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Review | Обзор

Adeno-associated virus vector-based gene therapy for hereditary diseases: current problems of application and approaches to solve them

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ABSTRACT

INTRODUCTION. Currently, gene therapy based on adeno-associated virus (AAV) vectors faces a number of barriers, both biomedical and technological, which require studying and overcoming for further development of this gene therapy technology.

AIM. This study aimed to analyse the use of gene therapy for a range of hereditary diseases, taking into account the barriers associated with its side effects and insufficient efficacy, the determination of the therapeutic window, and individual characteristics relevant to a particular hereditary disease; additionally, the study aimed to review the approaches to lifting these barriers and increasing the availability of gene therapy through the improvement of technological approaches to production and the reduction of production costs.

DISCUSSION. The authors reviewed the experience accumulated for gene therapy products that were approved or undergoing clinical trials. The study included a gene therapy applicability assessment using several hereditary diseases as a case study. The assessment showed that correct determination of the therapeutic window for a medicinal product and timely diagnosis of a hereditary disease were essential for effective and safe gene therapy. The study considered the strategies used to reduce the risks of adverse events and increase the effectiveness of AAV-based gene therapy. The authors assessed technological advancements in the manufacturing of AAV-based gene therapy products. The most perspective directions were the transition to suspension culture systems, the improvement of bioreactors, the use of new methods and materials for the purification of viral particles, the improvement of transfection systems, and the creation of new host cell lines. Ultimately, this can lead to lower production costs and an increased availability of gene therapy.

CONCLUSION. Currently, gene therapy is used only for a small range of hereditary diseases. Significant barriers to its use are due to insufficient efficacy, risks of adverse events, and high costs for treatment. Ongoing biomedical and technological development should lift many of these barriers and increase access to gene therapy.

Keywords: gene therapy; gene therapy product; adeno-associated virus; AAV; adeno-associated virus vectors; hereditary diseases; haemophilia; Duchenne muscular dystrophy; gene therapy efficacy; gene therapy safety; AAV production technology; AAV modification; triple-plasmid transfection; AAV-particle purification

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Генная терапия наследственных заболеваний на основе аденоассоциированных вирусных векторов: современные проблемы применения и пути их решения

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РЕЗЮМЕ

ВВЕДЕНИЕ. В настоящее время генная терапия на основе аденоассоциированных вирусов (adeno-associated viruses, AAV) сталкивается с рядом барьеров биомедицинского и технологического характера, анализ и преодоление которых необходимо для дальнейшего развития данного направления генной терапии.
ЦЕЛЬ. Провести анализ применения генной терапии ряда наследственных заболеваний с учетом ограничений, связанных с побочными эффектами или недостаточной эффективностью генной терапии, проблемой определения терапевтического окна и индивидуальной спецификой наследственного заболевания, а также рассмотреть подходы для снятия этих ограничений и повышения доступности генной терапии путем усовершенствования технологических подходов производства и снижения себестоимости генотерапевтических препаратов.
ОБСУЖДЕНИЕ. Рассмотрен опыт применения одобренных и находящихся на этапе клинических исследований генотерапевтических препаратов. Проведена оценка применимости генной терапии на примере ряда наследственных заболеваний и показано, что правильное определение терапевтического окна препаратов и своевременная диагностика наследственных заболеваний являются критически важными для эффективной и безопасной генной терапии. Рассмотрены современные стратегии снижения риска возникновения побочных эффектов и увеличения эффективности генной терапии на основе AAV, среди которых наиболее важны следующие: поиск новых серотипов AAV, модификация капсидов и генома AAV, подавление нецелевой экспрессии с помощью микроРНК, изменение содержания CpG, поиск новых промоторов для трансгена. Проведен анализ развития технологии производства генотерапевтических препаратов на основе AAV. Наиболее перспективным представляется переход на суспензионное культивирование, усовершенствование биореакторов, применение новых методов и материалов очистки вирусных частиц, совершенствование систем трансфекции и создание новых клеточных линий-продуцентов, что в конечном итоге может привести к снижению затрат на производство препаратов и повышению доступности генной терапии.
ЗАКЛЮЧЕНИЕ. В настоящее время генная терапия используется только для небольшого количества наследственных заболеваний. Существенные ограничения применения генотерапевтических препаратов связаны с недостаточной эффективностью, риском возникновения побоч-

ных эффектов и высокой стоимостью лечения. Ведущиеся в настоящее время биомедицинские и технологические разработки позволят решить указанные проблемы и повысить доступность генной терапии.

Ключевые слова: генная терапия; генотерапевтический лекарственный препарат; аденоассоциированный вирус; AAV; аденоассоциированные вирусные векторы; наследственные заболевания; гемофилия; миодистрофия Дюшенна; эффективность генной терапии; безопасность генной терапии; технология производства AAV; модификация AAV; трехплазмидная технология; очистка вирусных частиц AAV

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Финансирование. Работа выполнена без спонсорской поддержки.

Потенциальный конфликт интересов. Авторы являются сотрудниками компаний, занимающихся разработкой генотерапевтических лекарственных средств. Однако при написании рукописи авторы руководствовались соображениями научной и медицинской ценности обсуждаемых материалов и заявляют о беспристрастности оценки рассмотренных данных.

INTRODUCTION

Gene therapy (GT) is aimed at manipulating expression of a specific gene or changing biological properties of living cells for therapeutic purposes[1]. In turn, gene therapy medicinal products (GTMP) are drugs whose pharmaceutical substance is a recombinant nucleic acid or includes a recombinant nucleic acid that allows for control, repair, replacement, addition or removal of a genetic sequence[2]. The main GTMP groups are virus particles acting as a genetic vector; DNA oligonucleotides; and small interfering RNAs that modulate gene expression. In this review, the authors covered GTMPs using vectors based on adeno-associated viruses (AAV).

