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Reflection paper on quality, non-clinical and clinical issues related to the development of recombinant adeno-associated viral vectors

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1. Introduction

Recombinant adeno-associated viral (rAAV) vectors are derived from the single stranded DNA virus adeno-associated virus which belongs to the genus *dependovirus* within the *Parvoviridae* family. As the name suggests the wild type virus is incapable of independent replication and relies on co-infection of a helper virus to enable a lytic replication cycle (Gonclaves, 2005). Adenovirus (Ad), herpes simplex virus (HSV), pseudorabies virus (PrV) and human papilloma virus (HPV) are known to support wild type AAV replication.

Infection with wild-type AAV is not associated with any pathogenic disease, and in the absence of a helper virus co-infection, the virus may integrate into the host cell genome or remain as an extrachromosomal form (Schnepp, 2005). In both situations the virus appears to remain latent. *In-vitro* studies suggest that wild-type viral DNA integration can occur occasionally in a site specific manner (19q13.3) (Kotin, 1990 and 1991 and 1992), but only at very high multiplicities of infection (Hüser, 2002), and this was originally considered to be a safety feature of vectors derived from this virus. However, it has been subsequently shown that site specific integration is dependent on the presence of both the inverted terminal repeats (ITR) and the Rep gene products (Weitzman, 1994; Linden, 1996), the latter of which is not present in rAAV; as such the site specific integration feature of these vectors is lost. The level of integration of DNA into the cellular chromosome in *in-vivo* models, however remains contentious. Nonetheless, long term protein expression (*in-vivo*) from the gene of interest inserted into rAAV vectors has been observed (Flotte, 1993; Kaplitt, 1994; Conrad, 1996; Monahan, 1998; Donahue, 1999; Stieger, 2006), even in the absence of identifiable genetic integration (Miller, 2004; Song, 2004; Flotte, 1994). This persistence is thought to be derived from stable concatemerized duplex genome forms (circular or linear molecules) that are transcriptionally active (Duan, 1998; Yang, 1999; Fisher, 1997).

Examples of diseases studied include haemophilia B (Manno, 2006 and 2003), cystic fibrosis (Flotte, 2003), Parkinson's disease (Kaplitt, 2007), rheumatoid arthritis (*www.targen.com* [tgAAC94]), Leber's congenital amaurosis (Bainbridge, 2008; Maguire, 2008; Jacobson, 2006), infantile neuronal ceroid lipofuscinosis (Worgall, 2008) and muscular dystrophy (Xiao, 2000). Furthermore non-clinical studies indicate rAAV expressing heterologous antigenic sequences (HPV16 - Kuck, 2006; HIV – Xin, 2001 and 2002; SIV – Johnson, 2005; malaria – Logan, 2007) can illicit both humoral and cellular immune responses, and modest immunogenicity has been reported in a phase I/II study using rAAV2 encoding HIV antigens (Mehendal, 2008). However, it has been suggested that cellular responses to the transgene products of rAAV vectors may be impaired (Lin, 2007), as such the utility of these vectors when used for prophylactic purposes needs further investigation.

There are currently 6 confirmed serotypes of adeno-associated virus (AAV-1 to -6) and 2 tentative species (AAV-7 and 8) (source: International Committee on Taxonomy of Viruses [ICTV]). However there are a number of publications describing additional serotypes (i.e. 9 and 10) which are currently not recognized by the ICTV. It is likely therefore, that there are significantly more serotypes circulating that have currently not been formally identified or recognized (Pacak, 2006; Limberis, 2006; Gao, 2004). Nonetheless, the majority of the 67 clinical trials undertaken to date using rAAV for gene delivery have used serotype 2 (Gene Therapy Clinical Trials Worldwide. J. Gene Med. March 2009 Update, *http://www.wiley.co.uk/genmed/clinical*).

Evidence is accumulating which suggests that different AAV serotypes may have different tissue tropisms, for example AAV-8 is suggested to have a preferred tropism to the liver (Davidoff, 2005), while for AAV-1, -6 and -7 the preferred tropism is to skeletal muscle (Duan, 2001; Chao, 2000), AAV-4 is highly specific to the retinal pigmented epithelial cells in several animal species (Weber, 2003) and the ependymal cells (Zabner, 2000) and AAV-9 is described as being tropic to cardiac

muscle (Pacak, 2006), thought it also tranduces liver (Van den Driessche, 2007) and brain (Foust, 2009). Vectors based on these serotypes, in-vitro selected AAV with altered tropisms and hybrid vectors (i.e. ITR and Rep from AAV-2, Cap (protein coat) from another serotype i.e. 8) are being investigated (*in-vitro* and in animal models) to evaluate further the utility of the preferred tropisms and their potential for avoiding pre-existing immunity to AAV-2.

A new development in the field of AAV vectors is the use of self complementary (sc) AAV. Conventional rAAV vectors require 2nd strand synthesis before genes can be expressed, and it is theorized that scAAV bypass this step by delivering a duplex genome. This is achieved by deleting the nicking site of one ITR so that it no longer serves as a replication origin but still forms an AAV hairpin structure. The result is a single stranded, dimeric inverted repeat genome with the altered ITR sequence situated in the middle of the molecule and a wild-type ITR at each end. Following infection and uncoating, the DNA is folded to form a double stranded molecule. A closed hairpin end is formed from the altered ITR, and an open end formed from the two wild-type ITR's, thus mimicking the structure of a single stranded rAAV after 2nd strand synthesis (McCarty, 2003). It is anticipated that such vectors will improve transduction efficiency and improve the level of protein expression from the transgene. The coding capacity of these vectors, however, is reduced by a factor of two.

