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Review article

# The significance of chemical transfection/transduction enhancers in promoting the viral vectors-assisted gene delivery approaches: A focus on potentials for inherited retinal diseases ☆

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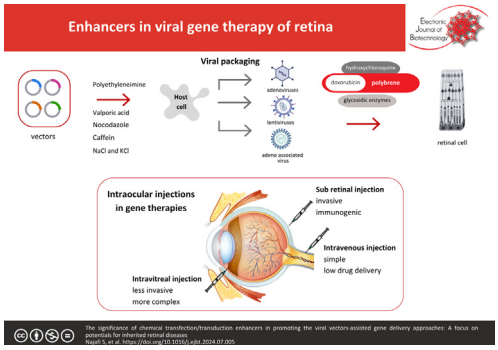
## GRAPHICAL ABSTRACT




Enhancers in viral gene therapy of retina

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## ABSTRACT

Viral vectors are among the main approaches currently used in studies for executing gene delivery to target cells. During the past decades of active studies in gene therapy, including viruses, adenoviruses (Ads), lentiviruses (LVs), and adeno-associated viruses (AAVs), have received the most attention among the biological approaches where potentially successful outcomes are recorded for numerous genetic conditions. The success of delivery methods, however, remains unsatisfactory. Using some additives that can improve transgene expression, transfection efficiency, viral particle production, and transduction efficiency is considered as a solution to overcoming the limitations of gene delivery approaches. These additives include caffeine, histone deacetylase (HDAC) inhibitors like sodium butyrate and valproic acid, and polycationic agents like polybrene and protamine sulfate. In this review article, we present an overview of viral vector-mediated retinal gene therapies and the application of some enhancers used to improve the outcomes of gene delivery. Three routes of administrating viral vectors into ocular compartments

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are employed for the delivery of target genes into impacted cells, and some additives have shown enhanced efficiency of gene delivery in retinal cells. The current study places a special focus on the viral vectors and enhancers used for gene therapies of inherited retinal diseases.

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**Contents**



## 1. Introduction

Hereditary diseases with more than 7,000 involved genes are identified to impact more than 30 million of the American population and several hundred million patients with rare genetic diseases around the globe [1]. For those diseases, repair or change of the genetic materials are suggested as potential therapeutic strategies with promising durable and effective outcomes [2]. Throughout 30 years, gene therapy has proven successful in opening new windows in fighting against human diseases that were previously considered with restricted or no treatments. Of note, those treatments engendered hope for patients and families and experienced various transitions, while some concerns were raised regarding their efficacy and safety [3]. Gene therapy is defined as a process that aims to correct genetic deficiencies at their origin by introducing exogenous genetic materials to repair, replace, or compensate pathogenic gene mutations [4,5,6]. Generally, the main strategies in gene therapy include gene replacement, gene addition, and gene silencing. Other strategies are either deleting a genetic sequence or using editing techniques to disable disease-causing genes or editing those sequences [7]. These therapeutic approaches can be theoretically conducted by inserting a healthy copy of a distorted gene, compensating a missed gene with substantial function, and targeting or editing the pathogenic genes [8]. Gene therapy is also considered a potential therapy for some acquired non-inherited diseases like cancer and infections [9,10,11]. The cell function-altering genetic materials used in gene therapy are multiple and can include double-stranded DNA (dsDNA), single-stranded DNA, and antisense oligonucleotides (ASON) [12,13]. Compared to protein replacement, gene therapy offers several advantages, such as overcoming the low bioavailability of proteins, lower costs of production, and less requirements for parenteral administration [6]. The first successful clinical translation of gene therapy was achieved in the early 1990s by Anderson and his colleagues [14] that was performed for Ashanthi DeSilva, who was diagnosed with adenosine deaminase deficiency severe combined immunodeficiency disease (ADA-SCID). That success was a product of several years of failure in trials during the 1970s. For instance, Dr. Stanfield Rogers was among the pioneers who planned the first trial for treating two sisters diagnosed with hyperargininaemia with Shope papilloma virus but did not receive any successful outcomes [15]. Although disappointing results in the next clinical trials shadowed the strategy success [4], gene therapy was proven to work for several recessive genetic disorders (e.g., cystic fibrosis and hemophilia), chimeric antigen receptor T (CAR-T) cells for cancer [16], and in some virus-caused diseases like the acquired immunodeficiency disease (AIDS) [17,18]. The successes of the application of gene therapies are mainly attributed to our accumulated knowledge about viruses [3]. Dozens of active clinical trials (more than 200 by 2022) were investigating the efficacy and safety of gene therapy systems to expand their potential application for the treatment of various human diseases [19]. As a result, more than 100 gene, cell, and RNA therapy products are approved for clinical use in patients with various diseases [20].

The key element in the success of gene therapy is the development of safe and efficient transfer vectors [21]. In addition to targeted delivery within the physiologic environment, transgene vectors can be delivered *ex vivo* to the extracted cells and then transfused back to the patient [3]. Development of transfer systems is an essential need to protect the therapeutic nucleic acid against biological barriers and ensure specific delivery to target tissues and subcellular compartments [22]. DNA materials have an anionic

charge, are sensitive to biological nucleases, and possess a large size, making them improper for passive transfer through the plasma membrane [23,24]. Generally, vehicles in gene delivery are divided into two classes of viral and non-viral vectors [25]; however, physical approaches including needle injection, electroporation, and gene guns are some approaches in addition to the conventional vectors [21]. A perfect vector should meet some requirements for an optimum gene therapy application. These include a lack of strong immunogenicity, the potential of transferring DNA at large sizes based on the construct sequence, the ability of sustained expression, targeting both dividing and non-dividing cells, and easy commercial processes of production [26,27]. Accordingly, virus-based vectors are the most commonly used vehicles, as they have demonstrated superior advantages to current non-viral transfer approaches (naked DNA, particle-based, and chemical-based) and thus, have attracted the majority of attention in research and clinical trials of gene therapy [28]. Notably, in this context, virotherapy used to target cancer cells has demonstrated promising achievements [29].

