site reactions with alipogene tiparvovec were initially explored through a number of good laboratory practice compliant safety studies in murine and feline models. The most noticeable observation after intramuscular (im.) administration of alipogene tiparvovec was bruising at the injection site within the first 2 weeks, which decreased in severity and occurrence after this time point. Similar reactions were observed in control animals, suggesting an administration effect rather than a drug effect. At the microscopic level, alipogene tiparvovec administration resulted in consistent and dose-dependent subacute inflammation and myodegenerative changes in the injected muscles; however, these were not associated with any overt necrosis, significant acute inflammation or exaggerated immune reaction. The muscle lesions were characterized as degeneration–regeneration with accompanying focal infiltrates of mononuclear leukocytes (predominantly lymphocytes), but as they were localized to the injection sites, they did not cause any clinical abnormalities (such as lameness) and tended to regress after 6 months. They are therefore likely to be of minimal clinical significance. Immune reactions to AAV and the local significant increase LPL enzymatic activity have emerged as the most plausible causes for this observation.Such inflammatory cell infiltrates have been described for several AAV1-based vectors containing various transgenesin animal studies [7–9]. Using an AAV1-based vector containing a dystrophin U1 exon-skipping transgene, these infiltrates were not associated with loss of efficacy and were found to be composed mainly of $CD11b^+$ cells, macrophages and neutrophils [10]. In one of these studies in a murine model, no $CD8^+$ T cells were detected in the muscles after single or multiple AAV1 injections, indicating that the infiltrates did not represent cytotoxic T-cell responses [10].

The administration of alipogene tiparvovec was well tolerated in all three interventional clinical studies, where multiple (40–60 sites) im. injections were administered during a single procedure under spinal anesthesia. The most frequent adverse events (AEs) were injection-associated local reactions that were directly related to the administration procedure. Reactions included myalgia, leg pain and edema, with bruising usually seen a few days post injection. Most of the adverse reactions were self-limiting within a few days after injection [3,11,12]. A 2-year follow-up of an open-label clinical trial (CT-AMT-011-01), in which alipogene tiparvovec was used for the treatment of 14 individuals with LPLD and prior pancreatitis, reported a number of acute AEs, particularly injection-related reactions directly after administration [3]. All 14 patients reported at least one mild-to-moderate AE and 12 subjects reported injection site events (local and transient burning, edema, sensitivity and/or pain lasting days or in some cases a few weeks). One subject developed a serious AE (fever 39.9°C) 10 h after injection, which resolved spontaneously after a further 10 h. Muscle biopsies taken from injected muscles showed local inflammatory responses in the majority of patients, and there was a trend toward a greater response in samples showing more LPL expression. However, a high variation was observed in the muscle biopsies, most likely dependent on the distance from the site of injection. As a consequence, it is highly likely that an increased acute site reaction in tissue samples with higher LPL is rather more dependent on the distance to the injection site than changes in the transgene expression. The more pronounced responses were characterized by infiltrates of $CD8^+$ T cells, $CD20^+$ B cells and $CD68^+$ macrophages [3]. However, there was no evidence of apoptosis, excess fibrosis or widespread necrosis in the samples.

Inflammatory cell infiltrates including $CD8^+$ T cells have also been observed in other clinical studies using im. injections. For example, in a study in nine individual who received im. injection with AAV1 vector expressing human α_1-antitrypsin, moderate-to-marked infiltrates including $CD8^+$ T cells and $CD4^+$ T cells as well as lower numbers of $CD20^+$ B cells and $CD68^+$ macrophages were observed in muscle biopsies taken 90 days following the vector injection [13]. These infiltrates observed in the human α_1-antitrypsin study appear to have a similar composition to those reported above for alipogene

tiparvovec [3]. In contrast, in a study of six individuals with Duchenne muscular dystrophy, im. injection of an AAV2.5 vector expressing mini-dystrophin did not result in greater infiltration of $CD8^+$ T cells compared with placebo (saline and empty capsids) [14].

Injection site reactions may differ according to different routes of administration and may well be directly linked to the im. route. For example, in one study of nine patients treated with AAV expressing α1-antitrypsin administered via the im. route, eight reported injection site reactions [13]. In one study with AAV expressing human factor IX (FIX), eight hemophilia B patients were administered the vector im. at multiple sites, but this did not seem to result in any local or systemic toxicity up to 40 months after injection [15], although the article does not describe how local reactions were assessed. In contrast, in another study with the same vector in seven subjects with hemophilia B injected intravenously (iv.) by a catheter inserted in the groin, three subjects developed small hematomas at the site of catheter insertion [16]. In a third, separate study examining intravenous (iv.) administration of AAV vector expressing a codon-optimized human FIX to six patients, there were no reported injection site reactions and no changes in vital signs during or following the infusion [17].

Immunological responses to the capsid & expressed gene protein product

GT medicinal products administered to humans may potentially elicit antibodies against the protein product expressed from the transgene and potentially also against its endogenous counterpart. Such immune reactions would represent a real safety concern. In contrast, the capsid being derived from AAV, which is a naturally occurring virus, will normally always elicit immune responses to some extent in the treated patients. Therefore, a potential concern about the use of AAV-based vectors for GT is that they may induce humoral and cellular immune responses in the recipient, which may impact efficacy and safety.

