Practice and Reflection on Non-clinical Safety Evaluation of AAV drugs

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Abstract: Due to the rapid development of biomedical technology, new high-tech therapeutic methods have also developed rapidly, among which gene therapy products as a representative of therapeutic drugs are booming and constantly iterated. In the process of drug development, non-clinical safety evaluation is a crucial part, which can provide preliminary exploration for subsequent clinical trials. In the past few years, there has been a significant rise in the FDA's approval of novel gene therapy medications. However, it is important to address certain safety concerns that have emerged during non-clinical trials. This paper mainly discusses the considerations of non-clinical safety evaluation of gene therapy drugs with AAV as an example and the concerns of AAV drugs in non-clinical evaluation.

1. Introduction

Adeno-associated viruses (AAV) belong to a group of defective DNA viruses characterized by their single-stranded linear genetic material. Its packaging capacity is about 4.7kb, without envelope, the shape is bare 20-hedral particles, and it depends on the co-infection of other auxiliary viruses (adenovirus or herpes virus) for self-replication [1-2]. It has been widely used for its advantages of non-pathogenicity, extremely low immunogenicity, wide range of host cells, and high safety [3]. Currently, numerous AAV-based gene therapy medications have been successfully introduced into the market. Nevertheless, a significant number of non-clinical and clinical investigations have been halted or discontinued without resulting in drug registration. Consequently, there is still an unmet need for treating various diseases, particularly those classified as rare conditions. For this kind of gene therapy product, it is necessary to carry out personalized non-clinical safety experiment design according to the preclinical evaluation of gene therapy drugs and their characteristics.

2. Characteristics of AAV carrier

(1) good safety. AAV is the safest viral vector available, it is replication-deficient and does not cause any human disease. Many studies have shown that the novel AAV vector derived from primates has the advantages of high transduction efficiency, low serum prevalence and weak immune response to muscle, liver and nervous tissue. In particular, AAV7, 8, 9 and other new vectors have a particularly outstanding performance in liver transgene. In recent years, with the development of cross-packaging and Self-complementary AAV (scAAV) carrier technology, These new AAVs are widely used in gene therapy studies of muscle, Central nervous system (CNS), liver and other tissues. In addition, AAV vector has a low risk of damaging the host genome. After transduction into target cells, AAV virus vector maintains a state of free DNA in the nucleus, which can reduce the risk of cancer caused by the destruction of the host genome, making it an ideal carrier for human experiments. (2) Wide host range. For commonly used mammalian cells and tissues from different sources, such as human, mouse, and rat, our AAV vectors can be easily and efficiently transduced by packaging them into serotypes for which they have affinity. (3) Small capacity. AAV is the smallest payload of all our viral vector systems. The maximum sequence length that can be accommodated between two ITRs of an AAV is 4.7kb. For example, it is difficult to transduce specific cell types, and when packaged with the right serotype, our AAV carrier system can transduce many different cell types, including non-dividing cells. (4) technological complexity. When using AAV virus vectors, the virus needs to be implanted in the packaging cell and then the virus titer is determined. Compared with conventional plasmid transfection, these processes have higher technical difficulty and longer cycle.

3. AAV gene therapy drugs

The initial gene therapy medication utilizing AAV that received clinical approval in Europe was Glybera, which aimed to address lipoprotein lipase deficiency (LPLD), an uncommon autosomal recessive disorder. However, due to high prices and low demand, it has been withdrawn from the market. There are presently 17 gene therapies that have received approval from the US FDA (Food and Drug