## 2. Viral vectors for gene therapy

Viruses were the first elements naturally developed to target specific cells and transfer genetic information while protecting their genetic content against degradation benefitting from their life cycle [21]. They can specifically infect some dividing or non-dividing cells and exploit the replication machinery in the host cells to preserve their genomes making them appropriate vehicles for therapeutic approaches [30]. Accordingly, viruses were also the earliest and still among the most commonly used vehicles for targeted gene delivery referred to as 'Trojan horses' offering employment for millions of years of evolution [31]. By eliminating the non-essential and pathogenic genetic elements (e.g., those in replication-defective viruses) and replacement with transgenes, recombinant viral vectors can be engineered as safe and efficient approaches with high capacities for transgenes in cell-specific gene therapies [32]. Additionally, these vectors can be manipulated through pseudotyping to engineer their cell targets [33]. Viral-based vectors mainly originate from several major viruses like retroviruses, adenoviruses (Ads), and herpes simplex virus (HSV). Some characteristics that are necessary for gene transfer, such as the efficient carriage of genes of interest and long-term expression have made viruses good candidates for gene therapy [34]. Three major components of viral vectors used in gene therapy include a capsid (plus a/or replaced by an envelope) responsible for virus tropism, the transgene of interest for therapy, and regulatory elements [35]. Occasionally, some functional proteins are also used in the packaging structure. Viral vectors possess superior properties to non-viral vehicles, making them good vehicles for gene therapy (Fig. 1). These properties include high transduction efficacy, targeting dividing or non-dividing cells, and the possibility of transgene expression regulation for either transient or persistent goals [36]. However, some major limitations of the viral vector application include genomic integration resulting in mutagenesis and carcinogenesis (insertion mutagenesis), immune responses in frequent administration, limitations in packaging capacity, and difficulties in manufacturing high titers [37,38,39]. The concerns regarding the safety of viral vectors were raised particularly when a patient named Jesse Gelsinger who underwent viral vector-assisted gene therapy died of immune responses [40]. Ads, lentiviruses (LVs), and adeno-associated viruses (AAVs), respectively have received a majority of research and clinical focus during the past couple of decades of translational gene therapy bringing a wave of preclinical and clinical achievements [3,41]. Among the

**Viral vectors strengths and weak points**

| Advantages | Disadvantages |
|---|---|
| Long term expression | Mutagenesis/carcinogenesis |
| High transduction efficacy | Immunogenicity |
| Targeting dividing and non-dividing cells | Limited packaging capacity |
| Transgene degradation prevention | Difficulty in high titer manufacturing |

**Viral specific vector comparison**

| | Advantages | Disadvantages |
|---|---|---|
| Ads | High coding capacity | High toxicity<br>High immunogenicity<br>Viral inactivation via blood components |
| LVs | Long term transduction<br>Moderate immunogenicity | Limited transgene capacity |
| AAVs | High transgene capacity | High toxicity<br>High immunogenicity<br>Viral inactivation via blood components |

**Fig. 1.** A summary of advantages and limitations of viral vectors versus non-viral vectors for gene therapy goals.

clinically approved gene therapy products, viral vectors have demonstrated an acceptable efficacy and safety for a number of inherited and infectious diseases [19].