In addition, many individuals have antibodies to various AAV serotypes following natural exposure to wild-type AAV (wtAAV), and adaptive B-cell immune responses to the AAV capsid may present a barrier to effective gene transfer [18]. The absence of acute clinical responses, however, such as changes in vital signs, nausea or vomiting following injection of AAV vectors suggest that innate immune responses are unlikely to cause major clinical concerns [19].There is some evidence from animal models for the recognition of AAV by elements of the innate immune response system [18]; however, immunological data from animal studies have only limited value for assessing outcomes in humans. The immune responses in animals can vary considerably depending on the tissue that is targeted, with outcomes ranging from almost no response to responsiveness. In animal models, cellular and humoral responses to AAV were generally found to be modest in intensity [18–20].

Immune responses following GT with AAV vectors have been assessed by measuring systemic and local cytotoxic reactions as well as antibodies against the AAV capsid and/or the expressed transgene protein in human clinical studies [11,15,17,21–29]. The eye and central nervous system are known to be immune privileged compartments of the body due to adaptations that limit immune responses. When AAV vectors were administered directly into the brain or subretinal space, no measureable immune responses, or only very limited immune responses, were observed [30,31].In contrast, humoral immune responses to AAV capsid proteins were reported in trials targeting AAV-based vectors to muscle or liver [3,13,17]. Results from these clinical studies indicate that antibody responses, especially pre-existing humoral responses to AAV capsid, may impact on the efficacy of the product but are unlikely to have an impact on the safety profile. It is likely that the extent of the impact depends on the particular AAV serotype and target organ as well as route of administration and dose.

Studies with AAV vectors targeting the liver and skeletal muscle have also shown the development of a T-cell response to the AAV capsid [18], which is most likely dose dependent. Such T-cell responses have been extensively discussed in relation to a study in hemophilia B patients. Following systemic injection of a rAAV that carried an expression cassette for coagulation FIX, an expansion of $CD8^+$ T cells was observed in parallel with an increase in circulating levels of liver transaminases [16,32]. This T-cell response was accompanied by a loss of FIX expression, suggesting an elimination of the transduced hepatocytes by AAV capsid directed T cells. This type of immunogenicity was also observed in a more recent clinical study in hemophilia B patients, which also utilized an AAV vector that targeted the liver [17]. Both of the participants in the high dose group (2×10^{12} vg/kg) experienced inflammatory responses marked by increased liver enzymes (aspartate aminotransferase and alanine aminotransferase) [17]. In both patients, the rise in liver enzymes occurred concomitantly with an increase in circulating AAV capsid-specific T cells and a parallel loss in FIX transgene expression. In contrast, even though such a (cytotoxic) cellular immune response was also noted in clinical studies in which rAAV vectors were administered locally, by im. injection, in these studies, sustained expression of α1-antitrypsin was observed for at least 1 year after delivery, suggesting that in this case the cellular immune responses to the AAV capsid had not eliminated transgene expression [13,22].

Three interventional clinical studies have been conducted with alipogene tiparvovec in patients with LPLD (CT-AMT-010, CT-AMT-011-01, CT-AMT-011-02). In all studies, alipogene tiparvovec was administered via multiple direct im. injections into the legs. Various doses and immunosuppressant regimens have been used in the studies. These are summarized in Table 1.

Following im. injection of alipogene tiparvovec, no treatment-related changes in C-reactive protein, an inflammatory marker; creatine phosphokinase, a marker of muscle damage; or lactase dehydrogenase, a marker of tissue turnover, were observed [3]. In this study (AMT-011-01), approximately half (n = 8) of patients had anti-AAV1 antibodies before alipogene tiparvovec administration, and all patients developed anti-AAV1 antibodies

Table 1. Summary of the clinical studies performed with alipogene tiparvovec.

Subjects (n), dose (gc/kg)	Immunosuppressant regimen
CT-AMT-010-01	
4, 1×10^{11}	None
4, 3×10^{11}	None
CT-AMT-011-01	
2, 3×10^{11}	None
4, 3×10^{11}	Neoral 3 mg/kg/day CellCept 2 g/day
8, 1×10^{12}	Neoral 3 mg/kg/day CellCept 2 g/day
CT-AMT-011-02	
5, 1×10^{12}	Solu-Medrone: single intravenous Bolus: 1 mg/kg Neoral: 3 mg/kg/day CellCept: 2 g/day