Despite decades of developing this GTMP type for the treatment of hereditary diseases, a number of objective factors restrain their widespread use. One of essential factors is extremely high cost, despite the fact that most cases require only a single GTMP administration. Other limitations include biomedical factors, such as GTMP safety issues primarily associated with immunogenicity, effective load limitations in AAV vectors, lack of efficacy for targeted delivery and transgene expression etc. In some cases, comorbidities, history of previous therapy, age, missed or suboptimal therapeutic window, as well as existing effective and safe standard therapy also hinder the use of even an already approved GTMP. Expert assessment of GTMP registration dossier is also a complex task, especially considering incomplete product description compared with traditional drugs [1]. Finally, GTMP production technology based on virus particles is characterised by an extremely high cost per dose. This review describes the challenges and the prospects of their solving.

This study aimed to analyse gene therapy used in a range of hereditary diseases, considering limitations associated with side effects and lack of efficacy, identification of the therapeutic window, and individual nature of a particular hereditary disease; additionally, the study aimed to review the approaches lifting these barriers and increasing availability of gene therapy with the help of improved technological approaches and reduced production costs.

MAIN PART

Medical and biological restrictions to the use of genetic therapy

Currently, several GTMPs have been registered for delivery of expression constructs *in vivo*. In majority of cases, these drugs are used in hereditary diseases *(Table 1)*.

Roctavian (valoctocogene roxaparvovec) for single administration is used to treat haemophilia A. The AAV vector used in this product provides expression of the functional coagulation factor VIII-SQ (FVIII) in liver cells. However, its use is limited by a number of factors. First, the patients still partly depend on injectable coagulation factors. The annual bleeding

[1] Chemistry, manufacturing, and control (CMC) information for human gene therapy Investigational New Drug Applications (INDs). Guidance for industry. 2008-D-0205. FDA; 2020.
[2] Federal law of the Russian Federation No. 61-FZ of 12.04.2010 “On circulation of medicines“.

rate has decreased from 5.4 to 2.6 cases per year, and the average number of coagulation factor infusions — from 136 to 5. However, the observation period after GTMP treatment was 3 years[3], thus the long-term stability of FVIII expression is not yet known. The level of transgenic FVIII is likely to drop over time, so the patients will again become dependent on frequent infusions of coagulation factors. Roctavian, like any other AAV with tropism for liver cells, is not indicated in children, since their liver is still growing. The same is true for patients with antibodies to AAV5 serotype, as well as with chronic liver diseases, since GTMP has pronounced hepatotoxicity. It is a serious limiting factor, since in Russia, the majority of haemophilia patients older than 20 years suffer from chronic hepatitis C. The reason is that until 2005, haemophilia patients were treated with blood components without viral inactivation[4]. Almost all patients receiving Roctavian had to complete a course of immunosuppressive therapy to prevent an autoimmune reaction caused by AAV administration. A survey of participants in the clinical trial (CT) of Roctavian revealed that for some of them, the immunosuppressive therapy they received either for prevention or treatment of transaminitis was the least tolerable part [2]. A significant number of patients have inhibitory haemophilia, showing a high level of neutralising antibodies, e.g. to FVIII, which makes GTMP ineffective. Such patients are switched to bypassing therapy (FVII, emicizumab) that allows to initiate the clotting cascade bypassing FVIII.

Haemophilia is an example of how effective and safe enzyme replacement therapy (ERT) with coagulation factors or bypass agents al-

Table 1. Approved gene therapy products (GTMP)
Таблица 1. Одобренные к применению генотерапевтические лекарственные препараты (ГенЛП)

GTP name *Наименование ГенЛП*	**Indication** *Показание*	**Description, delivery vector** *Описание, вектор доставки*	**Promoter** *Промотор*	**Dose** *Доза*	**Year of FDA/EMA approval** *Год одобрения FDA/EMA*	**List price**[5] *Стоимость терапии*[5]
Glybera (alipogene tiparvovec)[6] *Глибера (алипоген типарвовек)*[6]	Familial lipoprotein lipase deficiency (hyperlipoproteinaemia type I) *Наследственный дефицит липопротеинлипазы (гиперлипопротеинемия I типа)*	I.m. injection of the lipoprotein lipase gene, AAV2 *В/м инъекция гена липопротеинлипазы, AAV2*	CMV	1×10^{12} gc/kg *1×10^{12} гк/кг*	2012*	— (marketing authorisation not renewed) *— (регистрационное удостоверение не продлено)*
Luxturna (voretigene neparvovec-rzyl)[7] *Люкстурна (воретиген непарвовек)*[7]	Congenital retinitis pigmentosa (inherited blindness due to *RPE65* mutations) *Пигментный ретинит (наследственная дистрофии сетчатки, вызванная мутацией PRE65)*	Intra-ocular delivery of the functional *RPE65* gene, AAV2 *Внутриглазная доставка функционального гена RPE65, AAV2*	CAG	1.5×10^{11} gc/eye *$1{,}5\times10^{11}$ гк/глаз*	2017	425,000 USD *425 000 долларов США*
Zolgensma (onasemnogene abeparvovec-xioi)[8] *Золгенсма (онасемноген абепарвовек)*[8]	Spinal muscular atrophy due to *SMN1* mutations *Спинальная мышечная атрофия, вызванная мутациями SMN1*	I.v. infusion of the functional *SMN1* gene, AAV9 *В/в инфузия функционального гена SMN1, AAV9*	CAG	1.1×10^{14} gc/kg *$1{,}1\times10^{14}$ гк/кг*	2019	2,100,000 USD *2 100 000 долларов США*

[3] https://www.roctavian.com/en-us/roctavian-results
[4] https://medvestnik.ru/content/news/Bolee-90-bolnyh-gemofiliei-starshe-18-let-stradaut-hronicheskim-gepatitom-S.html
[5] https://www.globaldata.com/
[6] https://www.ema.europa.eu/en/documents/assessment-report/glybera-epar-public-assessment-report_en.pdf
[7] https://www.ema.europa.eu/en/medicines/human/EPAR/luxturna
[8] https://www.ema.europa.eu/en/documents/assessment-report/zolgensma-epar-public-assessment-report_en.pdf