## 3. Gene therapies in retinal diseases: How viral vectors can be beneficial?

Inherited retinal diseases (IRDs) are a group of heterogenous eye disorders characterized by degeneration of photoreceptors contributing to progressive loss of vision [42]. Various hereditary patterns are recognized for IRDs with more than 280 genes affected in monogenic IRDs (RetNet, https://sph.uth.edu/retnet/). Currently, no effective treatment is known for IRDs to modify the loss of retinal cells and the progressive visual dysfunction that eventually results in complete blindness in the affected patients [43]. As a potential therapy, targeted gene therapies, in particular gene replacement strategies, constitute a majority of preclinical models and active clinical trials seeking treatments to restore the visual function for many patients with IRDs [44]. The number of clinical trials in the past decade has rapidly increased with a hopeful vision of developing therapeutic strategies [45]. In addition to the significance of vision in human life and activities, several features regarding the IRDs make them noted for gene therapy studies. These features include access to various compartments of the retina, bypassing immunologic responses due to the presence of the blood-retina barrier that separates the retina from blood vessels to avoid systemic side effects, and the possibility of evaluations using non-invasive approaches like electroretinography (ERG) and optical coherence tomography (OCT) to monitor the transgene expression [46]. IRDs-associated genes mainly show specific expressions in the photoreceptor and retinal pigment epithelium (RPE) cells. Thus, cell-specific treatments for targeting some particular cells of the retina can enhance the safety and efficacy of gene therapies [46]. Using vehicles offering specific targeting of retinal cells can suggest a solution for this bottleneck of retinal gene therapy. Two conventional routes of injection are employed for the delivery of gene therapy materials into retinal compartments [47]. Subretinal injection that is most commonly used in clinical trials for accessing retinal targets through the delivery of genetic materials into the subretinal "bleb" under the fovea aims in reaching the photoreceptors and RPE, while reducing immunogenicity [1]. Although this is a more invasive approach and can cause mechanical damage to the retina further deteriorating the degenerate retina in IRDs, the bleb disappears upon absorption of the aqueous fluid [48,49]. Intravitreal injection is less invasive and protects degenerate retinas from further damage; however, it requires some special capsids to allow the viral vectors crossing the vitreous and penetrating the retina [1]. Non-viral vectors, such as polymeric and liposomal compounds, face the vitreous barrier restricting DNA uptake and diffusion to reach the retina and thus, their efficacy in retinal gene therapies remains challenging [50,51]. Employment of viral vectors particularly AAV-based vectors, and LVs in lesser extent, is actively being investigated for their ability to target photoreceptors in retinal gene therapies [52]. As AAVs are small in size, it supports their potential for effective and long-term transduction that is particularly shown in all layers of the retina including the photoreceptors and RPE [53,54]. AAV vectors, however, have limited capacity for transgenes and also, less probability to cross retinal barriers and causing immune responses [52]. LVs in contrast have larger capacities for transgenes and broad tropism particularly during development and in the degenerate retina [46]. Among AAVs, serotype 2 (AAV-2) is the most commonly used for gene delivery to retinal cells [55]. Gene replacement studies in animal models have revealed the feasibility and efficacy of AAVs in improving the outcomes of IRDs [56]. Although those studies in clinical trials had entered the advanced stages of evaluation for several IRDs like retinitis pigmentosa (RP), Leber's congenital amaurosis (LCA), and choroideremia (CHM), clinical translation still remains unattainable for a majority of other types of IRDs [57,58]. Development of cataract is an identified complication of vector administration to the retina [59]. The precise mechanism (s) responsible for those failures are not yet elucidated; however, weak transgene expression and immunologic responses are suggested as possible causes [1]. Further success of retinal gene therapies relies on the continued development of methods to ensure the specific targeting of primate retinal cells, and optimum expression of the transgene for identification of therapeutic doses that require examinations in large animals. Successful gene therapies also rely on the development of regulatory mechanisms for controlling transgene expression, investigation of vectors of relevant animals and appropriate patients, and production of viral vectors at therapeutic clinical scales [1,60].

Voretigene neparvovec-rzyl (Luxturna, Spark Therapeutics) is a recombinant AAV (rAAV)-based gene therapy product that received the United States food and drug administration (USFDA) approval in 2017 for patients with biallelic retinal pigment epithelium-specific 65 kDa (RPE65) mutation-associated LCA [61,62]. Subsequently, the European Commission and Health Canada granted Luxturna clinical approval in 2018, and 2020, respectively [63].

## 4. Transfection/Transduction efficacy in gene therapies

Theoretically, gene therapy is a simple approach and an efficient therapy for various human diseases. However, this process is complex in practice and requires several functioning solutions to overcome biological barriers for bringing the recombinant DNA intact to the nucleus of target cells [21,64]. Some requirements in delivery systems include avoiding contact with the blood cells and components, escaping uptake by surrounding vascular cells, and having a small size for facilitated transmembrane transportation [21]. The efficiency of gene delivery methods, biological or synthetic, is improved by using clinically relevant adjuncts, changing the delivery routes, employment of non-viral vectors, and application of specific promoters for the prolongation of transgene expression [64]. In particular, the employment of viral vectors has helped improve gene delivery efficiency *via* the transduction process [65]. The gene transfer efficiency, however, remains unsatisfactory since only one product has received approval for providing enough copies of the gene of interest at the target location [65]. This issue suggests that further requirements are needed for enhancing the efficiency and safety of gene transfer. Simple methods for expanding the production of viral particles in high titers can offer more extensive and effective potential of viral vectors for use in research and clinical applications [66]. Similarly, despite progress in the application of AAV and LV vectors for gene delivery to various cells in the retina, the success rate varies being affected by features of viral vectors [46]. To maximize the extent of cell uptake and delivery, and overcome limited transduction efficiency both for *in vitro* and *in vivo* conditions, some additives referred to as "enhancers" are currently being employed (Fig. 2). These additives have been selected from a wide spectrum of materials including chemical polymers, biological peptides, lipids, small molecules, and synthetic nanoparticles [67]. Mechanistically, these additives can act through the inhibition of histone deacetylases, induction of transgene expression, and improving transfection/transduction through impacting the cell uptake of genetic materials (Table 1). The significance of the chemical and biological enhancers in improving the