following treatment, which persisted throughout the post treatment follow-up [3]. This is consistent with the observations in another study using an earlier formulation of AAV1 vector expressing LPL (AMT-010-01), where one subject of the eight treated showed a rise in creatine phoposphokinase from Week 4 after im. vector injection. And where also humoral and T-cell immune responses to the AAV1 capsid but not the LPL protein were reported [11,12]. In the study reported by Gaudet *et al.* [3], local immune responses were observed at the injection site ranging from no or minor (two patients), slight (one patient), moderate (two patients) or more pronounced (two patients) response. As discussed earlier, the more pronounced responses were characterized by infiltrates of $CD8^+$ T cells, $CD20^+$ B cells and $CD68^+$ macrophages. Further work is being undertaken to characterize these cells but, initial as yet unpublished, data suggest that the CD8 T cells lack the functionality of mature CTLs which is in agreement with already published work in murine skeletal muscle where, after rAAV vector transduction, antigen-specific $CD8^+$ T cells were shown to be nonfunctional or have impaired proliferative capacity [33,34]. A moderate and transient T-cell response to the AAV1 capsid, but not the LPL transgene, was observed in peripheral blood mononuclear cells from 9 of the 14 patients [3]. This compares to four out of eight subjects who demonstrated detectable T-cell responses to AAV capsid in the earlier trial with a different formulation of AAV1 vector expressing LPL [12]. Despite these innate and induced immune responses, AAV1 vector DNA was found in all injection site muscle biopsies 26 weeks after administration. In addition, clinical efficacy in the form of reduction in plasma TG, sustained modification of the TG-rich lipoprotein characteristics, reduction in overall pancreatitis incidence or intensity and patient-reported improvements in quality of life were observed despite the presence of antibodies to AAV1 in approximately half of patients before alipogene tiparvovec administration.

Further information on the impact of immune responses on GT is provided by studies examining the expression of a mini-dystrophin construct to treat Duchenne muscular dystrophy targeted to skeletal muscle [14]. In the first study, in six patients, higher vector genome copy numbers were observed in patients without pre-existing neutralizing antibodies to the AAV capsid [31]. The two patients who received the highest vector DNA dose had the highest genome copy number but showed low or undetectable mini-dystrophin expression. Only one subject, who had lower vector DNA, had detectable mini-dystrophin expression. Although the small study size makes it difficult to make definitive conclusions, it appears that immune responses may be affecting the efficiency of gene transfer and expression in this study [14].

In summary, these data suggest that there are possible differences in immune responses to GT depending on variables such as the vector used, the injection site, the target tissue/organ and the transgene. As would be expected, administration of GT to immune-privileged sites has negligible or no impact on immune system. In studies targeting the expression of FIX to the liver, however, immune responses were observed along with indication of liver damage and loss of transgene expression [16,17]. A potential effect of immune responses in the efficacy of gene transfer and expression was also suggested by data from a study in muscular dystrophy [14]. In contrast, targeting alipogene tiparvovec to the skeletal muscle did not appear to be associated with increase in markers of inflammatory responses or tissue damage, and while transient local and systemic immune responses were observed in some patients' transgene expression and clinical efficacy were maintained [3].

Biodistribution & shedding

Biodistribution refers to the spread of the vector DNA after administration, and its localization and persistence in tissues, body fluids or organs. Shedding and germ-line transmission may be observed as a consequence of biodistribution. The former is the dissemination of the vector DNA via body secretions including excreta, and the latter more specifically concerns the presence of vector DNA in the gonads that could potentially be transferred to progeny. Both the EMA and the US FDA consider that although knowledge derived from studies using the same transgenes and/or AAV serotypes can be used to support new product-specific applications, assessment of biodistribution, shedding and germ-line transmission should be carried out on each particular GT product during its nonclinical and clinical evaluation stages [201–203].

In the case of AAV, biodistribution is primarily dictated by the virus-specific serotype tropism and the route of administration of the GT product. A number of preclinical and clinical studies have been performed investigating the tropism and biodistribution of natural AAV serotypes, and characteristic patterns have been identified [35]. For example, in various animal models, AAV8 appears to preferentially transduce liver and, to

a lesser extent, other organs [36–38]. AAV5 has been shown to be able to transduce both mouse and nonhuman primates' liver displaying a narrow tropism and thus displaying a preferred biodistribution of the vector to this organ and may therefore be preferable from a safety perspective [39–41].

Vector re-engineering has produced recombinant vectors with hybrid capsids resulting in altered or enhanced tropism and hence improved biodistribution [35,42–45] and a superior immunogenicity profile [46–48]. Immune responses directed against the AAV-vector components, the transgene product or both could potentially affect the biodistribution of the GT product, causing its rapid elimination [18]. Preclinical studies have also clearly highlighted that biodistribution is dependent on species as well as gender, and extrapolation to humans should therefore be approached with caution [39,49]. Such differences may be explained by differences between species and individual animals with regards to viral attachment and entry into cells, as well as the presence of neutralizing antibodies.

Three publications have attempted to collate and analyze AAV biodistribution data from a large number of preclinical studies, and they have confirmed that even though generalizations across AAV serotypes should not be made, particular patterns are evident [50–52]. For example, im. and iv. administration lead to widespread biodistribution, primarily in well-vascularized organs such as liver, lungs, spleen and kidneys. In turn, shedding of vector or its components also depends on the route of administration, with local routes (e.g., intraperitoneal or ocular) administration less likely to result in shedding in bodily fluids or excreta than systemic routes. The risk of shedding is also affected by the dose of the administered vector and the time elapsed since administration. In nonhuman primates, AAV vectors shed in fluids are no longer infectious after approximately 3 days, and in clinical trials, AAV vector DNA disappears from human bodily fluids several weeks after systemic administration [16,53].

