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Directed evolution for drug and nucleic acid delivery

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Abstract

Directed evolution is a term used to describe a variety of related techniques to rapidly evolve peptides and proteins into new forms that exhibit improved properties for specific applications. In this process, molecular biology techniques allow the creation of up to billions of mutants in a single experiment, which are then subjected to high-throughput screening to identify those with enhanced activity. Applications of directed evolution to drug and gene delivery have been recently described, including those that improve the effectiveness of therapeutic enzymes, targeting peptides and antibodies, and the effectiveness or tropism of viral vectors for use in gene therapy. This review first introduces fundamental concepts of directed evolution, and then discusses emerging applications in the field of drug and gene delivery.

Keywords: Molecular evolution; Directed evolution; Antibody affinity maturation; Viral gene therapy; ADEPT; GDEPT

Contents

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1. Introduction to directed evolution

Nature slowly evolves proteins and viruses toward new variations with improved abilities to carry out their functions. Directed evolution allows scientists and engineers to use nature's algorithm to evolve peptides, proteins and viruses on much shorter time scales—days or weeks compared to, in many cases, millions of years. For this reason, directed evolution is widely used in the field of protein engineering to improve the activities of proteins.

The power of directed evolution lies in the ability to rapidly engineer proteins without requiring an in-depth understanding of either the structure or the detailed mechanism of its action. Directed evolution involves the creation of a large number of variants (the library) of one or more peptides or proteins, including those found on viruses, using molecular biology techniques. A typical library consists of 10^4–10^9 variants of the parent. Creation of the library is followed by the identification of rare library members with improved properties through the use of carefully considered screening processes. In order to identify improved clones, it is essential to maintain a link between the genotype (i.e. the genetic sequence) and the phenotype (i.e. the observable property for which the protein is responsible).

This review begins with an overview of the various methods commonly used to generate libraries (Section 2), and is followed by a brief description of methods used to maintain the genotype–phenotype link (Section 3) and a section on the importance of carefully devising schemes by which to select new mutants with desired phenotypes (Section 4). Subsequent sections consider recent advances in the use of directed evolution in three major areas of importance to the drug and gene delivery field (Fig. 1): improving enzymes for cancer drug and gene therapy (Section 5), increasing antibody and targeting ligand specificities and affinities (Section 6), and engineering improved viruses for gene therapy applications (Section 7).

2. Techniques for generating diversity

The creation of a large library of diverse mutants is a key step in determining the success of the directed evolution approach, since a variant with improved properties cannot be found if it is not a member of the library. Several methods exist for creating libraries for directed evolution (see [1] for a recent review on this aspect). The most common and successful methods can be grouped into three general approaches (Fig. 2): random mutagenesis, cassette mutagenesis and gene recombination.

2.1. Random mutagenesis

For random mutagenesis (Fig. 2a), random mutations are introduced throughout the gene or a region of the gene. Most

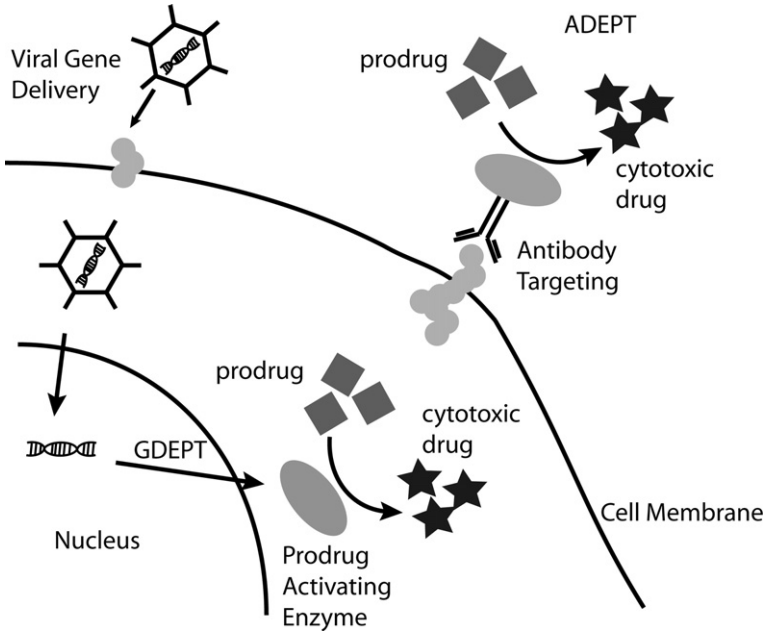

Fig. 1. Therapeutic strategies targeted by directed evolution include viral gene delivery, antibody/ligand mediated cell targeting, antibody-directed enzyme prodrug therapy (ADEPT) and gene-directed enzyme prodrug therapy (GDEPT).

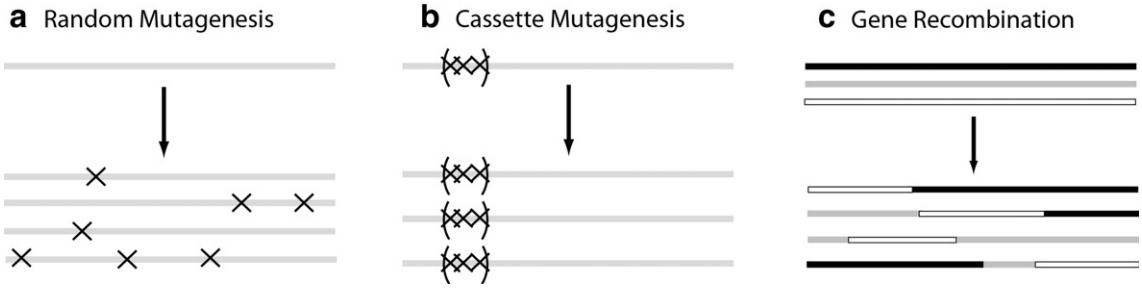

Fig. 2. Common methods used to generate diversity in DNA libraries for directed evolution. (a) Random mutagenesis introduces single nucleotide changes randomly throughout the gene (b) Cassette mutagenesis varies multiple nucleotides within a targeted region (c) Gene recombination reshuffles segments of closely related 'parent' (starting) genes.

commonly, mutations are introduced using error-prone PCR [2,3], which utilizes an error-prone DNA polymerase and reaction conditions (altered levels of Mn^{2+} or other additives, such as propanol [4]) that control the mutation rate. Despite the name "random mutagenesis," the method is not truly random in terms of the amino acid changes introduced at the protein level. It is extremely rare to introduce two mutations into a single codon (a three base sequence that codes for a single amino acid). Due to the nature of the genetic code, single-base changes within a codon typically change an amino acid to another amino acid with similar properties (hydrophobicity, charge, and size). A typical amino acid residue in a protein can only be changed to 5–6 other amino acids by a single change in a codon. The majority of mutations are detrimental to protein function, and the fraction of functional proteins in a library decreases exponentially with higher mutation frequency. However, an advantage of a high-error-rate library is the enhanced probability of finding proteins very different from the starting proteins. Optimal error rates, that balance the need for enhanced diversity (favored at higher error rates) with the need to achieve functional proteins with a reasonable probability (favored at lower error rates), will depend on both the protein and the mutagenesis protocol [5].

2.2. Cassette mutagenesis

Cassette mutagenesis (Fig. 2b) introduces multiple mutations, but the mutations are targeted to a single region (or small number of regions) [6–8]. Typically, mutations are directed to specific regions, such as ligand binding or catalytic sites. Libraries are created by methods involving short DNA sequences (oligonucleotides) synthesized with predetermined mixtures of DNA nucleotides at desired positions. The distribution of amino acids at a given position within the protein sequence can be varied widely since there are no restrictions on the types of amino acids the DNA encodes for (unlike the case with random mutagenesis by error-prone PCR). The use of completely random nucleotide sequences in cassette mutagenesis protocols will bias the library towards amino acids more commonly appearing in the genetic code and introduce stop codons (termination signal for protein translation) at a frequency of 3/64. Nucleotide mixture schemes have been developed that decrease the frequency of undesirable stop codons and bias the codons in a number of ways based on the hypotheses of what type of amino acid may be beneficial for improving desired function [9].

