Journal of Knowledge Learning and Science Technology
ISSN: 2959-6386 (Online)
2024, Vol. 4, No. 3, pp. 224–232
DOI: https://doi.org/10.60087/jklst.volx.ny.pz

Review

CRISPR-Cas9 as a gene editing tool using cardiac glycoside reductase operon for digoxin metabolism

Tanishka Nale[1], Karan Dhingra[2], Saloni Verma[3]

[1]Fravashi International Academy, Nashik, Maharashtra, India
[2]Department of Biomedical Engineering, University of Ottawa, Ontario, Canada
[8]Department of Biomedical Engineering, Cornell University, New York, United States of America

Abstract

Gene editing technology has gained popularity over the past two decades with the scientific advancements being made in genetics and computational biology. CRISPR-Cas9 technique allows us to target genetic material and perform accurate manipulation of targeted gene function. Cgr operon, commonly found in the gut microbiome bacteria of E. lenta has several attributes which can be modified using the CRISPR-Cas9 methodology. Here, we briefly describe how the Cas9 nuclease can be used to prevent Digoxin from being suppressed by the cgr gene, directly correlating to improved cardiac function. The editing tool is described to assist in mutating the genetic material of cgr gene discussing the inhibition of Na+/K+ ATPase in cardiac myocytes and CG-content. The principle of operation behind CRISPR-Cas9 is described using non-homologous end joining or homology-directed repair cellular mechanisms. Target site selection, designing sgRNAs and introducing various mutations are discussed with limitations of current technology and possible applications in the field of point of care diagnostics and biosensing developing therapeutic interventions using cgr operon.

Keywords

Genetic engineering, precision medicine, gene editing, CRISPR-Cas9, cgr operon, digoxin

1. Introduction

Clustered Regularly Interspaced Palindromic Repeats (CRISPR)-Cas9 was first discovered by a Japanese Research Group in 1987 while originally studying the genes involved in phosphate metabolism [1]. Post discovery, they tested nine nucleotide sequences to successfully find transcription sites [2]. However, not much was known about the biological implications for humans of this discovery. CRISPR was found to be an already-existing defense mechanism against viruses in bacteria. Later, similar repeated sequences were found in the archaeon *Haloferax mediterranei.* The researchers first suggested that CRISPR was responsible for the regulation of gene expression. However, this would not be possible in

*Corresponding author: Tanishka Nale

Email addresses:

drravikiransnale@gmail.com (Tanishka Nale), k2dhingr@gmail.com (Karan Dhingra), sv458@cornell.edu (Saloni Verma)

Received: 12-07-2024; **Accepted:** 25-09-2024; **Published:** 25-12-2024

bacteria [1]. In 2007, two food scientists, Rodolphe Barrangou and Philippe Horvath, learnt the first piece of experimental information about the working of CRISPR as they worked on cultures of yogurt.[3] A few years after this discovery, the use of the protein Cas-9 as molecular scissors in CRISPR was developed. Emmanuelle Charpentier and Jennifer Doudna first studied CRISPR as a genome editing tool in bacteria in 2012. Consequently, in 2020, they became the first two-women team ever to be awarded the Nobel prize for the essential molecule for nuclease activity named tracrRNA (trans-activating CRISPR RNA) [3].

CRISPR-Cas9 is a gene editing tool used in various fields of study to modify genomes [4]. The CRISPR-Cas systems are divided into two classes, I and II, based on the complexity of the Cas protein. Cas9 is a widely studied protein due to its relative simplicity. The Cas9 protein along with the guide RNA (gRNA) forms the CRISPR-Cas9 system. The gRNA guides the Cas9 protein to the desired gene sequence. The gRNA is specific to the target sequence. Then, the Cas9 protein acts like 'molecular scissors' and cuts the DNA forming complementary ends, also known as sticky ends. If another gene sequence is to be inserted, DNA ligase is used [5]. The CRISPR-Cas9 technology is highly specific and efficient compared to other gene editing tools [6]. Moreover, CRISPR is simpler and more versatile and hence has the potential to solve many pressing issues[7]. Firstly, CRISPR can be used to modify crops to make them resistant to drought and pests. Statistics show that 50% of the growth in the annual production of maize is due to genetic modification [8]. Additionally, CRISPR can be used to overcome drug resistance in breast cancer patients and improve the effectiveness of the treatment.[9] CRISPR's specificity also makes it the best technique to treat genetic disorders as it can precisely alter the mutated gene with lesser off-target effects and can also determine the genetic causes of certain phenotypes. [10] Lastly, CRISPR-Cas9 is pocket-friendly and easily accessible, which makes it a great tool for further research and developments of new treatments [10]. Today, CRISPR-Cas9 is being studied widely to truly understand the spectrum of opportunities with the gene editing tool. Recent developments showed that improving the Cas9 codons could improve the efficiency of gene editing as a whole. Later, a few methods were identified to reduce the off target effects. The protein Cas9n has shown to reduce off-target effects as it cuts only one strand of DNA, unlike Cas9. Furthermore, replacing Cas9 with the nuclease Cpf1 would add to the attempts to reduce off-target effects. xCas9, the result of mutation of Cas9, is more precise than Cas9 itself. Lastly, CRISPR-Cas9 now allows scientists to modify just a few bases in a genome instead of having to insert a completely new gene [11].