Single-dose alipogene tiparvovec biodistribution studies performed in mice demonstrated that im. administration resulted in the highest levels of vector DNA in the injected gastrocnemius and adductor muscles followed by the draining inguinal lymph nodes and the liver. Three months after administration of 1×10^{13} gc/kg, levels in the injected gastrocnemius and adductor muscles and inguinal lymph nodes decreased by <10-fold, whereas levels in the blood were decreased by >300-fold. Intermediate decreases were found for the other tissues analyzed. However, in the muscle vector, DNA levels at 6 months were similar to those at 3 months. In comparing different routes of administration, im. resulted in approximately 100-fold higher copy numbers in injected gastrocnemius and adductor muscles and 10-fold higher copy numbers in inguinal lymph nodes, testes and epididymides compared with iv. administration. In other tissues, comparable values were found after im. and iv. administration. No gender differences in biodistribution were evident following im. administration. However, females dosed iv. showed higher exposures in all tested tissues compared with males. Since the gene of interest is distributed to other organs than the target, it is important to assess any potential off-target expression. The use of an appropriate, tissue-specific promoter in the construct of the GT product helps limiting such risk.

Adverse reactions related to the biodistribution of GT products may arise due to the increased expression of the transgene at the target site or at other sites/organs. LPL overexpression in transgenic mice has been reported to cause myofiber degeneration, regeneration and cellular infiltrations [54,55]. However, the muscle lesions described in these mice, which are constitutively overexpressing human LPL present with a different picture to the alipogene tiparvovec-induced lesions. Such (alipogene tiparvovec) lesions are similar to those described in studies with AAV1-based vectors [7,10], whereby a high degree of mitochondrial and peroxisome proliferation is suggestive of the important role of free fatty acids in the biogenesis of these organelles. In transgenic rabbits, overexpressing LPL, no abnormalities such as muscle fiber degeneration or atrophy were observed by either light or electron microscopy [56]. These observations therefore do not support the hypothesis that increased LPL causes myopathies as originally described in transgenic mice. However, there are several important differences between LPL transgenic rabbits and mice hampering a direct comparison between both species including differences in lipid metabolism and lipoprotein composition. Furthermore, in mice, as in the injected LPLD patients, there seems to be no direct clinical effect of the observed histopathological findings, and no cases of chronic muscle dysfunction have been reported by patients after alipogene tiparvovec administration to date.

A consequence of potential spreading or distribution of the viral vector to the gonads is inadvertent germ-line (or vertical) transmission. Despite initial concerns regarding the predictability of animal models, subsequent preclinical and clinical studies with various AAV serotypes have shown that such viral vectors do not persist in the semen. Although vector DNA was found in the semen of rabbits treated with alipogene tiparvovec for some time after administration, subsequent breeding studies in mice showed that there is no risk of germ-line transmission via the male line and no maternal–fetal transmission was seen. In these studies, mice of both genders were treated once with alipogene tiparvovec by four im. injections into the hind limbs. Dosed males were paired with untreated females 6 days post-administration, and female mice were treated 4 weeks prior to mating with untreated males. Postmortem analysis showed that vector DNA was detected in the testes, epididymides and hind legs of treated males, and in the hind legs and reproductive organs (but not the placenta and foetus) of treated females. No vector DNA was detected in the uterus and placentas of non-treated females after mating with treated males [57].

In the clinical studies, following im. administration of alipogene tiparvovec, peak levels of vector DNA were detected at 24 h in serum, saliva and urine [3]. However, in the majority of samples, vector DNA levels dropped to undetectable levels by Weeks 4–6 following administration. At Week 1, vector DNA was detected in semen at low levels. Clearance from

semen occurred at 6–10 weeks, although in two of the five male subjects low levels of vector DNA, just above the limit of detections (1×10^1 gc/g DNA) were observed in a single sample at a later time point. The risk for AAV-based germ-line transmission is currently considered to be low and should be managed through patient instruction and monitoring [58].

DNA integration & risk of carcinogenicity upon administration

Integration

Recombinant AAVs are based on the ubiquitous, noncytopathic, replication incompetent. Despite the excellent safety profile for recombinant AAV vectors seen in numerous animal and clinical studies, and although wt AAV is not known to cause any disease in humans, the fact that vector DNA may integrate into human genomic DNA is recognized as a potential risk of carcinogenicity due to insertional mutagenesis.