The primary advantage of cassette mutagenesis is that the diversity in a library is focused in a region of the protein known or thought to be important for function. Furthermore, mutations not likely to occur by error-prone PCR random mutagenesis can be explored. On the other hand, the method precludes the identification of unexpected beneficial mutations in other regions of the protein. For instance, random mutagenesis studies have shown that mutations distal from the active site of a protein can have profound effects on the activity of a protein.

2.3. Gene recombination

In vitro gene recombination, best exemplified by the method DNA shuffling [10], occurs when a segment from one gene is exchanged for an analogous segment from a second homologous gene (Fig. 2c). In vitro gene recombination can be applied to construct a library of all possible combinations of beneficial mutations identified from error-prone PCR libraries [11]. The method can also be applied to recombine two or more homologous genes, in which case the method is referred to as family DNA shuffling or molecular breeding [12]. The basic process is the same for both cases. The gene(s) are fragmented (e.g. by digestion with a nonspecific nuclease) and subsequently reassembled using a PCR-like process that lacks external primers. Fragments from different genes become fused in a stochastic process that is directed by the sequence identity of the assembling fragments. A library of hybrid genes is created, in which various gene segments have been swapped between the original genes. In comparison to random mutagenesis and cassette mutagenesis, the method creates a more diverse library in terms of the sequence differences among its members. In addition, because every amino acid is acceptable (at least in the context of the original parent protein), and all the proteins recombined have similar structures, the "mutations" that are introduced by swapping between different homologs are enriched for functional mutations. The library consists of clones that are very different in sequence with each other and, potentially, very different in function from each other. This method has been used for a variety of purposes, including enhancing enzyme activity [13], improving protein folding [14,15], and changing substrate specificity [13]. Many alternatives and

modifications to the basic DNA shuffling scheme have been described [16,17].

3. From DNA to protein libraries: Linking genotype to phenotype

The creation of the library is performed at the DNA level, but the corresponding protein library requires a host system to produce the protein variants of the library. The host is typically *Escherichia coli* or yeast, although completely in vitro systems are feasible for some protein properties (i.e. ligand binding) [18–20]. For high-throughput screens (i.e. one well-one library member), this link is maintained because all library members are physically separated from each other. However, such an approach can be labor-intensive and success can depend on access to high-throughput robotic facilities. A variety of methods exists for maintaining the genotype–phenotype link without physically separating the library members [21]. If a genetic selection (i.e. only those cells carrying an improved gene are able to grow under certain defined growth conditions) can be devised [22], the link is preserved because any selected library members maintain the link within the cells selected (e.g. the protein conferring the ability to an *E. coli* cell to grow under the selection conditions remains in the cell with the gene). However, such selections are usually specific to the protein properties involved and it is difficult to create selection systems for many applications.

Display technologies are particularly useful for evolving proteins for binding activity (e.g. antibody affinity). A variety of display technologies exist, each with their own set of advantages and disadvantages. What they share is the display of the peptide or protein on the outside of a cell, virus or macromolecular assembly (e.g. the ribosome). The gene coding for the protein is either contained within the cell or virus or physically linked to the macromolecular assembly [23]. Display on the outside allows easy access to the protein, and selections, such as those based on chromatographic affinity separation or fluorescent probe binding, can be readily devised. Viruses have the advantage of a built in linkage of phenotype and genotype (e.g. modifications of viral coat proteins are displayed on the outside of the virus with the gene coding for the modification contained inside).

3.1. Phage display

One of the most frequently used display methods is phage display, developed from the initial discovery that foreign proteins could be displayed on the surface of filamentous bacteriophage as genetic fusions with the minor coat protein, pIII [24]. Phage display is a tool found in many laboratories, and extensive reviews on this topic are available [25–27]. Briefly, the gene (or library) for the proteins of interest is fused to a viral coat protein gene, and the gene fusion is encoded on a phagemid (a vector containing both plasmid and phage origins of replication). The phagemid DNA becomes packaged in the phage particle that displays the protein. The phage are then subjected to chromatographic separation or panning to select for desired binders. The recovered phage, which contain the phagemid DNA instead of the viral genome, are still infectious. Upon the infection of *E. coli* the phagemid is replicated as a plasmid and this DNA can be recovered for analysis or subsequent rounds of directed evolution. Phage display is simple and robust and can often be used in conditions that do not allow use of other display technologies (discussed below). Phage display has been successful in the isolation of human antibodies (self antigens, picomolar affinity antibodies) [28] and 10–12mer peptides targeting specific cell types [29–31].

3.2. Cell display (yeast, bacterial, mammalian)

Cell surface displayed libraries have also been explored as a complement to phage display [19,23,32,33]. Proteins are displayed on the surface of cells, most commonly yeast and *E. coli*, via fusion to a membrane-associated protein. The advantage of cell surface display lies in the ability to use fluorescence-activated cell sorting (FACS) for high-throughput screening, which cannot be used with phage libraries due to the small size of the bacteriophage. For example, a cell surface display library can be incubated with a fluorescently tagged target ligand and those library members with high affinity for the ligand can be isolated as the highly fluorescent cells by FACS.

Limitations of this method include susceptibility to surface proteases, conformational constraints or steric effects that can affect the function and utility of surface displayed polypeptides [34–36]. The positioning and length of the peptide linker also plays a critical role in the efficient surface display and recognition of the inserted epitopes [37–39].

3.3. In vitro display

The two major forms of in vitro display are ribosome display [18,40] and mRNA display [20,40]. The major advantage of these methods derives from avoiding the use of cells altogether. Instead, DNA libraries are converted to protein libraries using in vitro transcription/translation. Since cells are avoided, the inefficient step of transformation is eliminated and large libraries of up to 10^{14} members can be constructed [41]. Larger libraries translate into more possible proteins with greater improvements in function. In addition, in vitro display enables the evolution of proteins that are toxic to the cell.

Both methods start with the DNA library, which is converted to mRNA using in vitro transcription. Ribosome display and mRNA display are distinguished by the method for linking the mRNA to the protein. In ribosome display a stable protein–ribosome–mRNA complex is formed by stalling translation [18]. Gene constructs used for ribosome display lack a stop codon at the C-terminus, such that during protein translation, the ribosome stalls and remains attached to the newly synthesized protein. A spacer sequence is used to reduce any steric hindrance between the displayed protein and the ribosome to allow for the correct folding of the protein [42]. In mRNA display, the mRNA is linked to the protein via a puromycin tag that is introduced to the mRNA by attachment

of a synthesized oligonoculeotide [20]. During translation, the ribosome stalls at the synthesized oligonucleotide and a covalent amide linkage via the puromycin is formed between the mRNA and the peptide. This complex is then dissociated from the ribosome.

For both methods, the resulting complexes (which contain both the protein and the mRNA that encoded it) are subjected to chromatography or panning for binding to the desired target molecule. The mRNA from the recovered complexes is converted to DNA using reverse transcriptase and amplified using PCR. The resulting DNA can be used for subsequent rounds of evolution. Since cells are avoided, the slow step of growing cells is avoided resulting in shorter rounds of evolution. Since the mRNA is exposed during the panning step, panning on whole cells or membranes (which are likely to contain RNase that degrades RNA) is problematic. For those applications, phage or cell surface display is more applicable.