Продолжение таблицы 1
Table 1 (continued)

GTP name *Наименование ГенЛП*	**Indication** *Показание*	**Description, delivery vector** *Описание, вектор доставки*	**Promoter** *Промотор*	**Dose** *Доза*	**Year of FDA/EMA approval** *Год одобрения FDA/EMA*	**List price**[5] *Стоимость терапии*[5]
Elevidys (delandistrogene moxeparvovec-rokl)[9] *Элевидис (деландистроген моксепарвовек)*[9]	Duchenne muscular dystrophy *Мышечная дистрофия Дюшенна*	I.v. infusion of the functional *DMD* minigene, AAVrh74 *В/в инфузия функционального минигена DMD, AAVrh74*	MHCK7	1.33×10^{14} gc/kg *$1,33\times10^{14}$ гк/кг*	2023*	3,200,000 USD *3 200 000 долларов США*
Roctavian (valoctocogene roxaparvovec-rvox)[10] *Роктавиан (валоктокоген роксапарвовек)*[10]	Haemophilia A *Гемофилия А*	I.v. infusion of the functional FVIII gene, AAV5 *В/в инфузия функционального гена фактора FVIII, AAV5*	HLP	6×10^{13} gc/kg *6×10^{13} гк/кг*	2023*	2,900,000 USD *2 900 000 долларов США*
Hemgenix etranacogene dezaparvovec-drlb)[11] *Гемгеникс (этранакоген дезапарвовек)*[11]	Haemophilia B *Гемофилия В*	I.v. infusion of the functional FIX gene, AAV5 *В/в инфузия функционального гена фактора FIX, AAV5*	LP1	2×10^{13} gc/kg *2×10^{13} гк/кг*	2022*	3,500,000 USD *3 500 000 долларов США*
Vyjuvek (beremagene geperpavec-svdt)[12] *Виджувек (беремаген геперпавек)*[12]	Dystrophic epidermolysis bullosa due to mutations in the *COL71A* gene *Дистрофический буллезный эпидермолиз, вызванный мутациями гена COL7A1*	Topical cutaneous delivery of the functional collagen gene *COL7A1*, HSV-1 *Топическая накожная доставка функционального гена коллагена COL7A1, HSV-1*	COL7A1	4×10^{8}–1.2×10^{9} PFU *4×10^{8}–$1,2\times10^{9}$ БОЕ*	2023*	24,250 USD per vial (631,000 USD per patient per year) *24 250 долларов США за упаковку (631 000 – на пациента в год)*
Neovasculgen[13] *Неоваскулген*[13]	Atherosclerotic ischaemia of the lower limbs *Ишемия нижних конечностей атеросклеротического генеза*	I.m. injection of the vascular growth factor gene VEGF-165, plasmid *В/м введение гена фактора роста сосудов VEGF-165, плазмида*	CMV	1.2 mg *1,2 мг*	2011	78,600 ₽ *78 600 рублей*
Upstaza (eladocagene exuparvovec)[14] *Апстаза (эладокаген эксупарвовек)*[14]	Aromatic L-amino acid decarboxylase deficiency *Дефицит декарбоксилазы ароматических L-аминокислот*	Intraputaminal delivery of the decarboxylase gene, AAV2 *Введение внутри базального ядра головного мозга AAV2 с геном декарбоксилазы*	CMV	1.8×10^{11} gc (total dose) *$1,8\times10^{11}$ гк (общая доза)*	2022*	3,710,000 USD *3 710 000 долларов США*

The table is prepared by the authors / Таблица составлена авторами

Note. *, not approved in Russia; i.m., intramuscular; i.v., intravenous; AAV, adeno-associated virus vector; HSV-1, human herpes simplex type 1 virus vector; gc, genome copies; PFU, plaque forming units.
Примечание. * – не зарегистрирован в Российской Федерации; в/м – внутримышечно; в/в – внутривенно; AAV – вектор на основе аденоассоциированного вируса; HSV-1 – вектор на основе человеческого вируса герпеса 1 типа; гк – геномные копии; БОЕ – бляшкообразующие единицы.

[9] https://www.fda.gov/vaccines-blood-biologics/tissue-tissue-products/elevidys
[10] https://www.ema.europa.eu/en/documents/assessment-report/roctavian-epar-public-assessment-report_en.pdf
[11] https://www.ema.europa.eu/en/documents/assessment-report/hemgenix-epar-public-assessment-report_en.pdf
[12] https://www.fda.gov/media/169435/download
[13] https://neovasculgen.info/instruktsiya-po-primeneniyu
[14] https://www.ema.europa.eu/en/documents/assessment-report/upstaza-epar-public-assessment-report_en.pdf

lows controlling the disease and ensuring an acceptable quality of life even without GTMP. In this case, GT still has to prove its long-term effectiveness and safety, and above all, to reduce cost in order to become a treatment standard.

Other examples of effective ERT are certain lysosomal storage diseases (for example, Gaucher disease, Fabry disease etc.). It makes GTMP just one of the alternatives for the same indication. However, in a large number of hereditary diseases, classical ERT infusion of recombinant proteins is impossible by default and where delivery of a functional gene copy to the affected cells (or another method of correcting the mutations) is the only pathogenetic therapy. An example of these diseases is Duchenne muscular dystrophy (DMD).

One of the serious GTMP issues is AAV dose insufficient to restore the normal level of gene expression (associated with dose limitation due to toxicity), or decreased expression over time. The latter may have various causes: growth of the target tissue (as described above for the liver), natural cell death, the body's immune response, or transcriptional silencing of the transgene. In addition, low GTMP efficacy and safety may have less obvious causes, as is the case with Luxturna.

