Expert Opinion

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Endosomal disruptors in non-viral gene delivery

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Importance of the field: Non-viral gene delivery for the treatment of genetic and non-genetic diseases has been under investigation for several decades, but there has been very little application in patients because of poor gene expression and toxicity.

Areas covered in this review: As gene delivery almost invariably involves endocytosis, many of its limitations are related to compartmentalisation of the transgene within the endosomes. Gene expression enhancers have become an essential part of manipulating endosomal release, as well as protecting transgene from intracellular degradation. However, disruption of the endosomes can also release proteases that have been shown to activate apoptotic pathways.

What the reader will gain: An understanding of the role that endosomal release plays in the toxicity of gene delivery vehicles will help identify new approaches to minimise adverse effects while enhancing non-viral gene expression.

Take home message: The future of non-viral gene therapy needs to identify new approaches that limit endosome-induced toxicity while enhancing expression so that a pharmacological response can be reliably observed *in vivo*.

Keywords: gene delivery, expression enhancers, lysosomotropic, toxicity

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1. Introduction

Gene therapy has been a promising strategy for the treatment of various human diseases for more than three decades. As a field of study, it has increased our understanding of how macromolecules are internalised by cells and how transgenes can be expressed following uptake. Much of the translational research in gene therapy has involved viral delivery systems, in part because they use a highly refined mechanism that has evolved for viral infectivity over many thousands of years. However, non-viral gene therapy continues to attract much attention because of its apparent safety and versatility [1]. It has been less readily translated to the clinic because of the difficulty in delivering transgenes to the target site *in vivo* and maintaining gene expression for long enough to elicit a therapeutic response. The number of barriers that therapeutic genes are faced with *in vivo* is not trivial [2]. These include lipid membrane barriers, opsonising proteins in the circulation, nonspecific uptake in the reticuloendothelial system, and degradation by extracellular and intracellular DNases.

DNA used for gene therapy is usually in the form of a plasmid that is very loosely packed in solution. The condensation of the plasmid DNA with cationic counter-ions, forming a DNA complex of manageable size, is imperative for efficient delivery [3]. These counterions can be in the form of lipids, dendritic polymers or peptides that self-assemble into condensed structures in a manner similar to histone condensation of DNA in the nucleus of a cell [4]. The resultant complexes are still nanoparticle in size (~ 50 – 100 nm) [5]. However, they mainly traverse a membrane barrier using one of several endocytotic pathways that are available to

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Article highlights.

- Lysosomotropic agents, or gene expression enhancers, are an important component of non-viral gene delivery systems.
- Mammalian cells internalise macromolecules by several different endocytotic mechanisms. The pathway used to enter cells can markedly influence gene expression.
- Several different gene expression enhancers have been identified that can significantly increase the level of gene expression for non-viral gene delivery.
- Endosomal disruption is a major limitation for non-viral gene delivery because endosomal proteases (cathepsins) are proapoptotic.
- Gene expression enhancers can have a critical role in ensuring DNA escapes from the endosomes as well as assisting in DNA–carrier dissociation and protection from intracellular DNase degradation.
- As long as non-viral gene delivery relies on the endosomal system for gene entry, the need to disrupt the endosome compartment to release the transgene into the cytoplasm of the cell will remain a major obstacle.

This box summarises keypoints contained in the article.

cells [6]. Once internalised, the DNA–cation complex must escape from the endosomes, the complex must decondense to release the transgene, which then must translocate to the nucleus for expression to occur. Each of these steps is critically important for non-viral gene therapy.