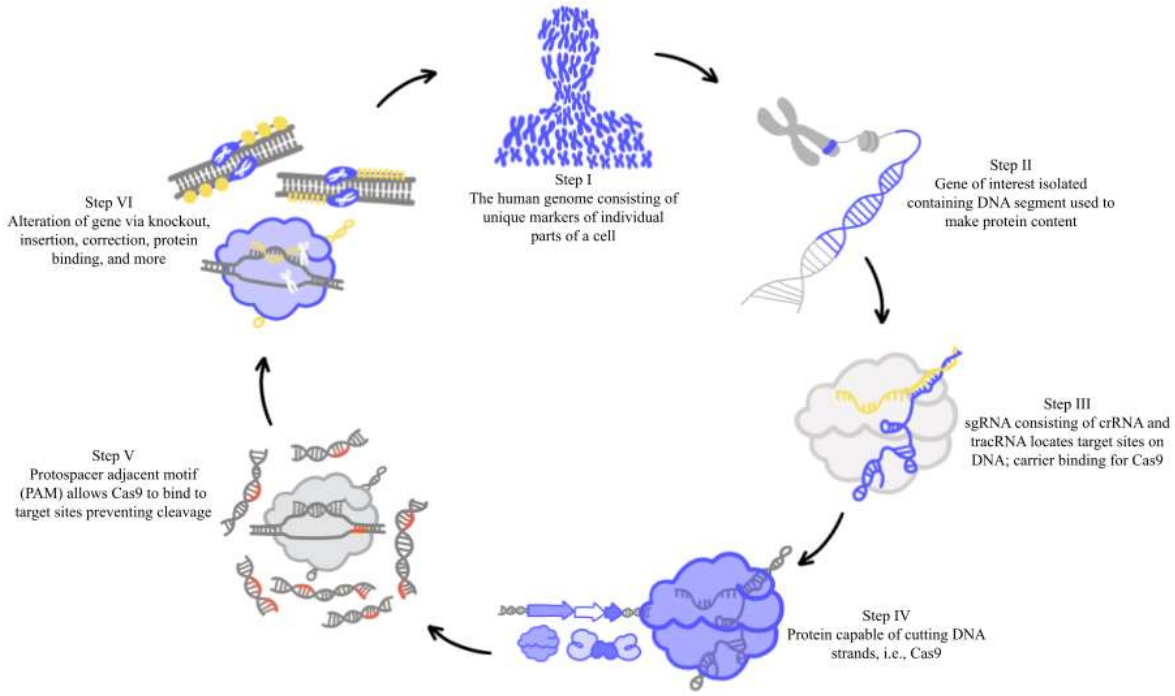

Figure 1. *3D Schematic of gene editing via CRISPR-Cas9.*

The cardiac glycoside reductase (*cgr*) gene is an essential part of an operon (commonly found in the gut bacterium *Eggerthella lenta*) responsible for the metabolism and inactivation of digoxin, a drug used to treat cardiac conditions.

The *cgr* operon contains multiple strains, cardiac glycoside reductase 1 (*cgr1*) and cardiac glycoside reductase 2 (*cgr2*) being the most researched genes. While *cgr1* supports electro transfer, *cgr2* is directly responsible for digoxin metabolism. In this review, we explore the possibilities of supporting characterization studies wherein CRISPR-Cas9 may be used to create a knockout of the *cgr2* gene for regulation, gene modification, in-vivo studies and therapeutic applications indicating the challenges and limitations to consider.

