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Sensitization of The Antibiotic Resistant M. smegmatis Bacteria Using CRISPR/FnCpf1 Gene Editing

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Abstract: The presence of antibiotic-resistant bacteria is a disease that is reported to cause the most deaths in the world in the coming years. Less and fewer antibiotics are being produced to kill antibiotic-resistant bacteria. This is because the antibiotic spectrum is about to expire. On the other hand, after these resistant bacteria infect our body, high doses and more effective antibiotics are used to break this resistance gained by the bacteria. However, since these antibiotics affect and kill our healthy microflora, there is a possibility that they may have side effects on our bodies. Mycobacterium tuberculosis bacterium causes Tuberculosis (TB), one of the oldest known human diseases, and it is still one of the most important causes of death, causing two million deaths each year. The resistance of M. tuberculosis bacteria causes the tuberculosis disease to become more insoluble. This thesis aims to show that antibiotic-resistant Mycobacterium smegmatis can be non-resistant with the CRISPR / Cas method in an in-vitro laboratory environment.

Key words: Gene editing, CRISPR/Cas, Mycobacterium smegmatis, antibiotics resistant, microflora, Mycobacterium Tuberculosis

1. Introduction

Mycobacterium tuberculosis is a human pathogen with an astonishing global impact (Talbot E et al., 2015a). 1.8 million people worldwide are thought to die from Human Tuberculosis (TB) each year, mostly in underdeveloped nations (World Health Organization 2016). Despite a recent reduction in TB incidence, 10.4 million new cases were envisaged in 2015, 0.48 million of which were multidrug resistant. Second-line drugs are needed, but they are ineffective, expensive, and toxic, and there is currently no advanced infrastructure for testing drugs for susceptibility, thus there is a need for these drugs, tuberculosis strains that are naturally resistant to many antibiotics are a significant global health concern (Gygli S et al., 2017).

Mycobacterium smegmatis is an environmentally friendly organism that does not mimic a human infection, budding directly and is commonly found in soil, water, and plants. It was determined that M. smegmatis was not pathogenic and developed faster than other Mycobacterium species. It is a Gram-positive bacterium defined by its dense cell-walled inner cell layer. With a higher Guanine-Cytosine content than Adenine-Thymine level, this bacterium possesses a distinctive property. M. smegmatis is an easy-to-study, doubling growth-rate bacterium, and is also a biosafety level-1 laboratory-requiring bacterium (T Joseph A S et.al, 2020). Therefore, M. smegmatis is shown as a valuable model for mycobacteria research due to its close association with M. tuberculosis. Since the 1940s, antibiotics, which are chemotherapeutic drugs, have been a very effective tool in the clinical therapy of bacterial illnesses. However, following the widespread appearance and dissemination of antibiotic-resistant bacteria, the advantages of antibiotics mostly vanished. Antibiotic resistance is seen in naturally occurring bacteria, even though it is obvious that indiscriminate and excessive use of antibiotics considerably contributes to the creation of resistant strains (Sengupta S et al., 2013). Antibiotic resistance is the capacity to withstand an antibiotic that gets rid of the bacteria or slows its growth. New antibiotic manufacture is required due to the rise of bacteria that are resistant to antibiotics in recent years.

New treatment options, such as antiviral tactics, bacteriophageal therapy, probiotics, therapeutic antibodies, and especially anti-resistance enzymes inhibitor synthetic inhibitor medicines, and oil acid biosynthesis, have been created to combat the issue of

antibiotic resistance (Schimmel et al., 1998; Su and Honek, 2007; Lock et al., 2008; Lu et al., 2009; Njoroge et al., 2009; Kohanski et al., 2010).

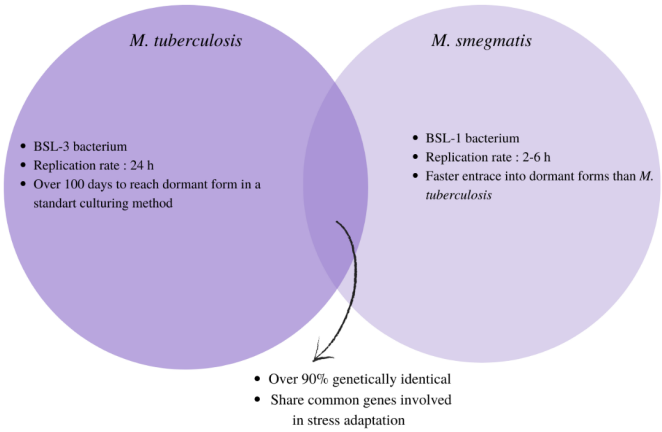

Figure 1: Differences and similarities between *M. tuberculosis* and *M. smegmatis bacteria*

However, these techniques have several drawbacks, including limited efficacy, off-target effects, and a lengthy production process for the new antibiotic (Linek et al., 2012; Nerys-Junior et al., 2018). An alternate strategy that was more focused and precise was now required. This technique uses the CRISPR/Cas system, which is a revolution in recombinant DNA technology. The CRISPR/Cas system is an essential part of the adaptive immune system created by bacteria and archaea to protect themselves against foreign DNA such as plasmids or phages that obliterate the foreign genome. Studies against antibiotic resistance, a serious issue of late years, utilise the CRISPR/Cas system. None of the previous approaches could match the gene editing system used by the CRISPR/Cas technique's speed, simplicity, great modification potential, and cost efficiency. When a foreign virus or piece of plasmid DNA enters a cell, the fundamental workings of the CRISPR/Cas system begin to operate. The CRISPR sequence is added upon when foreign nucleotides are identified by the Cas complex and broken up into pieces of around 30 base pairs in length. It has previously met tiny foreign DNA pieces from viruses or plasmids in this sequence. The guide RNA-focused area with repeated genes can accept foreign oligo DNA containing a PAM sequence. To create CRISPR RNAs, Cas proteins express and process the CRISPR region (crRNAs).

The cell now attempts to repair the damaged DNA after realizing that it has been damaged. In thesis, is aim to demonstrate that antibiotic-resistant *M. Smegmatis* bacteria can be made non-resistant with the CRISPR / Cas system. If I achieve this goal, I aim to use CRISPR as an antimicrobial agent against antibiotics or as an alternative to antibiotics in the future and to kill only the targeted antibiotic-resistant bacteria without harming the healthy bacteria in our microflora.

Resistance of Antibiotics

While the developments and successes in the medical world increased with the antibiotic revolution, over time, as a result of the wrong and excessive use of antibiotics, the development of resistance in bacteria caused a threat to the success of the antibiotic age. Towards the end of the 1900s, strains with low multidrug resistance began to emerge in pathogens frequently isolated from hospital infections such as Staphylococcus aureus and enterococci (Cöleri A, et al., 2008). Bacteria can resist the lethal or anti-proliferative effect of antibiotics and develop resistance with the biofilm they form (Donlan, R.M., 2008).

With the frequent misuse of antibiotics, the resistance selectivity of bacteria to antibiotics is increasing day by day. Infectious diseases that arise and re-emerge in the event of an increase in antibiotic resistance, and the lack of development of new classes of antibiotics are of concern. The Centers for Disease Control and Prevention (CDC) reported that between 2011 to 2020, there was a 23% decrease in the overall human use of antibiotics in the EU/EEA. The worrying is the increase in resistance to critically important antibiotics utilized to treat common diseases. Also, according to the CDC, millions of people are infected with antibiotic-resistant bacteria. More than 48,000 people die each year as a result of these infections in the U.S. (https://www.cdc.gov/drugresistanc, 2019). According to the WHO 2014 report, antimicrobial resistance could lead to up to 10 million deaths per year worldwide by 2050 unless serious action is taken. If no action is taken, it can cause economic losses of up to 100 trillion USD per year (O'Neill, J., 2016; Scarafile, G., 2016; Chokshi, A., et al., 2019). With the increase in resistance, treatments become more and more difficult. In addition, the treatments applied to require alternative drugs, which are more expensive and in high doses.

It has been determined that there are many resistance mechanisms in bacteria, and this resistance can occur with a single mechanism or with the combination of more than one mechanism. Resistance mechanisms in bacteria are examined under 2 main headings (Davies, J., et al., 2010).

Intrinsic Resistance

As a result of the studies carried out by the Central Research Institute of Agricultural Protection in the last three years, significant economic losses caused by WDV have been recorded in wheat and barley production areas, especially in the Central Anatolia Region (Ankara, Eskisehir, Kayseri, Kirsehir, Konya, Nevsehir, Sivas, Yozgat), Aegean Region (Afyon, Kütahya) and Marmara Region (Edirne, Kirklareli, Canakkale). In the Central Anatolian Region, in the areas where WDV has been detected in

recent years, it has been determined that there is a 20-90% yield loss in April with the decrease of the average precipitation compared to normal, the low temperatures in May and the effect of agricultural frost events.

Table 1: Examples of bacteria with intrinsic resistance (Reygaert WC., 2018).

Organism	**Intrinsic resistance**
Bacteroides (anaerobes)	aminoglycoside, many β-lactams, quinolones
All gram positives	aztreonam
Enterococci	aminoglycoside, cephalosporins, lincosamides
Listeria monocytogenes	cephalosporins
All gram negative	glycopeptides, lipopeptides
Escherichia coli	macrolides
Klebsiella spp.	ampicillin
Serratia marcescens	macrolides
Pseudomonas aeruginosa	sulfonamides, ampicillin, 1st and 2nd generation cephalosporins, chloramphenicol, tetracycline
Stenotrophomonas malrophilia	aminoglycosides, β-lactams, carbapenems, quinolones
Acinetobacter spp.	ampicillin, glycopeptides

Acquired Resistance

The condition of not being affected by an antibacterial agent to which bacteria are sensitive due to changes in their genetic characteristics is called acquired resistance. Acquired resistance mechanisms can vary in various bacterial species, and acquired resistance mechanisms are mainly studied biochemically and genetically. A bacterial strain can acquire resistance by one of the resistance mechanisms, or it can acquire resistance by more than one mechanism, and the diversity may vary depending on the nature of the antibiotic, the target region, the bacterial species and the presence of resistance plasmid or chromosomal mutation (Ciftci, A. et al., 2015).

Chromosomal Mutations Resistance

They are resistances that arise as a result of spontaneous mutations in the chromosome of bacteria. Chromosomal mutations can occur with some physical and chemical factors. Structural changes occur in the bacterial cell as a result of chemical and physical factors, but the resistance does not spread to other types of bacteria. Rifampin, isoniazid, quinolone resistance and methicillin resistance in staphylococci are examples of resistance that develop with chromosomal mutations and are important in medicine (Cesur. S., et al., 2013).

Resistance Due to Plasmids

Plasmids are extra-chromosomal DNA fragments that exist in bacteria before antibiotic administration and can replicate independently of the chromosome. Resistance due to plasmids is the main principle of resistance seen in the medical world. The resistance called R (resistance)-plasmid carries resistance genes against one or more antimicrobial drugs and heavy metals (Yüce., A., 2001).

Transposons Dependent Resistance

Transposons are DNA sequences that can move from chromosome to plasmid and from plasmid to chromosome. These DNA sequences, which cannot replicate independently, are responsible for the development of resistance to ampicillin, chloramphenicol, kanamycin, tetracyclines and trimethoprim. Transposons have a major role in the emergence and spread of multidrug-resistant strains. Genetic material and plasmids carrying resistance genes are transferred from one bacterium to another by mechanisms such as transduction, transformation, conjugation and transposition (Yuce A., 2001).

Mechanisms Of Resistance Against Antibiotics

Antimicrobial resistance mechanisms are divided into four main groups. These are limiting the uptake of a drug, modifying a drug target, inactivating a drug and active drug efflux. Limiting drug uptake, drug inactivation, and drug efflux are examples of intrinsic resistance strategies. Drug target modification, drug inactivation, and drug efflux are examples of acquired resistance strategies. The types of mechanisms utilized by gram-positive and gram-negative bacteria differ due to structural variations. All four major processes can be used by gram-negative bacteria, whereas gram-positive bacteria less frequently limit drug uptake and lack the ability to use some drug efflux mechanisms. (Chancey, S. T., 2012; Mahon CR et al., 2014).

Limiting Drug Uptake

Gram-negative bacteria have intrinsic resistance to some groups of strongly antimicrobial drugs because of the structure and workings of the LPS layer, which acts as a barrier to some types of compounds (Blair, J.M., et al., 2014). Since *Mycoplasma* and related bacteria don't have a cell wall, they are inherently immune to all drugs that target the cell wall, such as β-lactams and glycopeptides (Bébéar, C.M., et al., 2005). Porin channels are often how substances enter the cells of these bacteria with thick outer membranes, and in gram-negative bacteria, porin channels typically permit access to hydrophilic molecules (Blair, J.M., et al., 2014; Gill, M.J., et al., 1998). Porin alterations can restrict and limit drug uptake in two primary ways. These are

reductions in the number of available porins and mutations that alter the selectivity of the porin channel (Kumar, A., et al., 2005). When a pathogenic organism forms a biofilm, the bacteria are shielded from the host immune system's onslaught. Additionally, the development of biofilms offers a defence against antimicrobial substances. The closeness of bacterial cells, which is antimicrobial resistant, is an important finding regarding biofilms that facilitates horizontal gene transfer. This implies that certain bacterial populations may find it simpler to share genes (Mah, T.F., 2012; Soto, SM., 2013; Van Acker, H., et al., 2014).

Modification of Drug Targets

Antimicrobial medications may target several different parts of the bacterial cell, and the bacteria may also modify a number of those targets to confer resistance to the drugs. Modifications in the structure or quantity of penicillin-binding proteins are one mechanism of resistance to the β -lactam medicines, which are virtually exclusively employed by gram-positive bacteria. PBPs are transpeptidases that are involved to build peptidoglycan into the cell wall (Reygaert, W.C., 2009; Beceiro, A., et al., 2013). Ribosomal mutation, and ribosomal subunit methylation, most frequently involving erm genes or ribosomal protection can all lead to resistance to medicines that target the ribosomal subunits and the drug's ability to attach to the ribosome is hindered by these processes (Kumar, S., et al., 2013; Roberts, M.C., 2004). Resistance to medications that block metabolic processes occurs when folate biosynthesis pathway enzymes undergo mutations, or when resistant Dihydropteroate synthase and Dihydrofolate reductase enzymes are produced in excess. Resistance to medications that block metabolic processes occurs when folate biosynthesis pathway enzymes undergo mutations, or when resistant DHPS and DHFR enzymes are produced in excess. Because the sulfonamides and trimethoprim are structural analogues of the natural substrates, they attach to the corresponding enzymes. These medications work by competitive inhibition by attaching to the enzymes' active site. Most typically, these enzymes' active sites undergo mutations, leading to structural alterations in the enzyme that prevent drug binding while still enabling natural substrate to attach (Huovinen, P., et al., 1995; Vedantam, G., et al., 1998).

Drug Inactivation

There are primarily two ways that microorganisms render drugs inactive. These techniques involve the drug's real degradation or the addition of a chemical group. A very wide set of enzymes that hydrolyze drugs are known as β-lactamases. Tetracycline, through the tetX gene, is another medication that can be inactivated by hydrolyzation (Yang, S.J., et al., 2009; Blair, J.M., 2015). Most frequently, acetyl, phosphoryl, and adenyl groups are transferred to the medication to inactivate it. Acetylation is the mechanism with the widest use, and it has been utilized to combat aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones (Blair, J.M., 2015; Schwarz, S., et al., 2004).

Drug Efflux

Efflux pump genes are chromosomally encoded in bacteria. The main purpose of efflux pumps, which transport a wide range of chemicals, is to cleanse the bacterial cell of hazardous toxins. The available carbon source affects the resistance capacity of several of these pumps (Villagra, N.A., et al., 2014; Blair, J.M., et al., 2014). The five primary families of bacterial efflux pumps are categorized according to structure and energy source as follows: ATP-binding cassette family, multidrug and toxic compound extrusion family, small multidrug resistance family, major facilitator superfamily, and resistance-nodulation-cell division family. (Piddock, L.J., 2006; Poole, K., 2007). Fluoroquinolone efflux pumps, members of the MATE families and MFS families, and other efflux pumps may transfer intrinsic resistance to gram-positive bacteria due to their chromosomal encoding. Known plasmid-borne gram-positive efflux pumps are also present. The MFS family now makes up the described pumps in gram-positive bacteria (Jonas, B.M., et al., 2001; Truong Bolduc, Q.C., et al., 2005). The majority of clinically relevant efflux pumps are found in the RND family, however, they may be found in all five families of gram-negative bacteria (Blair, J.M., et al., 2014; Kourtesi, C., et al., 2013).

Mycobacterium Family

Mycobacteria are the only genus in the Mycobacteriaceae family and have a cell wall rich in mycolic acids and *mycosides*. They are also aerobic, immobile and Gram-positive rods. Mycobacteria (RGM), which grow under the pressure of oxygen, are ubiquitous in the environment. They can be isolated from soil and water and show visible growth in a culture medium within seven days. RGM includes *Mycobacterium chelonae-abscessus, Mycobacterium fortuitum* and *Mycobacterium smegmatis* groups (Quinn, P.J., et al., 1994).

Table 2: Reproduction rates of the Mycobacterium family (Durupınar B., 1996).

Group	**Types**
Slow Reproducing	*M.gordonae,M.asiaticum, M.gastri,M.terrae-triviale kompleksi*
Moderately fast Reproduced	*M.flavescens*
Fast Reproduced	*M.thermoresistibile,M.smegmatis, M.parafortuitum kompleksi, M.vaccae, M.phlei*

Mycobacterium smegmatis

The bacterium *Bacillus smegmatis* was first isolated from the syphilitic chancre by the scientist Lustgarten in 1884 (Trevisan, V., 1889). In 1889, scientists named Lehman and Neuman named the species isolated in 1884 *Mycobacterium smegmatis. M. smegmatis* are gram-positive bacteria commonly found in soil, water and plants (McCune, R.M., et al., 1966). *M. smegmatis* are known as a saprophytic group that is not pathogenic, rarely causes disorder and is not susceptible to living in mammals. It has been found to develop faster than other Mycobacterium species, as it begins to develop in about 48 hours (Newton, G., et al., 2002). In addition, *M .smegmatis*, an aerobic organism that donates the last electrons to oxygen during respiration, will obtain the maximum amount of energy by oxidative phosphorylation (T, J.A.S., et al., 2020).

Although *M. smegmatis* is a Biosafety Level 1 (BSL-1) microorganism, opportunistic infections have been reported. Also, partial 16S rRNA gene sequencing was used to identify phylogenetic relationships among mycobacterial species (Rogall, T., et al., 1990).

Cell Structure and Metabolism

M.smegmatis is a Gram-positive bacterium, which is indicated by the inner cell layer with a dense cell wall whose Guanine-Cytosine content is higher than Adenine-Thymine content. Cell division has a different character from other gram-positive microbes as it consists of mycolic acids, unsaturated fats normally found in slide microorganisms. *M.smegmatis* bacteria do not have motility and endospore regulation (Megehee, J.A., et al., 2007; Weber, I., et al., 2000; Park, S.W. et al., 2003).

Ecology and Pathology

Biofilms formed by *M. smegmatis* can use sterols from plants as a carbon source. Microorganisms will use androstenedione. If *M. smegmatis* uses the wide channel where it is found, the compound will have produced the asteroid hormone androstenedione (McLachlan, J.A., 2001). *M. smegmatis* is not persistently found in any mammal and is not a cause of contagious disease or infection, but some diseases can replace superficial cases. There are enormous types of disease-causing organisms. There are many varieties of *M. tuberculosis, Mycobacterium bovis and Mycobacterium leprae,* which are highly pathogenic. These pathogenic organisms will stay in the host for a long time, such as TB, disability, etc. why could it be. There are a few possible pathogens outside the host state, such as *M. avium*, which are harmful when downsized by the host (T, J.A.S., et al., 2020). *M. smegmatis* caused the death of an eight-year-old Italian boy named Tyke. As a result of the examination of heredity on the child, it was observed that a drastic change of four nucleotides occurred. However, although *M. smegmatis* is generally considered non-pathogenic, it has shown typical pathogenic characteristics in the juvenile's genome, and it was also discovered that a harmful microorganism has two mutant alleles (Branca, A.A., et al., 1981).

