Applications and Future Potential of Directed Evolution for Combatting Antifungal Resistance

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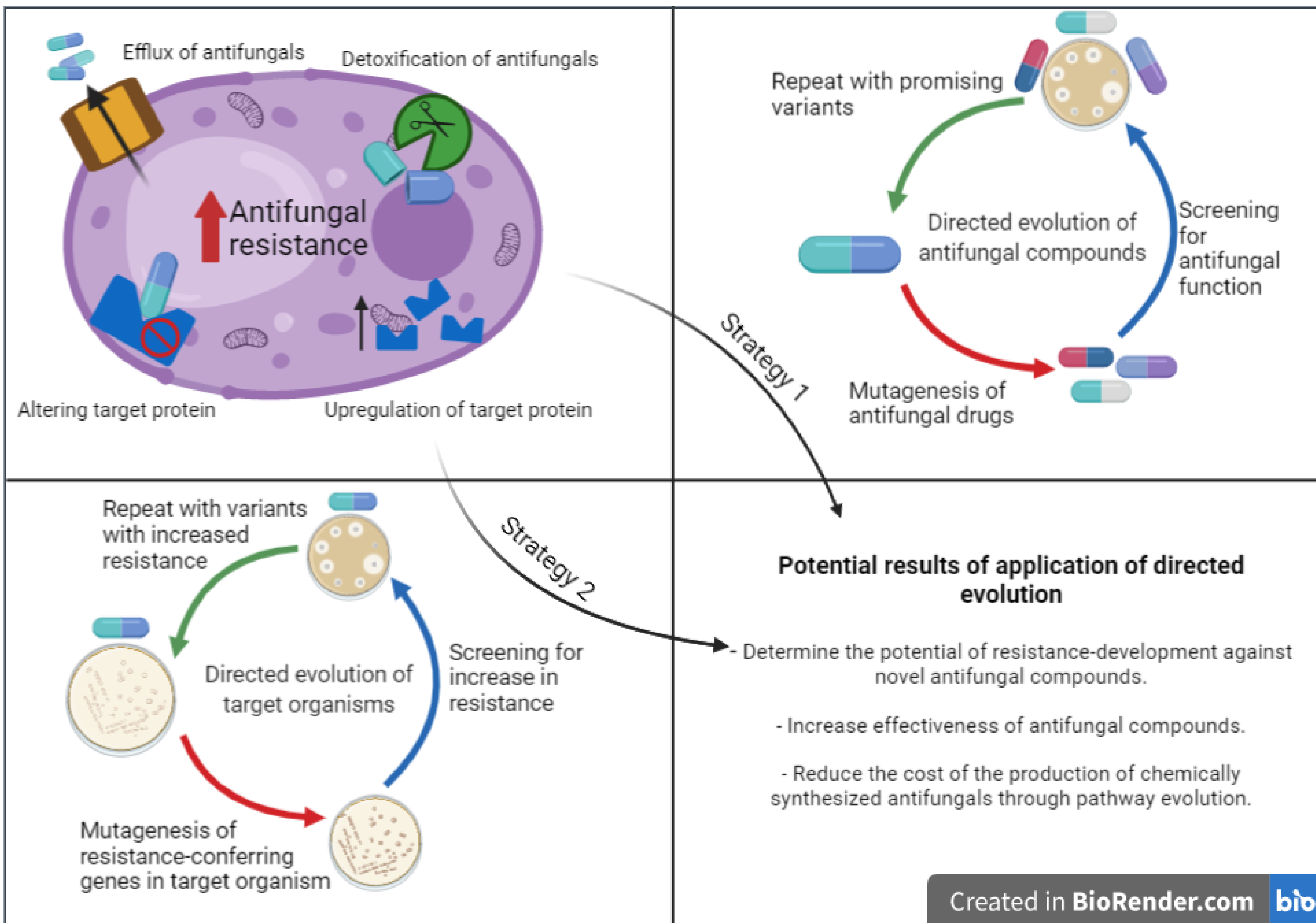

Simplified graphical summary of this review paper. The four causative mechanisms of antifungal resistance are shown. Additionally, two potential strategies of the use of directed evolution are shown. These strategies aim to enhance our efforts against pathogenic fungal species and their rampant increase in antifungal-resistance.

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Abstract

This review paper will explore different ways in which directed evolution can be used to combat the observed increase in antifungal resistance. In order to do this comprehensively, I describe the fungal kingdom, characteristics of different antifungal groups and the rise of antifungal resistance. After sketching the current fungal landscape, I will introduce directed evolution. In addition to exploring the general concept of directed evolution, its different methods, strengths and shortcomings will be discussed. Within the context of antimicrobials, examples of previous applications of directed evolution will be examined. Additionally, the potential for future uses of directed evolution within this field will be explored extensively. Conclusively, this review paper will explore the previous applications and future potential uses of directed evolution within the context of combatting antifungal resistance.