There has been a plethora of research into optimising gene uptake, mostly in immortalised cultured cells. For non-viral delivery, structural characteristics of the DNA complexes have been studied in detail. Charge density, degradability, rigidity, hydrophobicity, colloidal stability, size and toxicity have all been identified as potential determinants of transfection efficiency [7]. The addition of functional groups to the cation–DNA complex can assist in both targeting to specific cell types and internalisation. In this regards, cell penetrating peptides have received particular attention because of their ability to shuttle cargo across biological membranes [8]. However, more specific targeting has been demonstrated with functional groups that recognise surface receptors such as the folic acid transporter or the transferrin receptor. For each of these delivery strategies, the DNA complex is internalised into intracellular vesicles, usually endosomes. If the transgene is not released from the endosomal compartment, no expression is seen. Much of the early work on non-viral gene delivery used lysosomotropic agents such as chloroquine but it was later realised that the counterion used to complex the plasmid DNA, such as poly(ethylene imine) (PEI), could perform this function if appropriately engineered [9]. These agents have come to be termed 'gene expression enhancers'. Their primary function is to release the internalised DNA before it is degraded in the endosomal/lysosome compartment. However, recent work from several laboratories has suggested that this is only part of their role in enhancing gene expression (see below).

In this review, current knowledge about gene expression enhancers is summarised and an opinion is put forward of how these essential components in gene delivery systems may contribute to the current limitation in non-viral gene delivery. Alternative strategies are also proposed that deserve consideration if transgenes are eventually going to reach the clinic as a means of treating human diseases.

2. Pathways for internalisation

Until recently, little attention had been paid to the mechanism of DNA uptake by cells. However, there is now a realisation that the pathway used to enter cells can markedly influence gene expression [10]. Mammalian cells internalise macromolecules by several different endocytotic mechanisms. These are primarily phagocytosis, which is restricted to specialised cell types primarily in the reticuloendothelial system, and pinocytosis, a feature of most mammalian cells. Pinocytosis includes caveolar-dependent endocytosis, clathrin-dependent endocytosis and actin-dependent macropinocytosis. A fourth pathway, termed clathrin- and caveolae-independent endocytosis, has been described [11], but its role in gene delivery remains largely unexplored. The chief differences between these pathways are the regulatory mechanism that controls their formation, the size of the vesicle that is formed and the intracellular destination of the vesicle [6].

For DNA complexes, cellular uptake usually proceeds by means of pinocytosis. However, no one pathway appears to be dominant unless specific targeting molecules (such as transferrin) are attached to the DNA complex. Importantly, for any specific complex, different pathways may dominate in different cell types [12], adding a level of complexity when designing delivery systems to specific *in vivo* targets. Conceptionally, the delivery system for transgenes *in vitro* may not be the same as that *in vivo* because of this cell-to-cell variation. It is therefore important to test gene delivery systems in appropriate cell models that use the same internalisation pathway(s) as that *in vivo*.

The destination of the different intracellular vesicles that are formed following endocytosis is well understood for some pathways, but less clear for others. Endosomes formed by means of the different internalisation pathways can be distinguished by their structure and the associated accessory molecules that control their intracellular trafficking [11,13,14]. Both clathrin-dependent and caveolin-dependent pathways (and possibly macropinocytosis) deliver internalised vesicles to early endosomes where they are sorted for delivery to one of three destinations: lysosomes, the *trans*-golgi network or the plasma membrane [13]. Recent work has begun to identify the family of regulatory proteins involved in the trafficking of endosomes to these different destinations [14]. In addition, it is now clear that the fate of the endosomal contents is quite different for each pathway.

Delivery of extracellular cargoes to the lysosomes has been long known to result in degradation, whereas delivery to the

trans-golgi compartment, especially of transgenes used for gene therapy, is less well understood. Not unexpectedly, the fate of transgenes may be very different for each of the intracellular destinations.

To minimise degradation of transgenes taken up by endocytosis, gene expression enhancers have been used. These promote endosomal escape before delivery to the lysosomes. However, they are invariably toxic and have limited use *in vivo*.

As our understanding of the molecular machinery that drives the intracellular trafficking of endosomes becomes clearer, it may be possible to manipulate the destiny of genes delivered by endocytosis, in particular avoiding the lysosomal compartment. This could have profound effects on gene delivery efficiency as well as cellular toxicity.