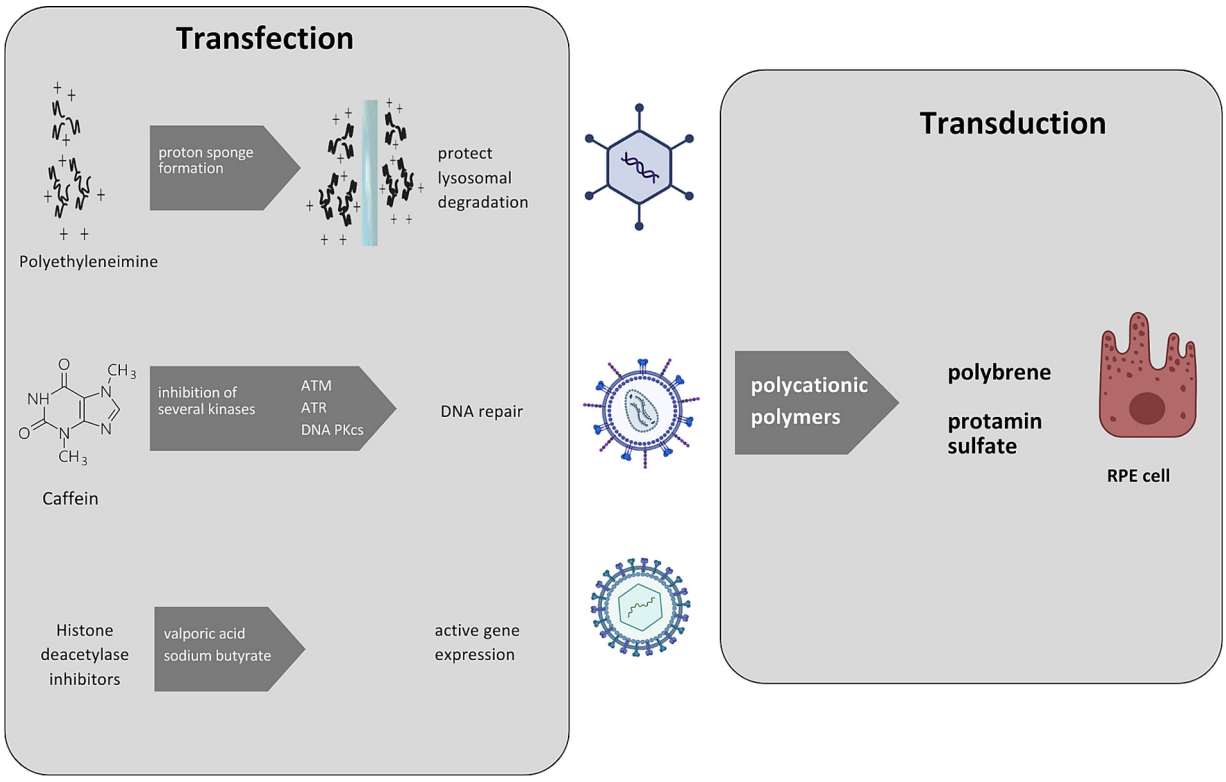


**Fig. 2. By employment of particularly various chemical agents, transfection and viral transduction efficiency can be improved for gene therapy.** Those materials act through different mechanisms like protection against lysosomal degradation by PEI, inhibition of kinases by caffeine, and inducing active gene expression, while polycationic agents can facilitate viral transduction by removing charge repulsions.

transfection and/or transduction with applications in retinal gene therapies is the scope of the current review.

### *4.1. Polyethylene imine as the gold standard agent of transfection*

Also referred to as cationic polymeric carriers, polyethyleneimine (PEI) has been considered the gold standard conduit of gene transfer for decades due to its efficacy proven in various studies [68]. It is a cheap, cost-effective, and easily available agent that has been universally used for the transient transfection of various eukaryotic cells and the production of recombinant proteins and viral packaging [69,70,71]. PEI acts through making complexes with DNA and helps endosome-mediated release *via* the proton sponge effect providing protection from lysosomal degradation and facilitates intracellular transportation of DNA [72]. The cargo is delivered to the cytoplasm without entering lysosomes [73]. PEI is produced in various molecular sizes and two forms, linear and branched. Each form offers its own advantages, such as improvement of molecular absorption of DNA for the branched form and higher releasing/dissecting potential for the linear form [74]. Although using low molecular weight PEI (LMW-PEI) is routinely helpful, PEI at high molecular weights faces a main drawback of cytotoxicity hampering cell growth [75,76]. To resolve this problem, copolymerization is recommended [67,77]. Owing to its features, PEI is commonly used as an efficient agent to facilitate eukaryotic cell transfection *in vitro* and *in vivo* and also viral packaging [78,79,80].

PEI-mediated gene delivery has also been tested for ocular gene therapies *in vitro*. Transfection of ocular cells, such as post-mitotic retinal neurons and RPE cells, using PEI has achieved satisfactory results with good efficacy and tolerance [81,82,83]. Other transfection reagents like Lipofectamine and liposomal compounds have also been used to assist in the process.

The employment of some products in combination with transfection agents has helped improve the transfection of target or host cells for viral packaging, promoting viral transduction efficacy, and increasing transgene expression (Table 1). Notably, the use of enhancers along with PEI is tested for enhancing viral production.

### *4.2. Enhancers of transfection efficiency*

#### *4.2.1. Caffeine*

Caffeine is an additive that has shown promising potential in viral vector production. It is believed that caffeine acts through inhibition of several kinases, including DNA-dependent protein kinase catalytic subunit (DNA-PKcs), ataxia telangiectasia mutated (ATM), ataxia telangiectasia, and Rad3-related protein (ATR) playing a role in DNA repair. Ellis et al. [66] introduced a simple and cost-effective approach for high-titer production of LVs using caffeine. They added 2–4 mM caffeine 17 to 41 h post-transfection during the standard process of LV production and found a 3- to 8-fold increase in lentiviral titers [66]. In comparison, the use of sodium butyrate (see section 4.2.4) exhibited half of the impact seen for caffeine on LV production. Although NU7026 as a DNA-PKcs inhibitor, demonstrated enhancement of viral titers, combined use with caffeine did not show an efficacy comparable to utilizing caffeine alone [66]. The positive impact of caffeine on LV titers is confirmed in another study [84]. In the latter experiment, adding caffeine at a concentration of 40 mg per 100 ml media one-day post-transfection greatly improved the viral titer.