2. Cardiac glycoside reductase

The cardiac glycoside reductase (*cgr*) gene is part of the gut bacteria *Eggerthella lenta*. Two genes encode proteins that are homologous to bacterial cytochromes. Cgr operon is predicted to encode a protein that is homologous to the NapC/NitT family of cytochrome c reductases, which is cardiac glycoside reductase 1 (*cgr1*) and encodes protein related to fumarate reductase, which is cardiac glycoside reductase 2 *(cgr2)*. These normal functions are known by sequence homology (PSI_BLAST) [12] and secondary structure predictions (HHPred) [13].

It has been biochemically confirmed that *cgr2* is responsible for reduction and inactivation of digoxin. Bioinformatics and network analysis show the uniqueness of *cgr2* as it employs a mechanism of substrate binding and reduction. Furthermore, microbial isolation and sequencing of human-associated isolates demonstrate that this gene is exclusive to a subset of *Eggerthella lenta* strains and is very well conserved at the amino acid sequence level [14]. The role of *cgr1* is to serve as a hemebinding domain in this process. In digoxin there are alpha, beta-unsaturated lactone and in fumarate there is an unsaturated carboxylic acid. These share structural and electronic similarities and thus this research is able to hypothesize that digoxin (and other related cardiac glycosides) are able to occupy the binding pocket of *cgr2* and undergo reduction by a similar mechanism. This is why they have the capability of using digoxin as an alternative electron acceptor. The genes signify a predictive microbial biomarker for the inactivation of digoxin [15]. To test the functionality of *cgr*, researchers grew *E. lenta* cultures in rich medium with low and high levels of arginine (arginine is known to inhibit reduction of digoxin) in the presence of absence of digoxin. RNA-Seq was performed on the cellular biomass and it was found that the two-gene operon (*cgr*) was highly up-regulated [16].

The FASTA sequences below are for *cgr1* and *cgr2* but they are named Elen_2528 and Elen_2529, respectively. This change may be due to the way that the authors decided to code the genes. As indicated in Table 1, gene Elen_2528 and Elen_2529 correspond to *cgr1* and *cgr2*.

FASTA (cgr1): `>NC_013204.1:c2958598-2957948 Eggerthella lenta DSM 2243, complete genome`

```
ATGGCTGAGGAACCTGTGGTGATCGGGGATCCCGCGCCCCG
GACGAGGAAGTGGCCCATCGTCGTGGGCG
TTGTCGTGGTTGTCTTGATTGCAGCCGGTGCCGGCTTCTGG
GTTTGGCACGAGCAGCCGAGCTTCTGCGC
CGCCATCTGCCACACGCCGATGGACGAGTACTTGGAAACGT
ACGAGCAGGAAGCAGGCACGGCGGGCGTC
GACAAGTGGGGCAACGAAGTTGCCAACACGAACGCGATGCT
GGCCGTCTCGCACAAGGCTCAGGGCAAGG
ACTGCATGGCCTGCCATGTGCCGACGCTGAGCGAGCAGATG
TCCGAGGGCATGAACTGGGTCACCGGCAA
CTACGTGTACCCGCTTGAAGAGCGCGACACGGAGATGCTGA
CCGAGGCTCGCGGCGTTGACGCCGACGAG
TTCTGCCTGAACGAGAGCTGCCACAACCTGACGCGCGACGA
CCTGATCAAGGCCACGAGCGACATGGAGT
TCAACCCGCATCAGCCGCAGCACGGCGAGATCGAGTGCAGC
GAGTGCCACAAGGCGCACCGCGCTTCCGT
GATGTACTGCACGCAGTGCCACAGCGAAGCCGAAGTGCCGG
AGGGCTGGCTGACCGTCGCCGAGGCCAAC
AAGCTTTCGACGGCGGCGTAG
```