Fungi

Fungi are defined as *"eukaryotic, spore-bearing, achlorophyllous organisms that generally reproduce sexually and asexually and whose usually filamentous, branched somatic structures are typically surrounded by cell walls containing chitin or cellulose, or both of these substances, together with many other complex organic molecules."* It is estimated that there are about 1,5 million different species of fungi. [1,3]

Fungi can grow on a remarkably wide variety of different surfaces, and show the ability to colonize plants, animals and humans. In agriculture, pathogenic fungi cause losses of up to 30% of crop yield annually. Additionally, an increase of fungicidal resistant strains is observed, worsened by the use of genetically similar mono-crop cultures. As colder climates start to heat up due to climate change, pathogenic fungi are able to thrive more broadly across the globe. [4]

In the medical world, pathogenic fungi can cause severe and sometimes fatal diseases. Annually, more than 1 million people are blinded by fungal keratitis, one billion people suffer from skin mycoses, over 10 million people suffer from serious airway diseases and approximately 1,6 million people die. In general, filamentous fungi often cause disease in a healthy population. Whereas yeasts are more opportunistic and are commonly encountered in immunocompromised patients. Additionally, fungal spores are often the cause of allergic reactions in humans. [2,3,5,6,43]

Antifungals

To combat these aforementioned problems, antifungal drugs are used. Most antifungals in nature are found as low-molecular-weight compounds, proteins and peptides. Hundreds of antifungal compounds are found in nature. [2]

However, developing antifungals is a complicated task, as fungi are closely related to plants and humans. Therefore, many potential drug targets within a fungus are also found in plant or human hosts, posing a high host-toxicity risk. The main cellular component in which fungi differ from plants and humans is the fungal cell wall, which primarily consists of mannoproteins, β(1,6) glucan, chitin and glycophosphatidylinositol. This cell wall is a common target that antifungals are used against, either its biosynthetic pathway or directly. [2,6]

Classes of antifungal drugs

Four molecular classes of antifungals are in use which targets three different metabolic pathways: fluoropyrimidine analogs, polyenes, azoles, echinocandins. Their origins, properties and modes of action will be discussed.

Fluoropyrimidine analogs

5-fluorocytosine and 5-fluorouracil are the two fluoropyrimidine analogs used as antifungals. They are synthetic structural analogs of cytosine and uracil, nucleotides in the DNA. They do not directly function as antifungal drugs, but are easily taken up by fungi. Through multiple enzymatic steps, they are converted into either 5-fluorouracill triphosphate or fluorodeoxyuridine monophosphate. 5-fluorouracil triphosphate inhibits protein synthesis, fluorodeoxyuridine monophosphate inhibits thymidylate synthase, halting cell replication. [3]

Polyenes

Polyenes are generally produced by the *Streptomyces* bacterium, even on an industrial scale due to economic reasons. They are produced in a known gene cluster within the *Streptomyces.* Polyenes are amphiphilic molecules built up out of a 20-40 carbon macrolactone ring bound to a d-mycosamine group. These drugs act on ergosterol, which is the main component of membranes in fungi. Due to its amphiphilic nature, it can cross the lipid bilayer, and form pores. Which in turn lyse the fungal cells.

Polyenes show a slight affinity for binding to cholesterol, the main component in mammalian outer membranes. Therefore, the use of polyenes is associated with side effects and these drugs are primarily used locally.

Only three polyenes are currently in use in medical practices, nystatin, natamycin and amphotericin B. Amphotericin B targets a broad range of fungal species. [3,6]

Azoles

Azoles are the most commonly used antifungal drugs in medical practices. Azoles are cyclic organic molecules with either two nitrogen atoms (imidazoles) or three nitrogen atoms (triazoles).

They target the biosynthetic pathway of ergosterol, the same molecule polyenes act upon. They inhibit the ergosterol biosynthesis by inhibiting the p450-dependent lanosterol 14-ɑ-demethylase. This leads to an increase in 14-ɑ methylated sterol molecules, which are toxic and fail to replace the function of ergosterol.

Slight inhibition of mammalian p450 is observed, which has caused toxicity. The newest generation of azoles have a greater affinity for the fungal p450 enzymes and show reduced toxicity. This new generation of triazoles also shows a broad range of antifungal activity.

Azoles are made chemically using organic chemistry. Which radically changes how directed evolution could potentially be used for azoles. This will be discussed under ‘applications for directed evolution’. [3,6,7,8]

Echinocandins

Echinocandins is the only novel group of antifungal drugs that have been introduced for the last 20 years. Three echinocandins are currently approved by the FDA. This portrays the need for efficient and effective ways of developing new antifungal drugs.