Given the basic biology of the 'parent' virus as described above, the methods for manufacture and quality control of product are complicated, and the long-term fate of the administered vector is at present unknown. There are a number of manufacturing strategies that can be used to produce rAAV vectors and these are discussed further below, however the basic functional requirements for manufacture are:

- The AAV ITR's flanking the 'gene of interest' (this construct contains the cis elements necessary for packaging and replication of its single stranded DNA genome).
- Genetic sequences (Rep and Cap) necessary for AAV replication and viral capsid proteins (generally provided in trans within a plasmid or in a packaging cell line).
- Helper virus functions: either co-infection of the helper virus or co-transfection/infection of a plasmid/chimeric virus encoding the helper genes (adenovirus: E1a/1b, E2a, E4orf6, VA1 RNA; herpes simplex virus: UL5, UL8, UL52 and UL29).
- A cell line capable of supporting helper virus and AAV replication.

The aim of this paper is to discuss quality, non-clinical and clinical issues that should be considered during the development of medicinal products derived from AAV, and to indicate requirements that might be expected the time of a market authorisation application (MAA). The issues raised are specific only to the development of rAAV vectors as medicinal products; general requirements for MAA are not within the scope of this paper. It is recommended that this paper is read in conjunction with the guidance documents referenced in section 4.2.

2. Discussion

2.1. Manufacturing Methodologies Used to Generate rAAV

2.1.1. Virus Containing Production Systems

2.1.1.1. Helper Virus

A cell line permissive to the helper virus (Ad is commonly used) is transfected with 2 plasmids, one containing the AAV ITR's flanking the 'gene of interest' ('transgene plasmid'); the other contains the Rep and Cap genes of AAV ('packaging plasmid'). Alternatively, cell lines stably transfected with the transgene cassette and/or the packaging elements can be used. The cell line is then infected with either wild-type Ad or a recombinant Ad (rAd). In the presence of Ad helper functions, the rAAV genome is subjected to the wild-type AAV lytic processes by being rescued from the plasmid backbone, replicated (using cellular DNA polymerases) and packaged into preformed AAV capsids as single-stranded molecules. 48-72 hours post infection the supernatant and cell lysate are harvested and rAAV purified. Inactivation of helper virus can be achieved by heat (56^{o}C for 1 hour for adenovirus) or any appropriate chemical method that has been suitably validated.

2.1.1.2. Chimeric virus production strategies

Chimeric virus production strategies utilize recombinant forms of the helper virus that encode some or all of the elements necessary for rAAV production (transgene plasmid and/or packaging plasmid), with the aim being to simplify rAAV manufacture and develop more scalable processes.

A recombinant HSV-1 virus (Δ-ICP27) encoding Rep/Cap from AAV has been used to manufacture rAAV using two different approaches. Firstly, 293 cells can be transfected with the transgene plasmid, followed by infection with the recombinant virus; or a stably transfected cell line containing the transgene plasmid can be infected with the hybrid virus (Conway, 1999).

Another herpes virus, Pseudorabies virus (PrV) (herpes virus of swine) is also a competent helper virus for AAV replication. A reduced virulence PrV (lacking glycoproteins D and E and the thymidine kinase gene) encoding Rep/Cap has been generated, and infection of 293 cells with this virus along with transfection of the transgene plasmid results in the generation of rAAV (Shiau, 2005).

Similar approaches have been used where the transgene construct is cloned into the E1 region of adenovirus. Infection of this virus and a wild-type or an E2b mutant Adenovirus into a packaging cell line stably transfected with the packaging plasmid results in the generation of rAAV (Lui, 1999; Gao, 1998; Farson, 2004).

Others have tried to harness the scalability of protein production using baculovirus as a means of rAAV production. To this end 3 recombinant baculoviruses encoding either packaging or transgene sequences are co-infected into Sf9 cells or adapted cell lines derived from Sf9. Three days post infection rAAV can be recovered (Urabe, 2002; DiMattia, 2005; Aucoin, 2007).

2.1.2. Virus-Free Production Systems

2.1.2.1. Tri-Plasmid Transfection

This strategy is very similar to that described in section 2.1.1 except that a 3rd plasmid is transfected (Xiao X et al, 1998), which contains the genetic elements from the helper virus required for AAV replication ('helper plasmid'), negating the need for co-infection of a helper virus. Alternatively, both

the packaging sequences and the minimal adenoviral helper functions are provided in a single plasmid (Grimm D et al, 1998). This results in a two-plasmid transfection protocol.

2.1.2.2. Strategies using packaging cell lines

A number of different strategies have been published in which the packaging sequences have been stably transfected into a range of different cells lines (Clark, 1996; Inoue, 1998). Some constructs have inducible promoters controlling Rep expression due to its cellular toxicity if over-expressed; another utilizes a strong heterologous promoter upstream of Cap, resulting in over-expression of the proteins from this gene. The transgene plasmid and helper plasmid are then transiently transfected into the packaging cell line as described in 2.2.1.

2.2. Quality Considerations

2.2.1. General points

All cell lines used in the manufacture of rAAV medicinal products should follow a cell bank system, and should be controlled using the principles described in the European pharmacopoeia monograph 5.2.3. Given that the cells are being used for rAAV manufacture the tests for adventitious agents should specifically address contamination by wild-type AAV and any viruses identified as helper virus for AAV replication, assuming such viruses can replicate in the cell line used for manufacture.