**Table 1**
Types of gene transfer enhancers and their mechanism of action.

| Enhancer | Mechanism of action | Significance | Target cells | Reference |
|---|---|---|---|---|
| **Transfection enhancement** | | | | |
| DOPE/CHEMS+ Tubastatin | Non-viral transfection enhancement | Enhanced transfection by 80% of post-mitotic cells | Neuronal cells | [135] |
| Fusogenic lipids + a HDAC inhibitor | Transfection enhancement | Increased LV transfection using PEIMAX as a gene carrier | HEK293 cells | [136] |
| Lithium acetate, valproic acid, and caffeine | Transfection enhancer Gene inhibition | Increased virus-like particles by 3.8-fold | HEK 293 cells | [94] |
| Nocodazole | Induction of G2/M cell cycle arrest | Enhanced transfection efficiency | HEK 293 cells | [94] |
| Sodium Chloride and Potassium chloride | Cationic agents | 5- to 10-fold enhancement of AAV production yield | HEK 293 cells | [137,138] |
| **Transgene expression** | | | | |
| VAI and 2-aminopurine (2-AP) Protein kinase R inhibitors | Suppression of antiviral defense | Up to 2-fold pAdVAntage plasmid titer | HEK 293 T producer cells | [139] |
| Caffein | Phosphodiesterase (PDE) inhibition ≫ increased transgene expression | 3- to 8-fold enhancement of LV titers | HEK 293FT cells | [66] |
| NU7026 | DNA-PKcs inhibition | Increased viral titer | HEK 293FT cells | [66] |
| CSC and PS | Lentiviral purification-concentration | Production of higher lentiviral titers compared with other purification protocols including protamine sulfate, polyethylene glycol, and ultracentrifugation | HEK293T and mMSC cells | [140] |
| HCl | Degrade the urothelium glycosaminoglycan barrier | 35% of the mice had AAV vector transduction in the urothelium | mice bladder urothelium | [141] |
| DDM or SDS | Degrade the urothelium glycosaminoglycan barrier | AV transduced >90% AV of the urothelial layer during 15 min | mice bladder urothelium | [142] |
| **Histone deacetylase inhibitors** | | | | |
| Sodium butyrate | Enhances expression of recombinant proteins via histone hyperacetylation | 100-fold elevated expression via retroviral vector | 293GPG packaging cell line | [143] |
| CHAP31 and FR901228 | HDAC inhibition | Increased adenovirus-mediated transgene expression | Rat fibroblasts | [86] |
| FR901228 | Histone deacetylase inhibitor/ increased AAV transgene expression | Increased cell surface expression of alpha v integrin, FGF-R1, and PDGF-R | Cancer cells | [87] |
| Sodium butyrate and trichostatin combination | HDAC inhibition | Inhibition of HDACs induced LV-mediated transgene expression<br>Enhanced production of both BIV and HIV vectors by 6- and 2.4-folds, respectively upon sodium butyrate treatment<br>Increase in those values were 4-fold and 2.4-fold, respectively for trichostatin, while it also caused enhancement of infectivity of both vectors.<br>Up to 4.5-fold and 3.8-fold enhanced transduction of viruses produced using sodium butyrate and trichostatin, respectively | HEK293T and HeLa<br>Canine fibroblasts | [106]<br>[144] |
| Valproate | HDAC inhibition | Enhanced the expression of zinc finger nucleases delivered by integrase-defective lentiviral vectors | K562 cells | [93] |
| **Transduction enhancers (TEs)** | | | | |
| Poloxamer synperonic F108 in combination with polybrene | Membrane modulator + charge protector | Enhanced transduction rate from 31.5% to 48.4% upon polybrene treatment<br>61.4% enhancement of cell transduction by F108 treatment<br>An additional 5% transduction enhancement by combinational treatment | HEK293T<br>——————————<br>KARPAS-299, SUDHL-1, PANC-1, SR-786 and SUP-M2 cancer cell lines | [145] |
| 8 compounds including LentiBOOST, PGE2, PS, Vectofusin-1, ViraDuctin, RetroNectin, and Stauro | Enhanced LV and alpharetroviral transduction | More than 2-fold enhancement of transduction<br>LentiBOOST, Stauro, and PGE2 showed the best impact on the transduction efficiency.<br>• 38.8% transduction in live cells with no TE<br>• 47–69% transduction with a single TE treatment<br>• 68–83% transduction with two TEs<br>• 84–91% transduction with three TEs | Hematopoietic stem cells | [146] |
| Bortezomib | Transduction enhancer; Proteasome inhibitor | Enhanced scAAV-mediated hFIX expression from 4+/-0.6 to 9+/-2 mg/ml in female mice | Liver cells | [147] |
| MG132 | Proteasome inhibition | Increased transduction efficiency of FIV particles | -TM-1 cells<br>-Monkey organ-cultured anterior segments | [148] |