The majority of AAV DNA exists as circular double-stranded DNA episomes in human tissues and, following naturally acquired infection, wt AAV DNA can persist for prolonged periods in infected cells [59]. *In vitro*, wt AAV has been shown to integrate specifically to a site on the long arm of chromosome 19 (19q13-qter), designated AAVS1 [60]. Although there are no large homologous regions between the AAVS1 site and the AAV genome, AAVS1 contains an active Rep binding element and the nonstructural AAV Rep protein (a DNA helicase) is required for site-specific integration of the vector [61]. Recently, other integration hotspots have been identified for wtAAV, where AAVS1 represented only 10% of total integration events [62].

rAAV vector transgenes, such as human LPL, are mainly expressed from episomal DNA, but also have the potential to originate from integrated vector genomes. rAAV vectors have the potential to integrate at nonhomologous sites in the host genome as single-copy concatemers –approximately 0.1% of rAAV vector genomes that enter cells integrate in this manner [63,64]. In some cases, both episomal and integrated vector genomes appear as high molecular weight DNA and can be difficult to distinguish from host cell DNA [65]. Even when host cell chromosomal DNA is removed, deletions and/or rearrangements of inverted terminal repeat (ITR) junctions and adjacent regions may further confuse the differentiation between molecular structures of the rAAV genome if, for example, the single cutting restriction endonuclease is located close to the ITR(s) [66].Early studies of rAAV vector integration used marker genes, such as neor, to select for cells that contained integrated proviruses. Southern blot, PCR and fluorescent *in situ* hybridization analysis of transduced human cell lines suggested that rAAV vectors integrated at apparently random genomic sites [67–69]. In subsequent studies, AAV shuttle vectors were used to rescue integrated proviruses in the form of bacterial plasmids to sequence vector–chromosome junctions. Russell and colleagues analyzed nine integration sites isolated from HeLa cells and 977 sites from unselected human fibroblasts, in which a significant preference for integrating within CpG islands and the first 1 kb of genes was found [70–72]. Integration sites were clustered throughout the genome with a preference for ribosomal DNA repeats. In such *in vitro* studies, chromosomal deletions, insertions and translocations are associated with rAAV vector integration. However, these *in vitro* analyses are presumably not predictive of what happens *in vivo*.

There are an increasing number of reports describing the integration profile for DNA delivered by rAAV*in vivo*. The first studies designed to estimate the fraction of rAAV vector DNA integrated into the host chromosome were performed in transduced mouse liver tissue. Southern blot analyses suggested that the rAAV vector integrated into the chromosomal DNA of wt mice livers, forming mainly head-to-tail concatemers with occasional deletions of the ITRs and their flanking sequences [73]. In a further analysis of vector integration, a shuttle vector system was developed to isolate rAAV vector–chromosomal DNA junctions. Sequencing analysis of 18 junctions revealed various rearrangements, including ITR deletions and amplifications of the vector and chromosomal DNA sequences. The breakpoints of the vector were mostly located within the ITRs, and chromosomal DNA sequences were recombined with the vector genome in a nonhomologous manner. Two rAAV-targeted DNA sequences were identified as the mouse rRNA gene and the α-1 collagen gene. A further 347 sites were identified from the livers of mice with hereditary tyrosinemia, in which transduced hepatocytes selectively proliferated [74,75].

Approximately 1,000 integration sites have been characterized from the liver, heart and skeletal muscle of mice [76], and five sites sequenced from mouse bone marrow showed that up to 30% rAAV integration events occurred in the vicinity of DNA palindromes with an arm length of 20 bp (total length 40 bp) [77]. Taken together, these studies represent the bulk of data of the sequenced rAAV vector integration sites. PCR and Southern blots also suggested that rAAV vector integration occurs in various tissues, including the brain, heart, skeletal muscle, liver, kidney and testicular tissue, although no provirus junctions were identified in these studies [78,79]. The frequency and spectrum of genomic integration of rAAV8 vectors in the liver following IP injection of 2.0×10^{11} vector genomes each at birth has also been investigated [80]. This dose was sufficient to transduce a majority of hepatocytes in the neonatal period. Mice were injected with a β-galactosidase-expressing vector at birth, and rAAV integration events were quantified by taking advantage of liver regeneration in a chronic hepatitis animal model following partial hepatectomy. Approximately 0.05% of hepatocytes contained rAAV integrants, whereas the average copy number of integrated double-stranded vector genomes per cell in the liver was approximately 0.2, suggesting integration of concatemers. Twenty-three of 34 integrations (68%) occurred in genes, but none of them were near the *mir*-341 locus, the common rAAV integration site found in mouse hepatocellular carcinomas (HCCs). Thus, rAAV8 vector integration occurs preferentially in genes at a frequency of 1 in approximately 10^3 hepatocytes when a majority of hepatocytes are transduced in the neonatal period.

A recent publication presented the analysis of the integration pattern in genomic DNA from liver samples derived from ornithine transcarbamylase (OTC) mice [81]. The samples were analyzed by LM-PCR and pyrosequencing as described by Li *et al.* and integration was seen at the *Dlk1–Dio3* locus both in healthy liver and tumors [82]. Analysis of the integration site distributions relative to the AAV serotypes could not demonstrate any differences between vectors due to the limited power of the study (two animals treated with each an AAV-OTC vector) and the poor controls (only one healthy liver sample analyzed). The original article by Bell *et al.* [83] described the incidence and histological analysis of liver nodules in OTCD mice and the key findings were that there was no increase in nodules/HCC in animals treated with AAV-based vector expressing OTC. In fact, the metabolic correction may have even diminished background tumors, consistent with the recent observation that OTCD is associated with an increased risk of spontaneous HCC [84].