4. Evolutionary pressure and selection for improved peptides, proteins and viral vectors

The ability to create millions of variants of a protein means nothing in the absence of an efficient means to identify improved variants. The identification of improved variants through selection or screening is analogous to evolutionary pressure in natural evolution (i.e. only the fittest survive). As the word 'directed' in directed evolution implies, laboratory evolutionary pressure is defined and applied by the researcher. It is important to appreciate that the selection method applied can affect the outcome. This is embodied in the phrase "you get what you select for." As a simple, illustrative example, consider performing directed evolution to improve the catalytic activity of a protein that confers antibiotic resistance to *E. coli*. A simple selection scheme would involve plating the bacteria containing the library on levels of the antibiotic at which the bacteria expressing the unmutated, parental protein cannot grow. However, improvement in the protein's catalytic activity is just one mechanism by which growth will be enabled at the higher level of antibiotic. Any mutation that improves the expression (e.g. increases transcription rate, increases mRNA stability, or increases translation rate) or increases the half-life of the protein in *E. coli* could also confer the ability to grow under these selective conditions.

Selections for binding affinity are generally easy to develop. In phage display, the most commonly used selection is affinity selection against either simple (immobilized protein) or complex (cell surface) targets. Phage are first allowed to bind to a solid support of interest followed by several washing steps using buffers of different stringencies to eliminate weak or nonspecific binders. The remaining attached phage particles are recovered and propagated in bacteria to be further enriched by repeated rounds of adsorption and recovery [25,26]. Biopanning can also be done on whole cell membranes [43,44]. However, many false positives may arise if the target protein is present in low concentrations relative to other cell surface proteins [45]. In other words, if a selection is for binding to a particular protein on the cell surface, one may select for binders to other proteins as well. Generally, one would employ a counter-selection against nondesired cells to eliminate these false positives.

Selections of cell surface display libraries generally begin with a fluorescently tagged target molecule that is allowed to bind to the displayed protein. Analysis of this library by flow cytometry allows one to get statistical information about all library members (i.e. what fraction of the library has improved binding to the target ligand). Furthermore, fluorescent activated cell sorting (FACS) allows for the enrichment of enhanced clones and the ability to discriminate directly between binders of different affinity and specificity. For example, the affinity of an antibody to *Bacillus anthracis* protective antigen was improved >200-fold by *E. coli* display [46].

Selection of improved viruses is facilitated by their replicative nature. Under selective pressure, viruses capable of infecting cells will predominate, and those with high efficiency will dominate over the less efficient viruses. However, one must ensure a low probability of forming chimeric viruses in which the encapsulated DNA sequence is not that which coded for the improved viral coat protein.

Identification of enzymes with the desired phenotype is a more difficult task [47]. Except in peculiar exceptions unique to the chemistry involved, display technologies cannot be used for the selection of enzymatic activity. If the researcher is fortunate or clever, the enzymatic activity might be able to be tied to a growth/no growth selection scheme (or some other easily selectable phenotype such as the one in which colonies of desired library members turn blue). Otherwise the researcher must employ a high-throughput strategy in which, for example, each library member is in a separate well of a multi-well plate and the library members are processed in parallel.

5. Engineered enzymes for cancer drug and gene therapy

One class of enzymes with potential for use in cancer therapies is prodrug activating enzymes—enzymes designed to activate a less toxic prodrug into a cytotoxic drug at the tumor site. The strategy is designed to minimize the damage done to nontarget tissues by localizing high drug concentrations at the tumor. Strategies under investigation include antibody-directed enzyme prodrug therapy (ADEPT) [48–50] and gene-directed enzyme prodrug therapy (GDEPT) [51–53]. In ADEPT, the enzyme is delivered as a fusion to an antibody. In GDEPT the enzyme's gene is delivered. Protein engineering has been used to address limitations in targeting the enzyme to the cell as well as increasing the effectiveness of the enzyme in catalyzing prodrug conversion.

In the ADEPT approach, targeting is achieved by fusing a tumor specific antibody to the enzyme. In the first step, the antibody-enzyme fusion binds an antigen expressed on the surface of tumor cells, leading to accumulation in the tumor. In the second step, a nontoxic prodrug is administered that is converted specifically by the enzyme at the tumor site into a low molecular weight toxic drug. Amplification is a key feature of this system. One antibody-enzyme conjugate molecule can catalyze the conversion of many molecules of the prodrug into the cytotoxic drug. Amplification leads to higher concentrations

of the drug at the tumor than typically obtained with systems in which an antibody is conjugated directly to a drug. ADEPT takes advantage of the bystander effect [54–56], in which cytotoxic drugs produced in the direct vicinity of the targeted tumor antigen diffuse to kill surrounding tumor cells that either do not express tumor antigen or do not bind the antibody-enzyme conjugate. Another advantage of this approach is that the antibody conjugate does not need to penetrate the solid tumor mass. Instead, diffusion of low molecular weight cytotoxic agent, which has significantly higher interstitial tumor transport rates, can provide significant tumor exposure. The main drawback of ADEPT remains the immunogenicity of the conjugate, which prevents repeated administration [57]. This can be potentially minimized by lowering the dose through improvements in enzyme activity or antibody specificity.

GDEPT is similar to ADEPT, but rather than delivering the enzyme directly, the gene encoding the enzyme is delivered to the cancer cell. Enzymes are subsequently expressed by the target cells and activate an administered prodrug. After gene delivery, prodrug administration must be delayed to permit protein expression within the targeted cells. The foreign gene should be expressed exclusively or with a relatively high specificity in tumor cells compared to normal cells for maximal clinical benefit. As in ADEPT systems, delivery of the bio-active agent to all diseased cells may not be required due to the bystander cytotoxic effect. Both the enzyme and the delivery system, most commonly viral vectors, are potential targets for directed evolution.

Chemoprotective enzymes, designed to ameliorate toxic side effects in healthy tissues, are another class of enzymes that may benefit from directed evolution approaches. Delivery of chemoprotective enzymes to bone marrow stem cells may be particularly useful since the doses of chemotherapeutic agents are often limited by the lethality to these cells [58]. Their function is opposite to that of the prodrug activating enzymes in that they are drug-inactivating enzymes designed to protect healthy cells. One approach to minimize toxicity is to transfect hematopoietic stem cells (HSCs) from the bone marrow with a gene coding for a chemoprotective enzyme. Transfection can be done ex vivo, followed by the retransplantation of transfected cells back into the patient [59]. In theory, only a small percentage of marrow cells need to be transfected, as cells expressing the drug-resistance gene have a selective advantage in the presence of administered chemotherapeutic drugs and therefore, outgrow unprotected marrow cells.

5.1. Improving prodrug activating enzymes by directed evolution

Several enzyme/prodrug combinations have been investigated both in vitro and in vivo. Among these, herpes simplex virus type 1 thymidine kinase (HSV-tk) is the most widely tested GDEPT system in clinical trials [60,61]. Human thymidine kinase (TK) catalyzes the phosphorylation of thymidine residues in DNA and a limited number of analogs; however HSV-tk can catalyze the phosphorylation of a wider variety of analogs including ganciclovir (GCV) and acycolovir (ACV) [62]. Upon phosphorylation of GCV or ACV by HSV-tk, a series of intracellular reactions result in the formation of a triphosphate that competes with deoxyguanosine triphosphate in DNA elongation during cell division. The result is the inhibition of DNA polymerase and the formation of single strand breaks in DNA, leading to cell cycle arrest [63–65].

In vitro studies have shown that stable expression of HSV-tk can increase cell sensitivity to GCV by up to 2000-fold [66]. The combination of HSV-tk and GCV has been demonstrated to be effective in many tumor models, using either stably transfected cell lines or following viral gene delivery. Despite promise, clinical successes have still been modest at best, due to poor transfection efficiencies, slower growth of human tumors compared to xenografts used in animal models and the immunosuppressive effects of the GCV doses required for tumor regression [60].

Directed evolution holds considerable promise to improve both the delivery method (improved targeting and viral vectors to be discussed later) and to enhance the activity of the enzyme itself. For example, one approach to lower the required GCV dose, and thereby minimize the immunosuppressive effect, is to engineer mutant HSV-tk with GCV as the preferred substrate over thymidine. Wild-type HSV-tk has a high affinity for thymidine with a K_m of 0.5 μM [67], but only a moderate affinity for GCV (HSV-tk has a K_m for GCV of 45 μM [68]). In other words, only a low concentration (0.5 μM) of thymidine is needed to reach 50% of the maximum rate of reaction, while a 90-fold higher concentration (45 μM) of GCV is needed to achieve the same reaction rate. Previous studies have shown that the mutation of glutamine to asparagine at position 125 on HSV-tk decreases its preference for thymidine (K_m of 20 μM) as a substrate while retaining the ability to phosphorylate GCV [69,70].