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Administration), one of which is Luxturna, an AAV vector called voretigene neparvovec rzyl (VN), developed by Spark Therapeutics in 2017. The AAV2 in VN is utilized for the treatment of biallelic RPE65-associated retinal dystrophy, a rare genetic condition characterized by deteriorating visual function that worsens over time and ultimately results in loss of vision. The FDA approved Onasemogene abeparvovec xioi (OA), an AAV-based gene therapy developed by AveXis and marketed as Zolgensma, making it the second such therapy to receive regulatory clearance in 2019. The treatment of type I spinal muscular atrophy (SMA1) in children under 2 years old involves the utilization of AAV9 to deliver a functional SMN1 transgene. SMA1 is distinguished by the degeneration of motor neurons in the brain stem and spinal cord, resulting in significant motor dysfunction and eventual demise due to respiratory insufficiency. It is the most prevalent genetic disorder leading to mortality in infants. In a Phase 3 clinical trial conducted in 2017, known as STR1VE (NCT03306277), it was observed that after 14 months of receiving the treatment, a significant proportion of patients (91%) did not require permanent respiratory support. Additionally, at the 18-month mark, approximately half of the participants (50%) demonstrated independent sitting ability [4].

Mechanism of AAV virus gene therapy: Recombinant AAV particles enter cells through clathrin-mediated endocytosis by binding to glycosylated receptors expressed on the surface of host cells. After the acidification of endosomes formed by endocytosis, the conformation of VP1/VP2 parts of the viral capsid changes, resulting in the virus escaping from the endosome and entering the nucleus through the nuclear pore. After entering the nucleus, the single-stranded DNA is released from the capsid. Single-stranded DNA can't be transcribed at this point, they need to become double-stranded DNA. Single-stranded DNA can be synthesized using the host cell's DNA polymerase, or two complementary strands released from different AAV particles can be annealed to form double-stranded DNA. The latest AAV genome design is able to design the single-stranded DNA carried in the capsid into a self-complementary sequence. The advantage of this sequence is that it can be transcribed without the step of copying single-stranded DNA into double-stranded DNA, and its gene expression is more rapid and higher than that of traditional single-stranded AAV genomes.

4. Strategies for non-clinical evaluation of AAV drugs

Similar to non-clinical development strategies for conventional drugs, AAV viral drugs should also be developed for research purposes. According to the guidelines of ICH M3 (R2) for nonclinical safety assessments in human clinical trials and pharmaceutical marketing authorization, as well as ICH S6 for preclinical safety evaluation of biotechnology-derived pharmaceuticals and ICH S8 for immunotoxicity studies in human pharmaceuticals, it is mandated that non-clinical studies be conducted in different stages to provide support for clinical trials or premarket investigations [5]; At the same time, it is necessary to combine the characteristics of AAV virus, and based on meeting the relevant regulatory requirements of gene therapy drugs and the principles of experimental design, confirm the immunological mechanism of candidate AAV virus drugs from basic research and proof of concept (POC) experiments, and then in the subsequent preclinical safety evaluation experiments, The focus is on the over activating effects of immune factors and potential safety concerns arising from viral replication.

At present, there are no specific guiding principles for non-clinical research on AAV virus in China, and the Technical Guiding Principles for Human Gene Therapy Research and Quality Control of Preparations drafted by the Drug Evaluation Center of the State Drug Administration in 2003 put forward some preliminary requirements for the safety of gene therapy. Some guidance documents of other international drug regulatory agencies have good reference significance: for example, the US FDA provided updated guidelines in 2007 regarding the considerations for plasmid DNA vaccines targeting infectious diseases. Furthermore, they released an updated industry guidance document in 2013: Preclinical Evaluation of Experimental Cellular and Gene Therapy Products, Factors to Consider in the Planning of Initial Clinical Trials for Cellular and Gene Therapy Products, and Development and Analysis of Shedding Studies for Virus or Bacteria-Based Gene Therapy and Oncolytic Products [6]; And then there's 2020. Follow-Up Study Conducted Over an Extended Period Following the Application of Human Gene Therapy Products. The EMA's publication in 2006 provides a guideline regarding non-clinical testing to prevent unintentional transmission of gene transfer vectors to germ cells. The 2008 release of the guideline concerning preclinical investigations necessary before the initial clinical application of gene therapy medicinal products. The 2019 release of the Quality Guideline focuses on the non-clinical and clinical prerequisites for conducting trials involving investigational advanced therapy medicinal products. Although there are no specific ICH guidelines for AAV viruses, there are several ICH Consideration documents that deserve researchers' attention. In 2006, General Principles were issued to tackle the potential risk of unintentional integration of gene therapy vectors into germline cells. Additionally, there are reflection papers available that address various aspects such as quality assurance and both non-clinical and clinical concerns about the advancement of RAAV-related viral vectors. Furthermore, another reflection paper focuses on similar matters but specifically related to the development process involving recombinant adeno-associated viral vectors.