Luxturna (voretigen neparvovec) is the first GTMP drug approved for the treatment of inherited retinal degeneration caused by biallelic mutations in the *RPE65* gene. The drug demonstrated an acceptable safety profile and efficacy in clinical trials, including observational post-marketing studies, allowing not only to stop the disease progression, but to partially restore lost vision in many cases. It is noteworthy that the observation period of the largest published post-marketing clinical trial to date (103 patients) has not exceeded 2.3 years [3]. This GT includes subretinal injection of AAV carrying the functional *RPE65* gene. The *RPE65* gene plays a key role in the renewal of 11-cis-retinal vital for the functioning of rods, retinal photoreceptors. The primary therapeutic target of Luxturna is retinal pigment epithelium cells, and photoreceptors are a secondary target for restoring visual function [4].

Following the approval of Luxturna, chorioretinal atrophy (CRA) was reported in many cases following GT, both around retinotomy site (injection site) and major retinal vessels. CRA is an atrophy of the outer retinal layers, the pigment epithelium, and the choriocapillaris. Growing CRA lesions were observed over several months, particularly in the youngest patients who initially had the best response to GT. CRA progression rate following Luxturna exceeded the natural course of the disease when compared with historical morphology in patients not receiving GT. Several CRA causes were excluded, such as surgical sequelae of injection, or immune response and inflammation. A further hypothesis was based on increased retinal metabolic activity following photoreceptor treatment with GT [4].

The degenerated retina has a lower oxygen supply and needs less oxygen due to cell loss. Most cases of retinal degeneration, including those mediated by *RPE65* mutations, are accompanied by depletion of the retinal vessels. If this hypometabolic state is suddenly reversed by an intervention that increases functional activity of the photoreceptors and hence all inner retinal cells, can a degenerated, low oxygen system cope with this after many years of depletion? Moreover, CRA cases were observed predominantly in young adults and adolescents with high levels of rods restored; this may indicate a cause-and-effect link between efficacy and safety. If so, functional restoration of degenerated tissue may lead to metabolic and oxidative imbalance that leads to cell death [4]. If this hypothesis is correct, in the worst case, not only would this GT fail to preserve vision throughout adulthood, but after the first years of apparent efficacy, it would also lead to accelerated vision loss compared to untreated patients. This assumption definitely requires more observational studies and a larger sample of patients. However, this example highlights both the risks of an overly simplistic approach to GT and the fact that functional restoration of a defective gene must be considered in terms of systems biology.

Determining the therapeutic window – an essential part of developing pathogenetic therapy for hereditary diseases

A lot is expected of GT for hereditary diseases as a universal tool that allows influencing the pathological causes. Timely GT is critical both for preserving life and its quality in patients. Many hereditary diseases progress rapidly and are associated with loss of functions and skills, dysregulation of normal cellular and tissue metabolism, and destruction of cells and tissues that

normally express the target protein. Use of GT may be ineffective in the late stages due to irreversibile changes caused by mutation and the loss of most cells that are the therapy target.

In this regard, it is necessary to study the natural history of a genetic disease in each individual case and determine the therapeutic window — the most sensitive period for treatment and functional restoration. For orphan diseases, study of the natural history is complicated by small samples and heterogenous patient groups, diverse mutations typical for some hereditary diseases, and the influence of modifier genes on the disease progression. It is almost impossible to predict the optimal age period for GT use based on preclinical studies. The consequences of pathogenic mutations are not always reproducible in animal models. In addition, it is quite difficult to approximate the age of animals and that of human patients.

The possibilities of applying GT at the disease onset, and especially at the presymptomatic stage, are inextricably linked with diagnosing possibilities. So far, the only drug, Elevidys (delandistrogen moxeparvovec), has been approved under an accelerated procedure by the US Food and Drug Administration (FDA) for administration in paediatric outpatients aged 4–5 years[15] as gene replacement therapy of Duchenne muscular dystrophy (DMD). The average (median) age of diagnosis among patients with DMD is 4.9 (4.8) years [7], and an average of 2.2 years has passed from the first signs of the disease to diagnosis. Late GT introduction may become not only ineffective, but even dangerous. There are known mortality cases after GT in patients with DMD at late stages of the disease (16 and 27 years) occurred during the clinical trials (NCT03362502, NCT05514249).

Zolgensma (onasemnogene abeparvovec) used to treat spinal muscular atrophy in a group of patients at presymptomatic stage illustrates exceptional GT effectiveness provided within the optimal time window. As shown in the pivotal clinical trial, 14 patients out of 14 treated with the GTMP showed motor development at the level of healthy children during 18 months of follow-up [6]. Thus, newborn screening is the most adequate strategy for GT application in rare hereditary diseases that allows diagnosing at the presymptomatic stage [6]. The ultra-rare hereditary diseases (with an incidence of less than 1 in 1,000,000 newborns) are worth mentioning, with attempts being made to develop patient-specific gene therapies. The time required to diagnose, including genetic diagnostics, and link the detected phenotype to ultra-rare genome mutations makes it difficult to develop and implement effective GT at the current scientific and technological level. In this regard, there is a need to create platforms allowing for the rapid discovery and screening of candidate formulations in patient-specific models to estimate their efficacy and safety [8].

Risk mitigation strategies for adverse effects when administrating GTMPs

Severe adverse events (SAE) caused by administration of AAV-based GTMPs are often associated with an immune response to a high dose of the virus vector or concomitant diseases in patients [9]. Thus, there are two ways of preventing possible side effects: careful selection of subjects and use of pharmacotherapy to reduce the potential immune response; or modification of the drug itself to reduce the dose and remove immunogenicity factors.