They are synthetic derivatives of lipopeptides, which are generally produced by members of the *Bacillus* genus.

Echinocandins function as noncompetitive inhibitors of β(1-3)-glucan synthase. This is an enzyme that catalyses the production of β(1-3)-glucan, which is an important molecule for upholding integrity and rigidity in the fungal cell wall. Inhibiting the production of β(1-3)-glucan causes the cell wall to destabilize, resulting in fungal cell lysis.

There are few side effects associated with the use of echinocandins. This is likely because of the molecular target echinocandins have. For fungi, the cell wall is necessary for survival. Yet there is no equivalence to the fungal cell wall in mammalian cells, which means that no off-target effects will take place. [3,6,7,9]

Protein and peptide antifungals

As previously mentioned, only four groups of antifungals are currently widely used in agriculture and medicine. However, hundreds of antifungal peptides are known to exist in nature, with novel antifungals being discovered daily.

As will be discussed later, the principle of directed evolution is most straightforward when used on peptide-based antifungal compounds. Another prominent way in which directed evolution could be applied is in antifungal compounds that are produced by engineerable biosynthetic pathways, as these can be engineered at the pathway level. Which will be discussed in more detail later. [2,33,34,37]

A short overview of the different classes of antifungal proteins and peptides will be given now. This group of antifungals could potentially be used as the ‘fifth’ group of antifungals. These peptides and proteins have been isolated from plant, fungal, bacterial, insect, vertebrate and invertebrate species alike. They vary widely in their modes of action. These range from pore formation, damaging ribosomes, inhibiting DNA synthesis and inhibiting the cell cycle of fungi. Many of these antifungals have a potency similar to that of most currently used antifungals in agriculture and medicine.

Plants have 11 different groups of pathogenesis-related (PR) proteins. These are called PR-1 up to PR-11 proteins, they have a wide range of molecular mechanisms that result in their antifungal properties, many of which are not yet fully understood.

Another group of antifungal peptides are the defensins, found across many different classes of organisms. Defensins can cause morphological changes in fungi, act fungistatic or have other unique modes of action.

Cyclyophillins are another group of antifungal proteins of which the mode of action is not understood, there are indications that they inhibit α- and β-glucosidases.

There is a group of glycine/histidine-rich proteins of which their antifungal mechanism is not understood.

Ribosome inactivating proteins (RIPs) is a group of antifungal proteins that depurinate rRNA, resulting in ribosomal damage. A topic of interest is how the organisms that secrete these RIPs prevent damage to their ribosomes.

Lipid Transfer Protein (LTPs) are a group of antifungals of which it is thought they form pores in the fungal cell membrane.

Killer toxins are a group of antifungals that have a broad and potent antifungal activity, their mechanism is based on halting cell wall synthesis, DNA synthesis, potassium channels and (1,3)β-glucan synthesis.

Protease inhibitors are effective against plant and animal pathogens, but their mechanisms are still largely unknown. [2,40]

The list of antifungal peptides is more extensive than portrayed here. Promisingly, some of these antifungal peptides are being tested in clinical trials currently. [2,40]

Developing new antifungals

In a manner similar to antibiotic resistance, antifungal resistance is on the rise. This calls for new and improved antifungal drugs. The arsenal of effective and safe antifungal drugs is limited in comparison to the wide availability of antibiotic drugs. This is due to the many similarities between fungi and mammalian and plant cells, resulting in a limited amount of molecular drug targets. Most current research is done towards finding molecular targets that are limited to fungi. This is done through various bioinformatics methods.

Traditionally, Identifying antifungal molecules was done by large screening assays of synthetic small molecules. This method has increased its efficiency in the past decades due to large high-throughput screening techniques.

More innovative methods have tried to identify antifungal activity in already approved drugs, which is economically more feasible. In addition, it is investigated whether FDA approved drugs can synergize with known antifungals and increase their effectiveness. [6,10]

In this review, it will be discussed whether *directed evolution* could be an effective method for developing or improving antifungal drugs, as to enter the arms race against antifungal resistance. Before I consider this directed evolution, it is necessary to discuss the rise of antimicrobial resistance, focussing on antifungal resistance.

Antifungal resistance

Antifungal resistance is defined as the growth of a pathogenic fungus being unaffected at a therapeutic concentration of the antifungal agents. [14]

Consequences of antifungal resistance

The rise of antifungal resistance is an increasing concern in both the medical and the agricultural world. Immunocompromised patients are at high risk of developing systemic fungal infections. While treatment options were limited, the accelerating development of antifungal resistance towards azoles is further diminishing our treatment options.