**Table 1** (*continued*)

| Enhancer | Mechanism of action | Significance | Target cells | Reference |
|---|---|---|---|---|
| **Specific retinal cells transduction enhancers** | | | | |
| Doxorubicin | Proteasome inhibitor | -33.8% increase in AAV transduction efficiency in retinal bipolar cells upon co-treatment of doxorubicin (300 μM) and dexrazoxane<br>-No cytotoxicity was seen upon enhancers treatment in all retinal layers | Retinal bipolar cells | [133] |
| Glycosidic enzymes | Degrade the inner limiting membrane and extracellular matrix proteoglycans acting as a barrier | - Enhanced AAV2 transduction efficiency in the retinal ganglion cell layer by adding heparinase III or chondroitin ABC lyase<br>- Improved transduction depth to the outer retina<br>- Enhanced retinal transduction *in vitro* and *in vivo*<br>- 17-fold increase in retinal transduction and robust transgene expression by combined administration of heparinase III and hyaluronan lyase<br>- Intact retinal function in treated mice | Outer retina, including photoreceptor cells<br>Degenerate retina of *rd1* mice | [116]<br>[134] |
| Hydroxychloroquine | Putative inhibition of TLR9 | - Safe and effective in the enhancement of gene expression<br>- Up to 3-fold increase in GFP expression in the human retinal explants<br>- 4.6-fold increased GFP expression in mouse eye for AAV2 and 5.9-fold impact for AAV8 | -Human retinal explants<br>-Mouse eye | [126] |
| Polybrene | Removing the charge repulsions | Enhanced MSCV transduction efficiency at different concentrations | Retinal stem cells | [122] |

PEI: Polyethylene imine; LV: Lentivirus; AAV: Adeno-associated virus; MSCV: murine stem cell retrovirus; FIV: Feline immunodeficiency virus; HDAC: Histone deacetylase; DNA-PKcs: DNA-dependent protein kinase catalytic subunit; CSC: Chondroitin sulfate C; PS: protamine sulfate; HCl: Hydrochloric acid; DDM: Dodecyl-beta-D-maltoside; SDS: sodium dodecyl sulfate; FGFR1: Fibroblast growth factor receptor 1; PDGF-R: Platelet-derived growth factor receptors; TLR9: Toll-like receptor; mMSC: mouse mesenchymal stem cells.

#### *4.2.2. Histone deacetylase inhibitors*

Several additives are known to act through inhibition of histone deacetylases (HDACs). Hyperacetylation of histones can unwind nucleosomes and allow active expression by providing access to the transcription machinery [85]. HDAC inhibitors (HDIs), thus, may enhance transgene expression resulting in improved yield of viral gene therapies. The main HDIs with the potential in enhancing transgene expression of viral vectors include valproic acid and sodium butyrate. Several other HDIs like CHAP31 and FR901228 (FK228) are also described with the same function for Ad and AAV vectors [86,87]. MicroRNAs (miRNAs/miRs) are endogenous, small non-protein coding molecules with an average length of 22 [19,21,22,23,24] nucleotides [88]. They are involved in various biological functions acting through posttranscriptional regulation of gene expression [89,90,91]. Among miRNAs, a study reports the role of miR-2861 in the inhibition of HDAC5 and improvement of recombinant protein expression in the Chinese hamster ovary (CHO) cells [92]. This study suggests a novel miRNA as an HDAC inhibitor with the potential for the improvement of transgene expression.

#### *4.2.3. Valproic acid*

Valproic acid is a known HDI agent that is frequently employed as an effective enhancer of transgene expression. Studies in LV vectors demonstrated enhanced transgene expression when treated with valproic acid at 1 mM concentration, while no cytotoxicity was reported [93]. Cervera et al. [94] evaluated the impact of combined use of transfection enhancers, such as nocodazole, lithium acetate, valproic acid, and caffeine, on the Gag-based virus-like particle (VLP) production levels in HEK293 cells. Among the eight compounds tested, several showed promising impacts. Nocodazole is a well-known transfection enhancer acting through arresting cells in the G2/M phase of the cell cycle that is already reported to have improved transfection by more than 94% when combined with PEI use [95,96,97]. It was shown that with a 2 μg/mL dose of nocodazole in the culture medium, 71% of the treated cells showed G2/M phase arrest following 24 h of treatment. Nocodazole; however, did not show any favorable impact on cell viability. Additionally, the use of dimethyl sulfoxide (DMSO), an organosulfur compound with various biological and pharmaceutical applications, enhanced the cell membrane porosity and improved transient transfection. Notably, VLP production was increased by 3.8-folds when a combination of valproic acid (3.36 mM) and caffeine (5.04 mM) was added to the culture medium 4 h post-PEI-mediated transfection [94].

#### *4.2.4. Sodium butyrate*

Sodium butyrate (NaBut) is an HDAC inhibitor, whose effect on improving transcription and expression of transgenes is well-documented in different cell lines [85,98,99,100]. NaBut is also shown to enhance the production of viral particles in HEK293 cells under PEI-mediated transient transfection. It is believed that NaBut acts through activation of the long terminal repeat (LTR)-mediated gene expression of the human immunodeficiency virus (HIV) [101]. Since the 1990s, NaBut has proven to help the optimization of LV and retroviral particle production at a concentration of 5 to 20 mM [102,103,104]. Ansorge et al. [105] added NaBut 16 h after transfection at a concentration range of 0.1–5.0 mM to the production process and analyzed its effect on the LV production yield along with several other factors, such as increasing cell density and optimization of media and transfection conditions. Incredibly, they found maximum LV titers of $10^8$ transducing units (tu)/ml 2 d post-transfection showing a 15-fold increase at 5 mM of NaBut and a 150-fold enhancement of

LV titers relative to non-optimized conditions [105]. Adding NaBut at a concentration of 10 mM in combination with trichostatin, an antifungal antibiotic acting through selective inhibition of HDAC class I and II, to the cell medium treated with 25 kDa linear PEI was shown to enhance transgene expression of integration-defective LV vectors in both dividing and non-dividing cells [106]. However, it may negatively affect cell viability at >5 mM concentrations [105].