Mycobacterium tuberculosis

In 1993, the World Health Organization designated the deadly illness known as human tuberculosis (TB), which is brought on by *Mycobacterium tuberculosis*, as a worldwide health emergency. With an estimated about 2 million fatalities each year, mostly in underdeveloped countries, TB is still one of the deadliest infectious illnesses (World Health Organization 2016). Despite a drop in TB prevalence over the previous few decades, an estimated about 11 million new cases were reported in 2015. 0.48 million of these were brought on by *M. tuberculosis* strains that were deemed to be multidrug-resistant. The treatment of multidrug-resistant *M. tuberculosis* strains, a significant global health concern, necessitates the use of second-line drugs, which are less effective, more expensive, and toxic, and more complex infrastructure for assessing drug susceptibility, which is not always available in resource-constrained settings. TB patients brought on by MDR/XDR strains of *M. tuberculosis* have shockingly low treatment success rates (World Health Organization, 2016).

Streptomycin, the first efficient antituberculous medication, was found in 1944 (Schatz et al., 1944). The newly found medication was employed right away to treat TB patients. Many streptomycin-treated tuberculosis patients experienced health gains in the early months of treatment, only for their situations to deteriorate once more over time. It was rapidly determined that the development of resistant *M. tuberculosis* strains, which rendered streptomycin worthless, was the cause of this (Crofton et al., 1948). MDR TB variants emerged in several situations in diverse parts of the world. Different public health systems' levels of quality were a factor in the proliferation of drug-resistant *M. TB* strains, which is why now see an uneven distribution of the global prevalence of drug-resistant variant incidence rates (World Health Organization, 2016).

Drug resistance mechanisms in *Mycobacterium tuberculosis*

Mycobacterium members are well known for having built-in resistance to a variety of antibiotics. This has mostly been attributed to the cell envelope, which is particularly thick and lipid-rich (Jarlier, V., et al., 1994). Some antibiotics may be structurally changed or cleaved enzymatically after entering the cell membrane, rendering them useless (Chambers, H.F., et al. 1995; Quinting, B., et al. 1997; Wang, F., et al., 2006; Warrier, T., et al. 2016). The apparent lack of continuing horizontal gene transfer is another feature of *M. tuberculosis* (Cole S.T et al. 1998; Gagneux S et al., 2007; Bolotin E et al., 2015). Although horizontal gene transfer between "species" of the genus Mycobacterium has been demonstrated. Although shown, it does not seem to make a significant contribution to the development of antibiotic resistance in *M. TB* (da Silva Rabello et al., 2012). In

M. tuberculosis, chromosomal changes can explain the great majority of drug-resistance phenotypes. Due to their frequently lower pathogenicity and lower biosafety requirements and quicker growth characteristics than *M. tuberculosis*, several investigations on a variety of other mycobacterial species have been conducted to investigate the causes of treatment resistance. The most often employed model is the environmental mycobacterium *M. smegmatis*, whose genome is around 1.5 times larger than that of *M. tuberculosis*.

The Mycobacterial Cell Wall and Drug Penetration

The unusual structure of the mycobacterial cell membrane has frequently been cited as the cause of the mycobacteria's innate resistance to a variety of medications. Because they contain a range of lipids, including mycolic acids, the family of the *Mycobacterium* have significantly thicker and more hydrophobic cell walls than other gram-positive bacteria. Numerous investigations carried out on numerous mycobacterial species revealed that the low compound permeability of the cell envelope is substantially influenced by the nature of the cell envelope and the modest amount of porins (Jarlier, V., et al., 1994; Brennan, P.J., et al.,1995; Nguyen L et al., 2009; Sarathy J.P et al., 2012; Mailaender C et al. 2004). The cell wall's lipid-rich composition makes it very hydrophobic and hinders the passage of hydrophilic substances. Small hydrophilic substances, such as many medications that are effective against *M. tuberculosis*, are believed to be able to enter cells exclusively through water-filled porins (Mailaender, C., et al. 2004).

The unique properties of the mycobacterial cell membrane prevent the diffusion of hydrophobic compounds, such as certain antibiotics from the macrolide, rifamycin, tetracycline, and fluoroquinolone groups (Brennan, P.J., et al., 1995). The hydrophobicity of the molecules does appear to have some bearing on the rate of diffusion, with hydrophobic compounds diffusing more readily across the mycobacterial cell membrane (Rastogi, N., et al., 1990; Nikaido, H., et al.,1993). Studies using mutants deficient in lipid production, which are vulnerable to medicines that the matching wild-type strain is resistant to, further support the idea that the cell envelope lipids are a key element in the intrinsic resistance of mycobacteria to many hydrophobic antibiotics (Liu, J., et al., 1999).

Drug inactivation by *Mycobacterium tuberculosis*

Antibiotics may be cleaved enzymatically after entering the cell wall as the first line of defense and becoming useless to them. The enzymatic degradation of β -lactam antibiotics by β -lactamases, which hydrolyze the β -lactam ring of the antibiotics, is one of the most noteworthy examples. Penicillin-based early studies revealed that *M. tuberculosis* is innately resistant to this class of antibiotics. (Abraham E.P et al. 1941). Only the M. tuberculosis genome encodes the BlaC class A β - lactamase, which is thought to localize to the periplasmatic area and can either be unbound or lipoprotein-anchored in the plasma membrane's outer leaflet. There has been a resurgence of interest in using β -lactam antibiotics to treat TB as a result of the rise in cases brought on by MDR/XDR *M. tuberculosis* strains. Further research is still required to determine the real benefit of β -lactam antibiotics in the treatment of drug-resistant *M. tuberculosis* strains. Given the good results of multiple studies, the shown safety profile of β -lactam antibiotics or β -lactamase inhibitors, and the lack of viable treatments for MDR/XDR TB, more research into treatment regimens involving this class of antibiotics is necessary. In addition to drug breakdown, antibiotics can also be rendered inactive by modification, such as methylation or acetylation. The mechanism of drug inactivation by chemical alteration in M. tuberculosis that has received the greatest attention to date involves Eis. Eis is the enhanced intracellular survival protein which is acetylated in various aminoglycoside/cyclic peptide antibiotics used to treat MDR TB. It is uncertain whether Eis overexpression alone causes capreomycin resistance at clinically important levels (Kambli, P., et al. 2016). As a result of Eis overexpression, high-level aminoglycoside/cyclic peptide resistance could develop.

Acquıred Drug Resıstance in *Mycobacterıum tuberculosis*

The bulk of clinically significant drug resistance in *M. tuberculosis*, including its resistance mechanisms of intrinsic, is brought about by chromosomal changes. These chromosomal alterations may impart varying degrees of resistance to drugs through a wide variety of different methods. There might be a variety of resistance mechanisms, depending on the antibiotic in the issue.

Drug Target Alteration

The most common form of antibiotic resistance in *M. tuberculosis* is drug target modification. Drug and drug target moieties interact with each other in very particular ways. Drug binding may be reduced or eliminated due to changes in the drug-medication target interaction sites, which would impart resistance to the drug in the issue. The drug targets used by antibiotics to carry out these crucial cellular activities are substantially conserved. The highly conserved nature of the drug targets restricts the mutational target size because the resistance mutation must achieve two goals. Firstly, it must stop the antibiotic from inhibiting the target. Secondly, it must make sure that the drug target's crucial function can still be carried out. This results in a decline in the bacterial cell's fitness without the medication in many but not all instances.

CRISPR/Cas Systems

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein are crucial components of the adaptive immune system that bacteria and archaea have created to defend themselves from foreign DNA,

such as plasmids or phage that obliterate the foreign genome (Jiang and Doudna, 2015). In addition, there is mounting proof that CRISPR sequences and Cas proteins have a role in gene regulation and genome editing, with different mechanisms in addition to immunization (Peters. J. M., et al., 2015). The CRISPR-Cas system was first identified in 1987 by scientist Yoshizumi Ishino and his colleagues during their research for the gene responsible for the alkaline phosphatase enzyme in *E.coli* bacteria, but since there were not enough DNA sequences in the gene bank at this time, it was not possible to compare these sequences with sequences in other genomes, (Ishino Y et al., 1987). In 1993, CRISPR was first observed in an archea, *Haloferax mediterranei*, and the discovery of DNA sequence similarities between the DNA sequences of plasmids, archeal viruses, bacteriophages and the spacer region of CRISPR in the early 2000s provided information that CRISPR has a function related to the defense system in bacteria (Mojica, M. J., et al., 1993). In the implementation of this hypothesis, Barrangou et al. cultured *Streptococcus thermophilus* with different phages and displayed phage-resistant mutants (Barrangou, R., et al., 2007). In these studies, it was shown that the CRISPR RNA (crRNA) complex could cut target DNA in vitro conditions in the future (Gasiunas, G., et al., 2012; Jinek, M., et al., 2012). The fundamental functions and processes of CRISPR systems started to be illuminated in 2010. Different research groups have utilized native CRISPR systems for various applications such as the production of phage-resistant strains and the phylogenetic classification of bacterial strains, but genome editing applications are still under investigation (Hsu, P.D., et al., 2014; Quiberoni, A., et al., 2010; Horvath, P., et al., 2008). In 2010, Garneau et al. reported that the gene, identified as cas9, encodes an enzyme among Cas genes that can cleave target DNA, and in 2012, following a striking study by Emmanuelle Charpentier and Jennifer Doudna the CRISPR-Cas system was able to replicate in prokaryotic cells. could be implemented using gene-specific gRNA designed for gene modifications (Garneau, J.E., et al., 2010). As a gene editing technique that will define the 21st century, this technology has led to advancements in numerous fields (Fichtner, F., et al., 2014; Hsu, P.D., et al., 2014; Sternberg, 2014).

Types of Cas

In terms of the variety of Protospacer Adjacent Motifs and the quantity and variety of Cas proteins, CRISPR systems are incredibly varied. There are two classes of CRISPR/Cas systems (Class 1 and 2) and are further subdivided into six categories (types I to VI). Class 2 systems (types II, V, and VI) use a single Cas protein, while Class 1 systems (types I, III, and IV) use several Cas proteins in their CRISPR ribonucleoprotein effector nucleases. 90% of all discovered CRISPR-Cas loci are class 1 CRISPR/Cas systems, which are most frequently seen in bacteria and archaea. A ribonucleoprotein complex made up of a CRISPR RNA (crRNA) and a Cas protein is assembled by the Class 2 CRISPR/Cas systems, which make up the remaining 10% of all CRISPR/Cas systems and also exists almost exclusively in bacteria (Gasiunas, G., et al., 2012; Shmakov, S., et al., 2017). Information to target a particular DNA sequence is contained in the crRNA (Horvath, P., et al., 2010). Type II subtype II-A CRISPR-Cas system, which employs the protein SpCas9 and is the most thoroughly researched CRISPR-Cas system, is present in Streptococcus pyogenes (Sp), and Cas9 was the first Cas-protein developed especially for gene editing. (Jinek, M., et al., 2012). Four other subtypes make up Class 2 Type V. (V-A, V-B, V-C, V-U). V-C and V-U are currently largely uncharacterized, and no structural data on these systems is available (Shmakov, S., et al., 2015). Cas12a, also known as Cpf1, is a protein that is encoded by V-A. Lately, numerous high-resolution structures of Cas12a have shed light on how it functions (Dong, D., et al., 2016; Stella, S., et al., 2017; Yamano, T., et al., 2016).

Cpf1/Cas12a

Cpf1 (CRISPR from Prevotella and Francisella1) or Cas12a is a new generation of CRISPR nucleases that Zhang and his team at MIT and the Broad Institute (USA) have found. Two components make up the CRISPR-Cas12a system: a protein/effector nuclease and a single crRNA. The sizes of the FnCas12a, LbCas12a and AsCas12a proteins are approximately 1300 amino acids, and they all have comparable domain arrangements. The spacer-derived region of the crRNA is base-paired with the complementary target DNA when Cpf1 detects a 5′-TTTV-3′ PAM in a DNA target. Cpf1 does not need tracrRNAs for crRNA synthesis since it also has RNAase and DNAase activity. Instead, the pre-crRNA forms a pseudoknot that Cpf1 recognizes and cleaves. Additionally, at the locations of cleavage, Cpf1 causes staggered ends (5 or 8 nucleotides 5′overhang regarding crRNA length) (Zetsche, B., et al., 2015). These distinguishing characteristics make Cpf1 an emerging tool for genome editing that can be applied to several biological techniques, including multiplex gene targeting, transcription, epigenetic modulation, and base editing. Additionally, the adaptability of Cpf1 has made it possible to use this promising tool in the modification of a variety of organisms.

Discussion

Antibiotics have become very important in today's world. Antibiotic-resistant bacteria increased. For this reason, producing antibiotics provides a very value-added effect for scientific research. On the other hand, since antibiotic production is very slow and difficult, CRISPR antimicrobial production can be used as a value-added agent in the future. The ability to sensitize *M. smegmatis* bacteria with this review will show us that we can sensitize the antibiotic-resistant bacteria of *M. tuberculosis* with the CRISPR / FnCpf1 method in the future.

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The Effect Of Beta-Lactam-Containing Drugs On The Development Of Drug Allergy

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Abstract: Allergy is the reaction of the immune system against the active substance entering the body. Drug allergy is caused by the entry of drug allergens into the body. Drug allergies are examined in two groups, Type A and Type B. Type A describes expected drug reactions and Type B describes unexpected drug reactions. Therefore, drug allergies are in the Type B category. Age, gender, past drug reactions, and genetic polymorphisms are important risk factors for drug allergy. Active drug substances are also particularly important for drug reactions, and drugs containing certain active substances are more prone to allergic reactions. Penicillin, cephalosporin, and fluoroquinolone active ingredients are especially important in beta-lactam antibiotics. At the same time, it is the examination of the use of different drugs belonging to the same group, without experiencing an allergic reaction, with only mild side effects. Especially if we think about the treatment method, CRISPR has been used successfully in various fields in recent years. According to a review study, there was a reduction in the rate of disease progression as a result of cloning research on mice with Type 1 diabetes using CRISPR technology and the disease-specific peptide. This technology may become available in the future by identifying the appropriate peptide group specific to the pharmacological groups for allergic reactions. In terms of diagnostic procedures, provocation tests and immunotherapy are still being studied clinically. As a future aspect of this review, targeted cell or drug content modification using CRISPR may become available for overcoming the allergy.

Key words: Drug Allergy, Beta-lactam, Nanomedicine, CRISPR, COVID Allergy

1. Introduction

Drug allergy is defined as the ability of the immune system to react to the active substance entering the body. According to the World Allergy Organization, reactions formed through IgE and T cells are considered drug allergies. Drug allergy counts for approximately 10- 15% of all undesirable drug reactions according to research (Erkocoglu et al., 2015). Undesirable drug reactions are classified into two groups separate by type A and type B. Type A defined as the 'expected drug reactions' and type B is defined as 'unexpected drug reactions'(Erkocoglu et al., 2015). Type B class contain drug allergies and it is classified according to Gell and Coombs' classification system. The Gell and Coombs classification system have 4 groups. These; Immunoglobulin E (IgE) antibodies such as immediate type reactions (type 1), cytotoxic reactions caused by immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies (type 2), immune complex reactions (type 3) and activation or aggregation of T cells. delayed-type hypersensitivity reactions (type 4) resulting from cellular immune mechanisms (Kilic et al., 2009). The system includes; reaction Type 1, fulminant reactions mediated by (IgE); type 2, cytotoxic reactions mediated by (IgG) or ımmunoglobulin M (IgM) (Kilic et al., 2009), Autoantibodies are formed against the antigenic protein/metabolite structure on the cell surface, and cell lysis occurs by stimulating the complement system in cytotoxic reactions (Kilic et al., 2009). Type 3, The reaction is called the immune complex reaction based on the combination of the antigenic structure of the drug freely in the circulation and the antibodies in the Ig G / M structure against it to form an immunocomplex and accumulate in postcapillary venules (Kilic et al., 2009). Type 4 includes retargeting-type reactions induced by cellular immune systems, such as the recruitment or activation of T cells. Four different subtypes for type 4 reactions. Type 4a involves reactions that are stimulated by Th1 cells and stimulate macrophages and

monocytes by intensely secreting IFN gamma and TNF alpha. In type 4b reactions Th2 cells are the main factor and eosinophilic inflammation occurs with secreted IL5 (Kilic et al., 2009). Type 4c reactions, CD8+ cytotoxic T cells directly lyse cells by releasing enzymes such as perforin and granzyme B. In type 4d reactions, while CXCL-8 released from T cells increases the lifespan of neutrophils, GM-CSF contributes to inflammation by preventing neutrophil apoptosis (Kilic et al., 2009).

Age, gender, viral infection, previous reaction to the drug, and genetic polymorphisms are risk factors for drug allergy. Furthermore, active ingredients are a noteworthy factor in allergy. Drugs with certain active ingredients are more prone to developing an allergic reaction (Erkocoglu et al., 2015).

Drug Allergy Affecting Chemical and Genetic Factors

A drug allergy may be associated with different factors such as age, gender, previous viral infections, genetic polymorphisms and drug active ingredients. It is affected by immunological reactions to drugs, susceptibility to drug allergy, and genetic polymorphisms in drug metabolism. Also, prolonged high doses or frequent doses cause hypersensitivity reactions more than a single large dose. Beta-lactam antibiotics and fluoroquinolones containing penicillin and cephalosporins are especially important in drug allergy. Patients generally have difficulty identifying the active ingredients in their medications and are often unaware of the concept of the active ingredient. In this case, when patients use more than one drug, the active ingredients may interact with each other and often cause serious or life-threatening complications.

Beta- Lactam Antibiotics

Beta-lactams are antibiotic species with beta-lactam rings in their structure. Beta-lactams are divided into 5 subgroups penicillins, cephalosporins, carbapenems, monobactams and beta-lactamase inhibitors (Warrington et al., 2011). Because penicillin (MW 356) has a low molecular weight, it must first covalently bond with tissue macromolecules (probably proteins) to form multivalent hapten-protein complexes, which are essential for the development of an immunological response. The penicillin group has the state of developing an allergic reaction to the drug, which includes all the main classes of antibodies and immunocytes. This occurs as a humoral immune response to antigens. The tendency of the patient to have an allergic reaction to penicillin content may vary with hapten specificity, isotypes and amount of antibodies formed, and differences in antibody affinities (Weiss et al., 1988). The other beta-lactam antibiotic subgroup is cephalosporins. This group is similar to penicillins in terms of the chemical structure and antibacterial action mechanisms (Misirligil Z., n.d.).

Its mechanisms of action inhibit bacterial cell wall synthesis and act by activating autolytic enzymes. 10% of patients who develop an allergy to penicillin also have allergies to cephalosporins because they have cross-reacting active ingredients. 3rd generation cephalosporins predominate in gram-negative activity (Warrington et al., 2011). Except for beta-lactam antibiotics, the allergic group is fluoroquinolones. Quinolones, unlike most antibiotics, are synthetic substances obtained by chemical means. Quinolones exert their effects by disrupting DNA synthesis in bacterial cells. They are concentration-dependent bactericidal effects. Very high concentrations of RNA inhibit protein synthesis (Hooper 2001, Leblebicioglu 2002, Jacoby 2005).