Patient-oriented approach

AAV is considered non-pathogenic for humans; however, a patient may have a history of contact with a natural AAV serotype, with memory immune cells formed. Given the rapid and strong immune reactions induced after formation of immunological memory, it is necessary to identify and exclude patients with formed adaptive immunity to a specific AAV serotype. Pre-existing immunity is determined by detecting antibody titres to a specific antigen, and titre cutoff values is one of eligibility criteria. It is noteworthy that GTMP developers have no uniform protocol to identify pre-existing antibodies cutoff levels. Both the type of assay used to detect different types of antibodies (neutralising or binding) and the cutoff values may vary [10]. Antibody titre limits are often defined in preclinical studies and then tested for clinical use. For example, transgene transduction analysis in rhesus macaques af-

[15] https://www.fda.gov/vaccines-blood-biologics/tissue-tissue-products/elevidys

ter isolated limb perfusion with AAVrh74.MCK.micro-dys.FLAG showed that animals with pre-existing AAVrh74 antibody titres >1:400 had reduced transgene expression compared to animals with AAVrh74 antibody titres ⩽1:400 [10]. These preclinical studies served as the basis for choosing selection criteria in subsequent clinical trials. The development of universal cutoff assessment protocols would allow optimising the use of GT and would likely expand the patient population covered by this therapy.

Possible immune responses to recombinant viral vectors are not limited to adaptive humoral immunity. Due to diversity of such responses, AAV GTMPs are administered in combination with systemic corticosteroids. For example, corticosteroids were administered for 7, 30, or 60 days to all patients receiving Luxturna, Zolgensma, and Elevidys, respectively. When applying GT in haemophilia (Hemgenix, Roctavian), corticosteroid immunosuppression is implemented when transaminases are elevated. This therapy can cause immune-mediated side effects, moreover, systemic immunosuppressants can also contribute to the development of adverse events. A lot of studies are devoted to searching more effective and safe pharmacotherapy to reduce the immune response to AAV GTMPs [11, 12].

Product modification approaches

To achieve efficient transduction of target cells, high doses of recombinant AAV are administered systemically, up to 1.33×10^{14} genome copies per kilogram of body weight (gc/kg) for Elevidys *(Table 1)*. Along with target cell transduction, the viral capsid and therapeutic gene can enter non-target tissues and organs, causing adverse reactions. For example, undesirable liver transduction can cause hepatocyte destruction, clinically manifested as increased serum transaminases levels (ALT, AST), the most common adverse effect of systemic administration of AAV-based drugs. Hepatotoxicity has caused several fatal cases following AAV-based GT in clinical trials in patients with X-linked myotubular myopathy and in observational post-marketing studies in patients with spinal muscular atrophy [9]. As a result, studies of AAV-based GTMPs with capsid modification are currently underway, as well as the attempts to introduce the new gene constructs with increased target transduction and expression and reduced off-target delivery. In the long term, such optimisation may help reduce the dosage required to achieve a therapeutic effect, decrease toxicity attributed to the drug, and consequently contribute to a reduction of AAV production costs.

Modifying amino acid sequence of capsid proteins. Currently approved GTMPs and the ones studied in advanced clinical trials have virus vectors based on capsids of natural human (2, 5, 9) and primate AAV serotypes, AAV (2, 5, 9) and AAV rh (74), respectively *(Table 1)*. New genetically engineered capsids based on natural AAV serotypes are being created to enhance tropism to target tissues and add other functions. Progress in this area was achieved by determining the three-dimensional structure of AAV capsids [13–15] and identifying positions in capsid proteins for insertion of exposed peptides required for cell infection by the virus [15]. For example, the position after amino acid residue Q588 in the VP1 protein, located in the variable region VR-VIII of the AAV9 capsid, was deemed optimal for inserting exposed peptides and further selection of modified variants with increased tropism for muscle cells. Two research groups have described variants that efficiently transduce cardiac, diaphragm, and skeletal muscle cells of mice and primates: MyoAAV 2A and MyoAAV 4E [15] and AAVMYO [17]. When administrating capsids at substantially lower doses than the standard ones, it was possible to achieve transgene expression level similar to unmodified AAV9. All the best selected capsid variants included the Arg-Gly-Asp (RGD) tripeptide in the first three positions of the inserted septapeptide. Laminin receptor, integrin $\alpha7\beta1$ abundant in all muscle types, is the most likely candidate for RGD peptide binding in muscle cells and is crucial for muscle cell development and function. However, a potential problem may concern the antigenic properties of RGD peptide. In this regard, higher immunogenicity risk of MYO modifications of AAV has been discussed [18]. Immunogenicity of the resulting capsids was not studied by any of the research groups that used RGD-exposing AAV variants. However, further investigation will determine whether potentially increased immunogenicity of RGD peptide-containing capsids negates the effect achieved by reducing the dose.

In pilot experiments on animal disease models, the above mentioned and other genetically engineered capsids were successfully used to correct the phenotype in DMD [16], X-linked myotubular myopathy [19], and Pompe

disease [20]. It has been demonstrated that increasing capsid tropism for muscle cells reduces non-specific hepatocyte infection, which may have a positive effect on GTMP clinical use [16].

Modification of the AAV2 capsid in order to increase cardiac tropism using the THGTPAD and NLPGSGD peptides showed significantly increased efficiency of cardiomyocyte infection, with simultaneous infection decrease of non-target tissues, as well as lower immunogenicity of these serotypes in mice [21]. In an *in vivo* experiment, this capsid was superior to AAV9 widely used to deliver transgenes to the heart [21].

Targeted modification of AAV9 capsids resulted in new MDV1A and MDV1B neurotropic serotypes that provide efficient delivery to neurons in a murine model, as well as PAL capsids (proline arginine loop) that showed high delivery to neurons in a primate model [22]. However, capsids efficient in mice did not show increased tropism in primates. PAL serotypes provided a small but significant increase in transgene expression compared to AAV9, with a reduced viral load in non-target organs such as liver, kidneys, lungs, and thymus.