The monoculture of crops in agriculture are prone to developing antifungal-resistant fungi, due to being genetically identical. This intensive agriculture was formerly protected by the use of antimicrobials. As antimicrobial resistance is on the rise, this leads us to lose our ability to control fungal infections and to uphold food security. [12,13,14]

Mechanisms behind the rise and spreading of antifungal resistance

Fungi are adept at developing resistance towards antifungals. Multiple, multi-drug resistant pathogenic fungi already circulate. Generally, there are four known mechanisms by which fungi increase resistance against antifungals. [13]

The first is altering the target protein by acquiring mutations in the protein's gene. This can decrease the binding affinity of the antifungal to the target protein. For instance, this is observed due to the extensive use of azoles. The aforementioned lanosterol 14α-demethylase, which is the target enzyme for azoles, is known to mutate quite extensively. *Zymoseptoria tritici* is found to have over 30 mutations in CYP51a, the gene encoding for lanosterol 14α-demethylase [11,12,16].

The second mechanism of acquiring resistance is through upregulation of the target protein. Therefore, despite the presence of antifungals, some proteins will remain functional. This increases the observed resistance to antifungal drugs. This mechanism does not seem to be the main contributor to azole resistance. [11,16]

Thirdly, the upregulation of antifungal efflux pumps to decrease intracellular antifungal concentration is a known mechanism for acquiring resistance. This is observed in *Candida albicans, Candida Glabrata* and *Candida dubliniensis.* Upregulation of i.e. MDR1, CDR1, CDR2 causes an increase in antifungal resistance. [16]

The last known mechanism is based on detoxification of the invading antifungal compounds. This detoxification is done by enzymes such as cytochrome p450 and glutathione transferase. This latter mechanism is not a common cause of the increase in resistance in the fungi. [16,17]

The mechanism behind the high rate at which resistance is spreading in fungi is attributed to fungi having a short life cycle, sporulating extensively and long-distance spore dispersal. [11]

It was expected by many researchers in the field that, in absence of antibiotics, antibiotic resistance would diminish due to an associated fitness cost. This proved not to be the case, and even in absence of antibiotics, resistance lingered. This indicates that effectively combatting antibiotics/antifungal resistance in the future, will depend on developing new or more effective antifungals. [16]

Directed evolution

Directed evolution was initially introduced in 1967. The method is based on Darwinian principles. In which rounds of mutation and selection are experimentally set up, with the end goal of improving or altering biocatalysts and other cellular properties. Generally, the method operates by inducing genetic changes within a population of cells, after which the population is screened to identify the cells that evolved towards the desired characteristics. [18]

However, there is an intrinsic difference between natural evolution and directed evolution. Firstly, natural evolution often takes multiple selective pressures into the equation, whereas directed evolution has tightly defined and controlled selective pressures. Secondly, natural evolution merely strives for the survival of the organism, whereas directed evolution can result in functions that do not necessarily have a practical use and can be developed by intelligently selecting in the screening phase. [18}

I will discuss the most common methods used in directed evolution, specifically focussing on their method of mutagenesis, applicability and their advantages and disadvantages.

In vitro methods of directed evolution

Error-Prone PCR

Error-Prone PCR is a technique that is often used for random mutagenesis induction in directed evolution experiments. During the Polymerase Chain Reaction (PCR), error-prone DNA polymerase is added, which randomly induced mutagenesis in the amplicon. Following this mutagenesis, the mutated DNA fragments are digested using restriction enzymes, separated on an agarose gel, ligated into a vector and finally, they are transformed into a competent host strain. These host strains can then be screened for their phenotypes. Host strains can be diverse and range from *Eschericha coli* to *Saccharomyces cerevisiae*. [20,21]

Using this technique, an increase in ceftazidime resistance of *E.Coli* was observed. The gene encoding for TEM-1 -lactamase was subjected to error-prone PCR and was scanned for resistance-conferring mutants. All 7 variants that showed an increase in resistance had similar mutations in the substrate-binding domain. These mutations are also known to occur in practice in ceftazidime resistance. This method has also been used to increase the thermostability of lipases and altering the substrate specificity of a glutaryl acylase to an adipyl acylase. [20]

The method is straightforward to use and does not require any specific or expensive equipment. The mutational rate can be tweaked by adding Mn^{2+} or dinucleotide analogues. This makes it a popular option for inducing random mutagenesis. [20}

The downsides of this method is the mutational bias, codon bias and amplification bias observed in these experiments. [19,20]

DNA shuffling

Evolution in nature not only occurs following mutations, there are other mechanisms by which genes can change. One such mechanism is through recombination, of which 4 different methods are known. [20,22]

The DNA shuffling technique capitalizes on this way of evolution by recombining different variants of genes with each other. These different variants all have random point mutations. A DNase is used to randomly restrict a gene, resulting in a variety of fragments of the target gene with slightly different genetic codes. Subsequently, an annealing reaction is run without primers, causing the different fragments to self-prime in regions of homology. The DNA polymerase extends the annealed fragments. This is repeated for multiple rounds until a full-length gene is formed. These are then amplified using PCR. These homologous recombinant genes can then be screened for their functionality.