Studies on the genetic factors of drug allergies are usually done on human leukocyte antigens (HLA). Human leukocyte antigens, commonly known as the Major histocompatibility complex (MHC), are a class of cell-surface proteins that may attach to foreign molecules and be recognized by matching T cells, inducing immunological responses (Thong et al.,2011). HLA is examined in different subgroups but the most important subgroups are Class 1 and Class 2. Class 1 are recognized by CD8 + T cells and consists of three major genes, HLA-A, HLA-B, and HLA-C. Class 2 include 6 main genes. These are HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRA and HLA-DRB1.

The class 1 HLA is found on all nucleated cell surfaces and is responsible for cell-present antigen presentation to CD8 + cytotoxic T cells. Class 2 HLA molecules are found in immune cells and present extracellular antigens to CD4 + helper T cells. For type 1-3 allergic reactions to occur, drug-specific B cells must differentiate into antibody-producing plasma cells via CD+4 Th2 cell stimulation. In the reaction case, an IgE antibody is produced. Most of these reactions can be symptomatic, from skin rash to anaphylaxis and these reaction sources are caused by Beta-lactam antibiotics, including penicillin (Thong et al., 2011). Especially in penicillin, antibiotic binds to high molecular weight proteins and forms a complex of molecules that are recognizable by IgE antibodies (Simper et al., 2017).

In some studies, it has been investigated that the antigen presentations of HLA molecules produced from the drug play an important role in the development of allergy and hypersensitivity. There are several hypotheses that can explain how tiny molecule synthesized chemicals are identified by T cells in an MHC-dependent way (Simper et al., 2017). These models are called the hapten concept/prohapten model, the pi model, and the modified repertoire model. It includes beta-lactam antibiotics, especially penicillin, hapten model and type 1 reaction. In HLA allergies, allergic-type B is in the ADR group. The hapten model is associated with the formation of new antigenic markers by binding small chemicals to proteins and peptides. These linked structures produce hapten-peptides that are capable of stimulating T cells depending on HLA. In

penicillins, haptened peptides are presented by HLA-DRB1 to CD4 + T cells (Simper et al., 2017). Further studies focused on the p-i model. The p-i model describes that hypersensitivity reactions can occur by binding the drug itself to the TCR and HLA molecules. The innate immune system and B cells are not included in this model (Simper et al., 2017). These structures are specific for allele and polymorphic regions, and immune responses can only be observed for carriers of certain HLA alleles. The connection between HLA and hypersensitivity reactions can be explained in more detail with the altered peptide repertoire model.

HLA is of great importance in genetic factors. Studies on gene polymorphisms, alleles and HLA are still ongoing. Genetic factors that vary according to population are effective in risk factors for drug allergy (Thong et al., 2011). IL-4; IL-4 plays an important role in the development of allergic inflammation **(Figure 1)**. IL-4 is required for the development of native T helper cells into T(h)2 effector cells that increase humoral immunity **(Figure 1)**. IL-4 is also involved in the pathophysiology of the ability of IL-4 and related cytokines to promote the differentiation and function of mast cells, basophils, and eosinophils suggesting that they may play a role in the pathogenesis of atopy (Genevose et al., 2010). On the other hand, an enhanced Th2 immune response and elaboration of cytokines such as IL-4, IL-13 and IL-5 influence the level of induction of allergy and asthma (Soyer et al., 2011). Interferon-gamma, a Th1 cytokine, acts together with Th2 (IL-4, IL-13 and IL-5) in the maintenance of chronic allergic inflammation **(Figure 1)**. IL-10; Interleukin-10 is a powerful inflammatory response receptor that is important in the regulation of allergic airway inflammation. Individuals with asthma have lower levels of IL-10 in bronchoalveolar lavage fluid and IL-10 secretion from alveolar macrophages as compared to non-asthmatics. In addition, polymorphisms in the IL10 gene that cause low IL-10 development have been linked to extreme asthma (Ogawa et al., 2008). Mast cells are also capable of producing IL-10. The alveolar macrophage is the primary source of constitutive IL-10 secretion in the healthy lung; moreover, the circulating monocyte produces more IL-10 than the alveolar macrophage. inhibits the IL-4-induced IgE transition in peripheral blood mononuclear cells while increasing IgE activity in B-cells that have already been switched to produce IgE (Jeannin et al., 1988). IL-13; The cytokine IL-13 is provided by various T-cell subsets and dendritic cells. Many biologic events are shared by IL-13 and IL-4. This is because IL-13– and IL-4–receptor complexes share the IL-4–receptor -chain, which is important for signal transduction However, IL-13 is necessary for optimal induction of IgE synthesis, particularly when IL-4 output is low or absent. Based on these findings, it is possible to infer that IL-13 is an anti-inflammatory cytokine that plays a distinct role in the activation and maintenance of IgE formation and IgE-mediated allergic responses (Bacharier et al., 2000). The various roles of IL-13 in controlling IgE formation, eosinophilic inflammation, airway-smooth-muscle hyperplasia, inducing goblet-cell hyperplasia with mucus production, and recruiting monocytes, macrophages, and T cells into the airway spaces make it a primary therapeutic target in allergy and asthma124. IL-13 binds to a low-affinity IL-13R1 subunit as well as a high-affinity cluster of IL-13R1 and IL-4R. Binding to this high-affinity complex activates Janus kinase 1, JAK2, and STAT6 through phosphorylation. IL-4R also stabilizes IL-13 binding to its receptor, enhancing IL-13-mediated responses (Marone et al., 2019). IL-18; An allergic disorder characterized by hypersensitivity mediated by complex immunologic pathways. Traditional allergic disorders are mediated by immunoglobulin IgE, but other cell-mediated pathways can also occur. IgE influences the classical level of an allergic reaction, while different types of leukocytes mediate both sensitization and the subsequent response to allergens (Cappella et al., 2012). The ability of IL-18 to induce a Th1 response, resulting in the development of IFN- and then inhibiting a Th2 response, was first identified. It is now understood that IL-18 may have antagonistic effects on the equilibrium of Th1 and Th2 activation and cytokine formation. Th1 cells abundantly express IL-18R, while Th2 cells express less, and IL-18 can also accelerate but not cause Th1 cell differentiation. IL-18 activates Th1 development while inhibiting IgE formation in an IFN-dependent manner when combined with IL-12. When given alone, IL-18 raises IgE levels independently of IFN-18 (Yoshimoto et al., 1999). Since IL-18 can raise IgE, IgG, and IgM levels, it can play a role in these potentially fatal reactions. Co-administration of these cytokines prevents the IL-4-induced stimulation of anaphylaxis, which is consistent with the antiallergenic action of the mixture of IL-18 and IL-12. There is no evidence to support the function of IL-18 in anaphylactic and anaphylactoid reactions. IL-18 polymorphisms, on the other hand, have been linked to latex and penicillin allergies (Jeannin et al., 1998).

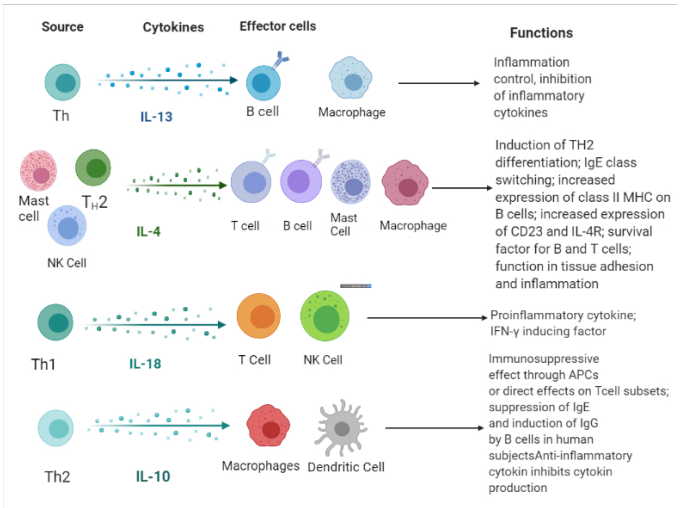

Figure 1. Effective Cytokines in Drug Allergy and Biological Roles

Drug Allergy Diagnosis Methods

Patient history is of great importance in drug allergy. If an allergic reaction is suspected in line with the anamnesis of the patient, different tests can be applied for a definitive diagnosis. Tests for diagnosis are evaluated in 3 basic categories. A) Skin tests B) in vitro tests C) drug provocation tests. Depending on the patient's history, the general sequencing starts from skin tests and can be forwarded to the drug provocation test to make a diagnosis (Erkocoglu et al., 2015).

A) Skin tests; Skin test methods are classified as skin prick test (SPT), intradermal test (IDT) and patch test (PT). These tests are chosen in themselves according to the drug hypersensitivity reaction. In drug allergies, skin prick and intradermal tests are recommended in early-type reactions, and patch tests are recommended in late reactions (Erkocoglu et al., 2015). Skin prick tests are important for early reactions. It has been determined as both the safest and fastest method. Major determinant (MDM) and Minor determinant are used in patients with a suspected beta-lactam allergy, but because there is no specific form of ampicillin, cephalosporin and other beta-lactam antibiotics in skin tests, parenteral forms of these drug active ingredients are used. Although the sensitivity rates of skin tests are generally high, the negative results are not sufficient to rule out the diagnosis of drug allergy. In patients with negative skin tests, advanced provocation tests should be performed to confirm the diagnosis or to exclude the drug group (Erkocoglu et al., 2015).

B) In vitro lab test; In vitro laboratory tests are safer for patients than in vivo tests, but most in vitro test methods are not yet standardized and are still used for research purposes. Specific IgE measurement and basophil activation test are applied in early-type reactions, while lymphocyte transformation test is applied in late reactions (Erkocoglu et al., 2015). Specific IgE measurement test; It is the most commonly used in vitro method for early allergic reactions. Basophil activation test; After the reaction experienced in early-type drug allergies, specific IgEs that develop in the body against the drug is located on the mast cells and basophils in the periphery (Erkocoglu et al., 2015). The Basophil activation test method is based on the display of specific activation markers on the cell surface or in the cell by flow cytometry method after the incubation period of basophils with the drug-causing allergy. The markers determined for intracellular are P-p38MAPK, for the cell surface CD63 and CD203c (Erkocoglu et al., 2015). There is a sensitivity rate of 33-67% for beta-lactam antibiotics with the basophil activation test and is still not used as routine clinical practice (Erkocoglu et al., 2015). Lymphocyte transformation test; It is used to determine specific IgE recognition in T cells and severe IgE-mediated reactions to drugs, especially in late drug reactions. In the test press, the patient's peripheral mononuclear cells are isolated, then incubated with the suspected drug to be tested, and proceed as the determination of drug-specific T cell clones (Erkocoglu et al., 2015). The sensitivity rate of this test is in the range of 30-70% (Erkocoglu et al., 2015).

C) Drug Provocation Test; Drug provocation tests are the most important test for the diagnosis of drug hypersensitivity reactions. Drug provocation tests are applied in case of negative skin and in vitro test results (Erkocoglu et al., 2015). Drug provocation test carries serious risks and must be done under the supervision of an allergist. The commercial forms of the drugs, i.e. the derivatives used by the patients, are used in the test. In combination drugs with more than one ingredient, each ingredient should be tested separately. In general, drug provocation tests are performed at least 4 weeks after the reaction (Erkocoglu et al., 2015). If there are objective findings, the test result is considered positive, but if subjective findings are found, a double-blind placebo-controlled provocation test may be required. The double-blind placebo-controlled provocation test is a method of taking the suspected allergen and the placebo dose. These two factors are given to the patient in different periods as separate days or hours. The result is re-evaluated according to this test (Erkocoglu et al., 2015).

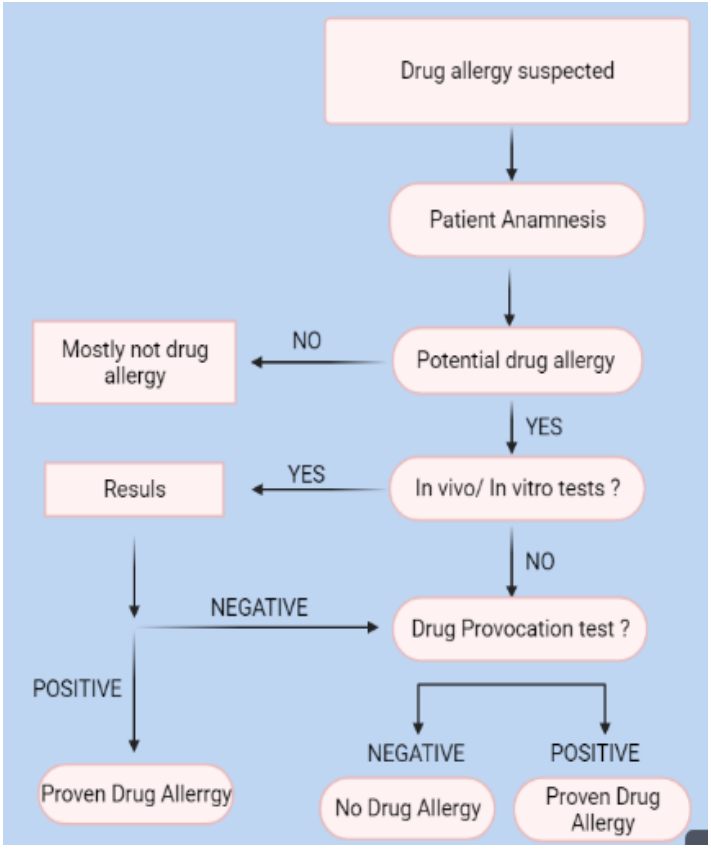

Figure 2: Drug Allergy Process Timeline

Proposed Treatment Methods

Two methods are used for the treatment of drug allergy, which is considered one of the undesirable consequences of drugs. The

first method is used as 'Desensitisation' and the second as 'immunotherapy'. Immunotherapy is generally a preferred method in cases where desensitisation fails or shows a more severe allergic reaction.

Desensitization

Desensitization; It is defined as the process of desensitization to the allergen (Mirakian et al., 2009). Desensitization may be preferred, especially when there is no alternative to the drug used and the patient needs to use the drug. Since these treatment methods are risky, they should be done under the supervision of a doctor. Despite the effectiveness of accelerated desensitizations, cellular and molecular inhibitory pathways are unknown (Mirakian et al.,2009). In vitro models of mast cells and basophils include the cellular signalling molecules syk and STAT6. Rapid desensitization procedures treat type I mast cell/IgE-related reactions such as anaphylaxis seen with penicillin, cephalosporins and other antibiotic sensitization. This method is still open to improvement and there are deficiencies in understanding its basic mechanisms (Castells M., 2006). In particular, the first described desensitization methods are used to treat type 1 (IgE-mediated) mast cell-mediated reactions.

Immunotherapy

Allergy immunotherapy is based on changing the response to the allergen by creating variability in immunological tolerance. Immune variance is a term that refers to a transformed immune response to allergen exposure in which allergen-specific T helper type 1 cells are mobilized and stimulated at the cost of Th2 cells (Robinson et al.,2004). Allergen-specific immunotherapy (SIT) provides induction of immunological tolerance and blocking IgG antibodies by re-exposure to allergens. This method also increases the level of IgA, and IgG antibodies and decreases the IgE antibodies specific to the allergen. It also stimulates CD4+CD25+FOXP3+ cells (Cappella et al., 2012). Treg cells with elevated levels of IL-10 and/or TGF, two cytokines believed to suppress allergen-specific TH2-cell responses. TGF's pleiotropic roles sustain a diverse and self-tolerant T-cell repertoire, like TReg cells, by suppressing mast-cell, eosinophil, and T-cell responses (Capella et al., 2012). Immunotherapy is effective in controlling the symptoms of allergic reactions and reducing the need for medication to suppress the reactions. The therapeutic effects of this method can continue after the treatment is stopped, so it is effective in preventing the development of new sensitivity and asthma. Successful immunotherapy treatment is based on a detailed and good knowledge of allergens, investigation of the cross-reactivity of these allergens, and experience in risk management. Different methods of immunotherapy under development can help make this treatment safer and more effective. These new methods include local immunotherapy, sublingual immunotherapy, peptide immunotherapy and DNA vaccination methods (Cappella et al., 2012). There are immunotherapeutic allergen vaccines used with different methods approved by the FDA, but there is open to development, especially in terms of its use in drug allergies.

Nanomedicine

The definition of nanoscience can be expressed as the study of structures and substances at the molecular level at nanometric scales ranging from 1 to 100 nm (Bayda et al., 2019). The most common studies in the field of nanoscience are carried out in the field of drug delivery and cancer. Nanoparticles of a diameter of 100 nm have been commonly used to increase drug accumulation, internalization, and therapeutic effectiveness (Seleci et al., 2016). Apart from that, nanoparticles have certain classifications within themselves. These classifications are generally made according to basic factors such as the type of substance and chemical properties. The most basic classification;

1. carbon-based nanoparticles (fullerenes, highly sensitive carbon nanotubes etc.)
2. metal-based nanoparticles (gold colloidal, nanoshells, iron oxide particles etc.)
3. Made as semiconductor-based nanoparticles (quantum dots etc.)(Lewinski et al.,2008).

In this review study, especially allergic reactions from drugs were emphasized. Another point that has been researched in the field of nanotechnology is their use in drug delivery systems and the potential of these regulated systems to develop allergic reactions. Apart from nanoparticles, different nanomaterials can be used as carriers. Nanocapsules, solid lipid particles, liposomes, fullerene, micelles, carbon nanotubes have an important place (Vega-Vásquez et al., 2020).

Drug delivery systems (DDS) are technologies designed to ensure targeted delivery or controlled release of therapeutic agents within the body. Recently, different ideas have been put forward regarding the individuality of the drugs used in the treatment of diseases. The main idea that causes this is that the drugs do not give the desired effect in every individual and the reactions they show. Drugs have a reaction mechanism that starts from side effects and progresses to allergies in patients. One of the planned points with drug delivery systems is to ensure the delivery of drugs to the right cells and organs in a faster and more efficient manner. (Vega-Vásquez et al., 2020).

So what are the features required for these drug delivery systems? This method should increase the bioavailability level of the drug. It should be able to efficiently deliver the drug to the area it needs to reach, by staying away from the non-infected host tissue. These systems can be divided into two main categories. It is classified as conventional DDS and novel DDS. The conventional drug delivery system mainly affects the rapid absorption of the drug and is used more in these situations. Although absorption is

rapid, rapid methods are required for its release. In this regard, oral, inhalation, or injection methods are preferred for administering the drug to the patient. The need to find novel DDS systems is due to the shortcomings of conventional methods. The most basic of these missing points is that the concentration of the drug cannot be kept at a constant level and requires dose repetition at regular intervals. To eliminate this necessity, novel generation methods have been directed. Novel drug delivery systems are also called controlled DDS. This is because this NDDS aims to control different areas such as drug release control, safer administration, innovation in administration doses, and new techniques to target the drug to a specific cell (Jain K., 2020).

Targeted drug delivery systems deliver the drug to a more specific area instead of spreading it to the whole body or organ. Polymers also contain different fields such as pharmacology. The goal of the TDD system is to manage and control the pharmacokinetics, pharmacodynamics, non-specific toxicity, immunogenicity, and biological recognition of therapeutic agents. While reducing the risk of side effects, the efficiency of the treatment is increased (Tewabe et al., 2021).

Targeted drug delivery method; It aims at an attempt to reduce the relative concentration of the treatment in other tissues while concentrating the drug in the relevant tissues and making it efficient. As a result, the drug concentrates at the targeted point and does not affect other adjacent tissues. On the other hand, as a result, drug loss is prevented and this results in optimum therapeutic effect (Allen T.M., 1998). There are two main approaches to targeting drug delivery systems. These are active and passive targeting. Passive targeting aims at the accumulation of a drug or drug delivery system in a specific area due to different reasons (pharmacological, chemical etc.). These systems can be passively targeted using pathophysiological and anatomical developments. The setup for passive targeting targets systemic circulation. The body's natural reaction to the physicochemical parameters of the drug or drug delivery nanosystem results in drug targeting. Passive targeting uses tissue permeability. Both biological and pharmacological factors can affect the accumulation of a drug at a certain point. Active targeting; It is based on the delivery of the drug delivery vehicle to the determined target site by conjugation method. In this way, the drug accumulates in a certain area. There are robust molecular interactions between moiety and carrier, as well as between receptor and ligand.