GCV is the most widely used prodrug with HSV-tk, however, ACV has a more mild toxicity profile and can be tolerated at high doses [71]. However, its use has been limited because HSV-tk has a poor K_m (>400 μM), almost 10-fold higher than for GCV [68,72]. Novel enzymes with either an increased specificity for phosphorylating GCV or ACV, or improved enzyme kinetics may result in enhanced cell killing and reduced prodrug-mediated toxicity.

Cassette mutagenesis (see Section 2.2) was used to target six amino acids (positions 159–161 and 168–170) within the HSV-tk gene to increase its substrate specificity for GCV or ACV over thymidine [73]. The DNA sequence corresponding to those six amino acids was completely randomized using primers made with a mixture of equal ratios of the four naturally occurring nucleotides. The library was subsequently transformed into *E. coli* cells deficient in thymidine kinase activity and screened for HSV-tk activity. GCV and ACV sensitive mutants were identified by plating on increasing concentrations of the prodrug. *E. coli* transformed with improved HSV-tk mutants are unable to grow due to the efficient conversion of GCV or ACV into a cytotoxic drug. A library of greater than one million HSV-tk was generated. Of this library, 426 mutants possessed HSV-tk activity, 26 of which had enhanced sensitivity to GCV and 54 to ACV.

One such mutant (mutant 30) identified in the study was further investigated in both an in vivo and an in vitro model [74]. Rat C6 glioma cells, a model system used to evaluate TK/GCV systems in brain tumors or subcutaneous tumors [75,76], were stably transfected with either wild-type HSV-tk or mutant 30 HSV-tk. Increasing doses of GCV or ACV were applied and cell survival assessed (Fig. 3), with a vector with no HSV-tk used as a control. Treatment with either GCV or ACV killed 50% of cells transfected with the mutant 30 HSV-tk at concentrations dramatically lower than that for wild-type [74]. Mutant 30 shows 25 nM IC_{50} values with GCV and ACV, compared to IC_{50} values of 30 μM for wild-type HSV-tk with GCV (~1000-fold enhancement) and >100 μM with ACV (~4000-fold enhancement).

In a mouse xenograft tumor model, the mutant 30 and wild-type HSV-tk stably transfected cells were subcutaneously injected into nude mice. Growth of the mutant 30 expressing tumor was restricted by GCV at doses at least 10-fold lower compared to wild-type HSV-tk expressing tumors. In the presence of GCV, a substantial bystander effect was observable when only 20% of the tumor cells expressed the mutant, while no restriction in tumor growth was observed with the wild-type HSV-tk under the same conditions [74]. Upon investigation of enzyme kinetics, the authors conclude that rather than enhancing the catalytic activity of the enzyme for GCV by decreasing its K_m for GCV, the mechanism of enhanced sensitization may be due to strongly reduced affinity of the enzyme for thymidine (35-fold increase in thymidine K_m). This reduces the competition between the prodrug and thymidine at the active site, giving the mutant HSV-tk a substantial kinetic advantage despite its high K_m values for both GCV and ACV.

In a second generation library, the sequences from the improved mutants of the initial library were used to design a smaller, semi-random library, in which five amino acids were targeted [77]. Rather than mutating the DNA sequence completely randomly, primers were made such that only a limited number of amino acids would be possible at each position, yielding 512 possible mutants. When the same rat C6 glioma cell and mouse xenograft studies were performed, one HSV-tk mutant (SR39), possessed an IC_{50} of 17 nM, an ~300 fold reduction

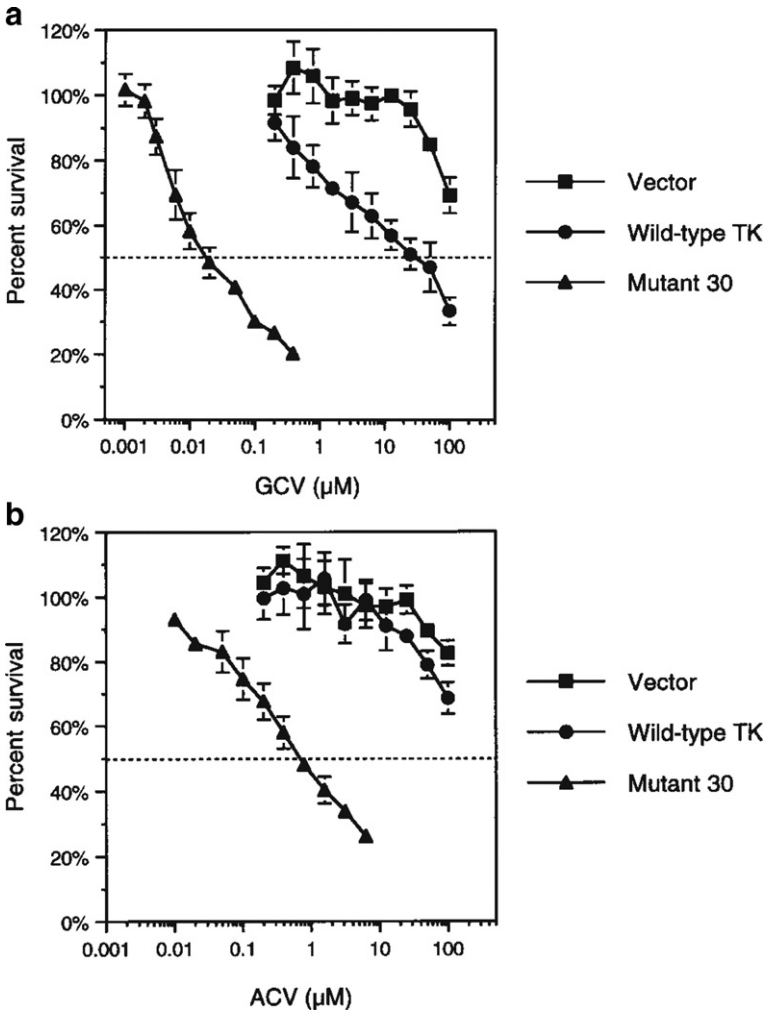

Fig. 3. Tumor cells (rat C6 glioma) transfected with a mutant HSV-tk (mutant 30) results in greater cytotoxicity upon treatment with (a) Ganciclovir (GCV) or (b) Acyclovir (ACV) than cells transfected with wild-type HSV-tk [74]. The mutant was produced by a cassette mutagenesis and screened on increasing GCV or ACV plates. Reprinted with permission from Macmillan Publishers Ltd: [Gene Therapy Vol.6 (8): p.1415–26, 1999].

compared to wild-type. SR39 tumor growth in mice was similarly restricted by GCV at doses of at least 10-fold lower compared to tumors transformed with wild-type. This approach demonstrates how results from a random mutagenic library can be used to design a more focused, smaller and easier to screen library, from which dramatically improved mutants can be identified.

Combining the results of these studies, Mercer et al. [78] mutated the previously identified asparagine to glutamine at amino acid position 125 [69,70] along with the mutated regions of two mutants identified by Kokoris et al. [74]. Sixteen such mutants were generated and the substrate preferences for each mutant enzyme were compared with wild-type HSV-tk. A single mutant was identified which had a lower K_m for GCV (40 μM) than thymidine (53 μM). Expression of this mutant in tumor cells (human colorectal cancer cell line HCT116) exhibited comparable metabolism to wild-type, an increased tumor sensitivity to GCV, and minimal thymidine phosphorylation compared to wild-type HSV-tk.