This method is easy to use and works on a similar timescale to error-prone PCR. However, when DNA shuffling is used in regions where there is a low DNA sequence homology, the efficiency decreases. Additionally, the resulting full-length sequences tend to be limited in their diversity. [20,23]

Staggered Extension Process (StEP)

This method of generating a diverse library uses a range of similar DNA sequences of a target gene. These sequences are used in a PCR reaction without primers and a short extension cycle. This is to ensure that after self-priming of the DNA, sequences are only partly extended. After a short extension cycle, they are denatured and bind to a different DNA fragment, to be partially extended further. Resulting in a variety of chimeric sequences. To prevent a mutational bias to occur, two DNA polymerases with opposite mutational spectra are used.

The disadvantages of this method are that it requires the full length of the DNA sequence as a template before the reaction can work. Additionally, for this method to work, there should be sufficient sequence overlap between the different variants of the DNA sequence to allow for the priming to occur efficiently and accurately. [20,23,42]

In vivo methods of directed evolution

Directed evolution with random genomic mutations (DIvERGE)

A relatively new method, specifically designed for identifying mutations that can confer antibiotic resistance, is the DIvERGE (directed evolution with random genomic mutations) method. This *in vivo* method is developed to assess if resistance develops against certain antimicrobials, and at what rate. By testing this early in clinical trials, resources can be saved by assessing the potential of long-term use of an antimicrobial.

The researchers behind this theory state that the usual fluctuation tests and serial passage experiments are too slow and therefore very limited in their scope of searching for resistance towards antimicrobials. More recent in vivo techniques have increased their speed but lack in controllability, maximum target region length, show a mutational bias and have limited throughput.

The DIvERGE method has broad and well controllable mutagenesis spectra, allows for high mutation rates of the targeted sequences, allows for a multitude of rounds of mutation and selection, is compatible with a wide range of host species (including fungi) and is cost-effective.

It is based on binding a pool of softly mutated oligonucleotides to the target sequence on the genomic DNA of the host species. These oligonucleotides will induce mutagenesis in the target DNA. This will result in a broad library of diversely mutated host genomes in the target DNA. This cycle is repeated multiple times to further amplify the diversity. This can increase the natural mutation rate by a factor of a million.

Most mutationally driven acquired resistance is dependent on single mutations since simultaneous mutations rarely occur in nature. The DIvERGE method was tested for its efficiency in determining single point mutations that would increase resistance to trimethoprim, an antibiotic compound. 17 mutations of which the majority was also encountered in clinical isolates were expected to develop using DIvERGE. Seven new mutations were predicted to arise under extensive evolutionary pressure of trimethoprim. This study proposes to use DIvERGE to filter out the antimicrobial compounds to which resistance is likely to develop.

The disadvantages of this technique are that the causative genes in resistance have to be known before performing mutagenesis since oligonucleotides have to be designed. Additionally, the method is currently not compatible in working with plasmids, which sometimes also can confer resistance. [19]

TY1 retrotransposon continuous evolution

The TY1 retrotransposon based *in vivo* method is mechanically radically different from the DIvERGE method. It is based on a natively present system in multiple yeast species and uses error-prone reverse transcriptase as its base mechanism for inducing mutations.

The mechanism of this *in vivo* continuous evolution (ICE) technique works via a tunable amount of mutational rounds. Initially, the genetic sequence in question is cloned into an inducible TY1 retrotransposon. When the TY1 retrotransposon is induced, it is transcribed, as is a reverse transcriptase. Consequently, the TY1 element, including the

genetic sequence of interest, is reverse transcribed in a mutational manner by the reverse transcriptase. This causes a slightly mutated version of the TY1 element in the coding DNA form to be re-integrated into the DNA, after which screening can be done. These cycles can be repeated to further mutate the target sequence.

This method allows for mutagenesis of DNA sequences up to 5kb, which means that it can be used to target an entire genetic pathway. Additionally, a mutation rate of 0.15/kb was observed, meaning ~1.6 x $10^{\wedge 7}$ mutants per litre of inoculated medium. Which is the highest *in vivo* library creation potential reported. The method was tested for its effectiveness in performing directed evolution in enzymes, regulatory proteins and entire pathways; it proved to be functional in all three cases. [24]

Orthorep

Orthorep is another *In vivo* technique, which allows for the continuous evolution of target genes. This method is based on transforming an orthogonal plasmid into a host. This plasmid functions orthogonally with regard to the host. This means that this plasmid system is replicated independently of the genomic DNA.