Micelles

Micellar is defined as the sum of amphiphilic surfactant molecules that spontaneously form when interacting with water and often form spherical vesicles. It has a hydrophobic core that can be released to different parts of the organism of the desired hydrophobic substances. It has a head with a hydrophilic or polar charge and a hydrophobic tail consisting of hydro-carbonate portions of long fatty acids. The sizes of the surfactants that make up the micelle vary according to their properties. Mixed micelles have several key properties that are required for nano-drug delivery systems. A hydrophilic coating around the drug-loading core protects the water-insoluble drug from being solubilized and the contained protein or nucleic acid from degradation at non-target sites. In addition, synthetic materials used to form micelles can precisely control the chemicals contained in polymeric materials, thereby efficiently improving the functional results required for the use of polymeric micelles, including circulatory durability, tissue deposition and penetration, and controlled intracellular mobility. gets. may affect the rates of spatially and temporally controlled release and reduced intrinsic toxicity (Kapare et al., 2020). Micellar is important for drug delivery systems, considering its beneficial effects on pharmaceutical agents in general, biocompatibility, longevity, and high stability in vitro and in vivo. It increases the yield rate by interacting with groups sensitive to stimuli, and by designing their surfaces with various ligands and fragments that can enter the cell to allow specific targeting and intracellular accumulation (Jhaveri et al., 2014).

Solid Lipid Nanoparticles

Solid lipid nanoparticles (SLN) are referred to as aqueous colloidal dispersions whose matrix consists of solid biodegradable lipids. SLN material is used in the field of nanomedicine with its advantages in physical stability, safety in the transport of drugs, efficiency in the transport of unstable drugs, controlled release, and tolerance level (Bozzuto et al., 2015). Lıpozomes are defined as spherical vesicles with a medium empty watery space surrounded by a phospholipid bilayer. Liposomes provide high efficiency in preventing or delaying the degradation of drugs and reducing their side effects. Drugs can be incorporated either within the aqueous space (hydrophilic drugs) or within the phospholipid bilayer of liposomes (hydrophobic drugs) for targeted drug delivery. Because of their biocompatibility and biodegradability, they are the first nano-drugs to be accepted for use in the nanomedicine field. In liposome-based drugs, the solubility of the transported drug is Liposomes have been modified with PEG due to their different disadvantages. PEGylated liposomes have been approved, but these modified nanomaterials have instability, polydispersity, and toxicity in repeated administration. There are disadvantages such as immunostimulation and the ability to induce complement activation (Bozzuto et al., 2015)

Immunomodulation Of Nanoparticles In Nanomedicine Applications

The composition, surface chemistry, size, structure, and protein binding abilities of nanoparticles are important factors in the

effect of immunomodulation. In particular, the compositions of these NPs are associated with the immune system. As an example, in the articles researched, QD material showed high immunotoxicity because it was combined with heavy metal ions. The different core structures found in nanoparticles can differentiate the effects seen in this immune system. For example, Carbon nanotubes have an adjuvant effect and cause a hypersensitivity reaction in allergic bodies, while fullerene NP has immunosuppression (Chen et al., 2017).

The investigation of the mechanisms between NPs and allergic reactions is still incomplete. Studies on this subject are continuing, but allergic reactions can be seen in the interaction of allergens, especially materials such as Au, ZnO, and Ag, with nanoparticles. Previous studies have identified pro-allergenic reactions in mice with materials such as Au, and SiO_2. At the same time, apart from the composition of the materials, the material dimensions are also effective in allergic reactions. Larger-sized particles are more likely to induce an allergic response than smaller-sized particles (Marquis et al., 2009).

COVID and Drug Allergy

Pneumonia of unknown origin was reported in the Chinese city of Wuhan in December 2019Severe Acute Respiratory Syndrome Coronavirus 2 was discovered when respiratory samples were injected into Vero E6 and Huh7 cell lines, which are used to study human airway epithelial cells. This novel respiratory virus was isolated as a consequence (SARS-CoV-2). The betacoronavirus SARS-CoV-2 belongs to the subgenus Sarbecovirus. The World Health Organization declared a pandemic on March 12, 2020, as a result of the global spread of SARS-CoV-2 and the hundreds of fatalities brought on by coronavirus disease (COVID-19) (Ciotti et al., 2020).

During this pandemic process, treatment and vaccination studies have started quickly. Vaccines have been produced in different countries and have been put into practice.

Pfizer/BioNTech BNT162B2, Moderna mRNA-1273 and AstraZeneca recombinant adenoviral ChAdOx1-S vaccines, which are among the vaccines developed for the Covid global epidemic affecting the whole world, started to be used after approval (Unsal et al., 2021).

After the first doses of several vaccines, allergic responses have been recorded. According to research organizations in the European Union, the United States of America, and the United Kingdom, these allergic reactions develop against one or more of the vaccine components and are most severe after the first dosage. According to the European Academy of Allergy and Clinical Immunology (EAACI), the outcome of this situation does not include any contraindications for administering the vaccine to individuals who have allergic responses. It is recommended in this situation to determine the type of allergic reaction during the vaccination process and to vaccinate with equipment and in postures that allow the patient to be controlled during the vaccination procedure (Sokolowska et al., 2021).

The Pfizer/BioNTech BNT162B2 COVID-19 vaccination caused 11.1 adverse reactions per million doses. The possibility of an allergic reaction should be considered with the patient's history. Anaphylaxis developed within 15 minutes in 71% of individuals who received the Pfizer/BioNTech BNT162B2 COVID-19 vaccination (Rüggeberg et al., 2007). In the results of the study conducted for the pathogenesis of allergic reactions observed in vaccines, results related to the PEG substance were found. The two mRNA-based vaccines studied are packaged in vials and need to be diluted. Since there is no extra adjuvant, there are axials such as lipid, salt, and sugar, which have the task of stabilizing the vaccine. The main task of the lipids in the vaccine is to form a nanoparticle carrier that acts as a protective shield for mRNA after administration and to easily provide cellular uptake from the plasma membranes (Sokolowska et al., 2021).

Lipids can be modified with Polyethylene Glycol (PEG) to increase the water solubility of the lipid nanoparticle carrier. PEG, also known as macrogol, is used in different molecular weights in different fields, from cosmetics to drugs, from food additives to vaccines. PEG with a molecular weight of 2000 g/mol was used in Moderna and Pfizer/Biontech vaccines. Although it is predicted to be safe for use in general drugs, it has been observed that allergic reactions in vaccines may be caused by PEG (Sokolowska et al.,2021). It contains amino acid L-histidine, ethanol, salt and most importantly polysorbate 80 as an excipient, which is the first vaccine study for COVID-19, and it is used in polysorbate 80 drug formulations. At the same time, it has the potential to cross-react with PEG, so if there are two substances in the vaccine, it can be overdone. may cause sensitivity reactions (Sokolowska et al., 2021). PEG without mRNA interacts with IgE antibodies that bind to the FcR1 receptor on cell surface lipid nanoparticles, mast cells, or basophils, resulting in the rapid release of inflammatory and proinflammatory mediators such as histamine, prostaglandins, and proteases (Selvaraj et al., 2021). Spike glycoprotein, an integrin-binding motif, an RB motif that binds to human angiotensin-converting enzyme 2 (ACE2B), is capable of making all two fusion peptides and two heptad repeats and is found in SARS-CoV-2. The Uniprot database was used to obtain the spike glycoprotein (P0DTC2) sequence (Selvaraj et al., 2021). The Aller-TOP results for RBD capable of binding to human ACE2B revealed that the receptor binding motif and two fusion peptides can cause an allergic reaction. As an allergen, the spike protein leads it to the host receptor ACE2B, and as a result, the amino acid residues of the vaccine containing spike protein may trigger

an allergic reaction coming from the ACE2B sequence (Selvaraj et al., 2021). SARS-COV-2 variations detected in spike protein important substitutions have been reported, and it is argued that amino acid residues may cause anaphylaxis as a result of these results, and that vaccine and therapeutic research should be expanded (Selvaraj et al., 2021). In a different study, when the VAERS database was examined, it was determined that 21 anaphylaxis reactions occurred at the first dose between 14 December and 23 December 2020 (Cabanillas et al., 2021). Anaphylaxis was also recorded in ten patients after receiving the first dosage of 4.041,396 of the mRNA-1273 vaccination between December 21, 2020, and January 10, 2021. Because these studies do not include information on first reactions such as rash, itching, vomiting, and shortness of breath, which are less common, the rates of adverse reactions reported following immunization are likely greater. This data also reveals a risk of anaphylaxis in the mRNA BNT 162b2 and mRNA-1273 Covid-19 vaccines, which is roughly 2 to 8.5 times higher than the incidence reported in the 2016 VSD trial (Cabanillas et al., 2021). Following all of these studies and approvals, the Federal Institute for Vaccines and Biomedicine in Germany and the European Medicines Agency (EMA) suggested that patients in the European Union who are allergic to any specific substance in vaccines should not be vaccinated. At the same time, it is underlined that the patient's allergy history should be evaluated before vaccination, and approval for the vaccination procedure should be granted once the appropriate controls are accomplished (Cabanillas et al., 2021).

Some vaccinations contain egg protein and ovalbumin in trace amounts. If a patient was allergic to eggs as a youngster, they are more likely to be allergic to vaccines containing these substances. Gelatin, which is used to stabilize both live and inactivated vaccines, is another component that might trigger adverse reactions in vaccines. Because of these factors, vaccines with differing ingredients may trigger allergic responses (Cabanillas et al., 2021). There is a possibility of anaphylaxis in addition to local allergic reactions and influenza, and typhoid vaccines with the task of stabilizing gelatin MMR. Neomycin; It is used as an antimicrobial in vaccines such as influenza, smallpox, MMR, and hepatitis A, and it is possible to cause an allergic reaction as anaphylaxis. Polyethylene Glycol was used as the surfactant of mRNA in the COVID-19 vaccine and, in the same way, it caused an anaphylaxis reaction after the vaccine(Cabanillas et al., 2021). Formaldehyde and beta-propiolactan (BPL) are used in the vaccine to neutralize the viruses used in vaccine production. Approved in China for the Covid-19 pandemic, SinoPharm's BBIBP-CorV and Sinovac Life Sciences' CoronaVac's COVID-19 vaccines used BPL as ingredients to neutralize viruses(Zhang et al., 2021). Trace amounts are sufficient for this substance to be effective, but in different vaccines, This ingredient may be related to allergic reactions in vaccines, since it causes dermatitis when used. The reason for this can be concluded that the carbonyl groups on the antigens of BPL affect the immunostaining of the entry site and cause different negative effects (Moghaddam et al., 2006).

PEG, which is one of the most important ingredients shown to cause allergic reactions in vaccines, is also known as E1521. It is widely used with its volume-increasing and stabilizing properties. It is used as an excipient in a wide range of pharmaceuticals, as well as in cosmetics and processed foods. Importantly, polyethylene glycol is present in both the Pfizer BioNTech and Moderna mRNA vaccines against SARS-CoV-2 which are being used worldwide to battle the current pandemic (Cox et al., 2021). So far, world regulatory organizations have approved BNT162b2 made by Pfizer-BioNTech, mRNA-1273 produced by Moderna Therapeutics, and AZD1222 produced by AstraZeneca-Oxford University for the prevention of COVID-19. BNT162b2 and mRNA-1273 are mRNA vaccines based on the same mRNA technology that encodes the viral spike (S) glycoprotein of SARS-CoV-2. To ensure the stability of the mRNA molecules, these vaccines wrap them with lipid nanoparticles (LNPs). LNPs have also been PEGylated, which involves chemically adding polyethylene glycol (PEG) to the surface of LNPs to boost their effectiveness and transport to target cells (Cabanillas et al., 2021). The AZD1222 vaccine is also based on the production of the glycoprotein spike (S) antigen of SARS-CoV-2, but it is a DNA vaccine made up of a replication-deficient chimp adenovirus vector (ChAdOx1). The AZD1222 vaccine ingredients do not include PEGylated LNPs or other PEG components but do include polyoxyethylene-80-sorbitan monooleate, also called polysorbate 80 or Tween 80, which has structural similarities to PEG. The molecular mass of PEG is also important for understanding allergic reactions. The molecular weight (MW) of PEGs can range from 200 to 35,000 g/mol; as a result, some patients react to PEG-20000 (g/mol) but tolerate PEG-6000 (g/mol), while others are sensitized to PEG-3350 (g/mol) but tolerate PEG-300 (g/mol). (34) Tween 80 (polyoxyethylene-80-sorbitan monooleate) is a non-ionic agent used as an emulsion and stabilizing in the pharmaceutical and food sectors, and it is applied in vaccine biotechnology to maximize vaccine solubility. Contains polysorbate 80 and 20 in vaccines used in AZD1222 and the COVID-19 pandemic. Allergic reactions known to occur against polysorbates are associated with drugs containing this excipient, but allergic reactions seen in polysorbate 80 are less common than with PEG (Cabanillas et al., 2021). In a study on Covid-19 vaccines and the allergic substance PEG; Women and people who have had a previous allergic reaction are more likely to have an mRNA vaccine allergy (Warren et al., 2021). Patients who have a systemic adverse reaction to vaccination should not get a second dose of that vaccine or other vaccines containing similar components. The European Medicines Agency (EMA) and the US Food and Drug Administration (FDA) have both

advised that the vaccination not be administered if there is an allergy to one of the vaccine components or if the first dose causes a severe allergic response. The vaccination should be injected under medical supervision also with appropriate medical assistance. Other vaccinations should be administered with caution to anyone who has contraindications to one of the already authorized COVID-19 vaccines. For instance, recipients of any of the mRNA vaccinations should not be those who have a contraindication to one of the mRNA Covid-19 vaccines. Both the mRNA and AstraZeneca-ChAdOx1-S versions of the Covid-19 vaccine include polyethylene glycol (PEG), which is a polysorbate. People may receive the Janssen COVID-19 vaccination and vice versa if the mRNA COVID-19 vaccine is contraindicated in a patient's case (Unsal et al., 2021)

Discussion

Allergy is defined as the reaction of the immune system against the allergen entering the body; It may differ according to age, genetic factors, and the substances contained in the drug. The incidence of drug allergic reactions is 10-15% and is increasing day by day. One of the biggest factors of this is by fits and starts drug use and outside the treatment process, drug use and environmental factors and changes in the immune system can be shown. Nowadays, an allergy reaction, which is an undesirable drug interaction, is observed especially from the beta-lactam antibiotic group. Beta-lactam group antibiotics are generally examined as penicillin, cephalosporin and fluoroquinolone groups. Immunologically based drug allergies are associated with IgE-secreting B cells. In this scan, interactions between drug ingredients and cell mechanisms were closely examined. First of all, the methods required to detect drug allergy are skin prick tests, which are defined as patch testing and in vitro laboratory tests. These tests may show results for the presence of drug allergy, but cannot pinpoint the substance due to allergy. By examining the drug content used in the allergy process, general information about allergen substances can be obtained. When the genetic basis of drug allergy is examined, IL-4, IL-10, IL-18 and IL-13 come to the fore among the HLA cytokines that play a role in our immune system. It can both trigger the cross-reaction of drugs and, through these cytokines, the body system can take the incoming substance as foreign and cause an allergic reaction for defense. There are different methods used in the diagnosis and treatment of allergic reactions. Starting with skin tests at the first stage, the analysis can be done with drug provocation tests at the last stage. For the treatment method, the desensitization method and immunotherapy application are at the forefront, but there are still drugs that are insufficient in diagnosis and treatment and patients who experience these allergies. Nanoparticles, which have started to be used in drug delivery systems, are classified according to their different properties. These properties can be factors such as size, permeability, toxicity, and chemical properties. In this review, these properties will be evaluated, and nanoparticles used in drug delivery systems were investigated. During this research, it was thought that nanoparticles used in drug delivery systems may affect allergy rates in the future. Nanoparticles used such as Micelles, solid lipid particles and the effects of allergic reactions on each other are still open to research. In some cases, patients may have an allergic reaction to nanoparticle fragments containing Au, and ZnO. It is important to make a detailed preliminary examination of the immunological effects of the nanoparticles used. One of the most important points in this review is the COVID-19 pandemic, which has an effect all over the world, and the tendency of vaccines to show allergic reactions during this pandemic. During the COVID-19 pandemic that started in 2019, more than one vaccine was produced. The most prominent vaccines with FDA approval, availability and production speed are Pfizer/BioNTech BNT162B2, Moderna mRNA-1273 and AstraZeneca recombinant adenoviral ChAdOx1-S. The Pfizer/BioNTech BNT162B2 COVID-19 vaccine has demonstrated an allergic reaction rate of 11 per million. In another study, it was stated that 21 anaphylaxis reactions were experienced within 9 days of the mRNA -1273 vaccine. When the causes of allergic reactions related to these vaccines were investigated, the most striking substance was PEG. PEG is a substance with different molecular weights, used in different areas such as cosmetics, vaccines, coating of daily products. PEG and polysorbates 80 have been used actively in vaccines and as a result of research, it has been thought that they may cause allergic reactions. It is recommended to be cautious against COVID-19 vaccines, especially in patients with a previous allergic reaction, and to be administered under surveillance in a health institution. Allergic reactions can occur in vaccines, drugs, foods and many different substances. It is thought that the genetic basis, the substances used and the ingredients that cause the allergic reaction should be investigated in detail and improvement should be made in this regard. There are still different opinions and uncertain results about drug allergy diagnosis and treatment methods and allergy reactions of COVID-19 vaccines.

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Making Antibiotic-Resistant E. coli Non-Resistant with CRISPR/Cas9 Gene Editing

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Abstract: Bacteria are becoming increasingly resistant to antibiotics used in recent years, and antibiotics used today cannot resist this resistance gained by bacteria. The presence of antibiotic-resistant bacteria is shown as a cause of disease that is reported to cause the most deaths in the world in the coming years. Because people resort to the use of more effective antibiotics in high doses to kill these resistant bacteria after they infect our bodies and to counter the resistance they have gained. However, since antibiotics in high doses also affect and kill the healthy microflora of the human body, they are likely to cause side effects and serious problems. Escherichia coli is a bacteria frequently discovered in both human and animal intestines. Some varieties of E. coli can enter the blood from the intestines even though they generally reside in your intestines. Serious sickness might develop as a result. In cases where it mixes with the blood, it can cause many infections such as diarrhoea, respiratory tract problems, urinary tract infections and blood infections, especially in children. Urinary tract and intestinal infections caused by antibiotic-resistant strains of this bacterium, which do not pose a threat to human health under normal conditions, are becoming more common and dangerous. Among the E. coli strains, O157:H7 is the most harmful and deadly strain. The inadequacy of antibiotic treatments applied against resistant bacteria can lead to more deadly results. Fewer and fewer antibiotics are being produced to kill antibiotic-resistant bacteria because the spectrum of antibiotics is about to run out. The thesis, it is aimed to render antibiotic-resistant E. coli bacteria non-resistant in vitro by using the CRISPR/Cas system, which is the genome editing tool of the 21st century, to prevent all these. Thanks to the CRISPR/Cas system, only methicillin antibiotic-resistant E. coli bacteria will be sensitized or killed without harming the healthy bacteria in the microflora. This project will prove that antibiotic-resistant bacteria, a global health problem, can be sensitized. Thus, CRISPR will be able to use as an alternative antimicrobial agent to antibiotics in the future and will facilitate the production of antimicrobials with CRISPR technology.