HSV-tk has also been subjected to directed evolution to improve anti-HIV treatment by zidovudine (AZT) phosphorylation. DNA shuffling (see Section 2.3) was performed on HSV-tk type 1 and 2 [79], the two most closely related herpes virus TK genes (they share 78% identity [62]). High-throughput robotic screening was used to identify new HSV-tk chimeras with improved AZT phosphorylation [79]. Briefly, robots were used to plate colonies of mutant HSV-tk transformed *E. coli* onto replicate filters containing various concentrations of AZT. After overnight incubation, visual inspection was used to identify clones that grew on plates with no AZT, but did not grow on plates with AZT. These selected clones have active HSV-tk enzymes. After HSV-tk active clones were identified, the gene was reshuffled, retransformed into *E. coli* and screened on progressively lower concentrations of AZT. After four rounds of evolution, two clones were isolated that sensitized *E. coli* to 32-fold less AZT compared with *E. coli* transformed with wild-type HSV-tk type 1, the best parental enzyme. Upon sequencing, both clones were shown to be hybrids with several amino acid changes from the two parental genes, along with several amino acid mutations found in neither parent. Kinetic measurements show the chimeric enzymes had acquired a ~3-fold improvement in its K_m for AZT as well as ~5 and ~13 fold decreased specificity for thymidine.

5.2. Improving chemoprotective enzymes by directed evolution

The dose of chemotherapeutics used to fight cancer is often limited by bone marrow toxicity, as is the case with the alkylating agents temozolomide and 1,3 bis(2-chloroethyl)1-nitrosourea (BCNU) [59,80]. BCNU treatment leads to the formation of O^6-alkylguanine, a cytotoxic lesion in DNA [81]. O^6-alkylguanine-DNA alkyltransferase (AGT) is a ubiquitously expressed DNA repair protein encoded by the methyl-guanine methyl transferase (MGMT) gene. AGT removes alkyl groups from the O^6 position of guanine in DNA [82], returning the DNA to its normal state. Bone marrow cells are particularly susceptible to O^6-alkylguanine lesions because they possess low levels of AGT [82]. Transplantation of bone marrow cells transduced using retroviral vectors containing the gene encoding for wild-type human MGMT has been shown to increase the resistance of hematopoietic stem cells (HSCs) to an alkylating agent, BCNU [83]. HSC's expressing a mutant AGT with greater than wild-type activity may give these cells a greater protective advantage over cancer cells expressing wild-type AGT.

Another approach to improve AGT efficacy is to engineer AGT mutants with greater resistance to drugs that inactivate endogenous AGT. For example, O^6 benzylguanine (BG) is capable of inactivating wild-type AGT and has been clinically tested [80] to deplete tumor cells of wild-type AGT, thereby increasing the cytotoxicity of administered alkylating agents. However, if BG is not delivered specifically to tumor cells, it could further reduce the already low levels of AGT in the bone marrow [82]. If AGT mutants can be engineered to be resistant to BG, it is hypothesized that the cytotoxic effects on cancer cells may be enhanced while minimizing the toxicity to bone marrow cells. Directed evolution provides the toolset necessary to create and identify mutants with either improved AGT activity, reduced susceptibility to BG, or even both. Currently, chemotherapy regimens of BG in combination with BCNU, temozolomide and Gliadel (a slow-release polymer wafer loaded with BCNU) are in clinical development [80].

To improve AGT activity, 12 amino acids near the active site cysteine residue were targeted for randomization [84]. The library of mutant AGT was selected for the ability to provide alkyltransferase deficient *E. coli* with resistance to a methylating agent, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG). Although few amino acid changes were tolerated (i.e. still produced a functional protein), one mutation (V139F: valine to a phenylalanine at amino acid 139) was found in 70% of the mutants. Cells were treated with varying doses of MNNG and the percentage of cells able to grow on plates containing rifampicin was used to assess the mutagensis rate. Resistance to rifampicin results from mutations in RNA polymerase and is a commonly used indicator of cellular mutation frequencies. The single amino acid change from Val to Phe provided *E. coli* with greater protection than wild-type, reducing the mutagenesis rate 2.7–5.5 fold.

BG-resistant AGT mutants were similarly identified from a library of 6.5×10^6 mutants with six random amino acid changes near the alkyl-accepting cysteine [85]. One of the mutants identified had both the highest resistance to MNNG and to BG. Treatment with 100 μM BG had no significant effect on this mutant's ability to protect *E. coli* from the cytotoxic effects of MNNG, whereas the wild-type *E. coli* with AGT was completely eliminated by this dose of BG (Fig. 4). This mutant was also able to protect *E. coli* better than the wild-type and equally well as the V139F single mutant, resulting in a mutant with the combined benefits of BG resistance and increased alkyltransferase activity.

The AGT protein has many naturally occurring variants, as well as the mutant forms generated in the laboratory [80]. There exists potential to further improve the properties of these enzymes by combining the three mutagenesis methods, error-prone PCR, cassette mutagenesis and DNA shuffling. Other enzymes that have been investigated for chemoprotection include thymidylate synthase and dihydrofolate reductase [58,59]. It may also be beneficial to evolve the enzymes discussed here for properties other

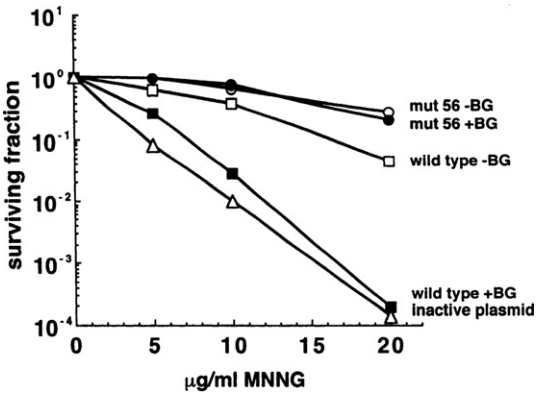

Fig. 4. Cassette mutagenesis evolves O^6-alkylguanine-DNA alkyltransferase (AGT) mutants with enhanced chemoprotective ability compared to wild-type AGT [85]. One isolated AGT mutant (mut 56) protected *E. coli* (GWR111) cells from the cytotoxic effects of an alkylating agent (MNNG) even upon treatment with benzylguanine (BG), an AGT inhibitor. Wild-type AGT provides little protection upon treatment with the same agents. Reprinted with permission from the American Association of Cancer Research: [Cancer Research Vol. 57 (10): p. 2007–2012, 1997].

than improved catalytic rates, such as low immunogenicity or improved stability.

6. Directed evolution for improved targeting

Antibodies have been fused to a wide range of therapeutic agents, such as cytotoxic drugs, peptides, proteins, enzymes and viruses, to improve targeting of these agents to specific disease sites [86–88], not to mention their other uses in cancer treatments [89,90]. Thus, the ability to engineer antibodies with high affinity, specificity, and stability is of considerable importance to the drug and gene delivery field. For example, cancer cells produce distinct antigens on the cell surface in the form of over-expressed receptors, tumor associated antigens (TAA), or differentiation antigens with distinct expression patterns [87]. The development of highly specific antibodies against these molecules is of interest in the field of tumor targeting. Antibody engineering has also become increasingly important in designing antibodies specific to tumor surface antigens, such as MHC class I peptide complexes presenting tumor specific antigens. Affinity for such receptors is typically low and a recent area of interest has been to bind such tumor peptide-MHC complexes at higher affinities [91,92]. Goals of antibody engineering include: the reduction of immunogenicity, the generation of smaller antibody fragments and engineering multiple specificities into single antibody molecules [41]. In this review, we will focus only on the improvement of antibody affinity and binding kinetics, an area in which directed evolution and library display has been widely applied.

6.1. Antibody affinity maturation by directed evolution

Antibodies generated in response to infection can have very high affinity for their antigens with dissociation constants in the 100 pM range [93,94]. However, antibodies isolated from naïve (i.e. non-immunized individuals) or synthetic libraries usually have higher dissociation constants, in the micromolar range [95]. Directed evolution has been used to improve the affinity of antibodies to the nanomolar [96–98] and even picomolar [99,100] range.