3. Transgene expression enhancers

There are several steps in the pathway from cell uptake to protein production where enhancers can increase the expression of exogenous genes delivered to cells by non-viral gene delivery methods. Although endosomal release by lysosomotropic agents has been most widely investigated, it is now clear that protection from DNA degradation and increasing transcriptional activity is also important.

Lysosomotropic agents are a group of chemicals that accumulate in the lysosomal compartment owing to its low pH. Many of these agents have different mechanisms of action but all achieve lysosomal disruption. Following accumulation, lysosomotropic agents reduce the normal pH gradient between the lysosomes and the cytoplasm, resulting in an osmotic inflow of water that causes lysosomal swelling and disruption. When non-viral DNA complexes are trapped inside the endosome/lysosome, addition of lysosomotropic agents has been shown to enhance their release into the cytosol as well as their delivery to the nucleus.

Several lysosomotropic agents have been used to enhance non-viral gene expression. Chloroquine, the most widely used gene expression enhancer, is a weak base with two protonation sites with pK_a of 8.3 and 10.2 [15]. It has been used in conjunction with a wide range of gene carriers [16-19]. The function of chloroquine has a close correlation to its structure. By modifying the key components of chloroquine, it was found that the aliphatic amine side chain, aromatic ring and the 7-chloroquinoline ring moiety were essential for its ability to enhance gene expression [20]. Most of the modifications made to these components diminish or reduce the effect of chloroquine. However, two chloroquine analogues, primaquine and quinacrine, are also able to enhance gene expression. Primaquine differs from chloroquine in that it has an aminoquinoline side chain at the 8-position of the aromatic ring whereas quinacrine is thought to intercalate more avidly with DNA [16,20]. In addition to chloroquine, sucrose, a sugar that causes lysosome swelling by osmotic pressure, has also been reported to enhance transgene expression in lipid-mediated and PEI-mediated transfection [21-23].

Interestingly, endosomal disruption alone is not sufficient to enhance gene expression, as several lysosomotropic agents failed to do so. It has been reported that monensin, a lysosomotropic agent that disturbs vacuole pH gradient by facilitating the transmembrane exchange of Na^+ for H^+, was ineffective at stimulating transferrin-polylysine-mediated gene delivery. By contrast, chloroquine significantly augmented gene expression of the same carrier [24]. In another study, chloroquine enhanced the gene expression of a PEI-transferrin carrier, but treatment with bafilomycin A1, a specific inhibitor of vacuolar type H^+-ATPase proton pump, reduced gene expression [25]. Using a gene carrier based on the cell penetrating peptide TAT, it was found that transgene expression is markedly enhanced by chloroquine, but not by monensin or bafilomycin A1 [26]. Although the possibility cannot be excluded that some transfection carriers may require acid pH to be released into cytosol [25], a more rational explanation is that chloroquine may have multiple functions as a gene expression enhancer. The authors favour the latter hypothesis because they have shown that, following release of the TAT peptide carrier/DNA complexes into the cytoplasm, transgene expression still relies on the presence of chloroquine [4]. The ability of chloroquine to interact directly with DNA has been known for many years [27]. In a study using TAT peptide-based gene carriers, it was found that chloroquine protected DNA from endonuclease degradation at a concentration equal to what was required for efficient gene expression (**Figure 1A**) [4]. When added to cells in conjunction with carrier/DNA complex, there was a significantly higher amount of intact exogenous DNA than in non-treated cells (**Figure 1B**). This protection is thought to result from the combined effect of direct interaction with DNA and inactivation of degradative enzymes during the buffering of the endosomal compartment. It was also found that chloroquine enhanced transcription of DNA condensed with the TAT peptide carrier, suggesting that it may also assist carrier/DNA unpacking, a known limiting factor in gene delivery [28]. The underlying mechanism for this is thought to be by competition between chloroquine and the peptide carrier for the negatively charged DNA. These findings were consistent with previous studies utilising other non-viral gene delivery carriers, such as a lactosylated polylysine carrier and a β-cyclodextrin polymer.