In the above-mentioned studies, modification of capsids to increase transduction rates of certain cell types also resulted in a decreased transduction level of non-target organs. At the same time, attempts are being made to perform point modifications of the capsid in order to decrease the delivery level (detargeting) to non-target organs, primarily the liver. A study by Adachi et al. [23] based on the screening of mutant AAV capsids allowed identifying amino acids on AAV9 surface responsible for certain tissue transduction, primarily the liver. Subsequently, new AAV capsids with lower liver accumulation were designed, for example, P504A/G505A mutants. At the same time, the modification reduced transduction efficiency of target organs, such as the heart. It was later demonstrated that when introduced, the mentioned mutations decrease delivery of other genetically engineered AAV9-based capsids to the liver. The AAVMYO-LD (liver detargeting, LD) serotype showed significantly lower liver accumulation in mice compared to the original AAVMYO serotype; at the same time, transduction efficiency of the diaphragm, heart, and skeletal muscles decreased. Moreover, introduction of LD mutations led to a lower yield of virus production in contrast to the original modification [17].

By changing the amino acid sequence of the exposed peptides, it was ensured that pre-existing antibodies to natural AAV serotypes do not recognise their modified variants. In the study by Han et al. [24], the rational design approach allowed to obtain mutants with increased transduction efficiency and reduced immunogenicity. The study objects were the AAV8 and AAVS3 capsids, which have a natural tropism for the liver. Screened were mutants with an altered PLA motif VP1/2, involved in AAV release from endosomes, and mutants with changed sequence of amino acids being glycosylated. The obtained variants provided higher transduction efficiency in liver carcinoma cell lines and primary hepatocytes, as well as in other human tissue cell lines. All test variants showed less sensitivity to neutralising antibodies *in vitro* and *in vivo*. Moreover, anti-capsid antibody profiles following *in vivo* transduction with modified capsids differed from their parent AAV capsids [24].

One of the most popular approaches for AAV modification involves *N*-acetylgalactosamine (GalNAc). The asialoglycoprotein receptor (ASGP-R), highly expressed on hepatocyte surface, is a carbohydrate-binding protein that recognises and binds GalNAc or galactose residues. Mével et al. [25] have developed a method for AAV chemical modification using GalNAc, and a significant increase in hepatocyte transduction *in vitro* was shown compared to unmodified AAV. When mice were administered modified AAV, fewer anti-capsid antibodies were induced compared to natural AAV2 vectors.

A universal bioconjugation-based approach is of considerable interest for regulation of AAV capsid immunoreactivity and tropism [25]. Another study also used chemical modification of reactive exposed lysine residues. *In vitro*, N-ethylmaleimide (NEM)-modified AAV9 capsid was shown to enhance gene expression in cultured human endothelial cells. *In vivo*, administration of AAV9-NEM resulted in increased bone marrow transduction and decreased liver tissue transduction compared to unmodified AAV9 [26].

To protect AAV epitopes from neutralising antibodies, an approach has been proposed to create AAV encapsulated in extracellular vesicles (EV-AAV). When using this medicine to deliver genes to cardiomyocytes, it was demonstrated that EV-AAV delivered significantly more genomic copies than unmodified AAV in the pres-

ence of neutralising antibodies in the human left ventricle, as well as in cardiomyocytes derived from human pluripotent stem cells *in vitro* and in the mouse heart *in vivo*. The efficacy of this delivery system has been demonstrated in the murine model of infarction with preliminary AAV immunisation [27].

It is noteworthy that over the past few years, production of a new genetically engineered capsid has ceased to be a labour-intensive and time-consuming task. More developers prefer these options hoping for higher efficiency and safety, as well as intellectual property protection.

Modification of the recombinant AAV genome. AAV genetic structure can be changed to increase the expression level of a target transgene, which can ultimately help decrease the required therapeutic dose. The minimal sufficient elements for controlling transgene expression are the promoter and the polyadenylation signal. In addition, enhancers, introns and post-transcriptional regulatory elements are widely used, certain combinations exponentially increasing transgene expression [28].

The effectiveness of gene therapy directly depends on the selected promoter that controls the target gene expression. Strong constitutive small-size promoters are capable of providing high expression levels. However, the expression triggered in non-target cells can activate cell-mediated and humoral immune responses to the transgene. In addition, viral promoters are quickly methylated in many cell types, over time reducing the effectiveness. The first approved AAV drugs used constitutive promoters (Glybera, Luxturna, Zolgensma), while the later GTMPs include tissue-specific liver (Roctavian, Hemgenix) and muscle (Elevidys) promoters *(Table 1)*. To increase transgene expression specificity, new highly effective tissue-specific promoters are being created [29].

Several *in vivo* studies have shown the developing immune tolerance to the transgene at a low level of its liver expression [30]. Colella et al. [31] have proposed an approach to combine various tissue-specific promoter elements for the expression of therapeutic transgenes both in target tissues and in hepatocytes. For this purpose, they used the apolipoprotein E enhancer, elements of the human alpha-1 antitrypsin promoter, and the short synthetic spC5.12 promoter, active in both cardiac and skeletal muscles and previously successfully used in large animal models of muscular dystrophy. The authors showed that the effective tandem promoters prevented immune responses to the transgene after systemic delivery of the acid alpha-glucosidase (GAA) gene using AAV in immunocompetent *Gaa*$^{-/-}$ mice. Neonatal gene therapy with AAV8 or AAV9 in *Gaa*$^{-/-}$ mice was also shown to have a durable therapeutic effect when using the tandem liver-muscle promoter (LiMP), which provided high and stable transgene expression. This promoter was also used to deliver transgene as part of a genetically engineered AAV-MT (muscle transduction) capsid. This capsid-promoter combination with improved muscle expression and specificity allowed for increased glycogen clearance in cardiac and skeletal muscles of adult *Gaa*$^{-/-}$ mice. In neonatal *Gaa*$^{-/-}$ mice, complete restoration of glycogen level and muscle strength was observed 6 months after AAV injection [32].