Libraries of antibody genes are often constructed from either naïve or immunized individuals, or synthetically generated. Cycles of mutation, display and selection, as shown schematically in Fig. 5a, have been far more effective at increasing antibody affinities than precisely designed alterations for affinity enhancement [100,101]. Mutations outside the complementarity determining region (CDR) loops have led to large increases in affinity, stability and expression [100–102]. Methods to introduce random mutations over the entire variable domain genes include homologous gene recombination and error-prone PCR with or without chain shuffling [103,104]. Recently highly mutagenic enzymes, such as mRNA reverse transcriptase and DNA polymerase with no proof reading activity has been used to generate mutations [105].

Phage display is one of the most widely used technologies for display and selection of antibodies with enhanced binding capacity [28,106], although ribosome and mRNA display have also been used with success in affinity maturation of antibodies (Fig. 5b). Cell surface display, in which antibodies are displayed on/in bacterial cells and screened by replica plating, is not as widely used because this screening method has a library size limit of $<10^8$ members [41]. However, recent development of high-speed flow cytometers has renewed interest in this area, leading to the isolation of several high-affinity antibodies by this method [101,107]. Details of all of these display methods are provided in Section 3.

An anti-c-erbB-2 (a tumor antigen) scFv antibody with an affinity of 13 pM was identified from a library of randomized nine amino acid residues in the V_L of a CDR followed by phage display [108]. The library was selected using decreasing concentrations of antigen. Relative to wild-type, a mutant was identified with a 1230-fold increase in the affinity, demonstrating the power of in vitro maturation technologies. Affinities as high as 48 fM have been identified after 4 cycles of mutagenesis (error-prone PCR and DNA shuffling) and selection (yeast display) to yield an anti-FITC antibody [101]. Promising results from cell display include scFvs displayed on 293T cells, where a 240-fold enrichment was seen in a single pass cell sorting from a large excess of cells expressing wild-type antibody with lower affinity [109].

As a reflection of the success of recombinant antibodies, several have been tested in clinical trials. For example, Humira an anti-TNFα for the treatment of an autoimmune disease has been approved for arthritis. In phase II clinical trials include an antibody targeted to interleukin 12 for the treatment of multiple sclerosis, and one targeted against epithelial cell adhesion molecule for breast and prostate cancer. For more information on in vitro affinity maturation, the reader is referred to recent reviews [41,103,104].

6.2. Identification of targeting ligands by directed evolution

The most commonly used tool for the identification of short peptide ligands for targeted delivery of therapeutics is phage display. A peptide sequence is expressed as a fusion with phage coat proteins or phage displayed proteins. Two peptides with high affinity to human umbilical vein endothelial cells (HUVEC), but

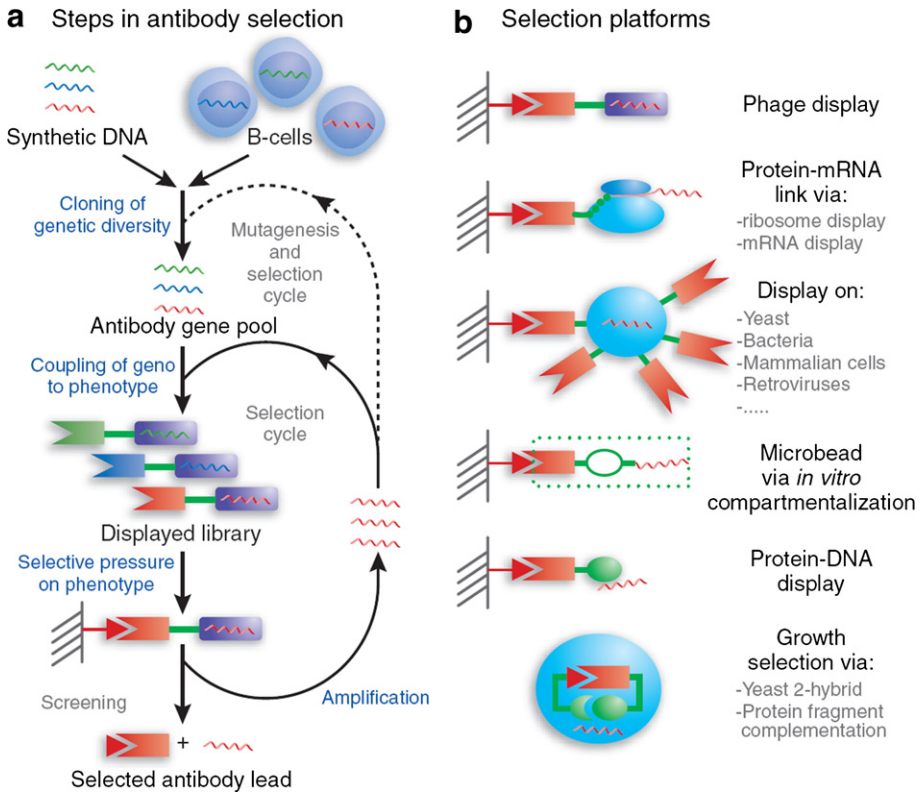

Fig. 5. Engineering improved antibodies using directed evolution. (a) antibody diversity is first generated, genotype and phenotype are coupled by a display method, and finally selective pressure applied to isolate desired clones. Steps are repeated for further amplification. (b) various selection platforms that have been successfully used for antibody affinity maturation [103]. Reprinted with permission from Macmillan Publishers Ltd: [Nature Biotechnology Vol.23 (9): p.1105–16, 2005].

low affinity towards hepatocytes and human saphenous vein smooth muscle cells (VSMCs) (two common targets of adeno-associated virus 2) was recently identified by phage display (Fig. 6a) [110]. These two peptides were inserted into an AAV2 at a position previously identified as an efficient site for the insertion of targeting peptides [111]. In vivo bio-distribution studies 1 h after infusion of modified AAV showed significantly lower liver accumulation and delayed blood clearance at early time points when compared to wild-type. One peptide insertion, (called AAVmtp) showed significantly higher accumulation than AAV wild-type (Fig. 6b) in the vena cava. In a follow up publication, in vivo biopanning led to the identification of a nonheparin binding peptide able to retarget the virus preferentially to the target endothelium [31]. More recently, methods to identify peptides that enhance cell uptake [112] or nuclear targeting for gene delivery [45] have also been developed.

6.2.1. Ligands to enhance cell uptake

To identify peptides that not only bind but facilitate internalization into cells, researchers recover phage from cell lysates instead of those bound to the outside of cells [112]. From a phage-display library with nine amino acid peptides displayed on the surface, phage internalized into human cells (HEp-2 and ECV304) were selected [30]. After three rounds of selection, isolated phage populations were found to be internalized 1000 to 100,000 fold more efficiently than the initial library.

Peptides that might enhance cell target specificity and efficiency to adenovirus 5 (Ad5) were identified by phage biopanning on monolayers of cystic fibrosis transmembrane receptor (CFTR) deficient human tracheal glandular cells (CF-KM4) [29]. The most efficiently internalized phage were isolated, leading to the recovery of three different ligands. These ligands were inserted into the extremity of the gene encoding the short fiber shaft of Ad5. One vector (QM10) with the showed enhanced gene transduction of CF-KM4 cells compared to control wild-type. The enhanced effect was not cell type specific, as many other cells were transduced with significantly higher efficiency. No negative selection step (on nondesired cell types) was used, showing the limitations of phage biopanning on living cells, outside the context of the in vivo system.