PEI and other pH-responsive polymers are another option to enhance endosomal escape of exogenous DNA. Owing to the 'proton sponge effect', PEI does not require any lysosomotropic agents to achieve efficient gene delivery. PEI is rich in secondary and tertiary amines, which largely remain unprotonated at physiological pH. Once taken up into an acidic compartment, the large number of nitrogens in PEI acts as a strong sponge that absorbs protons. Every third nitrogen of PEI can be protonated, which buffers endosomal acidification and leads to organelle swelling and disruption [29]. PEI differs

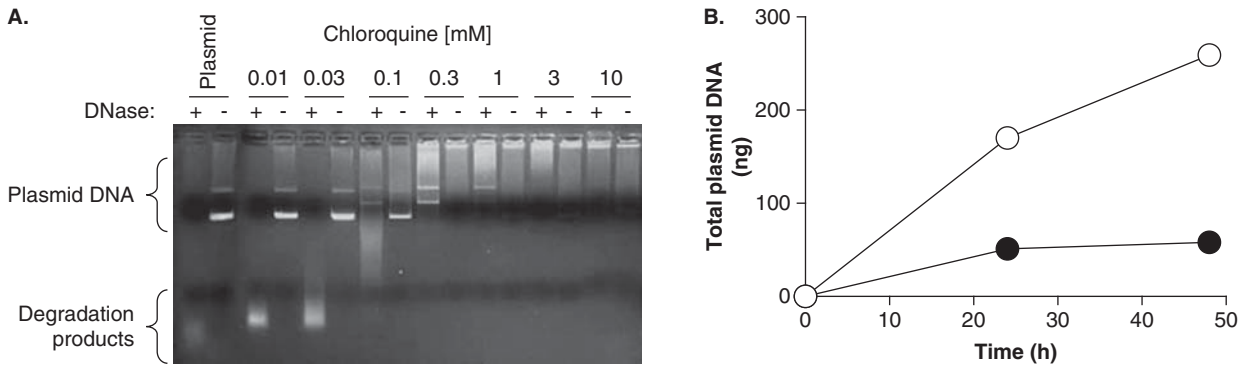

Figure 1. Protective effect of chloroquine in TAT-peptide-mediated gene delivery. A. Chloroquine protects DNA from DNase degradation. Plasmid DNA pGL3-control (400 ng) was incubated with chloroquine and DNase I. The DNA integrity was then examined by agarose gel electrophoresis, which showed loss of degradation products with increasing drug concentration. **B.** Quantification of intracellular plasmid DNA by real-time PCR. HeLa cells were transfected with DNA complexed with a peptide-based carrier [4] in the presence (○) or absence () of chloroquine. The increase in plasmid DNA in the presence of the drug was a result of decreased intracellular degradation.

Figure adapted with permission from Yang *et al.* [4].

from chloroquine in that it disrupts only the endosome/lysosome that it enters, whereas chloroquine disrupts potentially all endosome/lysosomes in the cell. PEI has been shown to be effective in a wide range of cells, including *in vivo* tissues in the central nervous system [30], kidney [31], lung [32] and tumours [33]. The major drawback that limits the use of PEI is toxicity, which is caused by membrane permeabilisation and excessive lysosomal disruption [34,35]. Interestingly, the toxicity of PEI is correlated with its molecular mass, with lower molecular mass polymer showing significantly less toxicity both *in vitro* [36] and *in vivo* [37]. Several other cationic polymers have been developed to achieve a similar pH-responsive effect. Polyamidoamine (PAMAM), for example, is a dendrimer that is also rich in tertiary amines [38]. Unlike PEI, PAMAM dendrimers disrupt anionic vesicles from outside the vesicles by bending the membrane and inducing bilayer packing stress by means of electrostatic force [39].