Furthermore helper/chimeric viruses used in the manufacture of rAAV medicinal products should also be produced from a seed lot system using a qualified cell line, and information relating to the viruses origin and subsequent manipulation should be provided. Unless satisfactorily justified, the viral stock should be controlled to ensure that there is no contamination with wild-type AAV. If a recombinant virus is being used for helper purposes, this stock should be controlled with respect to replication competent virus content.

Regardless of the manufacturing strategy used there is the potential for regeneration of wild-type AAV and even the generation of novel replication competent viruses, though it is accepted that such events might occur only rarely. Nonetheless, it is undesirable for a drug product to be contaminated with these types of impurity as such the likelihood of contamination by replication competent AAV should be assessed and evaluated as necessary. Furthermore when designing non-clinical and clinical studies the potential for contamination with these impurities should be considered along with their impact on the overall safety of product, given that the parental virus is not known to cause disease in man.

2.2.2. Virus Containing Production Systems

The main disadvantage of this system is the potential for contamination of the product with the helper/chimeric virus.

Before being used to manufacture rAAV, the helper (or chimeric) virus should be characterized and qualified for use. It is recommended that a quality specification for the helper/chimeric virus is set, and the testing strategy detailed in the Ph. Eur. (General chapter 5.14 Gene Transfer Medicinal Products for Human use) can be used for guidance in defining an appropriate testing program. In particular, if the helper/chimeric virus is considered to be replication incompetent, the specification of that starting material should include a limit for replication-competent virus contamination that may have arisen by recombination events during its manufacture.

It is not considered acceptable to administer rAAV contaminated with live helper/chimeric viruses, as such it is important that the method of inactivation of the helper/hybrid virus is shown to be effective. Even if helper/chimeric virus has been shown to be fully inactivated, there is still the potential for transfer of its DNA during administration of the rAAV product, as such consideration should also be given to the quantification of helper/chimeric virus DNA contamination present in the final bulk or drug product, with particular attention given to any sequences that might be considered to be oncogenic or have the potential to result in a physiological function following administration. New and emerging sequencing techniques have the potential to identify and relatively quantify all DNA sequences in the product. Assuming they are suitably validated, such methods could be introduced as standard release tests. Furthermore, if the helper/chimeric virus is non-enveloped, DNA quantification should be undertaken both before and after DNase treatment to ensure quantitative limits on encapsidated DNA are determined.

Some tissues can express endogenous helper-like functions, as such there is a theoretical risk that delivery of rAAV contaminated with intact Rep sequences could in theory result in inadvertent replication of the rAAV genome. Therefore, where a helper/chimeric virus is used that contains an intact Rep gene, DNA content in terms of contaminating Rep sequences should be quantified.

If helper/chimeric virus inactivation can not be validated, the product purification process should be validated for its removal, and a content limit of helper/hybrid virus should be included in the final bulk or drug product specification.

2.2.3. Virus-Free Production Systems

Limitations of a manufacturing approach that relies solely on plasmid transfection lie in the difficulties of process scale up and the consistency of manufacture due to the inherent variability of the transfection process itself. However, the advantage of such an approach is that the quality of the final product is improved as there will be no contamination of the product with a helper/chimeric virus - though there is still the potential for generation of replication competent AAV. Materials used in the production of the vector such as E. coli plasmid master cell banks, purified plasmid lots, and transfection reagents, should be qualified.

It is recommended that the transfection conditions are thoroughly evaluated and optimized at each scale of manufacture to assure consistency in product quality and yield, particularly as the scale of manufacture has been shown to impact on the amount of rcAAV generated. This was reported following scale up of the triple plasmid manufacturing process, while small scale manufacturing runs were free from contamination (Allen, 1997); however certain helper plasmid constructs appeared to reduce rcAAV production (Grimm, 1998). It is advisable therefore to design plasmids which minimize genetic homology and utilize strategies to minimize rcAAV production (i.e. alteration of transcriptional orientation of Rep/Cap, or the use of non-mammalian expression cassette systems and cell lines).

Quality issues specific to packaging cell lines are identical for those used to manufacture recombinant proteins in that the genetic stability of construct should be shown, at or beyond the expected number of population doublings required for manufacture. If some method of transcriptional control is being employed for example in relation to Rep expression, the purification strategy for the product should be validated with respect to removal of the induction agent, or a content limit should be included in the release specification. It would be advisable to qualify the level of 'leakiness' of the inducible promoter and ensure that the level does not change on extended culture of the cells (phenotypic characterization of the cell line).

2.2.4. scAAV

Vector stocks of scAAV, when analyzed by alkaline agarose gel electrophoresis, have been shown to contain 90-95% scAAV vectors. During production, virtually all the replicating vector DNA is in dimeric, or multimeric molecules. Therefore, purification tends to rely on methods relating to density separation. Given the potentially heterogeneous population of virus particles that might be present in such preparations, characterisation and quantification of all product forms and product related impurities will be necessary. As both dimeric or multimeric molecules could be present, the use of newly emerging sequencing techniques that can identify all genetic elements present may be necessary in order to fully characterization the product, as well as more common techniques such as restriction enzyme mapping, potency/biological activity determinations etc.