In addition to traditional approaches based on enhancing transduction and transcriptional specificity, miRNA-dependent post-transcriptional silencing is a new and increasingly effective tool used to regulate transgene expression [33, 34]. MiRNAs are small non-coding RNAs whose expression is often tissue- or differentiation-stage-specific. They typically regulate gene expression by binding to complementary sequences in the 3'-untranslated region (UTR) of mRNAs. To control exogenous transgene expression, tandem repeats of artificial miRNA-binding sites (miR-BS) are typically incorporated into the 3'-UTR region of the transgene expression cassette, resulting in subsequent transcript degradation in cells expressing the corresponding miRNA. Many suitable miRNAs selectively expressed in individual tissues have already been identified. For example, miRNA-122 is a miRNA almost exclusively expressed in liver tissue. Several studies have shown successful suppression of transgene expression in the liver mediated by miRNA-122 [33]. However, specific miRNA expression may be too weak to achieve strong miRNA-dependent suppression of transgene expression.

Antigen-presenting cells are key elements in the immune response. In order to reduce transgene-specific immune responses and develop safe gene constructs, a combination of miRNA-142BS and miRNA-652-5pBS was selected as a result of *in vitro* screening, which significantly suppressed transgene expression in antigen-presenting cells, but maintained a high expression level in myocytes. Intramuscular delivery of AAV1 vector carrying miRNA-142/652-

5pBS achieved stronger transgene expression than previous constructs, effective inhibition of cytotoxic T-lymphocyte activation, and suppression of Th17 response [35].

An RNA interference-based approach has been proposed for regulated transgene expression. In the work by Subramanian et al. [36], a construct containing both short hairpin RNA and its binding sites was developed. REVERSIR technology was used for transgene induction: a synthetic high-affinity oligonucleotide complementary to the guide strand of a short hairpin RNA that allows for RNA interference suppression and rapid restoration of transgene expression. These results create a basis for regulated RNA interference-based switches and dynamic modulation of gene therapy expression, with possible reduction of dosing frequency in clinical settings [36]. Tetracycline-inducible promoter elements were also tested to achieve regulated transgene expression. Thus, an AAV1-based reporter system was used to assess the duration of transgene expression in response to doxycycline. The authors demonstrated that expression of the target protein was maintained for up to 5 years after administration [37].

A muscone-induced transgene system (AAVMUSE) has been proposed by Wu et al. [38]. It is based on murine olfactory receptor coupled to the G protein (MOR215-1) and a synthetic promoter responsive to cAMP. When exposed to a trigger, muscone binds to MOR215-1 and activates cAMP signaling pathway, initiating transgene expression. Use of AAVMUSE provided dose- and time-dependent control of luciferase expression in the liver or lungs of mice for at least 20 weeks. Effectiveness of the AAVMUSE system for phenotype correction was demonstrated in murine models of chronic inflammatory diseases: non-alcoholic fatty liver disease and allergic asthma [38].

Adaptive immunity can be activated when the TLR9 (Toll-like receptor 9) recognises hypomethylated CpG motifs of the delivered gene construct, which then triggers formation of cytotoxic T-lymphocytes. Level of CpG motifs is often changed by codon optimisation of the constructs. Preclinical and clinical GTMP studies demonstrated a dependence of the cytotoxic immune response on CpG content in the product. In turn, the reaction leads to rapid loss of transgene [39, 40]. J.F. Wright et al. [41] have proposed a quantitation method of gene construct potential to activate TLR9 based on the content of CpG motifs. Therapeutic AAVs with the content of CpG motifs close to human genome are shown to be the safest for clinical use. Apart from reducing the content of CpG motifs, use of DNA sequences that block TLR9 receptors was proposed as another solution for this problem [42].

AAV production technology: past, present and future

Currently, AAVs are the gold standard for *in vivo* GT medicinal products. Since authorisation of the first GTMP, Glybera (2012, EMA)[16], the production process has been significantly improved. The main obstacles that drive the modernisation are limited production capacities with the current low process productivity, unable to meet the ever-growing demand for GTMPs, and the high cost.

Transient transfection of HEK-293 cell line by plasmid DNA remains the most common method for AAV production. Typically, three plasmids are used for transfection; the first plasmid carries the therapeutic gene of interest (GoI), the second — AAV capsid and replication genes (*rep/cap*), and the third one, called helper, — additional adenovirus genes necessary for AAV replication inside the HEK-293 host cell. The earliest technology involved cell cultures attached to the vessel wall (adherent culture) in a medium containing fetal bovine serum [43, 44]. Similar to evolution of monoclonal antibody (mAb) production, this technology is difficult to scale due to a large number of culture flasks, rotator bottles, CellStack flasks, etc. required [45, 46]. The first step towards scaling up was creating iCELLis™ (Pall) fixed-bed microcarrier bioreactors, with the culture surface area in one vessel up to 500 m^2 (equal to 3,000 roller flasks with an area of 1,700 cm^2), while providing some process control by maintaining sufficient gas exchange, nutrients, and reducing accumulation of cell culture metabolites such as ammonium and lactic acid [47].

However, these systems are also limited in terms of surface area, achievable cell density, and ultimately require further scaling to meet the demand. These limitations, as well as the controversial issue of using animal-derived components, have led to further technology improvements resulting in the development of special media for HEK-293 cell suspension

[16] https://www.ema.europa.eu/en/medicines/human/EPAR/glybera

cultures and scaling up the cultivation technology from bench-top systems to industrial-scale stirred tank bioreactors (up to 2,000 L) [48].

In parallel with the improved cultivation technology, the past decade saw significant changes in the purification process of virus particles. The following main trends include: increased percentage of full particles due to removal of both empty and partially filled capsids; more efficient purification from process impurities (residual host RNA and DNA); increased purification efficiency that cut down production costs. Recent years saw a significant success of novel affinity resins that allow for efficient isolation of virus particles from cell lysates virus particles are a minor component [49]. Moreover, new chromatography media have emerged, such as monolithic or membrane resins, which can provide higher throughput than traditional resins. One example is a liquid-liquid phase separation approach based on binding of a hydrophobic affinity sorbent to viral particles in a cell lysate. Combined with tangential flow filtration, it allows to obtain purified material [50]. Another example is a disposable flow system using chromatographic resin in a recirculating flow. In this system, various process buffers are mixed and can circulate in the flow along with the original lysate. When applied in an optimal sequence, the purified material is eluted into a separate container [51].