Another recent study by Li *et al.* addresses these questions using high-dose liver-directed AAV-mediated gene transfer in the adult C57Bl/6 mouse as a model (80 AAV-injected mice and 52 controls). After 18 months of follow-up, AAV-injected mice did not demonstrate a significantly higher rate of HCC compared with controls [82]. Tumors in mice treated with AAV vectors did not have significantly different amounts of vector DNA compared with adjacent normal tissue. Integration patterns in tumor tissue and adjacent normal tissue, analyzed by LM-PCR and pyrosequencing, were similar to each other, showing preferences for active genes, CpG islands and G-C-rich regions. Gene expression data showed that genes near integration sites did not show significant changes in expression patterns compared with genes more distal to integration sites. No integration events were identified as causing increased oncogene expression.

The evaluation of rAAV integration frequency in mouse muscle tissue has yielded far more conservative estimates for rAAV vector integration than those in the liver [74,75]. Three different assays were unable to detect integrated vector DNA at a sensitivity of <0.5% of total vector DNA [59].AAV vectors have also been shown to integrate inefficiently into the genome of myocytes and persist as episomal chromatin in nonhuman primate muscle [85]. Characterization of AAV vector genome structure and potential integration up to 22 months after im. delivery in nonhuman primates has shown that AAV predominantly persist as episomal and concatemeric circles in a head-to-tail configuration. Another study analyzed transgene expression after AAV8 delivery of the LacZgene to newborn rat liver and showed that it was persistent in small clusters of hepatocytes 3 months post-delivery [86]. Integration site analysis data obtained in transgene expressing hepatocyte clusters dissected 1 month post-injection supported the presence of AAV genomes that have integrated with low frequency, indicating that integration may be responsible for long-term expression in these cells.

Linear amplification-mediated PCR (LAM-PCR) has been developed for amplification and sequencing of unknown sequences flanking the integrated vector DNA and has been proven to be the most sensitive available technology to date to identify and characterize vector integration down to a single event [87]. LAM-PCR has been successfully coupled with next-generation pyrosequencing and subsequent (semi-) automated bioinformatical data mining, including trimming of sequences, alignment using UCSC BLAT analysis tools and identification of nearby genes and other integration features allowing high-throughput analysis of retroviral integration sites in preclinical and clinical settings [88–94].

AAV vectors are known to integrate at low frequency into host mammalian genomes, so performing a comprehensive integration site analysis from clinical samples can be difficult. In one study, LAM-PCR was combined with next-generation pyrosequencing to analyze alipogene tiparvovec integration and persistence in muscle biopsies from LPLD patients and in a murine model after im. and iv. delivery [95]. Furthermore, using a (semi-)automated bioinformatic data mining pipeline, the ratio between concatemers and integration sites was estimated to detect sequence logos around the integration sites and to discover preferential ITR breakpoints.In total, more than 600,000 AAV-derived LAM-PCR amplicon sequences were analyzed and the study identified 1,968 unique mappable AAV insertion site (IS) in patient muscle biopsies as well as 1735 IS and 892 IS in mouse muscle and liver samples, respectively. As expected, the majority of LAM-PCR amplicon sequences represented concatemeric AAV rearrangements and showed large deletions of the vector ITR sequences. These data show large-scale *in vivo* AAV analyses are feasible and in contrast to other AAV integration studies, there is a largely random AAV integration profile in muscle of individuals with LPLD without any integration within the AAV-HCC locus, the 6-kb genomic region on the mouse chromosome 12 in which integration of AAV was associated with the development of HCC [95].

In the analyzed patient samples, two AAV integration hotspots were found (~50-kb downstream of the gene *OR4F29* [33 IS] and in intron 2 of the *PCBD2* gene [20 IS]). A direct alignment of these AAV IS sequences to the approximately 16 kb mitochondrial (mt) DNA genome more often revealed a greater homology than to the nuclear genome. In the preclinical murine muscle samples, alipogene tiparvovec was also found to be clustered (10 IS) in mtDNA homologous sequences after im. delivery: approximately24 kb upstream of the *Col19a1* gene. Two other hotspots (both composed of 11 IS) were retained from the nuclear genomic muscle-specific gene Ttn and the Myh gene cluster. Interestingly, and in contrast to im. injection, after iv. injection of alipogene tiparvovec, no hotspots were retrieved in either nuclear DNA or mtDNA [95].

Carcinogenesis

Apart from the reported proliferation of rAAV-transduced hepatocytes in the mouse model for tyrosinemia [96], only one small study has described the occurrence of HCC following the iv. injection of 8^{10} infectious units/kg rAAV2 vector encoding the human enzyme β-glucuronidase (GUSB) into newborn

mutant mice [97]. HCC was first detected in a 35-week-old mouse and by 72 weeks of age, three out of five rAAV-treated MPS-VII mice had similar lesions. However, integrated rAAV sequences were not detected from these HCC, and it was unclear whether the tumourigenesis was linked to the rAAV2–GUSB vector, for a number of reasons. The dose of 8^{10} infectious units/kg was 15-times higher than previously shown to be therapeutic for the MPS-VII mouse model [98]. Injecting extremely high doses 2 days after birth, when hepatocytes are more actively dividing, potentially increases the frequency of integration, the amount of cell proliferation after integration and the length of time for tumor development, which occurred preferentially in older mice. Furthermore, without treatment, mutant MPS-VII mice have a limited lifespan (6–10 months) so generally that they may not live long enough to develop the neoplasms observed in this study. Since HCC was only detected between 35 and 72 weeks, one potential explanation is that only after receiving the therapeutic vector did their extended lifespan provide the necessary time for tumor formation. Finally, there is the possibility that the GUSB transgene activated neighboring genes or promoted cell proliferation in some manner, or the viral preparation contained carcinogenic impurities of infectious or chemical nature.