6.2.2. Ligands to enhance nuclear targeting and gene delivery

Ligands that can deliver genetic material to the nucleus can be identified by ligand identification via expression (LIVE) [45,113]. The phage genome is modified to carry a reporter GFP gene along with the ligand library to be displayed on the phage coat. Phage are then used to deliver genes to cells, where cells transfected are identified by expression of GFP. The most efficient phage are then isolated by cell sorting (FACS). PCR amplification can be used to recover the gene coding for the ligand that enables enhanced cell targeting and expression of the delivered gene. For further rounds of selection, identified sequences can be re-inserted into a new phage vector and the selection scheme repeated. A significant advantage of this strategy is the selection for ligands that not only bind cells and

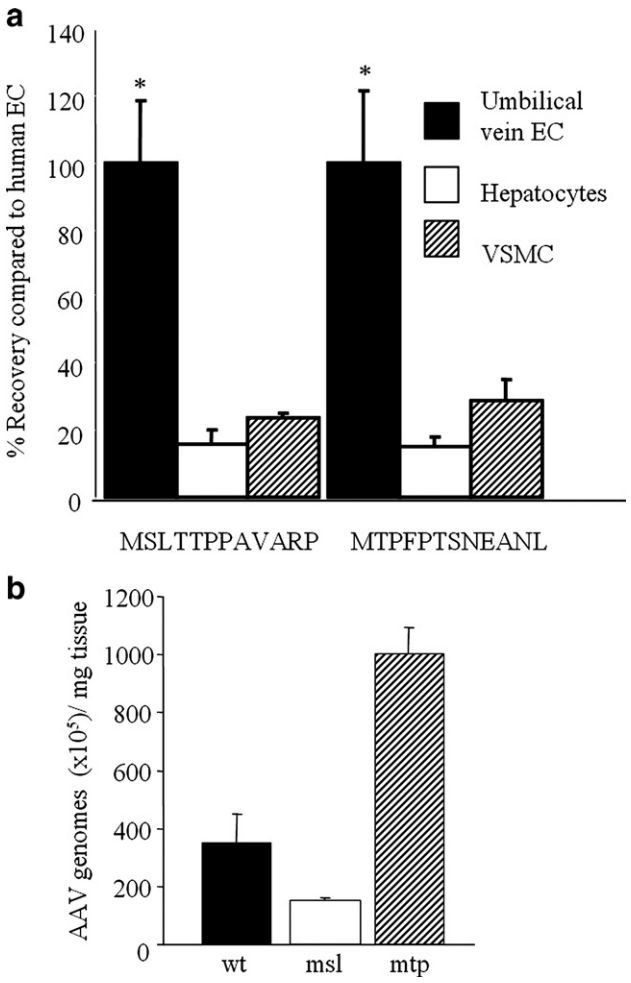

Fig. 6. Enhanced endothelial cells-targeting ligand identified by phage display [110]. (a) Two isolated peptides (MSLTTPPAVARP and MTPFPTSNEANL) facilitate phage binding to umbilical vein endothelial cells (EC) with a decrease in binding to hepatocytes and vein smooth muscle cells (VSMC), two common targets of AAV. b) AAV with identified peptide inserted (AAVmtp) shows greater accumulation in the vena cava compared to wild-type (AAV-wt), as quantified by real-time PCR. Reproduced with permission from Lippincott Williams & Wilkins: [Circulation Vol. 109 (4): p. 513–519, 2004].

become internalized but also traffic appropriately for gene delivery [45].

7. Directed evolution of viral vectors for gene therapy

Directed evolution has been applied to viruses in an effort to improve vectors for gene therapy. Although many vectors have shown promise in early clinical trials, there remains considerable room for improvement in decreasing viral immunogenicity and improving specificity. Improved targetability or specificity lowers the vector burden, decreasing vector-induced toxicities and immune response. Targeting has been modified by engineering changes to the capsid [114,115] or envelope proteins [116]. Incorporation of targeting moieties into the viral coat proteins with covalent linkage of antibodies [117,118] or peptide ligands identified via phage display [119–122] has also been attempted.

7.1. Adeno-associated virus (AAV): Modifying tropism and reducing immunogenicity

An increasingly utilized viral vector for gene therapy is adeno-associated virus, which despite a recent death of a patient enrolled in an AAV trial remains unique in its lack of known pathologies and low inflammatory potential. Although the native tropism for AAV is the airways, AAV has shown efficient gene delivery and sustained transgene expression in numerous tissues including muscle [123], liver [124,125], and lung [124]. Clinical trials have generally indicated a lack of vector-mediated toxicity [126–128]; however, repeated administration has not been capable of sustaining positive effects [124,129]. One major limitation of this system is the common presence of neutralizing antibodies in the human population. A majority of the human population has already been exposed to various AAV serotypes, so a significant fraction of the patient population may harbor antibodies that block gene delivery [130].

Previous studies have shown that the AAV2 capsid proteins can tolerate changes without disrupting its infectious properties [111]. Peptide insertion into specific capsid locations has also been shown to improve cell-type specificities [119–122]. However, ligands identified by phage display may not provide the same selectivity when inserted into AAV, partly due to the altered environment of surrounding amino acids or different 3-dimensional positioning relative to the surface. A method of displaying random peptides directly on the surface of the AAV capsid with

subsequent selection was devised by Perabo et al. [121]. A random seven amino acid peptide was inserted at position 587, a site known to tolerate insertions. A library of 4^{10} viral particles with modified capsids was subjected to repeated cycles of infection in a nonpermissive human megakaryocytic cell line. Forty-eight hours post infection, viral progeny were harvested. After five rounds, the peptide sequence RGDAVGV was identified. Due to the nature of the selective pressure viruses containing this peptide have the ability not only to bind to this new cell type, but also to internalize, un-coat, enter the nucleus, and replicate. Libraries displayed directly on viral vectors of interest for use in gene therapy may be more advantageous in the identification of targeting ligands because selection occurs within the context of the specific virus.

A random mutagenesis approach can generate larger scale changes to the capsid proteins to moderate cell specificities, including those that may be unidentifiable by insertion of targeting moieties. Since there are both linear and complex three-dimensional neutralizing antibody epitopes on AAV [131,132], rational design to eliminate such binding sites is very difficult.

Viral particles more infectious than wild-type following incubation with neutralizing serum, were identified by directed evolution. Perabo et al. [115] used error-prone PCR to introduce random DNA point mutations throughout the AAV2 viral capsid gene, thus generating a library containing 2.5×10^7 members. As described in more detail in Section 2.1, the mutations are not completely random since amino acid changes at any given position tend to be biased towards those with similar properties when error-prone PCR is used. Selection was performed by incubating the library with AAV2 neutralizing serum before infection of HeLa cells co-infected with adenovirus (which provides helper function for active replication). Viral progenies were then harvested 48 h after infection and applied to new cells for further selective pressure and selection. Thus, the viral population was progressively enriched for mutants better able to infect cells in the presence of neutralizing antibodies.

After three selection rounds, DNA was extracted and single clones were analyzed by sequencing. Mutations at two different amino acid positions were strongly selected, whereas mutations at 22 other sites only occurred once. Interestingly, the two amino acids strongly selected were separated by 92 amino acids in the primary sequence, but were located closely together on the surface of the three dimensional structure (accessible to antibody binding). Mapping the other, less frequently selected, sites revealed that 73% of all recovered mutation sites clustered in the same capsid region and that region was on the surface of the virus. It is of note that previous studies had failed to identify this region as immunogenic [132], perhaps due to the region being part of a complex structure composed of loops from different capsid proteins not easily mimicked by any single peptide fragment. Other mutations not on the surface may also affect antibody binding, as it has been previously shown that residues buried in the protein structure may still influence overall topology of the surface [133,134]. The increased ability of AAV mutants evolved by directed evolution to escape neutralization was confirmed by infection with recombinant GFP expressing AAV particles containing either one or both of the two strongly selected amino acid changes [115]. Although improved compared to WT, isolated mutants were still inactivated by relatively low sera concentrations, so further optimization appears necessary. However, the double mutant was more efficient than the single mutant, so it may be possible to engineer improved mutants by combining several mutations on a single capsid.