Separating empty and full capsids is also one of the areas of AAV purification that has made certain progress. In the early days of the technology, the only method was ultracentrifugation in a CsCl or iodixanol density gradient [52, 53], but this method is difficult to scale and causes an issue with GMP compliance. Difficulties in chromatography development were associated with the fact that separation of different AAV forms is possible only by charge. However, isoelectric points for empty, filled and partially filled capsids are very close. Nevertheless, progress has been made in recent years [53]. In particular, a joint study of JSC GENERIUM and Marlin Biotech LLC on the development of AAV-based GTMP made it possible to optimise purification technology based on anion-exchange chromatography, allowing to produce a drug with a 90% content of full AAV particles. After this stage, only one round of preparative centrifugation remains, on a much smaller scale, to achieve the purity the developers specified in the product quality profile. The proposed approach significantly improves process scalability and reproducibility, and complies with all current GMP requirements.

Despite the progress made in AAV production, the high production cost and, consequently, limited patient access to GT remains an open challenge. Current efforts are aimed at increasing the initial AAV titre during cultivation. Nowadays, several main trends are identified: improvement of the three-plasmid technology; use of a modified helper adenovirus; developing a host cell line with genome-integrated genes for AAV expression (to avoid cell transfection for each production cycle).

Improvement of plasmid technology involves searching for more efficient transfection reagents, culture media and feeds, further development of the parental HEK-293 cell line, and optimisation of genetic constructs of the pRepCap and pHelper plasmids. Xcite®AAV platform (Lonza) is an example of such technology. According to the developers, the platform allows for a 2–8-fold increase in AAV titre compared to other commercially available plasmids and cell lines[17].

Virus transduction as a method of delivering genetic material into a cell is more efficient and cost-effective than plasmid transfection. However, an additional virus agent in production is undesirable for safety reasons. Adenovirus life cycle consists of two phases: early and late; considering that, developers of TESSA™ technology (OXGENE Ltd) have constructed modified recombinant helper adenoviruses that self-inhibit the major late promoter (MLP), thereby blocking their own replication. Inserting tetracycline repressor binding site (TetR) makes normal viral replication possible only in the presence of doxycycline, while in its absence, only genome amplification and expression of early helper function genes occur. In the first production stage, two helper adenoviruses are obtained (one of which encodes the *rep* and *cap* genes of AAV, and the other – the target AAV gene) in the presence of doxycycline. In the second stage, only AAV is produced by co-infection of HEK-293 cells with two helper adenoviruses in the absence of doxycycline [55]. An important advantage of this technology is the possibility to avoid producing plasmids in large quantities. Ac-

[17] https://www.lonza.com/knowledge-center/cellgene/brief/AAV-production-HEK293-cell-line

cording to the developers, the technology is approximately 30 times more productive than the plasmid-based approach. Among the disadvantages is the formal need to purify the final product from helper adenoviruses, which theoretically still may be present, or validate their absence.

Creating stable cell lines producing AAV, similarly to mAbs production, is a promising approach enhancing productivity. A significant technical limitation is the toxicity of virus genes for the expression system. The developers of ELEVECTA™ platform (Cytiva) created inducible cell lines producing AAV based on HEK-293 cell line with genome-integrated adenovirus helper genes (*E2A*, *E4orf6*, and *VA RNA*), whereas the toxic AAV *rep* gene is induced by added doxycycline (Tet-On induction system). Genome integration of the *rep* gene became possible due to inactivation of the p19 promoter while maintaining the functionality of Rep78/68. Then, to obtain the final stable AAV producer, this cell line is transfected with a construct with the desired serotype-specific *cap* sequence, as well as a sequence of the gene of interest. According to the developers, the productivity of perfusion process on this platform is 10^{15} vg/l, with 30% filled capsids [56]. Promising platforms for AAV biosynthesis compared with the traditional three-plasmid technology are summarised in *Table 2* (published on the journal website[18]).

Thus, AAV production is a relatively new field that passes all development stages traditional for biotechnological production of recombinant products. In this context, it is similar to evolution of mAb production. AAV production technology has already experienced significant changes and is improving. The mainstream plasmid technology is the current standard, but replacing it with new approaches using recombinant helper viruses and stable cell lines becomes a trend. In addition, new media, culture feeds, resins, filters, and other raw materials are constantly emerging. The increasing demand for AAV-based GT-MPs will continue to drive technological improvements, increased productivity, and lower costs, ultimately making this product class more affordable.

CONCLUSIONS

The current analysis of gene therapy in hereditary diseases allowed to reveal a number of biomedical and technological challenges that need to be overcome for further development of this area. In order to justify the use of gene therapy and increase its availability to patients, it is necessary to solve a range of problems, namely:

- show the long-term effectiveness of gene therapy;
- identify the optimal therapeutic window for intervention, especially in cases of irreversible changes due to natural progression of a hereditary disease;
- implement an effective screening system for the early detection of hereditary diseases amenable to gene therapy;
- develop a patient stratification and routing program that considers the risk-benefit ratio for GTMP administration;
- increase GTMP safety (primarily by reducing immunogenicity), enhance the target and reduce off-target delivery of drugs, achieve the optimal therapeutic level of long-term targeted expression of the transgene by modifying viral capsids, the genome of viral vectors, suppressing off-target expression of the transgene, and using new specific promoters;
- to improve the quality of gene therapy drugs based on virus vectors and reduce their production costs by developing new cultivation, purification, and transfection methods, as well as by creating new producer cell lines.

These problems should be solved both at the preclinical stage of development and after the first demonstration of GTMP clinical efficacy. Without a systemic solution to these problems, GT based on viral vectors will not be able to enter the treatment standards not only because of the high financial burden on health care, but also because of limited efficiency.

[18] https://doi.org/10.30895/2221-996X-2024-24-2-123-139-table2

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