2.2.5. Quality Control of the Product

2.2.5.1. Product content / titre

The most commonly used titration methods for rAAV rely either on the quantitation of DNA amplification (Salvetti, 1998) or transgene expression following transduction and co-infection with the helper virus. In the most part quality control release specifications are likely to include both methods of titration in order to get an overall measure of 'infectivity' in terms of the ability of the DNA to enter cells, and biological activity in terms of transgene expression. It is more challenging, however to develop what might be considered 'true' bioassays such as $TCID_{50}$ given the need for co-infection with a helper virus and the inability to distinguish cpe from the helper and that of rAAV. Nonetheless from a regulatory point of view this is just the type of assay that would be preferred if at all possible.

It has been reported that the PrV based vector expressing Rep/Cap can be used to titrate rAAV using a more conventional $TCID_{50}$ assay (Shiau, 2005) as this virus is attenuated to the point where it is incapable of causing cpe on infection of a permissive cell line. Thus the actual infective dose given to a patient can be measured. The development of such strategies for rAAV titration should be encouraged as a combination of this type of assay and DNA quantification of DNase resistant particles would give a more precise measure of full particle to infectivity ratio. An essential component is the identification of cell lines that are permissive for each AAV serotype (i.e. cell lines that provide efficient infection and the greatest assay sensitivity).

Virus titre, upon which the dose is defined, is most frequently determined using PCR-based technology, with administrative doses being defined in terms of genome copies rather than virus particles or infectious titre. Wherever possible, quantitative PCR methods should be used for this measure of content.

2.2.5.2. Purity

Assays for process impurities and potential contaminants should be utilized to evaluate the purity of rAAV vector lots. These assays can be used to detect residuals such as nucleases, plasmid DNA, cellular proteins, helper/hybrid virus DNA or infectious virus and the AAV vector transgene protein product generated during rAAV manufacturing, many of which have the potential to induce immune responses.

It has been reported that rAAV particles also co-package plasmid (Wright, 2008) or helper virus DNA, used for production. Particles containing co-packaged DNA are likely to be considered a process related impurity and, the extent of this observation needs to be thoroughly characterised in order to support a MAA. The extent of the characterisation required is likely to depend on the amount of co-packaged DNA in the product. Characterisation to determine whether or not open reading frames are

present may be necessary, and if they are found to be present it should be investigated whether or not protein can be actively translated from those sequences in mammalian cells. When evaluating the potential for protein expression from the co-packaged DNA it is important that a range of cell lines that reflect the biodistribution profile of the rAAV are used. Ideally it should be demonstrated that the ratio of particles containing co-packaged DNA is consistent between batches throughout development, and that an appropriately justified release specification is included for batch release purposes. If possible the fate of virus genomes containing the co-packaged DNA should be investigated, as it is unclear if virus particles whose packaged DNA is greater than that of wild-type DNA are still capable of forming episomes, and thus persisting within the cell for extended periods. If long term persistence of both the gene of interest and co-package DNA is observed, a comprehensive risk assessment relating to the long term consequences of this eventuality will be required. It will also need to be demonstrated that the presence of such particles does not impact on overall product safety.

Recombinant AAV vector stocks are generally a heterogenous mixture of empty capsids (i.e. do not contain DNA), uninfectious particles (i.e. contain DNA, but DNA amplification in-vitro is not observed) and infectious particles (enters the cell and transgene expression/DNA amplification is observed in-vitro). It could be considered therefore, that empty particles and uninfectious particles are product related impurities, both of which can impact on the immunogenicity profile of the product when administered to patients. The product should therefore be characterised in terms of the content of all these virus particle types, and justified release specifications should be introduced to ensure a consistency in 'antigenic load' when administered to patients.

2.2.5.3. Reference materials

A Reference Standard Stock for AAV-2 is now available. This reference standard will be useful for other AAV serotypes since the vector genome and other physical characteristics will be applicable regardless of the serotype (Moullier, 2008). However a product specific reference will also be required as the biological activity of the transgene will need to be measured as part of the product specification. Such product specific reference standards should be fully characterised, with defined stability / performance monitoring strategies in place to determine when replacement references are required. Ideally laboratory or product-specific reference standards generated internally should be normalized, where possible, against a primary (community recognized) vector reference standard.

2.3. Non-Clinical Evaluation for Consideration

2.3.1. Choice of Animal Model

AAV is a species specific virus, therefore it is possible that the biodistribution of a human serotype derived vector in a mouse or rat may not correlate to that when administered to man as cellular/organ uptake may be different as a result of differences in, or differential expression of, the receptor used for entry. A number of animal species have been used in non-clinical evaluation of rAAV vectors (rats, mice, rhesus monkey, non-human primates, dogs, cats and pigs); however it is not clear which is the most appropriate model to use, and it may be necessary for more than one species to be used to complete a full non-clinical development program. Given these difficulties there may be scientific justification for using in pivotal non-clinical studies, a serotype of virus that is specific to the animal model of choice, rather than the human serotype that will be used in clinical studies. Such studies may provide more useful information in relation to biodistribution and the impact of pre-existing immunity to the vector to it.

For proof of concept/ efficacy studies in small animal models such as mice, consideration should be given to the injection volumes used. It is advisable to avoid large injection volumes of up to 20-50ul per injection, since these may not reflect the situation in the clinic where, relatively speaking, more moderate injection volumes are used.

The impact of immune responses to the transgene product will also need to be factored into the assessment of the suitability of the animal model particularly as the gene of interest is likely to be of human origin, and this may result in cells constitutively expressing the protein being cleared more readily by immune surveillance. It may therefore, be justifiable to use a rAAV containing the appropriate homologous animal gene rather than the human transgene that will be used clinically.