### 4.3. Transduction enhancers

The transduction efficiency of viral packaging systems determines the success of gene therapies. Currently, it is still unsatisfactory that necessitates optimization for various applications [3]. Endogenous transduction inhibitors in addition to basic biophysical constraints have some factors that may limit the transduction efficiency of viral vectors [107]. A variety of polycationic polymers, such as polybrene and protamine sulfate, are currently employed for improving the efficiency of transduction [108].

Polybrene (hexadimethrine bromide) is a cationic polymer that is frequently reported to enhance the transduction of retroviruses and LVs in various types of target cells, such as human and mouse somatic and stem cells [109,110,111]. Mechanistically, polybrene acts by making bridges between the viral particles and the cell membrane of the target cell, removing the charge repulsions to facilitate viral absorption [112]. Polybrene is routinely used in a concentration of <10 μg/mL resulting in the best performance on transduction [107,113]. Evidence shows the safety of polybrene without cytotoxicity at high concentrations by up to 40 μg/mL [113]. However, some studies show that polybrene in commonly used concentrations (1–8 μg/mL) is cytotoxic to some cell types like human mesenchymal stem cells (hMSCs) [114], and cochlear hair cells [115].

## 5. Enhancers for retinal gene therapies

Limited transduction is a major challenge for retinal gene therapies by the conventional approach of intravitreal viral vector delivery. The special retinal structure composed of an inner limiting membrane (ILM) and proteoglycans of the extracellular matrix (ECM) is considered a barrier obstructing the transfer of viral vectors across the retina [116].

Among the additives discussed above, polycationic polymers particularly polybrene have been proven to improve the transduction yield of retinal gene delivery methods (Fig. 3).

Polybrene (mainly at 8 μg/ml) has been successfully used for enhancing the transduction efficiency of human and mouse retinal cells since the 1990s [117,118]. Hashimoto et al. [119] evaluated the efficacy of an LV-based gene replacement strategy in the retinas of MYO7A-null mice. Polybrene at a concentration of 6 mg/ml in transduction media was used to help the infection of RPE cells as well as viral delivery *in vivo*. Results revealed the success of viral transduction by more than 95% [119]. Alsing et al. [120] reported successful transduction of RPE cell line ARPE19 using 8 μg/ml of polybrene in a vascular endothelial growth factor-A (VEGFA)-targeting gene therapy method by LV vectors. They found no negative impact on the cell viability in the cells treated with polybrene. At an 8 μg/ml concentration, polybrene was also employed for successful delivery of the *CHM* gene-containing AAV2/5 vector to the induced pluripotent stem cell (iPSC)-derived RPE cells that reconstructed the biochemical phenotype of the choroideremia cell model [121]. Additionally, polybrene in combination with ultrasound has shown an enhancing influence on the retroviral transduction to the human retinal stem cells [122]. Notably, LV-mediated gene delivery of *bcl-xL* to the corneal endothelial cells in the presence of polybrene (pre-incubating the graft with 6 μg/mL) is shown to protect against graft rejection [123].