The finding of HCC in a small number of treated mice was not immediately replicated with a larger sample size [97]. However, 6 years later a short report was published, where newborn MPS-VII mutant mice received 1.5^{11} genome copies each and six of 18 (33%) AAV-treated MPS-VII mice developed HCC [99]. Wild-type mice injected with the AAV2-GUSB vector or a version lacking the β-actin promoter also developed HCC at significantly higher rates (56% and 33%, respectively) compared with untreated normal mice (8.3%). No tumors were observed in transgenic mice overexpressing GUSB from the same expression cassette as that in AAV2-GUSB. In MPS-VII mutant mice that received the full vector, four integrated proviruses were identified in a region of chromosome 12 that contains multiple maternally and paternally imprinted genes. Microarray analysis showed upregulation of nearby transcripts, and although it is likely the observed tumourigenesis was due to the iv. administration of considerably high doses of rAAV2 into neonates, the precise cause of the remains unclear.

The reported HCC from the Donsante-authored articles prompted a meta-analysis of a number of gene transfer studies in mice to look for any correlation between rAAV administration and liver tumor development [100]. No macroscopic tumors were seen in 226 control mice, but out of 695 mice from five different strains that received rAAV2 vectors via portal vein injection, one tumor (a lipoma) was observed. However, quantitative PCR showed that it contained fewer vector genomes per total DNA than the surrounding liver tissue. Another treated mouse had a macroscopically visible nodule containing lymphocytes, but immunohistochemistry revealed the cells were not of monoclonal origin, and contained fewer AAV genomes than the surrounding hepatocytes. Compared with an average frequency of spontaneous liver tumors in C57BL/6 mice (0–10%) and given the absence of high levels of vector DNA in the observed tumor, the authors concluded AAV vectors do not predispose mice to the formation of liver tumors [101]. The negative association regarding rAAV gene transfer to the livers of mice and tumourigenesis was compared with data from the mouse tumor biology database [102]. This database allows the retrieval of information about the normal frequency of tumors for a given organ in a given mouse strain. In general, C57BL/6 mice are rated as having a 'moderate' frequency of liver tumors and the majority of the literature about the natural occurrence of liver tumors in C57BL/6 mice indicated a higher natural frequency than that reported for the different rAAV vector-treated C57BL/6 mice (0.35% vs 0.14%) [101]. A further liver study with rAAV2 and 7, 8 and 9 vectors expressing mouse OTC was performed in two mutant OTC-deficient mouse models (spf and spfash), both of which express <10% residual OTC activity in the liver [100]. After injection of the vector, numerous mice from all treatment groups (including untreated controls) were observed to have either nodules or discrete tumors in the liver after 250 days. Since the incidence of hepatic tumors was higher following rAAV vector administration into spfash and wt mice than in untreated, control littermates, these mice were further analyzed [101]. rAAV vectors were administered to mice on the B6C3F1 hybrid mouse background from which the OTC-deficient mice were derived [103]. In a report published by the FDA's National Center for Toxicological Research, 33% (16/48) B6C3F1 male mice developed either HCC or hepatocellular adenoma after 2 years [104]. For B6C3F1 mice that received rAAV vectors, there was an increased incidence of hepatic tumors associated with rAAV encoding the LacZ reporter gene, but not the OTC gene. Furthermore, isolated tumor tissue generally contained lower numbers of rAAV vector copies than the surrounding liver tissue and did not express the transgene. Only one of the four tumors evaluated by Southern blot analysis contained an integrated copy of the AAV vector, rendering it unlikely that integrative damage was linked to tumor development [105]. None of the tumors occurred in younger, three-month-old mice, consistent with age-related onset of the tumors at a frequency consistent with previous reports for the B6C3F1 hybrid mouse [103].

The long-term persistence of expression reported for rAAV vectors is due to maintenance of episomes that over months are converted into higher molecular weight concatemerized genomes that do not integrate into the host chromosomes, but assimilate with chromatin with a typical nucleosomal pattern. A frequency of 0.1% integration, if accurate, is not associated with cancer in adult rodents, nor in long-term studies in hemophilia B dog models and nonhuman primates [106,107]. Furthermore, from more than 200 human patients who have received rAAV vectors, none is reported to have developed any tumors. Although the reported HCC in the MPS-VII mouse model suggests advising caution regarding liver-directed gene transfer to neonates using particularly high doses, the tumourigenic potential of rAAV vectors seems to be minimal, especially in tissues that divide relatively slowly, such as muscle.