Such a library was attempted by Maheshri et al. [114]. Using error-prone PCR, followed by recombination using a DNA-shuffling-like staggered extension process [17], a library of $>10^6$ independent clones was created. This library was subjected to a selection process similar to Perabo et al. [115].

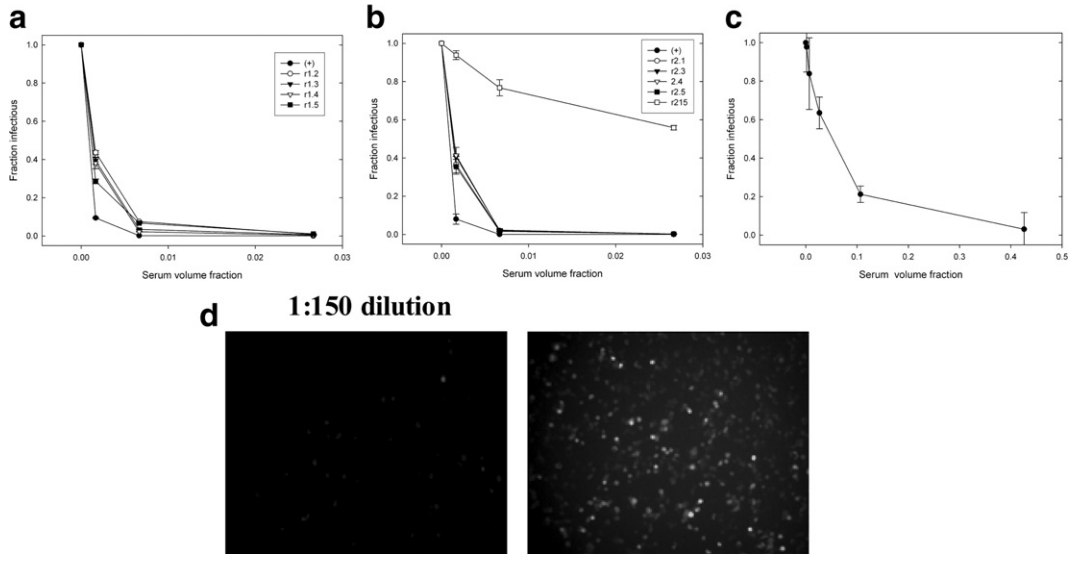

Fig. 7. Error-prone PCR followed by recombination yields improved neutralizing antibody escaping AAV2 [114]. (a) After one round of mutagenesis, GFP was packaged into the AAV library. After incubation in an anti-AAV2 serum, the fraction of infectious particles was quantified by flow cytometry and normalized to infectious particles in the absence of serum. (b) Five mutants isolated in (a) were subjected to another round of mutagenesis with r2.15 exhibiting significant further improvement. (c) Further characterization of best mutant (r2.15) shows 96-fold improved antiserum evasion. (d) At a 1:150 serum dilution, HEK 293 cells were transduced with mutant r2.15-AAV-GFP (left), while little transduction is visible with wild-type-AAV-GFP (right). Adapted by permission from Macmillan Publishers Ltd: [Nature Biotechnology Vol.24 (2): p.198–204, 2006].

Library members were pre-incubated with varying antiserum concentrations and, subsequently, added to the human embryonic kidney (HEK) 293 cells and rescued by adenovirus. The serum concentration was escalated after each round. One mutagenesis and three selection steps generated mutant capsids with the ability to transfect cells following incubation in three-fold higher neutralizing antibody concentrations, as compared to wild-type. These engineered viral vectors also provided an ~7.5% infectivity at serum levels that completely neutralized wild-type infectivity. Upon a second round of evolution (mutagenesis followed by three selection steps), a variant with five mutations was created capable of avoiding 96-fold higher neutralizing antibody concentration compared to wild-type (Fig. 7). Interestingly, two key mutations, different from those identified in Perabo et al. [115], were identified on the viral surface in twelve of the mutants. This suggests that the screening of libraries obtained by consecutive rounds of mutagenesis and gene shuffling of previously identified mutants, or with other AAV serotypes may yield chimeras with further enhanced neutralizing antibody escape properties.

An advantage of generating a random mutagenic library in which mutations are generated over the entire gene is the ability to perform selection against multiple criteria. For example, Maheshri et al. [114] screened the library for both antibody escape and affinity to heparin sulfate. Library members were loaded onto a heparin affinity chromatography column. Each low or high heparin affinity elution fraction was separately amplified by a low multiplicity of infection (low virus to cell ratio to decrease probability of multiple virions entering one cell) on HEK 293 cells, followed by rescue by adenovirus serotype 5. After two rounds of enrichment, two viral pools were identified which eluted from the column predominantly at 150 mM or 750 mM NaCl (wild-type typically elutes at 450–550 mM) [114]. For AAV serotype 2, which binds heparin sulfate [135], a commonly found proteoglycan on the surface of cells, the low heparin binding mutants may be beneficial in aiding tissue dispersion.

7.2. Murine leukemia virus (MLV): Modifying tropism

Recombination is the major driving force for the natural evolutionary change of retroviruses. Copackaged retroviral genomes have been estimated to recombine at a rate as high as 40% per replication cycle [136]. Such recombination allows genetic information to be shuffled efficiently leading to rapid generation of new phenotypes. An in vitro mimic of this process was performed by DNA shuffling of six homologous parental envelope sequences of murine leukemia viruses (MLV) [116].

Six parental strains of MLV, non-infectious for CHOK1 cells, were shuffled to generate a library of 1×10^6 clones by Soong et al. [116]. Infectivity for the new cell type was selected for on a mixture of predominantly CHOK1 cells with a small percentage of Lec8 cells. Lec8 cells are CHOK1 cells with a defect in glycosylation [137,138]. This defect results in more accessible receptors making these cells more permissive to certain MLV infections [139,140] and served as an intermediate so that viruses with very low infectivity of CHOK1 may be amplified as well. The shuffled library became progressively enriched by passage five [116].

The dominant sequence isolated was composed of four segments from three of the parents with a minimum of three recombination events. In vivo retroviral recombination alone would not have generated the dominant sequence isolated here since the parental strains have such differing infectivity of cell lines making it very unlikely for copackaging of certain strains to allow for retroviral recombination.

7.3. Adenovirus: Improving oncolytic properties

Yan et al. [141] generated a novel adenovirus (Ad) with improved oncolytic properties—illustrating the diversity of functions that may be improved by directed evolution. Derivatives of human adenovirus 5 have been developed to replicate and kill cancer cells selectively. One such virus, ONYX-015 has shown clinical benefit in several phase I and phase II clinical trials [142–144]. The goal is the improvement of the replication efficacy of Ad5 that replicate specifically in cancer cells leading to more efficient killing and enhanced spreading within the tumors.

Wild-type Ad5 was mutated by treatment with the chemical mutagen nitrous acid to generate a library of mutants [141]. Cells were then infected with the viral library at a one to one ratio of cell to virus. Upon the first sign of cytopathic effects, media was harvested and used as an inoculum for subsequent rounds of selection (6–20 rounds depending upon the cell type). Viral cytolytic activity was then identified by a standard MTT assay. Two mutants were identified that replicated more rapidly than wild-type, and lysed HT29 cells up to 1000-fold more efficiently, with the difference being most profound when a very low virus to cell ratio was used. Both mutants contained seven single-base pair mutations compared to wild-type. Cytotoxicity of both remained unaltered in normal primary human cells and improved oncolysis was observed in other human cancer cell lines as well, including another colorectal cancer cell line (HCT116) and a lung cancer cell line (A549) [141].

8. Conclusion

Directed evolution provides a powerful strategy to rapidly advance the identification and development of improved protein-based drugs, including enzymes and antibodies, peptide ligands and antibodies for specific targeting, and even viral vectors for improved gene therapy approaches. The molecular biology techniques that enable directed evolution have matured to the point that they are accessible to those in other fields. As such, directed evolution promises to play an increasingly important role in the advanced drug delivery discipline.

Acknowledgments

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