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6 # REFLECTION PAPER

7 # QUALITY, NON-CLINICAL AND CLINICAL ISSUES RELATING SPECIFICALLY TO
8 # RECOMBINANT ADENO-ASSOCIATED VIRAL VECTORS

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15 16 17 **REFLECTION PAPER ON QUALITY, NON-CLINICAL AND CLINICAL ISSUES RELATING SPECIFICALLY TO 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 takes place occasionally in a site specific manner (19q13.3) (Kotin, 1990 and 1991 and 1992), 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 vector DNA into the cellular chromosome (in *in-vivo* models) remains contentious, with reports ranging from no evidence of integration (cotton rat) to up to 10% in mouse liver (Afione, 1996; Nakai, 2002). 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 (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 a further serotype (9) which is currently not recognized by the ICTV (Pacak, 2006; Limberis, 2006). Nonetheless, the majority of 54 clinical trials undertaken to date using rAAV for gene delivery have used serotype 2 (Gene Therapy Clinical Trials Worldwide. *J. Gene Med.* March 2008 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). Vectors based on these serotypes 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.

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:

i. 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).
ii. 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).
iii. Helper virus functions: either co-infection of the helper virus or co-transfection of a plasmid encoding the helper genes (adenovirus: E1a/1b, E2a, E4orf6, VA1 RNA; herpes simplex virus: UL5, UL8, UL52 and UL29).
iv. 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 are specific only to the development of rAAV vectors as medicinal products. 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'). The cell line (HEK 293 cells are commonly used) is infected with either wild-type Ad or a recombinant Ad (rAd) prior to or following transfection. 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. Infectious helper virus inactivation is generally achieved by heat inactivation at 56°C for 1 hour.

2.1.1.2 Hybrid vectors

The development of hybrid virus systems, utilizing recombinant forms of the helper virus which encode some or all of the elements necessary for rAAV production (transgene plasmid and/or packaging plasmid), are also under investigation in order to simplify 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

143 line stably transfected with the packaging plasmid results in the generation of rAAV (Lui, 1999; Gao,
144 1998; Farson, 2004).

145 Others have tried to harness the scalability of protein production using baculovirus as a means of
146 rAAV production. To this end 3 recombinant baculoviruses encoding either packaging or transgene
147 sequences are co-infected into Sf9 cells. Three days post infection rAAV can be recovered (Urabe,
148 2002; DiMattia, 2005; Aucoin, 2007).

149 *2.1.2 Virus-Free Production Systems*

150 *2.1.2.1 Tri-Plasmid Transfection*

151 This strategy is very similar to that described in section 2.1 except that a 3rd plasmid is transfected
152 (Xiao X *et al,* 1998), which contains the genetic elements from the helper virus required for AAV
153 replication ('helper plasmid'), negating the need for co-infection of a helper virus. Alternatively, both
154 the packaging sequences and the minimal adenoviral helper functions are provided in a single plasmid
155 (Grimm D *et al*, 1998). This results in a two-plasmid transfection protocol.

156 *2.1.2.2 Strategies using packaging cell lines*

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

163 *2.1.3 Self-Complementary Recombinant Adeno-Associated Virus*

164 A new development in the field of AAV vectors is the use of self complementary (sc) AAV.
165 Conventional rAAV vectors require 2nd strand synthesis before genes can be expressed, and it is
166 theorized that scAAV bypass this step by delivering a duplex genome. This is achieved by deleting
167 the nicking site of one ITR so that it no longer serves as a replication origin but still forms an AAV
168 hairpin structure. The result is a single stranded, dimeric inverted repeat genome with the altered ITR
169 sequence situated in the middle of the molecule and a wild-type ITR at each end. Following infection
170 and uncoating, the DNA is folded to form a double stranded molecule. A closed hairpin end is formed
171 from the altered ITR, and an open end formed from the two wild-type ITR's, thus mimicking the
172 structure of a single stranded rAAV after 2nd strand synthesis (McCarty, 2003). These vectors are
173 currently manufactured using a double or triple plasmid transfection process.