Key words: Antibiotics, CRISPR-Cas9, antibiotic resistance, Escherichia coli, gene editing.

Introduction

Antibiotics target certain cellular processes or activities, such as protein or nucleic acid synthesis, cell membrane function, and cell wall formation, to impact specific bacterial mechanisms. They are drugs used to prevent and treat bacterial infections. In search of curing infections, biologist Paul Ehrlich found a substance in 1909 (Guillaume André Durand et al., 2019). He observed that this substance was effective on some infections. These experiments were later followed by Louis Pasteur's observations that some microorganisms kill others. In 1928, Scottish scientist Alexander Fleming discovered by chance that the fungus, which he named "*Penicillium notatum*", prevented the development of *staphylococci*. Later, in the 1940s, he discovered the miracle of "penicillin powder", which Howard Florey and Ernst Chain isolated from this culture and was a million times stronger than it. All these studies have inspired the production of antibiotics in the fight against infection. The invention of penicillin was the inspiration and golden age for antibiotics (Hutchings MI et al., 2019). After penicillins and sulfonamides, new antibiotics, especially broad-spectrum penicillins, began to be developed rapidly, especially between the 30s and 60s, and many infectious diseases were successfully treated. About 5000 antibiotics are known in the world today. Of these, the number of meticulously researched drugs is 1000, and about 100 of them have been actively used in clinical use today. These antibiotics used affect five basic features. Cell membranes,

bacterial protein synthesis, bacterial nucleic acid replication and repair, and bacterial metabolisms are a few examples of them. In recent years, we resort to the use of antibiotics in all areas of our lives. The health and growth of aquatic species like fish, the preservation of food in the food industry, human and animal health, as well as scientific research in hospitals and the pharmaceutical business, all make substantial use of antibiotics. However, as a result of this intensive, unconscious and unnecessary use of antibiotics, health problems are encountered in humans and other living things. Antibiotics are in first place among the drugs used all over the world. In addition, incorrect and excessive use of antibiotics is more common in developing and developed countries. Because antibiotics are sold in pharmacies, markets and similar places even without a prescription.

Antibiotic resistance occurs in bacteria in response to the use of these drugs. The ability of a microorganism to resist the effects of antimicrobial drugs is called resistance. Antibiotic resistance is an example of this, as bacteria are creatures that can adapt quickly to changes in their environment. The inability of an antibiotic to kill or stop the spread of resistant bacteria at the therapeutic dose is known as resistance to that antibiotic. The problem of antibiotic resistance, which has been occurring in microorganisms since ancient times, has become quite effective today. Antibiotic resistance in bacteria increases day by day due to many reasons such as intensive care patients, immune system patients and the increased careless use of antimicrobial drugs and the use of these drugs in other areas. Antibiotics used especially in hospitals contribute to this increase in resistance. The problem of resistance caused by Gram-negative microorganisms that have developed since the 1970s has become very important today (Guillaume André Durand et al., 2019). Since antibiotics are mostly used in hospitals, the problem of resistant bacteria is mostly encountered in hospitals. Microorganisms with the most common resistance problem in infections in Turkey; *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Coagulase Negative Staphylococci, Enterobacter spp, Enterococci, Pseudomonas aeruginosa* and *Acinetobacter spp.* The resistance mechanisms that bacteria develop against antibiotics can take different forms. For example, a bacterium may develop more than one mechanism of resistance to the same antibiotic. This is called cross-resistance. The development of resistance of bacteria to many antibiotics with different structures and effects is called multi-drug resistance. Bacteria with antibiotic resistance gain the advantage over non-resistant bacteria in the presence of antibiotics, and as a result, resistant bacteria do not allow bacteria without resistance genes to multiply, causing the resistance genes to spread in the bacterial population.

Antibiotic-resistant bacteria have increased in recent years and continue to increase. It poses a major health threat as it causes higher medical costs, increased hospital stays death and disease rates all over the world (Aslam et al., 2018). New infection mechanisms are emerging and spreading worldwide. Antibiotic resistance is accelerated by the misuse and overuse of antibiotics as well as the mismanagement of infection prevention and control. For this reason, there is a constant need to produce new antibiotics. But producing new antibiotics takes a long time. Because the antibiotic spectrum of bacteria has increased a lot. So much so that it's possible that in the near future, antibiotics may no longer be able to treat infectious illnesses at all, and even minor wound infections may prove fatal. In addition, current antibiotic strategies are not the specific strategy that targets only antibiotic-resistant bacteria. If this situation is not prevented, the danger that awaits us in the future is much greater than this. New projects are needed to reduce the impact and limit the spread of resistance. As a result of the gradual spread of multi-antibiotic-resistant microorganisms, serious problems are experienced in the treatment of bacterial infections due to both gram-positive and gram-negative microorganisms, which increases the need for new antibiotics (Frieri et al., 2017).

One of the bacterial species found in the big intestine of animals is the gram-negative bacterium *E. coli*. It belongs to the normal intestinal flora. It reproduces best at body temperature, as it is adapted to growth in the intestines of mammals. Between 100 billion and 10 trillion E. coli bacteria move through the human body through faeces each day. Bacteria often do not cause disease because the host organism and E. coli, a typical component of the gut flora, get along well. However, if it enters the environment, which may be another organ in the same organism or the gut of another host organism, *E. coli* becomes a disease agent. Many varieties can spread disease to people even though they are harmless to the animal they are in. These diseases include diarrheal diseases, but also urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia (Gomes et al., 2016). There are even strains such as O157:H7, which are much more dangerous and deadly when this bacterium is transmitted to humans. The appropriate treatment depends on the antibiotic susceptibility of the *E. coli* type causing the infection. *E. coli* strains that produce broad-spectrum beta-lactamases produce a beta-lactamase enzyme that is resistant to various antibiotics, and their treatment is much more difficult (Bajaj et al., 2016). Urinary tract and intestinal infections caused by methicillin-resistant *E. coli* are seen with increasing frequency and may lead to complications with treatment inadequacies. To combat resistance, new antimicrobial strategies should be developed against antibiotic-resistant bacteria. At this point, a more targeted and specific method is needed as an alternative to antibiotics to overcome the resistance problem. This needed method is the CRISPR/Cas system.

E. coli, a gram-negative bacterium, is one of the bacterial species living in the large intestine of mammals. It belongs to the normal intestinal flora. It reproduces best at body temperature, as it is adapted to growth in the intestines of mammals. The number of *E. coli* bacteria that pass through the human body through faeces in a day is between 100 billion and 10 trillion. Since there is a harmonious relationship between *E. coli*, a normal member of the intestinal flora, and the host organism, bacteria do not normally cause disease. However, if it enters the environment, which may be another organ in the same organism or the gut of another host organism, *E. coli* becomes a disease agent. Although they are harmless to the animal they are in, many types can cause disease when they pass on to humans. These diseases include diarrheal diseases, but also urinary tract infections, meningitis, peritonitis, mastitis, septicemia and gram-negative pneumonia (Gomes et al., 2016). There are even strains such as O157:H7, which are much more dangerous and deadly when this bacterium is transmitted to humans. The appropriate treatment depends on the antibiotic susceptibility of the *E. coli* type causing the infection. *E. coli* strains that produce broad-spectrum beta-lactamases produce a beta-lactamase enzyme that is resistant to various antibiotics, and their treatment is much more difficult (Bajaj et al., 2016). Urinary tract and intestinal infections caused by methicillin-resistant *E. coli* are seen with increasing frequency and may lead to complications with treatment inadequacies. To combat resistance, new antimicrobial strategies should be developed against antibiotic-resistant bacteria. At this point, a more targeted and specific method is needed as an alternative to antibiotics to overcome the resistance problem. This needed method is the CRISPR/Cas system.

The 2020 Nobel Prize in Chemistry has been awarded to Emanuelle Charpentier and Jennifer Doudna for developing the CRISPR/Cas9 gene editing method. But in fact, the history of all this technology and gene editing dates to 1953, when DNA was discovered to be double-stranded. Since this date, the whole world of science has started to work on different methods to ensure the deletion, addition or change of DNA sequences in genes. However, the efficiency of the developed methods was low and the usage area was limited. CRISPR, on the other hand, allows the editing of the genetic code in almost every organism. It's also simpler, cheaper, and more precise than previously developed gene-editing methods.

Antibiotics and Their Discovery

Antibiotics have been known and used for treatment since ancient Egypt, before modern medicine. Antibiotics used in medicine today are secretions of bacteria and fungi. The effects of fungi are seen in wound and burn treatments. Medieval healers used microorganisms such as fungi and moulds to treat diseases. This is because microorganisms must produce and develop antimicrobial agents to fight, survive and gain resistance in their habitat. In addition, microorganisms that naturally produce antibacterial products also have resistance genes that include a biosynthetic antibiotic against antibacterial to reduce and prevent the toxicity effect.

Antibiotics were hailed as very useful drugs when they first appeared, and both patients and doctors were surprised by their enormous impact on advanced bacterial infections. The medical world hoped that antibiotics would cure all bacterial infections. Before the discovery of antibiotics, surgery was applied to the infected area or that area was treated with compounds such as silver. But after the discovery of antibiotics, that is, the rapid diversification and increase of antibiotics in the twentieth century, it has made a great contribution and success to clinicians in the treatment of various bacterial infections.

The introduction of antibiotics into human life was one of the greatest discoveries to affect the 20th century. This discovery made possible many medical techniques such as cancer, organ transplants, and open surgery, as well as the treatment of bacterial and infectious infections.

However, because of all these, the rapid and excessive use of this valuable compound made some infections unable to treat effectively and caused a rapid rise in antimicrobial resistance (AMR).

Antibiotic Resistance

In recent years, antibiotic resistance gained by bacteria is one of the leading global health problems. Due to its negative effects on both public health and the country's economy, it has become one of the most important issues in the global agenda in general, not in the field of health. The rapidly increasing rates of antibiotic resistance have had effects in areas such as health, economy, development, and trade, and it is predicted that they will have more impacts in the coming years. Antibiotic resistance, since the discovery of antibiotics and the use of bacteria as pharmacological agents, can develop resistance and if precautions are not taken, the antibiotics we currently use will become ineffective in the treatment of diseases (Frieri M et al., 2017). As a result, it is foreseen that humanity will again encounter the period before the discovery of antibiotics. While antibiotics were used in the treatment of bacterial diseases and infections in the past, today bacteria can develop resistance to new antibiotics in a short and rapid period due to the use of incorrect and high doses of antibiotics. As a result of resistance, there will be no response to antibiotics used in the community, and high, incurable infections will be encountered throughout the world. As a result of all these, antibiotic-resistant bacteria have become a health problem that threatens humanity both today and in the future.

Many resistance mechanisms have been identified in bacteria. Resistance mechanisms in bacteria are examined under two main headings. The first is natural resistance and the second is acquired resistance. The acquired resistance is divided into many sub-branches among itself (Davies J et al., 2010). These are resistance mechanisms that develop because of antibiotic inactivation, resistance that develops as a result of changes in the target molecule, active pump systems, resistance that develops as a result of cell wall permeability changes, and other mechanisms. Innate resistance is expressed as the inability of all strains of a bacterial species to be affected by some antibiotics. This resistance mechanism is formed by structural and biochemical features depending on the nature of the bacteria. Bacteria may not contain a site where antibiotics can bind, or they may show lower permeability depending on the chemical structure of antibiotics. In addition, although the antibiotic cannot enter the bacteria it will affect, resistance occurs, and its effect is still limited. For example, Because macrolides are too large to reach the cytoplasmic target and pass through the cell wall, Gram-negative bacteria are inherently resistant to the activity of macrolides.

Acquired resistance is the fact that bacteria that are sensitive to antibiotics by nature are not affected by antibiotics, albeit in various ways. The mechanism of acquired resistance also differs according to the type of bacteria. This mechanism can be divided into two main headings, biochemical and genetic. Biochemical mechanisms vary greatly. A bacterial strain can acquire resistance by one of these resistance mechanisms or by more than one mechanism. Depending on the type of antibiotic, the target location, the bacterial species, the resistance plasmid, and other elements like mutations, this may change.

CRISPR/Cas9

Bacteria are creatures that can live in harsh and varied conditions, but they can be infected by phages. The infection causes 4%-50% of the death of bacteria (Karginov FV. et. al., 2010). Bacteria have developed many resistance mechanisms to protect themselves from this infecting and lethal effect of phages. One of them is innate immunity Cas9 technology. CRISPR/Cas9, Clustered regularly spaced palindromic repeats, is a gene editing technology (Fuguo Jiang et al., 2017). It is the immune system that bacteria have developed against infection by bacteriophages and plasmid transfer (Hyman P et al., 2010). This technology is mainly based on two components. The first catalyzes the formation of double-strand breaks in a desired region of the genome via the endonuclease Cas9, which causes a double-stranded DNA break and will alter the genome, and a guide RNA (sgRNA) for matching to the target gene (Redman M et al., 2016). Genome editing is carried out by using the DNA repair mechanisms of these breaks: HR: CRISPR/Cas9 technology has become very common today and has been used in many areas. Examples include the identification of bacterial strains, determination of gene and miRNA functions, insertion/removal of DNA fragments from the genome, gene silencing, transcriptional and epigenetic targeting, or establishment of disease models. The reason for this widespread use is that CRISPR technology allows to correction of the mutated region quickly, cheaply and easily in the genes of a cell and organisms (Gumus. N., et al., 2018).

Alternative Therapies CRISPR/Cas9

New novel anti-virulence strategy schemes to overcome and prevent the problem of antibiotic resistance, including bacteriophage treatments, probiotics, therapeutic antibodies, synthetic inhibitor drugs that specifically inhibit resistance enzymes, bacterial efflux pumps, biofilm formation, fatty acid and biosynthesis pathways, therapeutic solutions have been developed. Over the past two decades, genome modification methods have been developed to prevent and counteract infectious diseases (Aslam B et al., 2020). These developed methods had some disadvantages such as low efficiency, off-target effect and long-time consumption. In addition, because the development of new antibiotics takes a long time and is very costly, only a few companies are currently investing and working in antibiotic drug discovery. For all these reasons, there is an urgent need for new treatment methods to resist resistant bacteria. The CRISPR/Cas system to be used in this thesis is a fast, simple, low-cost and high-efficiency method. These advantages of CRISPR technology have been used and continue to be used in many areas of science. Alternative therapy CRISPR/Cas technology could revolutionize the way we treat infections in the future.

Conclusion

Producing antibiotics has become a very important need for humanity today and a problem that concerns the whole world. With this need, antibiotic-resistant bacteria have increased, and this increase is expected to cause great problems for humanity in the long run. However, since antibiotic production is very slow and difficult, alternative CRISPR antimicrobials to antibiotics will be produced and used as an agent in the future and will benefit indigenous added value. We aim to develop CRISPR/Cas as an antimicrobial agent against methicillin-resistant E. coli bacteria. Since this system will be more targeted, it will not harm the microflora and will also eliminate one of the current disadvantages of antibiotics. Thanks to the advanced genetic engineering technology CRISPR/Cas to be used in the project, new antimicrobial agents that can specifically target methicillin-resistant E. coli will be produced. Thus, CRISPR will be able to use as an alternative antimicrobial agent to antibiotics in the future and will facilitate the production of antimicrobials with CRISPR technology.

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Chaperonin Variants in The Genus Acetobacter impacts Resistance To The Heat-Shock Stress.

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Abstract: Extreme cold regions affect the kinetics of biochemical reactions in a stressful environment, the transport and properties of substrates, the mobility of membranes, and the structure of macromolecules such as DNA and proteins, and greatly alter the physicochemical environment of living cells. Organisms living in psychrophilic conditions adapt to low temperatures and the folding ability of the proteins. The cpn60 and cpn10 chaperones have a positive effect on growth at low temperatures by increasing the refolding capacity of proteins at low temperatures. HSP60 (Heat shock protein-60), also known as chaperonins (Cpn), has important roles in the regulation and protection of both prokaryotic and eukaryotic cells, especially during environmental stress. Here we discuss that it has been discussed that the bacteria in the genus Acetobacter can be compared with each other by phylogenetic analysis, determining which species are more resistant to cold. After the phylogenetic analysis, it will be discussed that variants in the Acetobacter genus that express a heat-shock protein or are resistant to heat-shock stress can be taken. Here we determine the most efficient temperature value by culturing at various temperatures.

Key words: Chaperonin, Acetobacter, Heat shock protein

Introduction

At temperatures below -5°C, terrestrial bacteria can maintain long-term survival by compensating for cellular and macromolecular damage from their low metabolic rate. Organisms living in psychrophilic conditions adapt to low temperatures, and the folding ability of the proteins that provide this is much easier than at high temperatures. What facilitates this is the molecular chaperones responsible for protein folding processes. In this regard, it is known that some proteins tend to deteriorate and precipitate in low-temperature environments. Under these conditions, chaperonins are as active as possible, able to preserve the proteins they help fold in a closed compartment so that cell survival is possible even when the temperature is lower. Chaperones of cold-adapted organisms can confer much more adaptive properties than cold-shock proteins produced at short-term cold temperatures (Varin et al., 2012; Nunn et al., 2014; Mei et al., 2016). The more cold-resistant *Acetobacter* species can be identified and the genes encoding cold-active enzymes can be cloned. Chaperonins; They are proteins found in mitochondria and chloroplasts of bacteria, palaeontology, and eukaryotes. In cases where natural folding occurs, some of the cellular proteins perform important activities such as folding. The main task of molecular chaperones is to selectively bind to protein recognition to form stable complexes. The variously named Hsp60/Cpn60/GroEL, and TF55/CCT families contain chaperonins, and these cellular proteins form relatively large protein complexes for specific protein folding or assembly. These protein complexes involved in the folding of some denatured or newly synthesized proteins grow or contract through ATP binding or hydrolysis and play an important role as chaperones. Under normal conditions, "specific" molecular chaperones are also involved, which are involved in the assembly of specific multiprotein, also known as heat shock (stress) proteins (Ferrer et al., 2004; Fink, 1999]. It prevents the aggregation and misfolding of newly synthesized chains during folding, preventing inefficient interactions with other cellular components, leading to the assembly of larger proteins and multiprotein complexes, and unfolding of pre-folded proteins during stress exerting their effects. The folding pathways appear to be similar in both in vivo and in vitro studies. These cellular proteins are large multimeric complexes and are responsible for the folding and proper assembly of newly synthesized proteins (Fink, 1999).

Molecular chaperones are involved in the folding of some newly synthesized proteins and recognize unnatural substrate proteins from their hydrophobic residues. In addition to known heat shock proteins, one of the few classified chaperones was recently

identified as hsp40 in mammalian and avian cells (Otsuka et al., 1990). This 40 kDa protein hsp has been shown to share limited homology with the human amino acid sequence to the bacterial DNAJ heat shock protein [Hattori et al., 1993]. Other members of the Hsp family are 60 kDa, 70 kDa and 90 kDa heat shock proteins. The 60 kDa heat shock protein is large and circular. These chaperonins bind to partially folded intermediates and prevent their assembly. This makes folding easier. It exhibits similar features to the 70 families, but its mechanism is different. The activities of both families are regulated by ATP binding and hydrolysis. They belong to group 1 companions and are also called HSPD1 or Cpn60 (Bavisotto et al., 2010; Decalis et al., 2006). They are found in all biological parts except the ER, and it has also been suggested that misfolded structures can unfold and refold to prevent clumping. It consists of GroEL-like proteins and the TCP-1 family. Hsp60 proteins are large, have a gap in their centers where unnatural protein structures are attached, and their three-dimensional structure is likened to a hollow sphere (Fink, 1999; Walter, S., & Buchner, J., 2002).