Conclusion

Alipogene tiparvovec is the first GT to be approved in Europe and it is indicated for use in individuals with LPLD who have experienced severe or multiple attacks of pancreatitis despite maintaining a low-fat diet. Although clinical studies indicate that alipogene tiparvovec is well tolerated and has a good safety profile, only a small number of patients have been treated due to the rarity of LPLD. Therefore, in order to obtain a more comprehensive overview of the biosafety of GT using AAV vectors, this review examined a wider range of data from animal models as well as preclinical and clinical studies. The most frequent acute responses observed with im. injection of alipogene tiparvovec were local and transient injection site reactions. It is thought that these injection site reactions reflect local immune responses to the GT. Pre-existing humoral immunity to AAV vectors is common due to exposure to wtAAV and most patients will develop humoral responses following treatment. Data from alipogene tiparvovec and other GTs suggest that the generation of T cell-mediated immune responses may be dependent on the vector used, the route of administration, the tissue or organ target and the transgene.

Following im. administration of alipogene tiparvovec peak levels of vector DNA were detected at 24 h in serum, saliva and urine. In the majority of semen samples, vector DNA levels dropped to undetectable levels by Weeks 4–6 following administration. Based on these data, the risk for AAV-based germ-line transmission is considered to be low.Alipogene tiparvovec mainly exists in the nucleus as episomal contatemers and only integrates at very low frequency into the mammalian genome in a largely random pattern and without any integration within the AAV-HCC locus. The tumourigenic potential of rAAV vectors appears to be minimal, especially in slowly dividing tissues such as muscle.

Based on currently available information, GT with AAV vectors appears to be well tolerated and is associated with a positive safety profile. The approval of alipogene tiparvovec for LPLD heralds an exciting time for the transition of GT approaches from the laboratory bench into the medical practice. As with all new therapeutic options, however, the safety profile of alipogene tiparvovec will only be fully elucidated as more patients are treated and the experience grows through thorough data collection such as in the LPLD registry that has been set up upon authorization of alipogene tiparvovec in Europe.

Expert commentary & five-year view

Alipogene tiparvovec (Glybera) is the first GT medicinal product ever approved in Europe and in the Western world. It is also the first AAV-based product that was brought beyond academic research into commercial scale. As with all new therapeutic options, however, the safety profile of alipogene tiparvovec will only be fully elucidated as more patients are treated and the experience grows through thorough collection of long-term follow-up data. A LPLD registry has been set up to this end upon authorization of alipogene tiparvovec in Europe and is aiming at collecting long-term safety data and better understanding of LPL deficiency.

The approval of Glybera for the treatment of individuals with LPLD is the latest milestone in the long and difficult path that GT has followed. Many thousands of scientists have contributed to this progress in many countries, and there have been setbacks as well as triumphs along the way since Rogers and Pfuderer demonstrated proof of concept for virus-mediated gene transfer, using use the tobacco mosaic virus in 1968 [108]. Inherited diseases account for a substantial burden of disease and many of the most common inherited disorders, including thalassemias, cystic fibrosis and sickle cell disease, still lack treatments beyond supportive care. With many clinical studies completed, ongoing or about to start (for a complete overview see www.clinicaltrials.gov), it can reasonably be expected that AAV-based GTs will become available for many other diseases in the future. Based on currently available information and clinical studies worldwide, AAV vectors GT medicinal products appear to be well tolerated and are associated so far with a positive safety profile. AAV vectors are therefore currently considered as the delivery tool of choice for treating inherited diseases of post-mitotic tissues.

Demonstrating efficacy, however, proves to be the main hurdle for many of the AAV-based products especially because they are aiming to treat rare diseases that are not always well described or well understood. From a strict regulatory standpoint, the standards for orphan drugs is identical to the standards required for all other drugs, namely that 'substantial evidence' demonstrates the effectiveness and the safety of the drug for its intended uses. With the most precious resource being the persons with the rare disorders who heroically volunteer to participate in a trial, usually under conditions where little is known about the safety and potential effectiveness of the investigational therapy. Furthermore, clinical evidence is in those cases often based on surrogate outcomes collected on a short-term rather than long-term basis and the relationship between the two may not be easy to prove. Improvements in post-marketing studies and development of registries that allow long-term follow-up on safety and effectiveness would go part way to address these issues.

The approval of alipogene tiparvovec for LPLD heralds an exciting time for the transition of GT approaches from the laboratory bench into daily medical practice.

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Key issues

- Intramuscular administration of alipogene tiparvovec in lipoprotein lipase deficiency patients is well tolerated. The most frequent adverse events observed in the clinical trials were injection-associated local reactions that were directly related to the administration procedure and were resolved within a few days after injection.
- Innate immune responses are unlikely to cause major clinical concerns for adeno-associated virus gene therapy products.
- Humoral responses and their impact on transgene expression are dependent on the particular vector used, route of administration and target organ.
- Administration of alipogene tiparvovec to skeletal muscle is not associated with increase in markers of inflammatory response or tissues damage and while transient local and systemic immune responses were observed in some patients, transgene expression and clinical efficacy were maintained.
- DNA integration analysis, preclinical studies and clinical experience to date demonstrate that no specific carcinogenic profile is associated with adeno-associated virus gene therapy products.

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