The replication of the orthogonal plasmid is done by an error-prone orthogonal DNA polymerase, resulting in a steady and well-definable mutation rate. Due to the orthogonal nature of this system, these mutations will stay limited to the plasmid carrying the gene(s) of interest. The host cell will grow and replicate in its conventional manner, while the orthogonal plasmid is continuously being evolved. Through screening for the desired traits of the host, mediated by the genes on the orthogonal plasmid, a method for continuous directed evolution is developed.

This method was tested using *S.cerevisiae as a host.* The TRP5 gene was deleted from the host genomic DNA, causing the host to rely on the *tm*TRPB gene, an ortholog present on the orthogonal plasmid. Through the use of error-prone DNA polymerase, the *tm*TRPB gene was evolved for more than 100 generations of *S.cerevisiae,* which ensured a great deal of diversity and evolutionary depth. [41]

Applications of directed evolution

I have sketched the current fungal landscape, the necessity of antifungals and the different origins and modes of actions of antifungals. An introduction to the broad concept of directed evolution was given by introducing different methods which can be applied in this field.

Now, I shall explore applications in which directed evolution has been used within the antimicrobial context, and discuss its multitude of potential uses.

Predicting the rise of resistance against antimicrobials

One of the ways in which this directed evolution could potentially prove very useful in the race against antimicrobial resistance is by predicting the development of resistance against novel compounds. The development of resistance against antimicrobials can stem from either alterations in an existing resistance gene or the activation of a previously hidden resistance gene that was present in the genome of microbes. [15]

The DNA shuffling technique explained earlier was used to increase the effectiveness of TEM-1 -lactamase by multiple thousand folds. However, this method did not accurately predict the natural evolution of resistance against antimicrobials. A method that effectively allows for this should have a mutational spectrum similar to the species against which the antimicrobial will be used, have a controllable mutagenic rate and be reproducible and easy to use. A method called the Barlow-Hall method meets these demands, it is a technique similar to error-prone PCR, which also takes into account the chronological order in which mutations arise. This technique was successfully used to predict in which way the TEM -lactamase family might evolve cefepime resistance.

This technique can also indicate whether certain resistance genes can develop further towards a higher antimicrobial resistance. This was the case in the aac-6'-Iaa gene. This gene, found in *Salmonella enterica* confers resistance to tobramycin and kanamycin and has not been shown to confer any resistance to gentamycin and amikacin. After multiple rounds of the Barlow-Hall workflow, it was concluded that no increase in resistance to these four compounds would develop. This indicates that there is no infinite room for improvement in resistance-conferring genes. [15]

This technique was applied to antibiotics, but similar assays could very well be compatible with antifungal compounds as well. To prevent investing in a compound to which resistance would arise quickly. [15,25}

As mentioned earlier, developing new antifungals is a challenging task, since there is a limited array of molecular targets that are not present in mammals. Thus, very few new antifungal compounds are introduced into the market. This decreases the potential for using directed evolution in this manner in antifungals.

However, as ultra-high-throughput screening methods are being introduced in the world of biology, the discovery of new antifungal compounds may also increase. And using directed evolution to predict the rate at which resistance evolves against these compounds could be used more broadly. [25]

Another potential application for predicting the development of resistance towards antimicrobials could develop in combination with peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs). PPMOs are synthetically made oligos with 6-sided morpholin rings conjugated by phosphorodiamidate linkages. These rings are attached to nucleotides and these nucleotides can bind to complementary sequences of mRNA for which they were designed. This blocks the translation of the mRNA and can therefore hinder protein translation.

Resistance to PPMOs was found to arise but was quickly dampened by altering the peptide to which the PPMOs were bound. [19,25,26]

It is suggested that these PPMOs should be used along with antimicrobials to increase their efficiency. If PPMOs can be designed to bind to antimicrobial resistance-conferring genes, the acquired resistance of the target pathogen will decrease drastically. Combining the application of PPMOs with the DIvERGE technique or the Barlow-Hall method to infer resistance-conferring genes before they arise, carries big potential. This way, PPMOs can be readily designed to counteract the development of an increase in resistance from a certain gene.