Viruses have evolved efficient cell invasion mechanisms that include endosomal escape. Consequently, viruses can be excellent carriers for delivering exogenous DNA to cells. Replication-deficient adenovirus serotype 5, for example, has been engineered to deliver DNA into cells [40]. A more refined approach is to mimic the mechanism that viruses use to escape from compartmentalisation during acidification. A short peptide sequence with ~ 20 amino acids derived from the N terminus of Haemophilus influenza haemagglutinin (HA) protein has been shown to be responsible for the fusogenic activity of the virus inside endosomes. The HA protein is a trimer containing two subunits, HA-1 and HA-2. Once the virus is taken up into the endosomes, the low pH (between 5 and 6) triggers the cleavage of the HA protein, exposing the HA-2 N-terminal sequence and activating fusion activity. As a consequence of the structural rearrangement, the HA-2 subunit inserts into membrane, generating a hydrophobic curvature that allows deeper insertion and eventually leads to pore formation [41]. In *in vitro* studies, synthetic HA-2 N-terminal peptide has been shown to possess fusion activity at low pH on model phosphatidylcholine membranes [42] and has been widely used in endocytosis-dependent gene delivery [43-46]. Several other synthetic peptides also possess fusogenic activity. GALA is a pH-dependent 30 amino acid synthetic peptide that contains a glutamic acid-alanine-leucine-alanine repeat, histidine and tryptophan residues [47]. The decrease in pH from 7 to 5 triggers conformational changes in GALA, enabling it to form aggregates within the vesicle membrane. These aggregates then form transbilayer pores in the membrane and cause vesicle leakage. The GALA derivative KALA contains a lysine-alanine-leucine-alanine motif to interact with DNA, making it more suitable for gene delivery purposes [48]. Numerous other amphiphilic peptides have been examined as potential fusogenic agents, but there has been only limited investigation into their mechanisms of action [49] The authors have synthesised a fusogenic peptide HA-2-TAT-polylysine carrier in order to achieve efficient uptake as well as endosome release [4]. It was found that incorporation of fusogenic peptide resulted in significant endosomal release. However, the endosome escape was associated with substantial toxicity. These data suggest that toxicity may be the result of endosomal rupture.

Histone deacetylase (HDAC) inhibitors have recently been identified as a new group of gene expression enhancer. The transcription activity of genes in chromatin is thought to be regulated by means of acetylation and deacetylation of core histones. These two mechanisms are modulated by two enzymes that act on lysine residues in the histone tails: histone acetyltransferases and HDACs, respectively. Acetylation of histones decreases their interaction with DNA, resulting in unfolding of DNA promoter region and activation of