If it is considered that using species specific vectors and/or transgenes is the only way to fully evaluate the safety of the vector prior to first in man studies, it is strongly recommended that advice is sought from the regulatory authorities before proceeding (http://www.ema.europa.eu/htms/human/sciadvice/Scientific.htm).

2.3.2. Vector Persistence

The safety of rAAV in terms of insertional mutagenesis is still under debate following a recent publication where an increased rate of hepatocellular carcinoma was observed in neonatal mice treated with a rAAV (Donsante, 2007). While this study is not definitive in confirming the oncogenic potential of these vectors (Kay, 2007), the implications of the study can not be ignored, and the extent of integration of the vector under investigation should be evaluated. Methods recently used to detect AAV integration *in-vivo* include PCR, which has been used to amplify AAV/AAVS1 junctions, as well as linear amplification mediated (LAM) PCR (Schnepp, 2005; 2009). At the time of a market authorisation the suitability of the method (or methods) used and its sensitivity should be discussed. However it should also be considered that *in vivo* studies to assess the oncogenic risk of AAV vectors is not known to be a reliable method to fully exclude this risk. *In vitro* studies may therefore be preferable, particularly where these can either show integration or can eliminate it.

At present there does not appear to be consensus of opinion relating to the expected frequency of random integration events following administration of rAAV. Vector integration has been detected in the rat liver (Flageul, 2009) and in human tissues (Schnepp, 2009), while episomal persistence of the vector has been observed in non-human primates following intramuscular injection (Penaud-Budloo, 2008). Furthermore it has been repeatedly observed that rAAV DNA can persist for weeks, months or even years, particularly at the site of administration, and it is transcriptionally active as such protein expression can be detected for extended periods post administration (Rivera, 2005; Stieger, 2007). Non-clinical studies should be considered which are designed to investigate how long-term gene expression is expected to be achieved i.e. episomal or integrated. If integration is observed, further studies may be necessary to evaluate whether there is evidence of targeting to transcriptionally active regions of the host chromosome, preferential integration in some tissues or whether there is the potential for 'outward' gene activation.

Persistent recombinant vector genomes have been observed in both target and non-target organs following administration to animals via numerous routes (Donahue, 1999). The level of expression of the 'gene of interest' in those tissues where vector persistence is observed should also be investigated, such that a decline in vector level in different organs can be correlated with protein levels from the ectopically expressed gene of interest. Episomal maintenance duration may also be dependent on the rate of cell renewal, and this may need to be factored into the duration of non-clinical studies evaluating vector persistence.

It has also been reported that DNA plasmid sequences co-packaged within the rAAV vector particles can be detected in-vivo up to 1.5 months after administration in the Macaque (EMEA/CHMP/183989/2004). This observation should be considered when designing biodistribution and shedding studies if the method of vector product uses transfection of plasmids. Where possible methods for detection of the persistence of such sequences should be introduced into non-clinical studies and the potential for the release of antibiotic resistance genes into the environment via shedding of virus particles contaminated with such sequences should be discussed within the context of the environmental risk assessment.

2.3.3. Tissue Tropism

Different serotypes of AAV have been associated with specific tissue tropisms, for example AAV 1, 6 and 7 are effective at transducing muscle cells; serotype 9 preferentially transducing the myocardium and AAV 5 is suggested to be more tropic to the airway epithelium and the central nervous system (at least in the mouse model). This preferential transduction activity does not mean however, that the vector is not distributed to other organs. Indeed the tropic behavior of a rAAV vector can also be specific to the animal model used; for instance, AAV 5 is neurotropic in the mouse (Zabner, 2000) whereas AAV 1 is more efficient in the cat brain (Vite, 2003). It is possible therefore, that tissue tropism defined non-clinically may not be observed following administration to humans, and it is recommended that a cautious approach is taken when translating non-clinical data to humans.

2.3.4. Reactivation of Productive Infection

When developing rAAV vectors as medicinal products the consequence of long-term episomal maintenance and the potential for re-activation of virus if the subject is infected with both wild-type AAV and a helper virus should be considered. Where possible or relevant, this should be investigated in non-clinical studies such as those described by Afione et al (Afione, 1996).

In addition, following AAV serotype 1 injection into muscle, viral particles have been identified in the serum for up to 3 months (Toromanoff, 2008). It is unclear whether these particles are infectious; however, extensive periods of circulating viral particles may have implications on the immune response post administration, as such this observation should be considered when designing both non-clinical and clinical studies.

Associated treatment during clinical studies i.e. chemotherapy, immuno-suppression, anti-inflammatory medicines, may also impact on virus biodistribution and maybe even the likelihood of viral reactivation, particularly if immuno-suppression is being given. Where possible these additional treatments should be addressed during non-clinical evaluation of the product.

2.3.5. Germ-line Transmission

Biodistribution studies have shown in the mouse and the rat that rAAV DNA can be detected in gonadal DNA (Arruda, 2001) for a variable duration. Furthermore following hepatic artery delivery of a rAAV for the treatment of hemophilia B, transient dissemination to the semen in 1 patient was observed (Schuettrumpt, 2006). The potential for germ-line transmission can not therefore be entirely ruled out (Honaramooz, 2008), as such it is recommended that germ-line transmission studies are undertaken prior to first in man studies (refer also to 'Guideline on the Non-Clinical Studies Required Before the First Clinical Use of Gene Therapy Medicinal Products' (EMEA/CHMP/GTWP/125459/2006) and 'Guideline on Non-Clinical testing for Inadvertent Germ-line transmission of Gene Transfer Vectors' (EMEA/273974/05)).