5. AAV carrier drugs are not the focus of clinical research

5.1 General consideration

Due to the production of neutralizing antibodies, clinical AAV therapy can usually only be administered once, so

the clinical starting dose must be the effective dose. Many clinical cases involve children, and the use of young animals should be considered in preclinical protocols. Although wild-type AAV is not pathogenic to humans, it may cause adverse reactions to recombinant AAV vectors [7]. The administration route, frequency, and cycle in non-clinical studies should align as closely as possible with the intended clinical use.

5.2 Selection of relevant animals

We should simulate the immune state of the human body as much as possible, considering the influence of preexisting immunity, and be sensitive to the viral vector, have biological effects on the expression product, including distribution, expression, functional activity, etc., and the feasibility of clinical administration of the proposed drug. In some cases, we need to choose some unconventional animal models, such as transgenic animals, disease animal models, etc. In addition, the selection of animals, can not only stay at the species level but also take into account animal strain differences. In terms of animal species, one related animal species is fine, usually two species are not required, and large animals are not necessary. But sometimes both animals, especially NHP, are more reflective of the human condition[8]. Conducting toxicity studies with disease model animals helps understand the safety of disease development. For example, when two animal tests are planned, one animal can be selected as a model animal. For large animals, lower titers should be screened. If model animals are used, a suitable model should be selected to facilitate the observation of toxicity and ensure the quality of the study as much as possible.

5.3 Dose design considerations

Considering that AAV gene therapy can only be administered once, clinical FIHD should be effective, not a subclinical dose (antibodies can not be administered again), and the maximum dose is also the maximum effective dose, so preclinical studies should provide sufficient safety information. We need to focus on both pharmacological and toxicological studies. Pharmacological studies provide the effective dose and optimal effective dose. The high dose in toxicological studies should be several times the expected clinically optimal effective dose, usually 10 times. When it becomes challenging to attain this dosage, the alternative option is to administer the maximum achievable dose (MAD).

5.4 Biological distribution of viruses

The results of biological distribution experiments can provide a basis for the selection of drug administration schedule, monitoring plan of drug activity and safety indexes in preclinical studies, and time point of animal euthanasia [9]. Information obtained from biodistribution experiments, in conjunction with additional safety measures such as clinicopathology and pathology assessments, can aid in assessing the potential correlation between carrier distribution or gene expression and any observed toxicity or detrimental effects. Biological distribution experiments generally require the use of animals of two genders, if a single sex needs to be explained. Rodents consider 5 animals per sex at each time point, and non-rodents 3 to 5 animals per sex. At the same time, the experimental design should take into account the animal's age, physiological state, and other factors that may affect the carrier distribution. The clinical application should be considered when determining the administration route, frequency, and cycle. The dosage of AAV viruses is often limited by the choice of route of administration and the size of the animal. It is advisable to opt for the highest achievable dosage (HAD) or the dose suggested by clinical professionals. Biological distribution generally adopts real-time fluorescence quantitative PCR, ddPCR, dye method, UV/Vis, and other methods. Real-time fluorescence quantitative PCR (qPCR) is one of the widely used methods for quantitative analysis of AAV vectors. The method has the characteristics of high specificity, high sensitivity, repeatability, high throughput, easy execution, and standardization of analysis. Based on the amplification of target-specific primers, the detection can amplify and detect DNA sequences in the same reaction by combining the fluorescent signal sent by the target-specific primers or probes labeled with dye or fluorescence. The key to the qPCR method is to synthesize primers and probes with high specificity and sensitivity [10]. However, qPCR is sensitive to PCR inhibitors, which may also be introduced into samples during DNA extraction, such as organic solvents, salt ions, polysaccharides, hemoglobin, etc. In addition, there is also a preference for primer amplification efficiency in qPCR, and the difference in extraction efficiency leads to the difference in the amount of harvested products, resulting in inconsistent results. The secondary structure of DNA, sample preparation, and so on can significantly affect the efficiency of DNA amplification. Moreover, the qPCR method does not distinguish between intact viruses and non-infectious or degraded viruses.