Hsp60 is controlled by the cpn10 chaperone. Cpn-10 is also known as GroES in E. coli. GroEL recognizes polypeptides as potential substrates due to the exposed hydrophobic surface property of unfolded or misfolded proteins (Fink, 1999; Walter, S., & Buchner, J., 2002). The Hsp60 monomer consists of three structural domains. From these three parts, apical, middle, and equatorial, the apical domain substrates bind co-chaperones and participate in ATP conversion. The intermediate zone connects the apical and equatorial zones. The equatorial space facilitates communication between the two heptamer rings of the chaperone (Bavisotto et al., 2020). Self-refolding of proteins is better at low temperatures than at high temperatures, and for this to happen, molecular chaperones are needed, which are responsible for the folding of organisms under psychrophilic conditions. Several proteins are cold-denatured and tend to precipitate. Chaperones, which are active at low temperatures, protect the folded proteins in these harsh conditions. They do this by providing closed compartments and increasing cell viability (Ferrer et al., 2004).

Methods

Comparison of Growth Temperatures of *Acetobacter* Species

Bacteria being investigated (**Table 1**), *Gluconacetobacter xylinus* (Taxonomy ID: 28448, GroEL protein GenBank: CAY85843.1) , *Gluconacetobacter oboediens* (Taxonomy ID: 65958, Hsp60 chaperonine GenBank: AGH09511.1), *Acetobacter tropicalis* (Taxonomy ID: 104102, Hsp60 GenBank: AHK26727.1), *Acetobacter senegalensis* (Taxonomy ID: 446692, Hsp60 GenBank: AHK26723.1), *Acetobacter orientalis* (Taxonomy ID: 146474, Hsp60 GenBank: AHK26717.1), *Acetobacter indonesiensis* (Taxonomy ID: 104101, Hsp60 GenBank: AHK26720.1), *Acetobacter cibinongensis* (Taxonomy ID: 146475, Hsp60 GenBank: AHK26715.1), *Acetobacter orleanensis* (Taxonomy ID: 104099, Hsp60 GenBank: AHK26730.1), *Acetobacter farinalis* (Taxonomy ID: 1260984, Hsp60 GenBank: AHK26732.1), *Acetobacter malorum* (Taxonomy ID: 178901, Hsp60 GenBank: AHK26733.1), *Acetobacter cerevisiae* (Taxonomy ID: 178900, Hsp60 GenBank: AHK26739.1), *Acetobacter aceti* (Taxonomy ID: 435, Hsp60 GenBank: AHK26699.1), *Acetobacter estunensis* (Taxonomy ID: 104097, Hsp60 GenBank: AHK26694.1), *Acetobacter oeni* (Taxonomy ID: 304077, Hsp60 GenBank: AHK26691.1), *Acetobacter nitrogenifigens* (Taxonomy ID: 1120919, Hsp60 GenBank: AHK26690.1), *Acetobacter peroxydans* (Taxonomy ID: 104098, Hsp60 GenBank: AHK26705.1), *Acetobacter lovaniensis* (Taxonomy ID: 104100, GroEL GenBank: AGC10738.1), *Acetobacter fabarum* (Taxonomy ID: 483199, Hsp60 GenBank: AHK26714.1), *Acetobacter ghanensis* (Taxonomy ID: 431306, GroEL GenBank: AGC10737.1), *Acetobacter syzygii* (Taxonomy ID: 146476, GroEL GenBank: AGC10736.1), *Acetobacter pomorum* (Taxonomy ID: 65959, Hsp60 GenBank: AHK26706.1) and *Acetobacter pasteurianus* (Taxonomy ID: 438, Hsp60 GenBank: AHK26713.1). Since Hsp60 protein information is not available, GroEL partial sequences of *Acetobacter ghanensis*, *Acetobacter syzygii*, *Acetobacter lovaniensis,* and *Gluconacetobacter xylinus* bacteria were used. In **Table 1**, the growth conditions of *Acetobacter*-type bacteria were investigated. Some of the references belong to the Leibniz Institute German Microorganisms and Cell Cultures Collection (DSMZ) and Japanese Microorganism Collection (JCM). DSM no is the number of bacteria registered with the Leibniz Institute. It is aimed to analyze the protein sequence information of the bacterial species obtained from the NCBI database in FASTA format and observe their relationship with each other and obtain a phylogenetic tree from the BLAST database.

Table 1: Growth temperatures of *Acetobacter* species

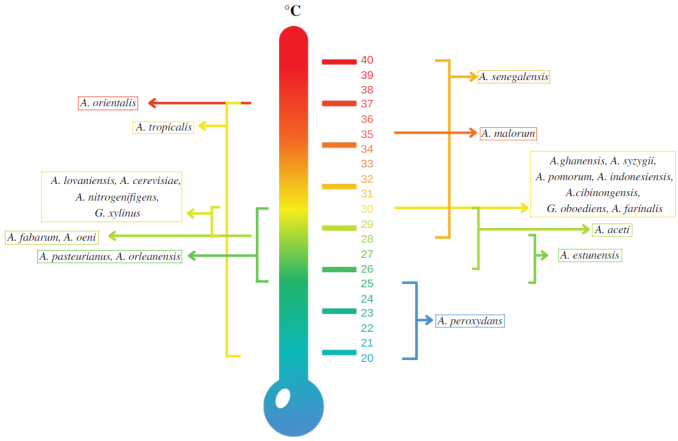
Organism	**Growth temperature (°C)**	**Temperature tolerance**	**Source and/or reference**
Acetobacter tropicalis	20-37	17	Bartowski et al., 2008
Acetobacter senegalensis	28-40	12	Ndoye et al., 2007

Acetobacter orientalis	37	0	Leibniz Institute DSMZ, DSM no: 15550 Chen et al., 2010
Acetobacter indonesiensis	30	0	Leibniz Institute DSMZ, DSM no: 15552
Acetobacter cibinongensis	30	0	Leibniz Institute DSMZ, DSM number: 15549
Acetobacter orleanensis	25-30	5	Boulton et al., 1996
Acetobacter farinalis	30	0	Tanasupawa t et al., 2011
Acetobacter malorum	35	0	Cleenwerck et al., 2002
Acetobacter cerevisiae	28-30	2	Leibniz Institute DSMZ,
Acetobacter aceti	26-30	4	DSM no: 14362
Acetobacter estunensis	25-28	3	JCM
Acetobacter oeni	28	0	König et al., 2009
Acetobacter nitrogenifigens	28-30	2	Leibniz Institute DSMZ,
Acetobacter peroxydans	20-25	5	DSM no: 23921
Acetobacter lovaniensis	28-30	2	JCM
Acetobacter fabarum	28	0	
Acetobacter ghanensis	30	0	Viticulture& Enology-UC Davis
Acetobacter syzygii	30	0	Leibniz Institute DSMZ,
Acetobacter pomorum	30	0	DSM number: 4491
Acetobacter pasteurianus	25-30	5	Leibniz Institute DSMZ,
Gluconacetobacter xylinus	28-30	2	DSM no: 19596
Gluconacetobacter oboediens	30	0	Leibniz Institute DSMZ, DSM no: 11826

Results

Temperature tolerance of the *Acetobacter* genus was investigated to determine the resistance against heat shock stress in a temperature-dependent manner. In **Figure 1**, the growth conditions of *Acetobacter* spp. bacteria according to temperature are shown with colour tones from blue to red (cold to hot). *A. peroxydans* can grow in colder conditions than other bacteria, at 20-25°C. *A. senegalensis* and *A. tropicalis* bacteria are more thermotolerant than other bacteria, with growth temperatures of 20-37°C and 28-40°C, respectively. *A. pasteurianus, A. orleanensis, A. estunensis, A. aceti, A. lovaniensis, A. cerevisiae, A. nitrogenifigens, G. xylinus, A. ghanensis, A. syzygii, A. pomorum, A. indonesiensis, A. cibinongensis, G. oboediens* and *A. farinalis* bacteria have an average growth temperature (25-30°C) compared to other bacteria. *A. malorum* (35°C) and *A. orientalis* (37°C) bacteria can grow at high temperatures.

Figure 1. Displaying the temperatures at which bacterial species



can multiply on the coloured thermometer.

Multiple Sequence Alignment and Phylogenetic Analysis of *Acetobacter* Species by Hsp60 Sequences

The 22 reference strains used for sequence comparison of Hsp60 partial sequences are listed in **Table 2**. The Hsp60 sequences of all strains were aligned **(Figure 3)** and specific regions were screened using the Clustal X program, version 1.8.

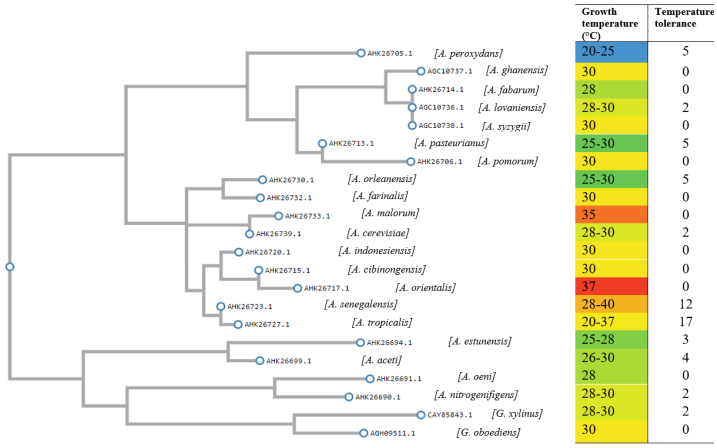
Table 2 Similarities of partial hsp60 sequences among type strains of the *Acetobacter* and *Gluconacetobacter* group*

Similarity of *hsp60* nucleotide sequences (%)	*G. xylinus*	*G. oboediens*	*A. tropicalis*	*A. senegalensis*	*A. orientalis*	*A. indonesiensis*	*A. cibinongensis*	*A. orleanensis*	*A. farinalis*	*A. malorum*	*A. cerevisiae*	*A. aceti*	*A. estunensis*	*A. oeni*	*A. nitrogenifigens*	*A. peroxydans*	*A. lovaniensis*	*A. fabarum*	*A. ghanensis*	*A. syzygii*	*A. pomorum*	*A. pasteurianus*
G.xylinus **"CAY85843.1	96	91	91	91	91	90	90	90	91	90	92	90	91	92	91	90	90	91	90	91	88	
G. oboediens "AGH09511.1		91	92	92	92	91	91	91	91	90	93	91	92	92	90	90	91	89	90	91	89	
A. tropicalis "AHK26727.1			99	96	99	98	97	97	97	96	98	91	91	91	94	93	93	93	93	95	93	
A. senegalensis "AHK26723.1				98	99	99	97	98	97	98	92	92	92	92	95	94	94	93	94	95	94	
A. orientalis "AHK26717.1					97	96	91	93	97	86	93	91	91	81	89	91	92	90	91	91	94	
A. indonesiensis "AHK26720.1						99	98	98	96	97	92	91	92	91	94	93	93	93	93	95	93	
A. cibinongensis "AHK26715.1							95	97	94	90	93	91	91	84	92	94	93	93	94	95	95	
A. orleanensis "AHK26730.1								98	92	92	92	92	90	90	94	94	93	93	93	94	93	
A. farinalis "AHK26732.1									93	92	93	91	91	86	94	94	93	93	94	94	92	
A. malorum "AHK26733.1										86	88	90	89	78	89	76	81	76	76	92	94	
A. cerevisiae "AHK26739.1											93	90	90	91	89	94	93	94	94	94	92	
A. aceti "AHK26699.1												96	92	93	91	78	92	77	78	92	91	
A. estunensis "AHK26694.1													93	92	91	91	90	89	91	90	88	
A. oeni "AHK26691.1														97	89	89	88	88	89	89	87	
A. nitrogenifigens "AHK26690.1															85	89	89	88	89	88	87	
A. peroxydans "AHK26705.1																95	95	94	95	95	95	
A. lovaniensis "AGC10738.1																	98	99	100	96	96	
A. fabarum "AHK26714.1																		96	98	95	95	
A. ghanensis "AGC10737.1																			99	98	96	
A. syzygii "AGC10736.1																				96	96	
A. pomorum "AHK26706.1																					96	
A. pasteurianus "AHK26713.1																						

*The numbers shown in the boxes are the ClustalW alignment scores.

** The hsp60 sequences of these bacterial strains were obtained from the NCBI database.

The neighbor-joining method was used with calculated genetic distances and the phylogenetic tree was constructed with the PhyML computer program package **(Figure 2)** [Guindon et al., 2010]. The tree was constructed using the neighbor-joining method. The scale bar represents a 0.01% sequence difference.



Growth temperature (°C)	Temperature tolerance
20-25	5
30	0
28	0
28-30	2
30	0
25-30	5
30	0
25-30	5
30	0
35	0
28-30	2
30	0
30	0
37	0
28-40	12
20-37	17
25-28	3
26-30	4
28	0
28-30	2
28-30	2
30	0

Figure 2 Phylogenetic tree based on hsp60 sequences of *Acetobacter* spp.

Discussion

This research aims to investigate the growth conditions of some bacterial species, *Acetobacter* and *Gluconacetobacter*, according to temperature, and to test whether variants in the cpn60 gene may be phylogenetically related to temperature tolerance in bacterial species that are close to each other. For this, firstly, research was carried out on the living conditions of bacterial species **(Table 1),** and the bacterium that was more resistant to cold was determined as *Acetobacter peroxydans*. As seen in Table 1, bacteria were ranked according to temperature tolerance and viable conditions reported in the literature, and colour adjustment was made on the thermometer figure **(Table 1 and Figure 1)**. Temperature values and tolerances of bacterial species are found in Table 1. It was investigated that while *A. tropicalis* can grow at 20°C, it can also grow at 37°C and *A. senegalensis* can grow between 28-40°C. It was concluded that the temperature tolerances of these two bacteria were much higher than those of the other bacteria. It was concluded that *A. orientalis* grows at 37°C and *Acetobacter malorum* at 35°C, together with *A. tropicalis* and *A. senegalensis*, in much warmer environments than other bacterial species. In Table 2, multiple sequence alignments were performed with partial Hsp60 sequences of bacterial species, and their similarities to each other were subtracted. In sequence alignment, 76-99% sequence similarity was observed between bacterial species. A few of them were selected and compared. For example, the sequence similarity of *G. xylinus* and *A. lovaniensis* bacteria is 90%, and their temperature tolerance and living conditions are the same. The sequence similarity between *A. peroxydans*, which is more cold-resistant than other bacteria, and *A. pasteurianus*, which has an average growth temperature, is 95% and their temperature tolerance is the same. In addition, *A. tropicalis* and *A.*

senegalensis bacteria, which have a much higher temperature tolerance than other bacteria, can grow at very low and very high temperatures, and their sequence similarity is 99%. A phylogenetic tree showing the relationships of bacterial species with each other through sequence similarities was extracted using the ClustalX program **(Figure 2)**. The distances of bacterial species from each other were observed.

Comparing the living conditions and interrelationships of bacterial species screened and studied in the literature is important to understand the functioning and activity of hsp60. When the temperature-dependent living conditions of *Acetobacter* and *Gluconacteobecter* bacteria species are examined, it has been revealed that they can grow and multiply at different temperatures, even though they are similar to each other. It has been reported that some bacteria can survive in a single degree of reproduction. For example, for *A. orientalis*, which can grow at 37°C, not saying that it can grow at 30-40°C may prevent a definite talk about tolerance. Comparisons may vary since there are bacteria whose tolerance we know, as well as bacteria whose tolerance we do not know. However, we can show a grouping according to the information given. Phylogenetic trees are simple diagrams depicting the origin and evolution of groups of organisms and give us information on how living/extinct species are related to each other [Kaplı et al., 2020; McLennan, 2010] The phylogenetic tree was created and examined with the ClustalX program and hsp60/GroEL sequence information of the examined bacterial species. Through this phylogenetic tree, the proximity and distance information of bacterial species of the genus *Acetobacter* and *Gluconacetobacter* has become visible **(Figure 2)**. When the temperature conditions and temperature tolerances of bacterial species are compared together in the phylogenetic tree, the first thing that stands out is that the temperature conditions they can live in are overlapping with each other. While *A. peroxydans* is a cold-resistant bacterium, it has a high affinity with other bacteria with an average temperature. Although *A. cerevisiae* and *A. malorum* are closely related to each other in the phylogenetic tree, *A. malorum* can live in much warmer conditions. This relationship is also valid between *A. orientalis*, which can grow in a much warmer environment, and *A. cibinongensis*, which has an average temperature. Considering the temperature tolerances, *A. orientalis*, *A. senegalensis* and *A. tropicalis* are three heat-resistant bacteria and it is seen that their relationships are close in the phylogenetic tree. However, the temperature tolerance of *A. orientalis* is not as high as the tolerance of the other two bacteria, or it may not be. Finally, after *A. tropicalis*, which has a high temperature tolerance, the branch is separated and it is observed that there is a direct transition to bacteria with an average temperature. While few of the question marks are like this, it is of great importance to understand the analysis of the phylogenetic tree and to compare the hsp60 protein information with the temperature and temperature tolerance information of the bacteria.

It requires systematic study of the genome and reprogramming of genes in cells, accurate and predictable ways to target a range of genes for expression or repression. The CRISPR-Cas9 system is a genome editing tool and genetic engineering approach based on a bacterial defense system against foreign genetic elements and this system provides a potential platform for targeted gene regulation. The CRISPR-Cas system offers precise, targeted, widespread use in genome editing of prokaryotes as well as eukaryotes. Some of the most important features of this system are low cost, robustness, originality, simplicity, and efficiency, it offers more advantages over other traditional genetic engineering tools [Parveen et al., 2020; LS et al., 2013]. By using the genome editing tool CRISPR, it is possible to transfer this gene to targeted bacteria with high production efficiency, together with the modifications that can be made to the hsp60 genes of these bacteria by identifying the bacterial species that are more resistant to cold. Thus, the targeted bacterial species can become more resistant and resistant to stress. An example of increasing production efficiency in bacteria is bacteria that can produce cellulose. Bacterial cellulose, which is the cellulose of bacterial origin; attracts great attention with its mechanical properties, non-toxicity, renewability and biodegradability. Bacterial cellulose is produced as an extracellular primary metabolite by many bacterial species, including the genus *Acetobacter*. The most effective producer is *Acetobacter xylinum*, the gram-negative acetic acid bacterium, which is included in the *Gluconacetobacter* genus as *G. xylinus* [Vazquez et al., 2013] In the last decade, there has been a great increase in bacterial cellulose-based activities. The usage areas of bacterial cellulose are also quite wide and continue to develop as time goes on. Bacterial cellulose, which can be used in wound healing systems, tissue engineering, cosmetics, immobilization applications, drug delivery system and pharmaceutical coatings, can also be used in environmental and agricultural areas, electronics, food applications, industrial applications and many other areas [Mohite et al., 2014]. Based on this information, modifications to the hsp60 gene of *G. xylinus* bacteria, which can produce bacterial cellulose, may allow the bacteria to become more resistant to harsh conditions and increase production efficiency.

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Author Contributions

M.T. and C.T. designed and completed the bioinformatic studies. M.T. and C.T. wrote the manuscript and contributed to the

editing of the manuscript. C.T. led the investigation and development study and contributed to the design and interpretation of the data.