174 ***2.2 Quality Considerations***

175 *2.2.1 General points*

176 All cell lines used in the manufacture of rAAV medicinal products should follow a cell bank system,
177 and should be controlled using the principles described in the European pharmacopoeia monograph
178 5.2.3. Given that the cells are being used for rAAV manufacture the tests for adventitious agents
179 should specifically address contamination by wild-type AAV and any viruses identified as helper virus
180 for AAV replication.

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

186 Regardless of the manufacturing strategy used there is the potential for regeneration of wild-type AAV
187 and even the generation of novel replication competent viruses, though it is accepted that such events
188 might occur only rarely. Nonetheless, it is undesirable for a drug product to be contaminated with
189 replication competent viruses, as such it is recommended that an appropriate assay(s) capable of
190 detecting such contaminants is developed. Furthermore when designing non-clinical and clinical
191 studies the presence of contaminating replication competent wild-type AAV should be considered.
192 Wild-type AAV has the potential for integration into cellular DNA. Evaluation of the nature of the
193 integration event (be it site specific or otherwise) and the long term consequences associated with
194 integration of the virus should be considered.

195 *2.2.2 Virus Containing Production Systems*

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

198 Before being used to manufacture rAAV, the helper (or hybrid) virus should be characterized and
199 qualified for use. It is recommended that a quality specification for the helper/hybrid virus is set, and
200 the testing strategy detailed in the Ph. Eur. (Monograph 5.14 Gene Transfer Medicinal Products for
201 Human use) can be used for guidance in defining an appropriate testing program. In particular, if the
202 helper/hybrid virus is considered to be replication incompetent, the specification should include a test
203 for replication-competent virus contamination.

204 It is not considered acceptable to administer rAAV contaminated with live helper/hybrid viruses, as
205 such it is important that the method of inactivation of the helper/hybrid virus is shown to be effective.
206 Even if helper/hybrid virus has been shown to be fully inactivated, there is still the potential for
207 transfer of its DNA during administration of the rAAV product, as such consideration should also be
208 given to the quantification of helper/hybrid virus DNA contamination present in the final bulk or drug
209 product, with particular attention given to any sequences that might be considered to be oncogenic or
210 have the potential to result in a physiological function following administration. Furthermore, if the
211 helper/hybrid virus is non-enveloped, DNA quantification should be undertaken both before and after
212 DNase treatment to ensure quantitative limits on encapsidated DNA are determined.

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

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

220 *2.2.3 Virus-Free Production Systems*

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

228 It is recommended that the transfection conditions are thoroughly evaluated and optimized at each
229 scale of manufacture to assure consistency in product quality and yield. Following each
230 manufacturing change product characterization should be undertaken to assure that the introduced
231 changes do not impact on product quality. Furthermore, the purification process should be sufficiently
232 robust to assure removal of excess plasmid from the final product. The scale of manufacture has been
233 shown to impact on the amount of rcAAV generated, as it has been reported that on scale up of the

234 triple plasmid manufacturing process rcAAV was observed, whereas small scale manufacturing runs
235 were free from contamination (Allen, 1997); however certain helper plasmid constructs appeared to
236 reduce rcAAV production (Grimm, 1998). It is advisable therefore to design plasmids which minimize
237 genetic homology and utilize strategies to minimize rcAAV production (i.e. alteration of
238 transcriptional orientation of Rep/Cap).

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

247 *2.2.4 scAAV*

248 Vector stocks of scAAV, when analyzed by alkaline agarose gel electrophoresis, have been shown to
249 contain 90-95% scAAV vectors. During production, virtually all the replicating vector DNA is in
250 dimeric, or multimeric molecules. Therefore, purification can rely on methods other than density
251 separation such as chromatography. However, as part of the product characterization, analysis and
252 quantification of all product forms and product related impurities will be necessary.

253 *2.2.5 Quality Control of the Product*

254 If at all possible, an infectivity based titration method should be employed to quantify the amount of
255 infectious virus present. This is quite difficult for rAAV preparations as infection of a cell line with
256 rAAV alone will not lead to cytopathic effect (cpe) because the virus is incapable of replication without
257 a helper, however co-infection of most helper viruses results in excessive cpe as these viruses are
258 themselves lytic. The most commonly used titration method relies either on the quantitation of DNA
259 amplification (Salvetti, 1998) or transgene expression following transduction and co-infection with the
260 helper virus.