2.4. Environment risk considerations

There is a substantial amount of literature available suggesting that shedding of rAAV is dependent on the dose and route of administration, and that vector DNA can be detected for a number of weeks in serum, and early times i.e. day 1 post administration, in saliva, serum, urine and semen (Favre, 2001; Manno, 2006; Provost, 2005). Ideally, if positive DNA signals are observed, the samples should be followed up for infectious virus quantification (refer also the ICH considerations general principles to address virus and vector shedding CHMP/ICH/449035/09). The data derived from non-clinical shedding studies and from early phase clinical studies can then be used to assess the likelihood of transmission and to justify the extent of viral shedding evaluation in subsequent trials.

All available data that can be used to estimate the extent of viral shedding and the likelihood and consequences of viral transmission, should be used in the environmental risk assessment presented as part of any future market authorization application. For further information on environmental risk assessment, refer to the guideline 'Guideline on Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products' (CHMP/GTWP/125491/06).

2.5. Clinical considerations

2.5.1. Biodistribution and shedding studies

The extrapolation of biodistribution data from animal models to humans is not straight forward. It is recommended that wherever possible an investigation into the biodistribution of the vector, by screening for DNA sequences in the first instance, should be included within the clinical trial protocol.

The biodistribution of the vector may depend on the route of administration, however extensive dissemination of vector has been observed following what is generally considered to a local route of administration i.e. intra-muscular. The appropriate samples to be taken during clinical studies may not always therefore reflect the route of administration. Examples of samples that could be taken include tissue biopsy's (if possible), blood/serum, tears, urine, semen, buccal swabs/sputum, lung lavage and faeces, however it is up to the product developer to justify the sample types chosen in relation to the non-clinical data obtained, as well as the practical feasibility and ethical justification of sampling. A sufficient number of patients should be included in these studies in order to draw robust conclusions and the time interval between samples needs to be justified in accordance with known shedding profiles of the parental virus and non-clinical experience (refer also to ICH Considerations: General Principles to Address Viral / Vector Shedding (CHMP/ICH/449035/09)

Furthermore, if virus reactivation (refer to section 2.3.4) is observed during non-clinical studies, it is recommended that the clinical protocol design is optimized to investigate this further in humans.

2.5.2. Immunogenicity

Equally the extrapolation of immunogenicity data for therapeutic applications of AAV vectors from animal models to humans is not straightforward, and the route of administration may also impact on the immunogenic profile of the product. It is, therefore, recommended that consideration is given to the potential of subjects having pre-existing antibodies to the serotype of AAV under investigation, and that evaluation of the immunogenicity of both the vector and the transgene is assessed in terms of neutralizing and non-neutralizing antibody formation after administration during clinical trials. The relationship (or lack thereof) between safety or efficacy and any response should be evaluated and discussed. This will be of particular importance if the aim is to re-administer the vector, and if long-

term expression of the 'gene of interest' is observed. In addition to the antibody responses, it is also important to consider the cellular immune responses directed against the vector particles and/or the transgene product. The AAV vector particles can be processed by the transduced cells and presented as antigenic peptides in association with major histocompatibility class I antigens. Consequently, this may evoke a $CD8^+$ cytotoxic T cell response that eliminates the transduced cells, resulting in a decline in transgene expression (Manno et al., 2006, Mingozzi et al., 2009, VandenDriessche et al., 2009).

2.5.3. Germ-line Transmission

The question of germ-line transmission in humans has not been fully resolved and short term DNA persistence has been observed in semen (serotype 2), therefore it is recommended that germline transmission is investigated during clinical studies and that the use of barrier contraception for a minimum of 3 months (in accordance with a normal spermatogenesis cycle) for individuals enrolled in clinical trials, is included in study protocols.

2.5.4. Concomitant use of Immunosuppressive Agents

Given the potential for pre-existing immunity in the patient population to a number of AAV serotypes, which might limit efficacy and/or re-administration of the product, or the development of a immune reaction to the expressed transgene product, clinical studies incorporating the use of immunosuppressive regimens prior to administration of the rAAV are being, or have been, carried out. The choice of regimen to be used is likely to depend on the disease to be treated and associated morbidities, as such defined guidance on the best combination of agents to use can not be given. Nonetheless, what ever immunosuppression regimen is used, its effectiveness must be demonstrated, and so standardisation of the regimen across all study sites will be required.

2.5.5. Long-Term Follow-Up

Non-clinical studies may indicate long-term persistence of the vector, be it due to viral DNA integration or episomal maintenance, in which case long-term follow-up of the patients treated with a rAAV product could be necessary, not only in terms of safety evaluation but also efficacy. It should also be considered that where these vectors are being investigated for preventive vaccination uses, long term expression of the antigenic proteins may be a safety risk rather than a desired outcome.

For further information on long-term follow-up requirements refer to the CHMP guidelines: 'Guideline on Follow-up of Patients Administered with Gene Therapy Medicinal Products' (CHMP/GTWP/60436/07) and 'Guideline on Safety and Efficacy Follow-up – Risk Management of Advanced Therapy Medicinal Products' (EMEA/149995/2008).

For evaluation of efficacy, reference to the relevant guidelines of the specific disease under investigation is also recommended e.g. 'Guideline on the Evaluation of Anticancer Medicinal Products in Man' (CPMP/EWP/205/95/Rev.3/Corr.2) if the indication is cancer.