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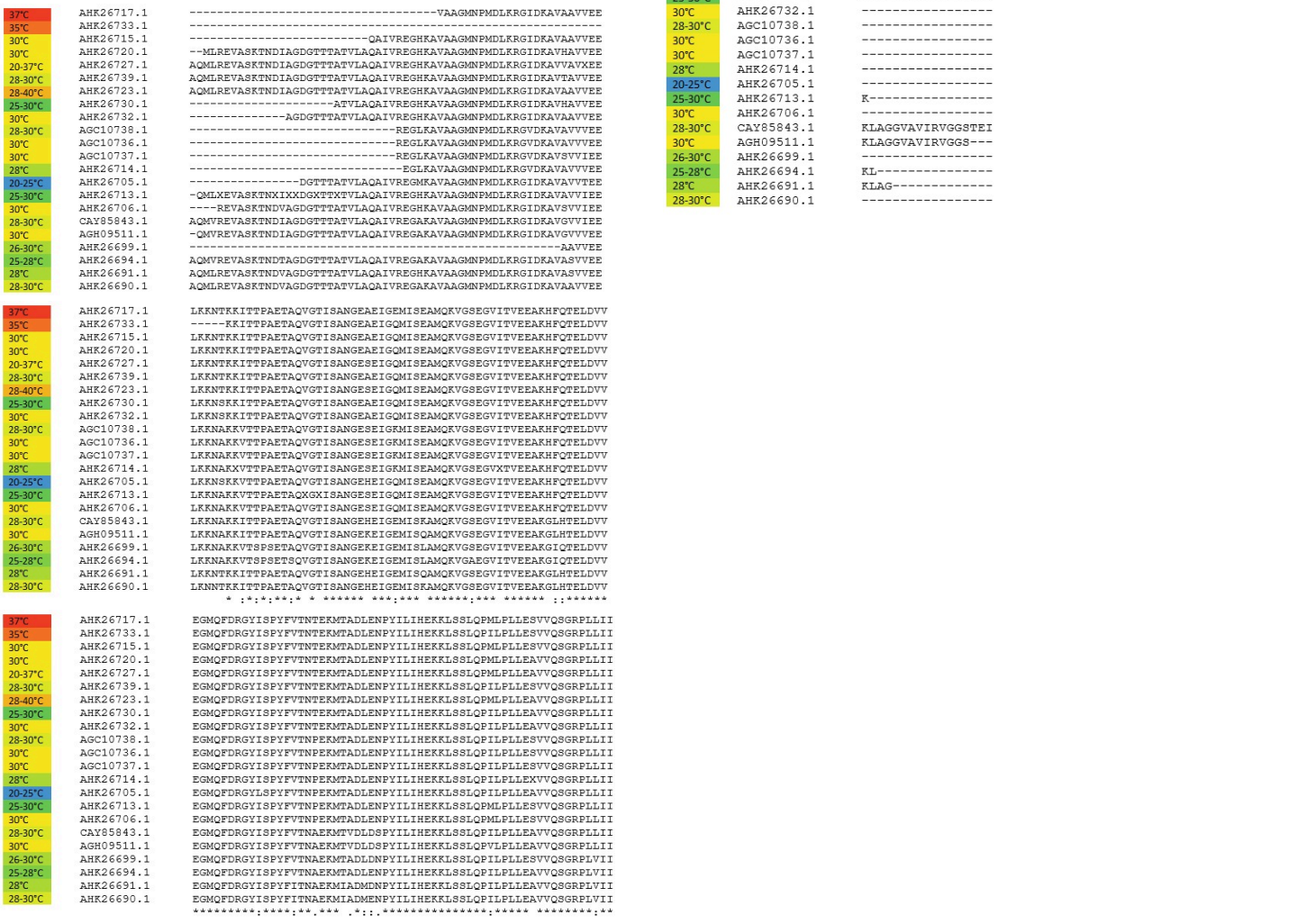
Supplementary Material

Multiple sequence alignment of partial hsp60, GroEL, and chaperone genes from 22 different bacteria. Protected regions are indicated by asterisks.



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Fanzor: A Promising CRISPR-like Gene Editing Tool in Eukaryotes

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Abstract: The genetic editing landscape has witnessed a transformation with the revelation of Fanzor, an innovative CRISPR-like system identified within eukaryotic organisms. Discovery of Fanzor introduces a new dimension to gene editing research, presenting an alternative to the widely recognized CRISPR/Cas systems. In this review, we explore the critical facets and ramifications of the Fanzor system, shedding light on its potential applications and advantages for precise gene manipulation in complex organisms.

Key words: Fanzor, CRISPR, Gene editing

1. Introduction

The revolutionary breakthrough of CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated) gene-editing systems, derived from prokaryotic organisms, has transformed the landscape of genetic engineering (reviewed in 1-2). Particularly, the Cas9 nuclease within these systems serves as a versatile and precise tool for manipulating DNA sequences across diverse life domains. The impact of CRISPR/Cas reaches far beyond its initial discovery, finding applications in a multitude of scientific, medical, and agricultural pursuits (reviewed in 3-5) . From targeted gene therapies to the development of genetically modified organisms, CRISPR/Cas stands as a cornerstone, facilitating unprecedented achievements in the field of genome editing and medicine (6).

Although CRISPR/Cas systems are renowned for their effectiveness, their primary elucidation occurred in prokaryotic organisms, notably bacteria and archaea. This has led to a longstanding question that has intrigued researchers: do analogous systems exist in eukaryotes? Eukaryotic organisms, encompassing fungi, plants, and animals, exhibit more intricate cellular structures and functions. Consequently, the pursuit of CRISPR-like systems within eukaryotes has become a compelling scientific endeavor.

In a significant leap forward, researchers have disclosed the existence of the inaugural programmable RNA-guided system in eukaryotes, introducing Fanzor (Fz) as a groundbreaking discovery (7). Fanzor utilizes RNA as a guide to precisely target DNA, mirroring the mechanism of CRISPR/Cas systems. This revelation carries profound implications for the field of genetic engineering, as Fanzor can be reprogrammed to edit the human genome, presenting an exciting alternative to established CRISPR/Cas systems. Moreover, Fanzor's compact nature and potential for enhanced targeting efficiency position it as a promising candidate for genome editing in complex organisms.

2. Discovery of Fanzor in Eukaryotes

In 2013, the initial characterization of Fanzor identified it as a eukaryotic TnpB-IS200/IS605-like protein, encoded by transposable elements (7). Early assumptions suggested that Fz, akin to its prokaryotic counterpart TnpB, might be involved in regulating transposable element activity, possibly through its methyltransferase activity. However, the dynamic landscape of genetic research has unveiled new links between TnpB and a recently discovered class of RNA-guided systems called OMEGA (Obligate Mobile Element-guided Activity) (8). These OMEGA systems feature an RNA-guided endonuclease protein, such as TnpB, IscB, or IsrB, working in tandem with a non-coding RNA known as ωRNA, transcribed from the transposon end region (8).

What heightens the fascination surrounding these findings is the realization that OMEGA systems serve as early models for the widely acclaimed CRISPR–Cas systems. The progression from TnpB to the singular RNA-guided endonuclease, Cas12, is particularly noteworthy (8). The intriguing aspect of TnpB's distant homology with Fz provocatively suggests that Fanzor could potentially serve as a eukaryotic counterpart to CRISPR–Cas or OMEGA systems. This enticing connection opens up a realm of innovative possibilities for genome editing within the eukaryotic domain. The prospect of Fanzor aligning with these groundbreaking systems adds an exciting dimension to the

ongoing exploration of genetic manipulation, fueling curiosity and paving the way for future advancements in the field.

2.1 Unveiling Fanzor: An RNA-Guided Genome Editing Mechanism

Unraveling the nuances of Fanzor's enzymatic activity and functional mechanism demanded a comprehensive approach, incorporating phylogenomic, biochemical, and structural analyses. This multifaceted investigation not only illuminated the diverse manifestations of Fz across various life forms but also uncovered the potential independent origins of two distinct Fz systems—designated as Fz1 and Fz2 (9-10).

Fz1 predominantly inhabits the realm of fungi, making occasional appearances in protists, arthropods, plants, and even eukaryotic viruses, notably giant viruses. In contrast, Fz2 exhibits a broader presence in fungi, with sporadic occurrences observed in mollusks, choanoflagellates, and eukaryotic viruses, predominantly giant viruses. The consistent presence of TnpB within both Fz branches, often coexisting in branches hosting giant viruses and sporadically interspersed among SAR (Stramenopiles, Alveolates, and Rhizaria) and fungi, compellingly suggests horizontal transfer events from prokaryotes to eukaryotic hosts (10). The intricate connections between eukaryotic radiations and lifestyles intertwined with bacterial species serve to emphasize the adaptability and resilience of these systems. With the potential to reshape the terrain of genome engineering within complex eukaryotic organisms, Fanzor emerges as a key player in the dynamic landscape of genetic manipulation.

Fanzor's genetic origins are intertwined with its integration within transposable elements, which are genomic entities known for their mobility and the ability to move from one location to another within an organism's genome (10). The presence of Fanzor within these elements has a profound impact on its functionality and distribution. Transposable elements often serve as reservoirs for diverse genetic material, including functional genes and regulatory sequences. Fanzor's association with transposons might be a consequence of its role in regulating the activity of these mobile elements.

In a recent groundbreaking study (2021), a novel family of RNA-guided DNA nucleases, termed IscBs, was uncovered (11). These enzymes share relations with Cas9 and TnpB nucleases found in bacteria and archaea, playing roles in transposon mobility. The widespread distribution of IscBs in prokaryotes suggests significant implications in genome editing and defense mechanisms. The study identified over 600 IscB genes in prokaryotic genomes, associating them with various transposons, including IS200/IS605, IS10, and IS110, as well as in viruses, including phages. Demonstrating their programmability to cleave DNA in vitro, IscBs were found in diverse prokaryotic genomes, spanning bacteria, archaea, and eukaryotes, hinting at their potential as a new and crucial class of RNA-guided nucleases.

Similarly, in a study by Jiang et al. (2023), a distinct family of RNA-guided DNA nucleases, Fanzors, was revealed (12). The study identified over 3,600 Fanzor genes across eukaryotic and viral genomes, associating them with various transposons like hAT, LINE, and Mariner/Tc1, as well as in viruses such as Phycodnaviridae, Ascoviridae, and Mimiviridae.

While both IscBs and Fanzors share the ability to programmably cleave DNA as RNA-guided nucleases, notable differences exist (11). IscBs are predominantly found in prokaryotes, associated with transposons, and require a 3' target-adjacent motif (TAM) for activity. In contrast, Fanzors are primarily located in eukaryotes, lack association with transposons, and do not necessitate a TAM. Structurally, IscBs exhibit greater similarity to Cas9, while Fanzors align more closely with TnpB. Despite these distinctions, both IscBs and Fanzors present promising prospects for genome editing and defense. IscBs, with their prevalence in prokaryotes, offer potential applications in this domain, while Fanzors' versatility without a TAM broadens their usability. Ongoing research is crucial to comprehensively understand these enzymes' mechanisms and develop innovative tools and strategies for their effective utilization.

2.2 Versatile Associations of Fanzor Nucleases with Diverse Transposons and noncoding RNAs

Fanzor nucleases exhibit diverse associations with transposons (12). The analysis, conducted within a 10 kb range of Fanzor loci, uncovers connections to various eukaryotic transposons, including well-known families such as Mariner/Tc1, Helitron, and Sola, as well as newly identified associations with hAT and CMCDNA transposons. Fanzors are linked with both autonomous and non-autonomous transposons, and they frequently interact with the DNA transposon hAT. Different Fanzor clades display preferences for specific transposon families. For example, Fanzor1a, b, and d are often associated with hAT, while Fanzor1c predominantly links with LINE, CMC, and Mariner/Tc1 transposons. Fanzor2 shows varied associations with transposons, including Helitron, hAT, and IS607, which carries a TnpA-like transposase, strengthening the connection between Fanzor2 and TnpBs. These findings highlight Fanzors' multifaceted roles in genome dynamics and interactions.

Fanzor nucleases have conserved, structured noncoding RNAs similar to the OMEGA system. Conserved regions, longer in some Fanzor families than TnpB and IscB, extend beyond the

Fanzor ORF. These regions are found in an IS607 transposon in Acanthamoeba polyphaga mimivirus (ApmFNuc) (12). In silico analysis predicted a stable fold, suggesting their role as Fanzor-associated guide RNAs (fRNA). The conserved structure extends into the coding region, indicating potential RNA processing sites. This resembles the OMEGA system, where both IscB and TnpB families exhibit limited structural variation, and mRNA's upstream region releases functional guide RNAs.

2.3 Unlocking the Nuclear Localization Signals and Mammalian Genome Editing

The study by Jiang et al (2023) investigated Fanzor nucleases and their potential for genome editing in mammalian cells (12). It was hypothesized that Fanzor nucleases might contain nuclear localization signals (NLS) to facilitate their entry into the cell nucleus for accessing genomic targets. In the case of ApmFNuc, a positively charged NLS was identified within the N-terminal region. Fusion of this NLS with superfolder green fluorescent protein (sfGFP) confirmed its functionality by causing strong nuclear localization.

A broader search for NLS sequences across various Fanzor families revealed that approximately 60% of Fanzor open reading frames contained readily identifiable NLS sequences (12). Subsequently, a subset of 22 Fanzor proteins was tested, and 21 of them exhibited functional N-terminal NLS sequences for nuclear localization in mammalian cells.

Furthermore, Fanzor nucleases, including DpFNuc, MmFNuc, and ApmFNuc, were codon-optimized for mammalian expression and engineered for optimal U6-based expression in mammalian cells (12). These modified nucleases demonstrated detectable genome editing activity on plasmids within human cells, primarily resulting in large deletions near the 3' end of the target site. Additionally, a panel of guides was designed for endogenous genomic targets, resulting in varying levels of editing efficiency, validating Fanzors as RNA-guided nucleases with activity in mammalian cells. Fanzor1 orthologs, including KnFNuc, also showed functional genome editing capabilities, further confirming their potential for genome editing in humans.

Study by Saito et al (2023) also described Fanzor as a new type of RNA-guided endonuclease (9). The authors show that Fz has a similar structure to other RNA-guided endonucleases, such as Cas9 and Cas12 (9). The authors of the paper conducted a phylogenetic analysis of Fz proteins and found that they are present in a wide range of eukaryotic organisms, including fungi, protists, arthropods, plants, and viruses (9). Two main branches of Fz proteins were identified, which the authors named Fz1 and Fz2. Fz1 is more common in fungi, while Fz2 is more common in other eukaryotic organisms. The authors also found that Fz proteins can be found in eukaryotic viruses, including giant viruses. This suggests that Fz proteins have been horizontally transferred between different eukaryotic species and viruses.

2.4 ωRNA: A novel non-coding RNA that guides Fz

The researchers identified a newly recognized category of non-coding RNA known as ωRNA, originating from Fanzor (Fz) loci. The study demonstrated the essential role of ωRNA in facilitating Fz-mediated DNA cleavage in controlled laboratory conditions (9, 13-14). In order to assess the significance of ωRNA in guiding Fz-mediated DNA cleavage, the investigators conducted in vitro cleavage experiments employing purified Fz protein along with synthetic ωRNA. Notably, their results indicated that the Fz protein alone lacked the capability to cleave DNA in the absence of ωRNA. Moreover, through systematic mutational analysis of different nucleotides within the ωRNA sequence, the researchers observed that these mutations adversely affected Fz-mediated DNA cleavage. These findings collectively underscore the indispensable role of ωRNA as a pivotal component within the Fz RNA-guided endonuclease complex.

Subsequently, the researchers conducted experiments involving the cultivation of human cells, where synthetic ωRNA and Fz protein were transfected into the cells for the purpose of examining Fz-mediated DNA cleavage (reference 9). The outcomes of these experiments revealed that Fz demonstrated the capability to cleave the targeted DNA sequences within human cells, resulting in precise modifications to the genome. In a conclusive phase of the study, the authors employed cryo-electron microscopy to unravel the structural intricacies of the SpuFz1–ωRNA–target DNA complex. Their observations disclosed that Fz occupied a central position within the complex, with ωRNA bound to its WED region and the target DNA sequence bound to its REC region. Notably, Fz displayed a comparatively smaller size and greater resistance to specific types of inhibitors compared to other RNA-guided endonucleases like Cas9 and Cas12. This observation implies that genome editing tools based on Fz may exhibit a reduced likelihood of causing off-target effects.

3. Conclusion and Future Prospects

The discovery of Fanzor , a groundbreaking RNA-guided endonuclease found in eukaryotes, marks a significant development in the field of genetic editing (reviewed in 10&15). Unlike previously identified programmable endonucleases predominantly in prokaryotes, Fanzor has expanded the realm of genome editing to eukaryotic organisms. Fanzor shares structural and functional characteristics with prokaryotic transposon proteins from the IS200/605 family, particularly the Obligate Mobile Element-guided Activity (OMEGA) systems.

These OMEGA systems demonstrate RNA-guided cleavage of DNA in a manner reminiscent of CRISPR/Cas systems, with Fanzor proteins exhibiting a remarkable resemblance to type V CRISPR effector Cas12. Horizontal gene transfer is a key factor in Fanzor's presence in eukaryotes. Two distinct types of Fanzor, Fanzor1 and Fanzor2 , have been identified in various eukaryotic species, including fungi, arthropods, protists, plants, and eukaryotic viruses. Their diverse distribution implies multiple horizontal gene transfer events, particularly in eukaryotic hosts living in close association with bacteria and fungi.

Fanzor's programmable RNA-guided DNA cleavage activity is highly specific, enabling precise genetic modifications. This specificity has significant implications, ranging from clinical therapeutics to gene therapy. The potential to generate staggered ends in DNA through Fanzor-mediated cleavage allows for targeted integration of specific DNA fragments at defined loci, fostering applications in homology-directed repair and mutagenesis.

These novel RNA-guided, programmable DNA nucleases exhibit unique features. Most notably, they have acquired NLS to actively cross the nuclear membrane in eukaryotic cells. The existence of functional NLS sequences in a majority of Fanzors emphasizes their capability to access the genome within the cell nucleus and execute their genomic functions. Furthermore, findings demonstrated that Fanzor nucleases can be harnessed for efficient genome editing in mammalian systems. With successful codon optimization and engineering of their fRNA/ωRNA guides for optimal expression, these nucleases were shown to be effective in generating insertions and deletions within target DNA sequences. The ability to induce precise modifications in the genome holds significant promise for various biotechnological applications.

However, the application of Fanzor is not without its challenges, particularly regarding cytotoxicity and potential off-target effects. The biological mechanism underlying Fanzor's function requires further investigation, along with its potential impact on essential cellular pathways. In summary, Fanzor's discovery represents a breakthrough in genetic editing. The broad distribution and versatility among eukaryotes highlight the potential for harnessing these miniature RNA-guided endonucleases for a wide range of applications, from precise genome editing to clinical therapeutics, gene therapy, and advancements in synthetic and applied biology.

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Revolutionizing Healthcare: Breakthroughs in Gene and Cell Therapy Products in 2023

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Abstract: Gene and cell therapy hold transformative potential for treating various illnesses by modifying or replacing genetic material or cells. In 2023, significant strides were made in the approval of new gene and cell therapy products, notably Lyfgenia, a gene therapy for sickle cell disease. Using a modified virus, Lyfgenia introduces a functional beta-globin gene into the patient's blood stem cells. This approval, alongside advancements in precise gene-editing techniques like CRISPR-Cas9, signifies substantial progress in correcting genetic abnormalities. Moreover, novel cell therapy products, including chimeric antigen receptor-like T cell (CAR-T) treatments for cancer, gained approval in 2023. These breakthroughs mark a transformative era in medicine, with the potential to enhance millions of lives and revolutionize medical practices.

Key words: Gene Therapy Products, Cell Therapy Products, CRISPR/Cas9 Gene Editing Technology

Introduction

Using genes to cure genetic disorders or fix genetic anomalies is known as gene therapy. Gene therapy can be used to replace mutant genes that act negatively to the cell or to repair damaged genes. It can also be used to introduce therapeutic genes into particular genomic areas [1]. Gene therapy has significant promise for the management of hereditary illnesses. There are now 26 RNA treatments, 29 gene therapies (including genetically modified cell therapies), and 65 non-genetically engineered cell treatments that are approved [2].