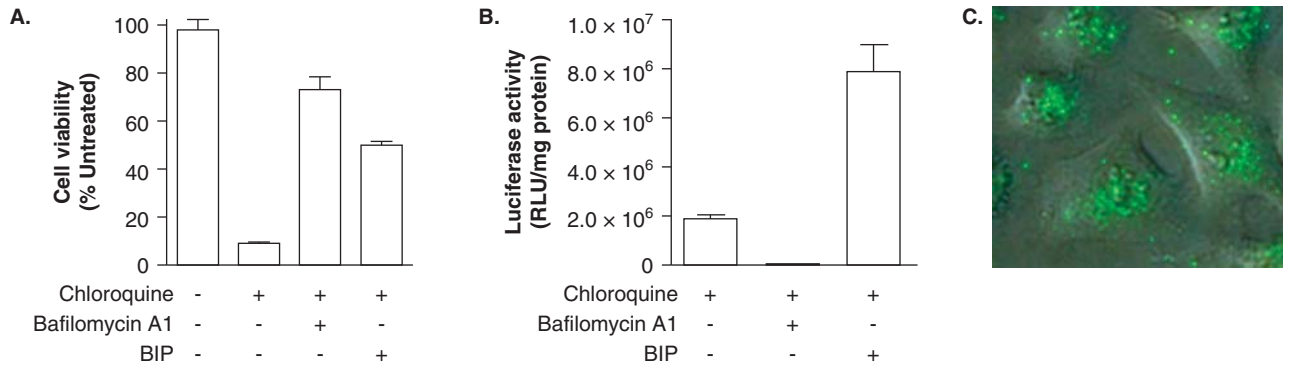

Figure 2. Mechanism of toxicity of chloroquine in cells. A. Effect of bafilomycin A1 (100 nM) and BIP (200 μM) on the cytotoxicity of chloroquine (200 μM) in HeLa cells. After 48 h, cell viability was determined using an MTT-based assay. **B.** Effect of bafilomycin A1 (100 nM) and BIP (200 μM) on the gene expression enhancer properties of chloroquine. HeLa cells were treated with a complex of pGL3–control (luciferase reporter gene) and a TAT-containing dendritic peptide, which has previously been shown to be taken up into endosomes. The results are mean ± s.e.m, n = 3. **C.** Plasmid DNA delivered to HeLa cells accumulates in numerous endocytic vesicles. pGL3–control was labelled with YoYo-1 and cells were viewed after 8 h by fluorescence microscopy [25].

transcription [50]. It has been shown that low transgene expression is partially due to the binding of unacetylated histones to the exogenous DNA in the chromosomal domains in which the gene resides. Addition of HDAC inhibitors increases histone acetylation and markedly enhances transgene expression [51]. This approach has been used successfully with various gene delivery systems including retroviral vector [52], adeno-associated viral vector [53] and liposomal transfection agents [54,55]. Typical HDAC inhibitors used in gene delivery studies include trichostatin A, FK228, FR901228 and cyclic hydroxamic acid-containing peptide [53-55].

So far, almost all of our understanding of the function of gene expression enhancers has relied on *in vitro* studies. Although escape from the endosomal compartment *in vivo* may still be important, it is at present unknown whether this is a rate-limiting process.

4. Toxicity of transgene expression enhancers

Although gene expression enhancers such as chloroquine and PEI have had a substantial impact on transgene expression *in vitro*, their usefulness *in vivo* has been limited. This is mainly because they show substantial toxicity. However, the basis for their toxicity has not been systematically investigated. Above, it has been described how transgene expression enhancers may function at several different levels. Nevertheless, enhancement of endosomal escape appears to be a common and necessary feature of these compounds. If toxicity is a consequence of the disruption of the endosomes, then avoiding this side effect may not be possible and different strategies for either the internalisation or the release of transgenes into the cytoplasm would be required. There is now considerable circumstantial evidence to suggest that lysosomotropic agents decrease cell viability, at least in part, by releasing the contents of the endosome/lysosome vesicles into the cytoplasm [56-58].

For chloroquine, it has become increasingly clear that much of its cytotoxicity is related to the release of proteases from the endosomal compartment. At concentrations that do not promote endosomal disruption (< 100 μM for most cell types), chloroquine increases the number of intracellular lysosomal vesicles [59] and the appearance of prelysosomal autophagic vacuoles [60]. It also inhibits cathepsin activity, an important family of lysosome-associated proteases [61]. At higher concentrations, endosomal disruption results in the release of the cathepsins into the cytoplasm. Using the chloroquine analogue, hydroxychloroquine, cathepsin release has been shown to initiate apoptosis in a Bax- and Bak-dependent manner [56]. Recently, lysosomal cathepsins have been shown to degrade the antiapototic Bcl-2 while activating proapoptotic Bid to trigger mitochondrial-dependent apoptosis [62]. These results suggest that any agent that releases sufficient amounts of lysosomal proteases into the cytoplasm will probably induce cell death.

This was tested for chloroquine by examining the cytotoxicity of the drug in the presence of bafilomycin A1, a proton pump inhibitor that prevents chloroquine-induced endosome disruption, or BIP (VPMLK-OH), a membrane permeable Bax-inhibiting peptide (**Figure 2A**). Chloroquine induced > 90% cell death by 48 h of treatment. However, both interventions significantly inhibited this, suggesting that endosomal disruption followed by Bax activation was required for the chloroquine cytotoxicity. When the effects of these two agents on the transgene expression enhancer properties of chloroquine were examined, it was found that bafilomycin A1 completely inhibited transgene expression, consistent with its ability to prevent endosome escape. By contrast, BIP enhanced expression fourfold (**Figure 2B**). These results provide

preliminary insight into new approaches that might be used in the future to allow the combination of gene delivery systems and transgene expression enhancers to facilitate better and safer expression *in vivo*.