3. Conclusion

This paper reviews the current status in the development of recombinant adeno-associated virus vectors, and raises regulatory points for consideration for pharmaceutical companies developing these products with the aim of submitting market authorisation applications (MAA).

Given the basic biology of the parent virus, there are a number of issues that should be thoroughly investigated in non-clinical studies, such as the potential for germline transmission, the potential for reactivation of infection and what impact contamination with wild-type AAV might have on product safety etc, as well as any associated risk of persistent expression of the gene product, which has been delivered using these vectors. The outcome of these studies should then be taken into consideration when designing subsequent clinical trial protocols. However, one of the main problems with this vector system is determining what is the most useful animal model for pivotal non-clinical studies, as it appears that extrapolation from animals to the human situation is not straightforward. Therefore determining what sampling/analysis is included or excluded from clinical protocols on the basis of non-clinical data will need to be scientifically justified.

Like retroviruses and lentiviruses, these vectors are of particular interest for gene therapy application due to their long term persistence and thus, for the potential long term correction of genetic disease. However, unlike the retrovirus and lentivirus particle, integration into the cell genome does not appear to be a prerequisite for this activity, though there is some uncertainty as to the extent of integration, and the exact mechanism of vector persistence. This activity can impact on the overall safety of these products therefore a thorough understanding of the mechanism of action of the vector and its gene product, and their associated risks, needs to be determined in non-clinical and clinical studies prior to submission of market authorisation applications. This may also result in the need for long-term follow-up of the patient post-administration to fully understand not only the long-term safety of these products, but also to confirm long-term efficacy if that is the ultimate goal of treatment.

The development of these vectors as medicinal products is at a relatively early stage, and both pharmaceutical companies developing the products and the regulatory agencies involved in giving advice and assessing MAA, have much to learn. To ensure a straightforward pathway through the regulatory process it is recommended that there is open dialogue between the two parties throughout product development, with the hope that in the not too distant future the first rAAV vector will be licensed for commercial use.

4. References

4.1. Literature References

Afione, S.A. et al (1996): J. Virol. 70(5): 3235-41.

Allen, J. M. (1997): J Virol. 71(9): 6816-22.

Arruda V.R. et al (2001): Mol Ther. 4(6): 586-92.

Aucoin, M.G, Pierner, M and Kamen, A.A. (2007): Biotechnol. Bioeng. 97(6): 1501-9.

Bainbridge, J.W. et al (2008): N Engl J Med. 358(21): 2231-9.

Chao, H. et al (2000): Mol. Ther. 2(6): 619-23.

Clark K.R., Voulgaropoulou, F. & Johnson, P.R. (1996): Gene Therapy. 3912): 1124-32

Conrad, C.K. et al, (1996): Gene Ther. 3 (8): 658-68

Conway, J.E. et al (1999): Gene. Ther. 6(6): 986-93.

Davidoff, A.M. et al (2005): Mol. Ther. 11(6): 875-88

DiMattia, M. et al (2005): Acta Crystallogr Sect F Struct Biol Cryst Commun. 61(10): 917-21.

Donahue, B.A. et al (1999): J Gene Med. 1(1): 31-42.

Donsante et al (2007): Science 317: 477

Duan, D. et al (1998): J Virol. 72(11): 8568-77.

Duan, D. et al (2001): J. Virol. 75(16): 7662-71.

Farson, D. et al (2004): J. Gene. Med. 6(12) : 1369-81.

Favre, D. et al (2001): Mol Ther. 4(6): 559-66.

Fisher, K.J. et al, (1997): Nat Med. 3(3): 306-12.

Flageul, M et al (2009): J. Gene Medicine. 11(8): 689-696.

Flotte, T.R. et al, (1993): PNAS. 90(22): 10613-7.

Flotte, T.R., Afione, S.A. & Zeitlin, P.L. (1994): Am J Respir Cell Mol Biol. 11(5): 517-21.

Flotte, T.R. et al (2003): Hu. Gene. Ther. 14(11): 1079-88.

Foust, K.D. et al (2009): Nat. Biotechnol. 27(1): 59-65.

Gao, G.P et al (1998): Hum Gene Ther. 9(16): 2353-62

Gao, G. P. Et al (2004): J. Virol. 78(12): 6381-8

Goncalves, M. (2005): J. Virol. 2 (43)

Grimm, D. et al (1998): Hum. Gene Ther. 9(18): 2745-60.

Honaramooz et al (2008): FASEB J. 22(2): 374-82.

Hüser, D., Weger, S. & Heilbronn, R. (2002): J. Virol. 76: 7554-7559.

Inoue, N. & Russell, D.W. (1998): J. Virol. 72(9): 7024-31.

Jacobson, S.G. et al (2006): Hu. Gene. Ther. 17(8): 845-58.

Johnson, P. R. (2005): J. Virol 79: 955-68.

Kaplitt, M.G. et al (1994): Nat Genet. 8(2): 148-54.

Kaplitt, M.G. et al (2007): Lancet. 369(9579): 2097-105.

Kay, M.A. (2007): Nature Biotechnology 25(10): 1111-3.

Kotin, R.M. et al (1990): PNAS. 87(6): 2211-5.

Kotin, R.M. et al (1991): Genomics. 10(3): 831-4.

Kotin, R.M. et al (1992): EMBO J. 11(13): 5071-8.

Kuck, D. et al (2006): J. Virol. 80(6): 2621-30.