Directed evolution of antifungal compounds

Although the methods for discovering new antimicrobials have increased their efficiency in recent years, these methods will always be restricted. This is because novel antimicrobial compounds have already evolved in nature and thus cannot be further improved easily. Directed evolution might allow us to take control of this evolutionary progress of antimicrobials, to enhance and specify their antimicrobial activity. [27]

Directed evolution of antimicrobials produced by a phylum such as the *Actinobacteria* could allow us to modify antimicrobials such that resistance to them is reduced. Additionally, both unknown or unused pathways for the production of new antimicrobials could be upregulated, as could anti-resistance compounds be upregulated. [27,28]

The molecular basis for proposing this method is that in groups of antimicrobials, a similar ‘core scaffold’ is often observed. A variety of side chains are attached to this core, which strongly affects the compound’s antimicrobial properties. Therefore, by placing an evolutionary pressure on the antimicrobial-producing species to evolve their antimicrobials, this theoretically will happen through altering these side chains. This will allow for the antimicrobials to be altered and evolve without the need for rational design. [27]

The method relies on applying a carefully designed selection pressure to an antimicrobial producing strain. This pressure could be the growth of the antimicrobial producing strain in coculture with a different species with resistance against the producers’ antimicrobials. The evolutionary pressure applied could be competition for the availability of nutrients. This competition-based directed evolution can be used to create mutants of known antibiotics and discover new antimicrobial compounds as well. The *Streptomyces* family is thought to produce upwards of 100.000 different antibacterial compounds, of which only a small fraction is explored. Similarly, there are hundreds of natural antifungal peptides found in nature, which are currently being investigated for their potential use in agriculture and medicine. Therefore there is a large potential of using directed evolution in this manner. [2,30,31]

This choice for the producer strain can be both bacteria (lactic acid bacteria and propionibacteria are known to produce antifungal metabolites) and fungi (Multiple *Aspergillus* and *Penicillium* species are known to produce antifungal substances). If no producer strain can be found which results in the expected production of evolved antimicrobial compounds, gene clusters of biosynthetic pathways of antimicrobials can be horizontally transferred into a suitable host strain to analyse its evolution. [30,36]

It should be noted that non-peptide antimicrobials are not easily compatible with a method such as this. The aforementioned azoles are all chemically produced and therefore not suited for this method. While Echinocandins and polyenes are non-peptide compounds, they are produced naturally. Therefore, adaptations to the compound can arise through alterations in the enzymatic pathways producing them through this evolutionary pressure. Applying this pressure to enzymatic and biosynthetic pathways will be discussed in the next section. [2,30]

Applying directed evolution on enzymes and biosynthetic pathways

Directed evolution can straightforwardly alter peptide-based antimicrobials; by applying selective pressure, the DNA sequence encoding for the antimicrobial-peptide might evolve by acquiring mutations, deletions or additions.

A large part of the group of antimicrobial compounds consists of secondary metabolites, which are not necessarily proteins but are compounds processed by enzymes within natural species. By applying selective pressure to enzymes or entire enzymatic pathways to evolve the secondary metabolites they produce, novel antifungals could be produced. Despite the specialized catalytic role enzymes have, they often show the ability for catalyzing alternative reactions. Literature indicates that the best starting point for using directed evolution is a protein close to the desired result. This indicates that applying directed evolution on enzymes within a metabolic pathway can be an efficient way of evolving novel enzymes. Thus, this could significantly alter the metabolic pathway this enzyme functions in, and consequently, evolve antimicrobials. [32,34,35,37,41]

Several properties of enzymes have been altered using directed evolution; their catalytic activity, stability of the enzyme, their substrate specificity, and stereoselectivity. [33,38]

By using an *E.coli XL-1 red* mutator strain, the substrate specificity of an amine oxidase isolated from *Aspergillus niger* was subjected to directed evolution. The wild-type amine oxidase oxidized benzylamine. The experiment aimed to increase its specificity against α-methylbenzylamine. A seven-fold enantioselective increase was observed in the best variant. This indicates that through directed evolution, enzymes and enzymatic pathways can be altered by changing substrate specificity. [39]

Additionally, stereoselectivity was also shown to be susceptible to directed evolution. Stereoselectivity is a term regarding the reaction products of a reaction, in which the reaction products are stereoisomers. Stereoselectivity is a term used for the distribution of stereoisomers in a reaction. Directed evolution, using error-prone PCR and DNA shuffling, was shown to alter the stereoselectivity of a lipase from *Pseudomonas aeruginosa*. An inversion of the enantioselectivity from an (S)-selectivity towards an (R)-selectivity by a factor of 30 was observed. [33,34]

Another instance in which enzymes were evolved using directed evolution, was introduced in the *in vivo* Orthorep method. Using this method, a large library of variations of the *tm*TRB gene was made. Some of these variants showed increased thermostability and higher activity than the wild-type enzyme, whereas others showed that alternative catalytic functions performed by the enzyme were increased significantly. This strengthens the potential for directed evolution to improve and alter enzymes. [41]