Transgene expression enhancers such as PEI or replication-deficient adenoviruses disrupt only those endosomes into which they are taken up. In theory, they should be less toxic than chloroquine, which is much less selective. For PEI, there have been several studies showing that modification of the dendritic structure or size can affect toxicity [10,37]. Low-molecular-mass PEI was less toxic *in vitro* than high-molecular-mass PEI, but it was also less efficient in promoting transgene expression [37]. This is consistent with a decrease in endosome disruption by the low-molecular-mass PEI. Interestingly, *in vivo*, the low-molecular-mass PEI produced the highest transgene expression [37]. Taken together, these results suggest that limiting toxicity by limiting endosomal disruption may not necessarily lead to a decrease in gene expression *in vivo*.

Viruses have evolved some elaborate mechanisms to escape from the endosomes following internalisation. However, they do not produce significant cell death as this would be counterproductive for their survival. So why can a virus disrupt an endosome with no apparent toxicity while lysosomotropic agents do not? The answer to this may be related to the absolute number of endosomes involved. Viruses rarely infect cells with more than one or two viral particles, whereas non-viral gene delivery systems can result in several thousand copies of plasmids within a single cell, trapped within numerous vesicles. This is exemplified in **Figure 2C**, which shows internalised plasmid DNA labelled with YoYo-1 delivered to HeLa cells using a Tat-dependent dendrimer [4]. There can be > 50 endocytic bodies in a single cell containing exogenous plasmid. Disruption of these either from outside (e.g., using chloroquine), or from within (e.g., using PEI or replication-deficient adenoviruses) will release far more endosomal proteases than that following viral infection.

5. Conclusion

The delivery of exogenous DNA to cells either *in vitro* or *in vivo* is restricted by the many barriers that the DNA must traverse before expression is seen. Transgene expression enhancers have become a necessary part of designing gene delivery systems, although our understanding of how they function is limited. If DNA is taken up, it almost invariably is delivered to an endosomal compartment, regardless of the mechanism of uptake. Targeting specific uptake pathways such as macropinocytosis as opposed to caveolae or clathrin-coated pits may reduce the degradation by limiting delivery to the lysosomes. Nevertheless, escape from these vesicles is essential and how to achieve this *in vivo* still requires optimisation. Gene expression enhancers can have a critical role in ensuring DNA escapes from the endosomes as well as assisting in DNA–carrier dissociation and protection from intracellular DNase degradation. By releasing cathepsins capable of inducing apoptosis, endosomal disruption contributes significantly to the toxicity of DNA complexes used to deliver transgenes to cells.

6. Expert opinion

As long as non-viral gene delivery relies on the endosomal system for gene entry, the need to disrupt the endosome compartment to release the transgene into the cytoplasm of the cell will remain a major obstacle. It is now clear that endosomal disruption is responsible for much of the toxicity associated with non-viral gene therapy. Gene therapists have limited options: either a non-endosomal pathway needs to be discovered to avoid toxicity, or approaches that minimise the number of endosomes disrupted for robust gene expression must be explored. Alternatively, through an understanding of the molecular events following endosomal disruption, toxicity may be minimised using innovative approaches to inhibit these molecular events. For example, the authors found that Bax inhibition not only decreases chloroquine toxicity, but also increases transgene expression. It may be possible that this approach is far more effective for gene expression enhancers such as PEI, which disrupt fewer endosomes, than the less specific enhancers, such as chloroquine. Non-endosomal delivery of transgenes has been reported sporadically, but there is at present a lack of understanding of these potential uptake pathways.

Declaration of interest

The authors state no conflicts of interest and have received no payment for preparation of this manuscript.

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- **An important study that begins to map the molecular mechaism underlying endosomal disruption and cytotoxicity.**

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