5.5 Support shedding test

The copy number of carrier DNA can be determined by qPCR or ddPCR, and the infectivity analysis of the excreted components is generally detected by cell-based in vitro tests. This method detects only intact and potentially transmissible viruses and vectors [11]. However, because the detection depends on cell infection, the operation is more complicated, the method cycle is long, the flux is low, and the detection sensitivity is poor. Each matrix type has its own unique challenges in terms of qPCR and/or ddPCR, as well as cell-based assay development, which should be considered throughout assay development and validation. For example, infectivity analysis in urine can be used in cell-based assays to stain advanced viral replication proteins using alternative markers. Taking AAV8 as an example, combined with the investigation of shedding in the toxicity test, the shedding of viral vectors was dose-dependent and the shedding duration was long, so

attention should be paid to extending the collection time. If the drug is administered locally to the eye, the main route of shedding is tear and nasal cavity. The duration of falling off was short, which was lower than the lower limit of detection after 7 days.

5.6 Safety testing

For single-dose gene therapy drugs, extended single-dose toxicity tests can be carried out. If the action time in animals is obviously short, repeated drug toxicity tests can be considered if the drug is used multiple times in humans and clinical practice. The duration of the toxicity test was determined according to the level and duration of the vector and the expression product, including the peak time point and the homeostasis time point of the imported gene. Focus on humoral or cellular immune responses, carrier distribution, abnormal/ectopic hyperplasia, etc. Group design: control group such as solvent, excipients and empty carrier can be selected, and the carrier control group is conducive to the analysis and interpretation of toxicity. The administration group is designed to incorporate indications, safety risk assessment, and usually 3 dose groups. Dose design: Limited dose (maximum dose concentration, maximum dose volume). Design of observation time: According to the distribution of vectors and the expression of transgenes, design the duration of the experiment and the recovery duration. Animals need to be euthanized at the point of peak biological distribution or when toxicity is evident.

5.7 long period observation test

On account of the long-term existence of AAV vector in tissues and the long-term expression of the transferred gene, in order to investigate the safety of the product more comprehensively, a 3-month toxicity test should be carried out at the same time, a 12-month long cycle test should be conducted to observe the toxicity and tissue distribution, including a long-term follow-up test at a later stage [12]. At the same time, the clinicopathological indicators, the distribution of antibodies and viral vectors, the expression of products and histopathology were examined.

5. Conclusion

Generally speaking, the AAV virus is a kind of natural replication defect disease with the simplest structure, which cannot replicate independently, and can only complete the replication of gene therapy products when auxiliary viruses such as herpes simplex virus exist. Personalized non-clinical experiments are carried out. Reasonable and moderate non-clinical studies are designed to provide valuable reference data for clinical practice. Effectiveness research runs through the whole non-clinical evaluation, and the effectiveness index should also be paid attention to in the safety evaluation. The integrity of the toxicity study design and the scientific analysis of results are the basis of clinical risk control. Precise methodology (virus and expression product detection and immunogenicity methods) ensures the reliability of test results. Scientific preclinical experimental design, accurate preclinical research methods, and objective and comprehensive results analysis can effectively reduce the risk of clinical research.

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