A total of 5 gene therapy products were approved by the FDA in 2023. The year 2023 has gone down in history as a turning point in gene and cell therapy products. On December 8, 2023, the FDA announced that it approved 2 new gene therapy products for SCD. These gene therapy products were the first products approved for SCD. At the same time, for the first time in history, a gene therapy product prepared using CRISPR/Cas9 gene editing technology was approved. This year, the FDA and the United Kingdom authorized CASVERY, a gene therapy product, for the treatment of SCD [3].

Among other important developments this year is that Hemgenix (Etranacogene dezaparvovec), whose hemophilia B treatment was approved by the FDA in 2022, was approved by both the EU and the UK this year [4]. In addition, Roctavian (Valoctocogene roxaparvovec), which was approved by the EU in 2022 for the treatment of hemophilia A developed by BioMarin Pharmaceutical, was approved by the FDA this year [5]. In this articles, we explained the gene and cell therapy products that will be approved in 2023. In addition, we also talked about the ADSTILADRIN product approved by the FDA on December 16, 2022.

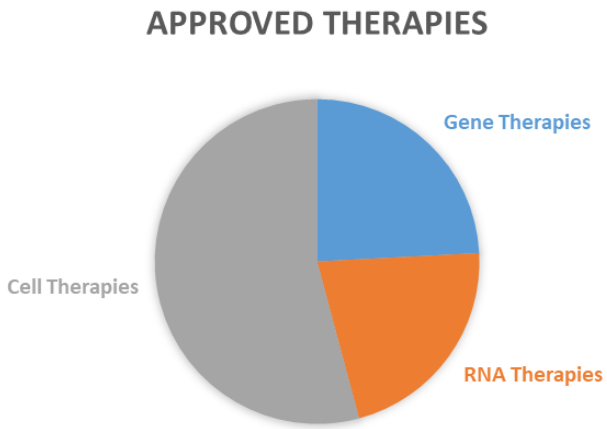

Figure 1. Pie Charts of approved gene, cell and RNA therapy products.

Approved Gene Therapies in 2023

ELEVIDYS™

Elevidys™ (delandistrogene moxeparvovec-rokl) was approved by the FDA on June 22, 2023, for the treatment of Duchenne Muscular Dystrophy (DMD). Elevisdys™, developed by Sarepta Therapeutics, is the first gene therapy product approved by the FDA for the treatment of DMD [6]. The accelerated approval of Elevidys™ means that the company must complete validation trials to evaluate the clinical benefits and safety concerns associated with this treatment.

DMD is an X chromosome-linked recessive hereditary muscle disease that mostly affects men. DMD disease is life-threatening by causing the muscles in the body to weaken and become damaged over time. Due to the mutation in the dystrophin gene caused by the disease, the muscles cannot maintain their strength and integrity because the dystrophin protein cannot be produced in the muscles or can only be partially produced [7].

Adeno-associated gene therapy, or Elevidys™, is used to treat children patients with DMD with mutations in the DMD genes who are between the ages of 4 and 5 [8]. The treatment works by directly delivering the gene encoding Elevidys™ microdystrophin, a dystrophin protein that is functionally shortened compared to the dystrophin protein in normal muscle cells. Selected domains of the 427 kDa dystrophin produced in normal muscle cells are present in the 138 kDa micro-dystrophin protein generated by Elevidys™, which is a reduced form of the dystrophin protein [6].

As a result of clinical studies, acute severe liver damage was observed in patients treated with Elevidys™ [9]. Therefore, patients treated with Elevidys™ should have liver enzymes or total bilirubin levels monitored for eight weeks. One of the priciest gene therapy treatments is the one approved to treat DMD, with a cost estimated by Sarepta Therapeutics Inc. at $3.2 million [10].

VYJUVEK™

In order to treat wounds in individuals suffering from dystrophic epidermolysis bullosa (DEB), a rare and dangerous illness affecting the skin and mucosal tissues, the FDA authorized VYJUVEK™ (beremagene geperpavec-svdt), a topical gene therapy developed by Krystal Biotech [11]. The treatment creates anchoring fibrils that keep the epidermis and dermis together by delivering healthy copies of the COL7A1 gene to wounds. It's the first redosable gene therapy ever and the only medication the FDA has licensed for treating recessive and dominant DEB. With VYJUVEK™, patients six months of age and up receive a normal copy of the COL7A1 gene using a genetically engineered herpes simplex virus-1 (HSV-1) vector [12]. The collagen type VII protein, which is produced by the COL7A1 gene, is required for the development of connective fibrils, which bind the epidermis to the dermis. Type VII collagen cannot be produced in patients with DEB due to a mutation in the COL7A1 gene. As a result, the skin becomes sensitive and prone to blisters and rips. In addition to skin-related symptoms, DEB can impact other organs and systems including the mouth, eyes, gastrointestinal tract, and urinary tract. In individuals with DEB, VYJUVEK™ may help heal injured skin and lessen the quantity of blisters and sores [13]. For the treatment of DEB, VYJUVEK™ is the first and only gene therapy authorized. While there is no known treatment for DEB, VYJUVEK™ may help people live better.

VYJUVEK™ treatment is applied directly to the patient's DEB lesions. First, in wounds, the genetically modified HSV-1 vector reaches the skin cells, so the skin cells receive a copy of the COL7A1 gene. Type VII collagen protein begins to be produced in skin cells, as a result of which the skin cells can form collagen protein type VII anchoring filaments. The dermis and epidermis are joined by anchor fibers. It reduces the amount of blisters and ulcers and helps wound healing. Many patients' quality of life may be enhanced by the potential benefits of VYJUVEKTM, a potentially new DEB medication.

31 individuals (with dominant or recessive DEB) between the ages of 1 and 44 participated in the trial, and the results showed that after three months, 26% of the wounds treated with placebo had healed, compared to 65% of the wounds treated with VYJUVEK™. Six months later, 23% of the wounds treated with a placebo had healed, compared to 68% of the wounds treated with VYJUVEK™ [14].

Lantidra

Type 1 diabetes, a chronic autoimmune condition necessitating lifelong insulin administration, poses daily challenges for patients in managing blood glucose levels [15].

Lantidra (donislecel) has received approval by the U.S. Food and Drug Administration (FDA), representing a groundbreaking development as the first cellular allogeneic pancreatic islet therapy derived from pancreatic cells of deceased donors for treating type 1 diabetes. Specifically intended for mature people unable to attain target glycated hemoglobin due to recurrent severe hypoglycemia despite rigorous diabetes management, Lantidra provides a pioneering cell therapy option. The therapy's primary mechanism involves the secretion of insulin by infused allogeneic islet beta cells, demonstrating promising outcomes in non-randomized studies where a substantial number of participants achieved extended periods of insulin independence. However, the safety profile of Lantidra, assessed in two non-randomized studies involving 30 participants, revealed varying unfavorable responses, emphasizing the importance of considering these events when assessing the therapy's benefits and risks [16].

Lantidra offers a potential solution for individuals struggling with insulin dosing, particularly those with difficulties

preventing hyperglycemia without inducing hypoglycemia. Evaluated after being administered as a sole infusion into the hepatic portal vein, Lantidra's effectiveness was assessed in participants receiving one to three infusions, with notable outcomes indicating prolonged periods of insulin independence for a significant proportion. Despite the potential benefits, serious adverse reactions associated with the procedure and immunosuppressive medications pose considerations for patient safety. The FDA's approval of Lantidra with patient-directed labeling underscores the necessity for informed decision-making, providing patients with comprehensive information on the therapy's benefits and risks. As the pioneering cell therapy, Lantidra stands for a groundbreaking advancement in treatment options, signaling a new frontier in addressing the complexities of this chronic condition [17].

The recommended administration plan for Lantidra entails an initial transplant infusion at a minimum dosage of 5,000 equivalent islet number (EIN) per kg of body weight, followed by subsequent infusions at 4,500 EIN/kg. It is essential to deliver the cells through the hepatic portal vein, and the anticipated tissue volume should not exceed 10 cc per transplant infusion. Lantidra is designed for adults contending with Type 1 diabetes and encountering difficulties in achieving the target HbA1c due to recurrent severe hypoglycemia. Its use should be combined with concomitant immune suppression medications for optimal therapeutic outcomes. [18,19].

Prime predicts the typical yearly expenditure exceeding for Donislecel will surpass $300,000. Despite the elevated expense associated with this drug, the projection is that the limited number of users will mitigate its impact on significant net new expenditure.

Omisirge

Approximately 8,000 patients aged 12 and above, diagnosed with hematologic malignancies, underwent allogeneic stem cell transplantation in 2019 in the US, while an additional 1,200 eligible patients struggled to find suitable donors [20]. Omidubicel's recent approval signifies a major advancement, offering potential to improve clinical outcomes and increase transplant accessibility for patients facing donor challenges. This regulatory endorsement highlights omidubicel's potential to address unmet needs in the transplantation of stem cells from a donor into a recipient to treat blood cancers or malignancies affecting the blood-forming tissues contributing significantly to scholarly discourse on hematopoietic cell therapies.

Omidubicel, a leading cell therapy candidate, demonstrates remarkable efficacy in allogeneic hematopoietic stem cell transplantation for patients with blood cancer. In a rigorous Phase 3 study (NCT0273029), omidubicel significantly reduces time to neutrophil and platelet engraftment compared to UCB, with favorable outcomes including infections and reduced hospitalization days. Notably, one-year post-transplant data reveals sustained clinical benefits, making omidubicel a breakthrough as the first stem cell donor source with FDA Breakthrough Therapy Designation and Orphan Drug Designation in the US and EU [21].

The primary objective of the investigation focused on omidubicel's impact on time to neutrophil engraftment in hematologic malignancy patients undergoing allogeneic bone marrow transplant. Results indicate a significant reduction to 12 days compared to the 22 days in the standard umbilical cord blood group ($p < 0.001$). Successful achievement of secondary endpoints, including platelet engraftment and infection rate, further supports omidubicel's favorable tolerability, positioning it as an effective intervention in allogeneic bone marrow transplant for hematologic malignancies [22].

Omisirge is priced at a wholesale acquisition cost (WAC) of $338,000 for a single treatment and is currently accessible to transplant centers. The manufacturing process has a turnaround time of 30 days. IPD Analytics suggests employing management tools like prior authorization to ensure the proper use of Omisirge [23].

Fucaso

Multiple myeloma is a prevalent hematological malignancy with a high incidence rate, often leading to relapse and refractory conditions despite current treatments. First-line drug therapies for multiple myeloma patients typically involve immunomodulatory drugs, proteasome inhibitors, and alkylating agents. Although these treatments can prompt remission, it's almost inevitable for patients to experience relapse or refractory stages, highlighting a critical unmet need for a well-tolerated and long-lasting solution [24,25]. FUCASO® (Equecabtagene Autoleucel) offers an advanced solution by presenting an innovative fully-human anti-BCMA CAR-T cell therapy employing lentivirus as a gene vector for autologous T cell transfection. Its CAR design incorporates a fully-human single-chain variable fragment (scFv), CD8a hinge and transmembrane, along with 4-1BB-mediated co-stimulation and CD3ζ activation domains. Demonstrating rapid, potent efficacy, and prolonged persistence, FUCASO® addresses the critical need in relapsed and refractory multiple myeloma (RRMM) patients. The approval by China's National Medical Products Administration (NMPA) for RRMM treatment reflects the collaborative efforts of Innovent and IASO Bio in mainland China. [26].

Furthermore, Equecabtagene Autoleucel has garnered recognition from the U.S. Food and Drug Administration (FDA), obtaining Orphan Drug Designation (ODD) for RRMM and securing FDA Investigational New Drug (IND) approval. In

February 2023, the FDA also granted Regenerative Medicine Advanced Therapy (RMAT) and Fast Track (FT) Designations to Equecabtagene Autoleucel. Beyond multiple myeloma, the NMPA has accepted another Investigational New Drug (IND) application, extending the therapeutic scope to include Neuromyelitis Optica Spectrum Disorder (NMOSD) [27].

In a study involving 103 subjects who were administered 1.0×10^6 CAR-T cells/kg, Equecabtagene Autoleucel exhibited impressive outcomes. Of the 101 assessable patients, the comprehensive response ratio was 96%, with a rigorously achieved complete response/complete response rate of 74.3%. Adverse events were limited, with only one patient experiencing grade ≥3 cytokine release syndrome and 2 with grade 1-2 immune effector cell-associated neurotoxicity syndrome, all recovering after treatment. Equecabtagene Autoleucel demonstrated persistence in 50% and 40% of patients at the 12-month and 24-month follow-ups, respectively. Importantly, only 19.4% of patients exhibited anti-drug antibodies after infusion, underscoring the therapy's effectiveness, safety, and enduring impact. [28,29].

Adstiladrin

Non-Muscle Invasive Bladder Cancer (NMIBC) is a bladder cancer superficial form, comprising 75% of the 81,180 new instances in the United States in 2024. Despite initial treatment with intravesical BCG, over 50% of high-risk NMIBC patients experience recurrence, leading to limited options, the complete removal of the bladder [30,31].

Adstiladrin® (nadofaragene firadenovec-vncg), a cutting-edge gene therapy utilizing an adenovirus vector, has been approved by the U.S. Food and Drug Administration (FDA) on December 16, 2022. This approval is intended for treating adult patients with high-risk, Bacillus Calmette-Guérin (BCG)-unresponsive non-muscle invasive bladder cancer (NMIBC), manifesting as carcinoma in situ (CIS) with or without accompanying papillary tumors. [32,33].

Adstiladrin® (nadofaragene firadenovec-vncg) is a groundbreaking gene therapy crafted for adults with BCG-unresponsive non-muscle invasive bladder cancer (NMIBC). Administered every three months through a catheter, the therapy employs a non-replicating adenovirus vector carrying the interferon alfa-2b gene. This vector infiltrates bladder wall cells, releasing the active gene, which is then assimilated by the cells' internal gene/DNA machinery. Consequently, the cells produce heightened levels of interferon alfa-2b protein, a natural anti-cancer agent. In this process, the gene therapy reshapes the patient's bladder wall cells into interferon microfactories, strengthening the body's natural defenses against cancer. The clinical trials involved high-grade 221 individuals, BCG-unresponsive non-muscle invasive bladder cancer (NMIBC), who had previously experienced ineffective BCG treatment.[34].

Adstiladrin is not intended for intravenous, topical, or oral use. The prescribed dosage is 75 mL of Adstiladrin with a 3 x 10^11 viral particles (vp)/mL dosage, administered every quarter. The cost for Adstiladrin intravesical suspension vncg is approximately $63,190 for a supply of 4 suspensions, applicable to cash-paying customers and not valid with insurance plans [35,36].

Casgevy

Exagamglogene autotemcel, market name Casgevy, was approved by the United Kingdom for the treatment of SCD and transfusion-dependent beta thalassemia in November 2023, and by the FDA for the treatment of SDC on December 8, 2023 [37]. Casgevy is the first cell-based gene therapy product approved using CRISPR/Cas9 gene editing technology. This gene therapy product was jointly developed by Vertex Pharmaceuticals and CRISPR Therapeutics [38].

With CRISPR-Cas9 technology, the target DNA sequence is cut from the desired section. There are two important molecules in this. CAS9 enzyme and guide RNA. While the Cas9 enzyme acts as a scissor and cuts the double-stranded DNA in the target region, the guide RNA Cas9 enables the enzyme to move to the target region. Thus, with this technology, changes can be made in the gene in the desired region of DNA [39]. The inventors of this technology, French microbiologist Emmanuelle Charpentier and American biochemist Jennifer A. Doudna, became the winners of the Nobel Prize in Chemistry in 2020 [40].

Sickle cell anemia is an autosomal recessive genetic disease that occurs when the red blood cells take a sickle shape as a result of the abnormality of hemoglobin, the oxygen-carrying protein in the red blood cells. Bone marrow transplant was currently the only treatment for sickle cell disease [41]. But with Casgevy's confirmation, that changed.

It received approval from both the United Kingdom and the FDA this year for use in people with sickle cell disease aged 12 and over who are suitable for stem cell transplantation but cannot find a suitable donor. Casgevy will be available in the United States with a list price of $2.2 million [42].

Lyfgenia

Bluebird Bio, Inc. created the ground-breaking gene therapy Lyfgenia, commonly referred to as lovotibeglogene autotemcel, to treat sickle cell disease (SCD) [43]. Sickle cell disease (SCD) is a chronic, hereditary blood illness that affects approximately one hundred thousand individuals in the US and millions of others worldwide. Abnormal hemoglobin molecules are the disease's hallmark; these molecules cause red blood cells to become malformed and inflexible, which can cause pain, damage to organs, and early death [44].

The FDA authorized Lyfgenia on December 8, 2023 for the treatment of SCD in individuals 12 years of age and older who have experienced vaso-occlusive events (VOEs), the most severe consequences associated with the illness. Lyfgenia is a cell-based gene therapy that transfers a gene that causes the patient's blood stem cells to make HbAT87Q via a lentiviral vector . Hemoglobin generated by gene therapy, HbAT87Q, operates similarly to hemoglobin A. HbAT87Q-containing red blood cells are less likely to sickle and obstruct blood flow. This makes it possible for the patient's body to create enough healthy hemoglobin to stop the excruciating episodes known as sickle cell crises, which are the main characteristic of the illness. Patients with SCD with a history of vaso-occlusive episodes 12 years of age and older are eligible to get Lyfgenia medication. The patient is subsequently given the altered stem cells [37] .

Patients using Lyfgenia therapy have been observed to have hematological malignancy, called blood cancer. The label of Lyfgenia includes information on this danger. Patients who use this medicine should have ongoing cancer screenings. As a one-time therapy that can prevent sickle cell crises and enhance general health, Lyfgenia holds the potential to change the lives of those who suffer from sickle cell disease [45].

The biotechnology company that created the medication said in a statement that Lyfgenia will retail for $3.1 million when it starts [46].

Conclusion

2023 marks a historic milestone in the field of gene and cell therapy products, with significant advancements and FDA approvals shaping a new era. The approval of two gene therapy products for sickle cell disease on December 8, 2023, including the groundbreaking CASVERY, crafted using CRISPR/Cas9 gene editing technology, stands out as a transformative moment. Other notable approvals include Elevidys™, the first gene therapy for Duchenne Muscular Dystrophy, and VYJUVEK™, a redosable gene therapy for dystrophic epidermolysis bullosa, presenting a novel approach to address recessive and dominant forms of the disease.

Lantidra, an allogeneic pancreatic islet cellular therapy for type 1 diabetes, introduces a groundbreaking option for adults unable to achieve target glycated hemoglobin due to recurrent severe hypoglycemia. Despite safety considerations, Lantidra signifies a paradigm shift in type 1 diabetes treatment. In the realm of multiple myeloma, FUCASO® (Equecabtagene Autoleucel) emerges as a promising CAR-T cell therapy, showcasing rapid efficacy and prolonged persistence, addressing the critical unmet need for well-tolerated and long-lasting solutions.

Furthermore, Adstiladrin, a gene therapy for BCG-unresponsive non-muscle invasive bladder cancer, received FDA approval on December 16, 2022, marking a crucial advancement for patients facing limited options. The recent approval of Casgevy, the first cell-based gene therapy product using CRISPR/Cas9 technology for sickle cell disease and transfusion-dependent beta thalassemia, adds another dimension to the transformative developments in 2023. Finally, the FDA authorization of Lyfgenia as a gene therapy for sickle cell disease, with cautionary notes on hematological malignancy risks, further underlines the dynamic landscape of gene and cell therapy products in this pivotal year.

The approved gene and cell therapies of 2023 represent a paradigm shift in medical treatments, offering targeted solutions for various conditions, from genetic disorders to cancer. These innovations bring not only groundbreaking efficacy but also the potential to reshape healthcare by improving patient outcomes and reducing long-term costs. As these therapies become integral to patient care, the transformative impact on health systems holds the promise of a future where cutting-edge treatments redefine medical standards for the benefit of both patients and the broader healthcare landscape.

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