Limberis, M.S. & Wilson, J.M. (2006): PNAS. 103(35): 12993-8.

Lin, S-W. et al (2007): J. Clin. Invest. 117(12): 3958.

Linden, R.M. et al (1996): PNAS. 93(21): 11288-94.

Logan, G. J. et al (2007): Vaccine. 25(6): 1014-22.

Lui, X.L., Clark, K.R. & Johnson, P.R. (1999): Gene Therapy. 6(2): 293-9.

Maguire, A.M. (2008): N. Engl. J. Med. 358(21): 2240-8.

Manno, C.S. et al (2003): Blood. 101(8): 2963-72.

Manno, C.S. et al (2006): Nat. Med. 12(3): 342-7.

McCarty, D.M. et al (2003): Gene Ther. 10(26): 2112-8.

Mehendal, S. et al (2008): AIDS Res. Hum. Retroviruses. 24(6): 873-80.

Miller, D.G., Petek, L. M. & Russell, D.W. (2004): Nat Genet: 36(7): 767-73.

Mingozzi F. et al, (2007): Nat Med. 13(4): 419-22.

Mingozzi F. et al, (2009): Blood. 114(10): 2077-86.

Monahan, P.E. et al, (1998): Gene Ther. 5(1): 40-9.

Moullier, P. & Synder, R.O. (2008): Mol. Ther. 16(7): 1185-88.

Pacak, C.A. et al (2006): Circ. Res. 99(4): e3-e9.

Penaud-Budloo, M. et al (2008): J. Virol. 82(16): 7875-7885.

Provost, N. et al (2005): Mol Ther : 11(2) : 275-83.

Rivera, V. M. et al (2005): Blood. 105(4): 1424-30.

Salvetti, A. et al (1998): Hu. Gene Ther. 9(5): 695-706.

Schnepp, B.C. et al (2005): J. Virol. 79(23): 14739-14803.

Schnepp, B.C. et al (2009): J. Virol. 83(3): 1456-1464.

Schuettrumpf, J. et al (2006): Mol. Ther. 14(6): 893.

Shiau, A.L., Liu, P.S. & Wu, C.L. (2005): J. Virol. 79(1): 193-201.

Song, S. et al, (2004): PNAS. 101(7): 2112-6.

Stieger, K. et al, (2006): Mol Ther: 13(5): 967-75.

Stieger, K et al, (2007): Gene Ther: 14(4): 292-303.

Toromanoff A. et al (2008): Mol Ther. 16(7): 1291-9.

Urabe, M., Ding, C. & Kotin, R.M. (2002): Hum. Gene Ther. 13(16): 1935-43.

VandenDriesssche, T. et al (2007): J Thromb Haemost. 5(1): 16-24.

VandenDriessche, T. (2009): Blood. 114(10): 2009-10.

Vite, C.H. et al (2003): Gene Ther. 10(22): 1874-81.

Weber, M. et al (2003): Mol. Ther. 7(6): 774-81.

Weitzman M.D. et al (1994): PNAS. 91(13): 5808-12.

Worgall, S. et al (2008): Hu. Gene. Ther. 19(5): 463-74.

Wright, J.F.(2008): Gene Therapy 15(11): 840-848.

Xiao, X., Li, J. & Samulski, R.J. (1998): J. Virol. 72(3): 2224-32.

Xiao, X. et al (2000): J. Virol. 74(3): 1436-42.

Xin, K. et al (2001): Gen. Ther. 12: 1047-61.

Xin, K. et al (2002): Gen. Ther. 13: 1047-61.

Yang, J. et al, (1999): J Virol. 73(11): 9468-77.

Zabner, J. et al (2000): J. Virol. 74(8): 3852-8.

4.2. Guideline References

Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99)

Guideline on the Non-Clinical Studies Required Before the First Clinical Use of Gene Therapy Medicinal Products (EMEA/CHMP/GTWP/125459/2006)

Guideline on Non-Clinical testing for Inadvertent Germ-line Transmission of Gene Transfer Vectors (EMEA/273974/05)

Guideline on Scientific Requirements for the Environmental Risk Assessment of Gene Therapy Medicinal Products (CHMP/GTWP/125491/06)

Guideline on Follow-Up of Patients Administered With Gene Therapy Medicinal Products (CHMP/GTWP/60436/07)

Guideline on Safety and Efficacy Follow-Up – Risk Management of Advanced Therapy Medicinal Products (EMEA/149995/2008)

Gene Transfer Medicinal Products for Human Use (General chapter 5.14 of the European Pharmacopoeia)

ICH Considerations: General Principles to Address Viral / Vector Shedding (CHMP/ICH/449035/09)

Report from the CHMP gene therapy expert group meeting (EMEA/CHMP/1839890/2004)

5. Glossary

AAV	Adeno-Associated Virus
Ad	Adenovirus
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
ICTV	International Committee on Taxonomy of Viruses
ITR	Inverted Terminal Repeats
MAA	Market Authorisation Application
PCR	Polymerase Chain Reaction

Ph. Eur.	European Pharmacopoeia
PrV	Pseudorabies Virus
rAAV	Recombinant Adeno-Associated Virus
rAd	Recombinant Adenovirus
rcAAV	Replication Competent Adeno-Associated Virus
scAAV	Self-Complimentary Adeno-Associated Virus
Sf9	Spodoptera frugiperda cells
SIV	Simian Immunodeficiency Virus
TCID50	Tissue Culture Infectious Dose
wtAAV	wild-type AAV