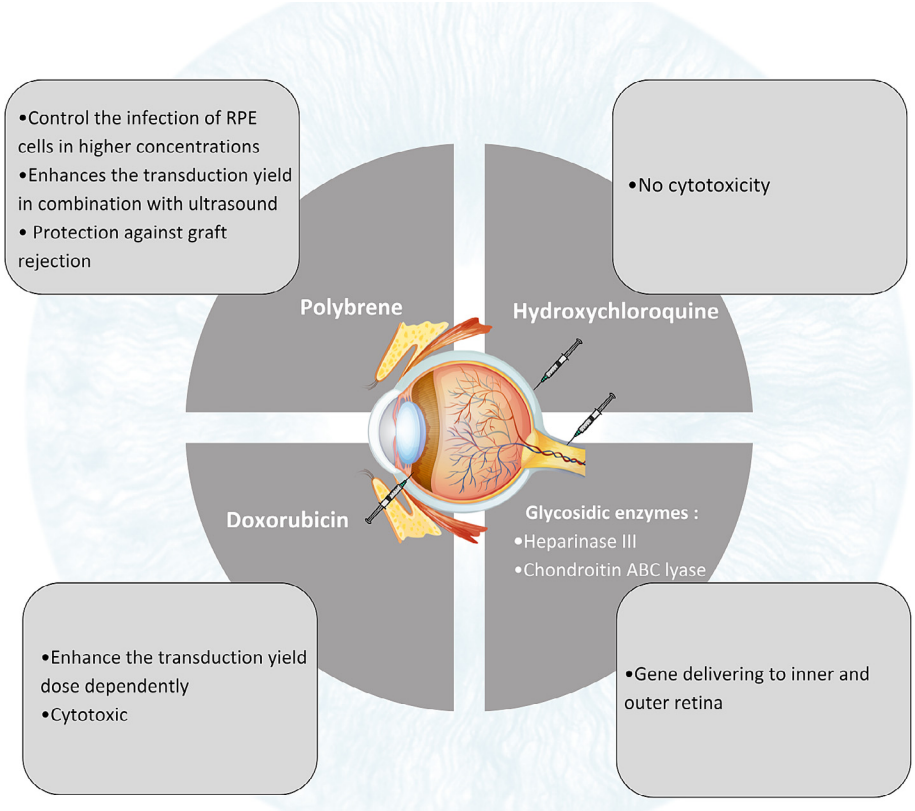


**Fig. 3.** A schematic illustration of four major enhancers used for the improvement of viral transduction efficiency in the retinal cells.

Hydroxychloroquine (HCQ) is a synthetic anti-malarial drug that received its approval for clinical use in 1955 [124]. HCQ, which has been used for the prevention and treatment of malaria, has also shown numerous therapeutic influences, such as anti-rheumatic properties [125]. It has been shown to inhibit the Toll-like receptor 9 (TLR9), an anti-viral pattern recognition receptor, and improve the efficacy of AAV-mediated gene therapy efficacy [126]. The impact of HCQ on the viral transduction and its potential in retinal gene therapy is a study's subject by Chandler et al. [126]. They treated RPE cells and human retinal explants with HCQ 1 h before transduction with a green fluorescence protein (GFP)-encoding AAV2 vector. Subretinal injections of AAV2 vector combined with 18.75 μM HCQ demonstrated a 4.6-fold increase in transduction *in vivo* without retinal toxicity. The authors concluded that a single pulse of adjunctive HCQ may help improve AAV transduction efficiency and thus, can suggest an effective and safe strategy for the enhancement of clinical outcomes in gene therapies [126].

Doxorubicin is an anthracycline antibiotic recognized as an effective anticancer drug and approved for the treatment of various human cancers [127,128]. Although the responsible mechanism is not elucidated, doxorubicin has been used for improving the transduction ability of viral vectors, particularly AAVs in several types of cells including lung and airway epithelia [129,130], rat neuronal cells [131], and neuronal cells [132]. In the context of the retina, the impact of doxorubicin was also evaluated on the transduction efficiency of AAV vectors in mouse retinal bipolar cells [133]. Their results demonstrated a dose-dependent increase of transduction; however, doxorubicin demonstrated cytotoxicity. To solve the problem, the researchers designed a co-application of doxorubicin (300 μM) with dexrazoxane that revealed 33.8% enhanced viral transduction with no cytotoxicity. Accordingly, the co-application of doxorubicin and dexrazoxane was suggested as an adjuvant treatment for enhancing the transduction of AAV vectors in retinal cells [133].

Glycosidic enzymes can help spread the injected viral vectors by degrading the glycosaminoglycans (GAGs) and collagen across the retina [116]. Co-injection of GFP-encoding AAV vectors and several glycosidic enzymes, including heparinase III or chondroitin ABC lyase, into the mouse vitreous significantly improved the transduction rate of the retinal ganglion cell layer and enhanced the depth of transduction into the outer retina. Administration of AAV vectors and heparinase III or chondroitin ABC lyase was suggested as a preferred injection route for gene delivery to both the inner and outer retina [116]. The efficacy and safety of those enzymes for intravitreal injection are confirmed in another study by the same group of researchers. They found enhanced transduction of AAV2 and intact retinal morphology and functions at least 12 months following administration of glycosidic enzymes in the treated mice [134].

## 6. Concluding remarks

Gene therapy has emerged as a promising therapeutic approach for a wide variety of human genetic disorders. Through various strategies, gene therapy can help restoration of essential functions affected by mutations. Owing to advances in methodologies and approaches, gene therapies have yielded several products for clinical use. In IRDs, gene therapies are the subject of various experiments and clinical trials. During the previous decades, exceptional hopes have been developed for treating IRDs. Viral vectors have been more conventionally employed for preclinical and clinical gene therapies compared to non-viral vectors. Although promising reports are documented for viral vectors, to bring results to clinics researchers should find solutions for overcoming barriers. Unsatisfactory efficacy of transduction is among the major bottlenecks of gene therapies that need proper addressing. As a solution, researchers have used some chemical agents or pharmacologic compounds to enhance the transfection and transduction efficiency. These particularly include polycationic agents like polyethylene imine and protamine sulfate, and inhibitors of histone deacetylases. Studies in retinal gene therapies have demonstrated the positive impact of several enhancers like polybrene, doxorubicin, hydroxychloroquine, and glycosidic enzymes on the yield of gene therapy. Although promising findings suggest the potential application of those agents for gene therapy goals, *in vivo* application at effective doses remains questionable and requires further studies to ensure the lack of toxicity.

## CRediT authorship contribution statement

**Sajad Najafi:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Azam Rahimpour:** Writing – review & editing, Conceptualization. **Hamid Ahmadieh:** Writing – review & editing, Supervision. **Maryam Maleki Tehrani:** Writing – original draft. **Mohammad Amin Khalilzad:** Methodology. **Fatemeh Suri:** Writing – review & editing, Conceptualization. **Javad Ranjbari:** Writing – review & editing, Conceptualization.

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## Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Supplementary material

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## Data availability

No data was used for the research described in the article.

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