An example of entire metabolic pathways being altered and tweaked using directed evolution can be seen in experiments within carotenoid producing pathways. Extensive research on the metabolic pathways of carotenoids has been done in recent years. Using error-prone PCR and DNA shuffling, a large library of mutations was made within the enzymes vital in carotenoid producing pathways. Through a combination of directing the evolution of enzymes and targeted implementation of enzymes within related pathways, more than 30 entirely new carotenoid compounds have been created that have never been observed before. Literature suggested that similar techniques could be applied to entirely unrelated pathways, which means this approach could have enormous potential within the field of antifungals. [34,37]

Previously, the production of carotenoids was done chemically. Directed evolution could allow researchers to produce carotenoids that are either difficult to synthesize chemically, difficult to isolate from natural sources or entirely new carotenoid compounds. If similar methods could be applied to the chemically produced azoles, the biologically produced echinocandins and polyenes, or peptide/protein antifungals, it could give a significant boost in our efforts for combatting antifungals. [2,34,37,38]

Discussion

In this review article, an overview regarding the current landscape of directed evolution in the context of antifungal resistance has been provided. To do this, the extraordinary kingdom of the fungi was briefly discussed, including the detrimental effects that pathogenic fungi cause in the agricultural and medicinal world.

The need and function of the four major groups of antifungals were discussed, which are the azoles, polyenes, echinocandins and fluoropyrimidine analogs. Additionally, antifungal peptides and proteins were posed as an untapped ‘fifth group’, and their many functionalities and origins were discussed.

An introduction to the alarming increase in antifungal resistance was provided, followed by the four major mechanisms which cause this increase in resistance. These mechanisms can be summarized as altering the target protein of antifungal, upregulating the target protein, upregulating antifungal efflux pumps and detoxifying antifungals.

Now that the issue of antifungal resistance was portrayed, and placed into the larger context of fungi and the interaction between fungi and humans and plants, directed evolution was introduced conceptually. The basic Darwinian principle of directed evolution was explained, which consists of rounds of mutagenesis and screening, as was the difference between directed evolution and natural evolution.

Next, the focus was shifted to different methods of conducting directed evolution in a laboratory. This was done by focussing on the primary methods of mutagenesis and explaining their advantages and disadvantages. Mutagenesis can be performed *in vitro*, by error-prone PCR, DNA shuffling, or staggered extension process. More advanced methods of directed evolution can be performed *in vivo*, such as the ‘directed evolution with random genomic mutations’ (DIvERGE) method, the ‘TY1 retrotransposon continuous evolution’, or the ‘Orthorep’ method.

Finally, previous applications of directed evolution in the field of antimicrobials were explored. Directed evolution to infer the potential resistance development against antifungal compounds was discussed. In which the DIvERGE method and the Barlow-Hall method showed promising predictory properties. Predicting the potential development of resistance against novel antifungals saves resources when performed in early clinical trials. Additionally, the potential use of combatting the emergence of resistance by using PPMOs combined with these predictory techniques was pointed out.

Directed evolution and its ability to improve existing antimicrobial compounds were explored. This application of directed evolution relies on making a coculture of an antimicrobial producing strain and an antimicrobial sensitive strain, in which these species are competing for the availability of nutrients. Using this method, an antimicrobial producing strain acquired the ability to produce a novel antibiotic, which is a promising result for further applications. A method such as this is explained to be restricted to antifungals that are produced by microbes, such as echinocandins and polyenes. The azoles are produced using synthetic chemistry and are less convenient as a target for this technique.

Lastly, directed evolution and its ability to alter enzymes and enzymatic pathways were explored. Enzymes show to have many ‘alternative’ catalytic functions, thus, they are suitable candidates for applying directed evolution to enhance these alternative functions.

Directed evolution has shown the ability to alter the stability, substrate specificity and stereoselectivity of enzymes.

Regarding the directed evolution of entire enzymatic pathways of antimicrobials, relatively little data is known. However, directed evolution has been applied to the well-understood carotenoid biosynthesis pathways, in which it shows enormous potential for altering the carotenoids produced. On top of that, more than 30 entirely novel carotenoids were produced that have not been made chemically.

The study on carotenoids concludes that directed evolution can be used for the production of carotenoids that are difficult to synthesize chemically, difficult to isolate from nature or entirely novel carotenoids. It is posed that these techniques can be applied to entirely different classes of compounds as well. This indicates that there is a big potential for its use for the production of (novel) azoles, echinocandins, polyenes and antifungal proteins and peptides.

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Graphical abstract was created with BioRender.com

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