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

269 Physical measures of virus titre most frequently used are PCR-based, with administrative doses being
270 determined using genome copies rather than infectious titre. Wherever possible, quantitative PCR
271 methods should be used to determine the physical titre.

272 Currently there are no commercially available reference materials for rAAV products, though at the
273 time of writing a Reference Standard Stock for AAV-2 was in preparation. This reference standard,
274 when available, will be useful for other AAV serotypes since the vector genome and other physical
275 characteristics will be applicable regardless of the serotype (Moullier, 2008). However a product
276 specific reference will also be required as the biological activity of the transgene will need to be
277 measured as part of the product specification. Such product specific reference standards should be fully
278 characterised, with defined stability / performance monitoring strategies in place to determine when
279 replacement references are required. Ideally laboratory or product-specific reference standards
280 generated internally should be normalized, where possible, against a primary (community recognized)
281 vector reference standard.

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

287 *2.3 Non-Clinical Evaluation for Consideration*

288 *2.3.1 Choice of Animal Model*

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

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

305 If it is considered that using species specific vectors and/or transgenes is the only way to fully evaluate
306 the safety of the vector prior to first in man studies, it is strongly recommended that advice is sought
307 from the regulatory authorities before proceeding.

308 (www.emea.europa.eu/htms/human/sciadvice/Scientific.htm)

309 *2.3.2 Vector Persistence*

310 The safety of rAAV in terms of insertional mutagenesis is still under debate following a recent
311 publication where an increased rate of hepatocellular carcinoma was observed in neonatal mice treated
312 with a rAAV (Donsante, 2007). While this study is not definitive in confirming the oncogenic
313 potential of these vectors (Kay, 2007), the implications of the study can not be ignored, and the level
314 of integration of the vector under investigation should be evaluated.

315 At present there does not appear to be consensus of opinion relating to the expected frequency of
316 random integration events following administration of rAAV, however it has been repeatedly observed
317 that rAAV DNA can persist for weeks, months or even years, particularly at the site of administration,
318 as episomal concatemeric forms of the vector. This form of the vector is transcriptionally active as
319 such protein expression can be detected for extended periods post infection (Rivera, 2005; Stieger,
320 2007). Non-clinical studies should be considered which are designed to investigate how long-term
321 gene expression is expected to be achieved i.e. episomal or integration. If integration is observed,
322 further studies may be necessary to evaluate whether there is evidence of targeting to transcriptionally
323 active regions of the host chromosome, preferential integration in some tissues or whether there is the
324 potential for 'outward' gene activation.

325 Persistent recombinant vector genomes have been observed in both target and non-target organs
326 following administration to animals via numerous routes (Donahue, 1999). The level of expression of
327 the 'gene of interest' in those tissues where vector persistence is observed should also be investigated,

328 such that a decline in vector level in different organs can be correlated with protein levels from the
329 ectopically expressed gene of interest. Episomal maintenance duration may also be dependent on the
330 rate of cell renewal, and this may need to be factored into the duration of non-clinical studies
331 evaluating vector persistence.

332 *2.3.3 Tissue Tropism*

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

342 *2.3.4 Reactivation of Productive Infection*

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

347 In addition, following AAV serotype 1 injection into muscle, viral particles have been identified in the
348 serum for up to 3 months (Toromanoff, 2008). One hypothesis to explain this observation is the
349 continual release of intact particles from the muscle, suggesting either replication by some means, or
350 remote sites where the injected AAV particles remain intact for extended period of time. It is unclear
351 whether these particles are infectious; however, extensive periods of circulating viral particles may
352 have implications on the immune response post administration, as such this observation should be
353 considered when designing non-clinical studies.

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

358 *2.3.5 Germ-line Transmission*

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

368 ***2.4 Environment risk considerations***

369 There is a substantial amount of literature available suggesting that shedding of rAAV is dependent on
370 the dose and route of administration, and that vector DNA can be detected for a number of weeks in
371 serum, and early times i.e. day 1 post administration, in saliva, serum, urine and semen (Favre, 2001;
372 Manno, 2006; Provost, 2005). Ideally, if positive DNA signals are observed, the samples should be
373 followed up for infectious virus quantification. The data derived from non-clinical shedding studies

374 and from early phase clinical studies can then be used to assess the likelihood of transmission and to
375 justify the extent of viral shedding evaluation in subsequent trials.

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

381 *2.5 Clinical considerations*

382 *2.5.1 Biodistribution and shedding studies*

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

387 The biodistribution of the vector may depend on the route of administration, however extensive
388 dissemination of vector has been observed following what is generally considered to a local route of
389 administration i.e. intra-muscular. The appropriate samples to be taken during clinical studies may not
390 always therefore reflect the route of administration. Examples of samples that could be taken include
391 tissue biopsy's (if possible), blood/serum, tears, urine, semen, buccal swabs/sputum, lung lavage and
392 faeces. A sufficient number of patients should be included in these studies in order to draw robust
393 conclusions and the time interval between samples needs to be fully justified.

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

396 *2.5.2 Immunogenicity*

397 Equally the extrapolation of immunogenicity data for therapeutic applications of AAV vectors from
398 animal models to humans is not simple, and the route of administration may also impact on the
399 immunogenic profile of the product. It is recommended therefore that consideration is given to the
400 potential of subjects having pre-existing antibodies to the serotype of AAV under investigation, and
401 that evaluation of the immunogenicity of both the vector and the transgene is assessed in terms of
402 neutralizing and non-neutralizing antibody formation during clinical trials. If possible it should be
403 determined if there is a correlation between pre-existing immunity / neutralizing antibody formation
404 and efficacy. This will be of particular importance if the aim is to re-administer the vector, and if
405 long-term expression of the 'gene of interest' is observed.

406 *2.5.3 Germ-line Transmission*

407 The question of germ-line transmission in humans has not been fully resolved and short term DNA
408 persistence has been observed in semen (serotype 2), therefore it is recommended that germline
409 transmission is investigated during clinical studies and that the use of barrier contraception for
410 individuals enrolled in clinical trials is included in study protocols.

411 *2.5.4 Long-Term Follow-Up*

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

417 For further information on long-term follow-up requirements refer to the CHMP guidelines:
418 'Guideline on Follow-up of Patients Administered with Gene Therapy Medicinal Products'
419 (CHMP/GTWP/60436/07) and 'Guideline on Safety and Efficacy Follow-up – Risk Management of
420 Advanced Therapy Medicinal Products' (EMEA/149995/2008).
421
422 For evaluation of efficacy, reference to the relevant guidelines of the specific disease under
423 investigation is also recommended e.g. 'Guideline on the Evaluation of Anticancer Medicinal Products
424 in Man' (CPMP/EWP/205/95/Rev.3/Corr.2) if the indication is cancer.

425 3. CONCLUSION

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

429 Given the basic biology of the parent virus, there are a number of issues that should be thoroughly
430 investigated in non-clinical studies, such as the potential for germline transmission, the potential for
431 reactivation of infection and what impact contamination with wild-type AAV might have on product
432 safety etc. The outcome of these studies should then be taken into consideration when designing
433 subsequent clinical trial protocols. However, one of the main problems with this vector is determining
434 what is the most useful animal model for pivotal non-clinical studies, and it would appear that
435 extrapolation from animals to the human situation is not straightforward. Therefore determining what
436 sampling/analysis is included or excluded from clinical protocols on the basis of non-clinical data will
437 need to be scientifically justified.

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

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

454 4. REFERENCES

455 *4.1 Literature References*

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526 ### *4.2 Guideline References*

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

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

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

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

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

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

539 Gene Transfer Medicinal Products for Human Use (Monograph 5.14 of the European Pharmacopoeia)

540 ICH Considerations: General Principles to Address Viral / Vector Shedding (in preparation at the time
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