FASTA (cgr2): `>NC_013204.1:c2960290-2958608 Eggerthella lenta DSM 2243, complete genome`

```
ATGGAATACGGAAAGTGCCGCGGCATCGAACGGGGAATGGG
AAGGCGAGACTTCCTCAAGGCGGCCACGC
TGCTCGGCGCCACGGCGGCAGGAGCAGGCATGTTGGCCGGT
TGCGCGCCGAAAAGCGCATCCGAAGCACA
GGCTCAGACCGCGCCTGCGGCAACCGGGGGCCTCGACCCGG
CCGACGTCGACTGGAAGTACGAGACTGAT
GTCGTCATCGTCGGCTCGGGCAGCGGCGGCACGTGCGCGGC
CATCGAGGCGGCAGAGGCCGGCGCCGACG
TCGTCGTGTTCGAGAAGGACAAGGCGATGTACGGCGGCAAC
TCGGCACTATGCGGTGGATACATGCTGGC
AGCGGGTTGGTCCACTCAGGAAGAGATCACCGGCTACGCGG
GCGATACGGGCGAAGCGTTCGCGAACCAG
ATGCTGCGCTGGTCGCAAGGTCTGGGAAACCAGGATATGAT
CCGCGAGGCGTGCTTGCGCTCCGGCGAGG
CCGTCGATTGGATGATGGACACCGGTCGCACGTACGAGGGC
GCAAGCCCGCTGCCCCCGGTCTGGTCATG
CGGCGACACGGAGGCCGATGTCGTGCCGCGCTCGGTGTACA
ACCACAATGCCTACGGTGCGACGGAAGGC
CATATGGCAACGCTGAAAAAGCGCGCCGAAAGCCTGTCGAA
CATCGAAATCGAGATGGGCTGCGAGGTGG
CGCACATCCTGAAGAACGCCGAAGGCTCCGTTATCGGCGTG
CAGCTGGCCGACGGCTCCTTCGCCAAGGC
```

GCGAAAAGGCGTGGTCATGGCGTGCGCTTCGGTGGACAACA
ACCTTGAGATGTCTAAGGACCTGGGCCTC
ATGCAGAACGTCTGGGGCCTAACGCTCGAAGGCGCAGGGCT
GCTCGCTCCGGGCAACCCCGATATGGACT
CGAATACCGGTGACGGGGTGCGGATGCTGCGCGAGATCGGT
GCTGAGCTTTGCATGCAGCAGGCCGTGTG
CATGAACGATTCTATCTACGTAGGCGGCATCAGCGACTGGG
GTATGAGCGAGATCCTGGGCAAGGACGTC
AATATCCACGACTCGTCGAACATAGACGCCATCCTCGTGGA
CAAGACCGGCAGACGGTTTTGCCAGGACG
ATGCCGAGTGGGGCTATGTCATGCACGAGTGCGCGCAAGCT
GCATGGAAGCAGGGTTTCACCCCCGACGA
TCCGACTACGGGTTATATCTTCTATGTGTACGATGCGACCG
GGGCGCCTTTTTTCGAGATGAAAGGGCAT
ACGCCCGACACGTGCGATACTACGTTCTCGGCCGATTCGGT
TGATGGCCTCGCCGAGTTTATCGGCTGCG
ATCCGACCGCCCTCGCCTCCGAAGTGGAGAGGTGGAACTCT
TTCTGCGAGGCCGGTTTGGATGCCGACTT
CGGCCGCAGGGCCAACATGGCCCCCATCGCGACGCCGCCGT
TCTACTGCGATGTCGTGCGCCCCGGCCCT
ATGGGCACCTTCGCCGGCGCGAAGTCCAACGTGGAAGCCGA
GATCATCGGCCTCGACGGCAACCCCATTC
CACGGCTGTACGGCGCCGGGTGCATCATCGGGGGTAACGTC
TCGGGTGCCTTCTACTTCGGCTGCGGCTG
GTCCATCACGAACACCGTCGTCTGGGGCCGCGAGGCCGGGC
GGAACGTGGCCGCCCTCGAGCCGTGGGAGTAG

2.1. Survival

E. lenta has a total of 3,181 genes but since it is a difficult bacterium to cultivate, its survival and effects are rarely reported [17]. A reason for this is because *E. lenta* is involved in mixed infections with less fastidious bacteria. Thus it is difficult to conclude whether the cgr operon affects its survival. However, by understanding the mechanisms of cgr1, cgr2, and activation or deactivation of the drug, one can make hypothetical inferences based on the literature. In this two-gene operon, cgr2 is the soluble reductase partner that interacts with the hemebinding domain of cgr1 (in either a transient or stable complex on the extracytoplasmic side of the membrane). Thus cgr2 requires cgr1 to fully function. However, in terms of the survival of either cgr genes, cgr2 can survive without cgr1 because cgr2 is necessary (exclusive to *E. lenta*) but cgr1 can be replaced with any other operon that has a hemebinding domain. Heme-containing proteins are found in all living species [18]. Thus this hemebinding domain (cgr1) can be justifiably replaced after thorough experimentation to attain an adequate alternative.

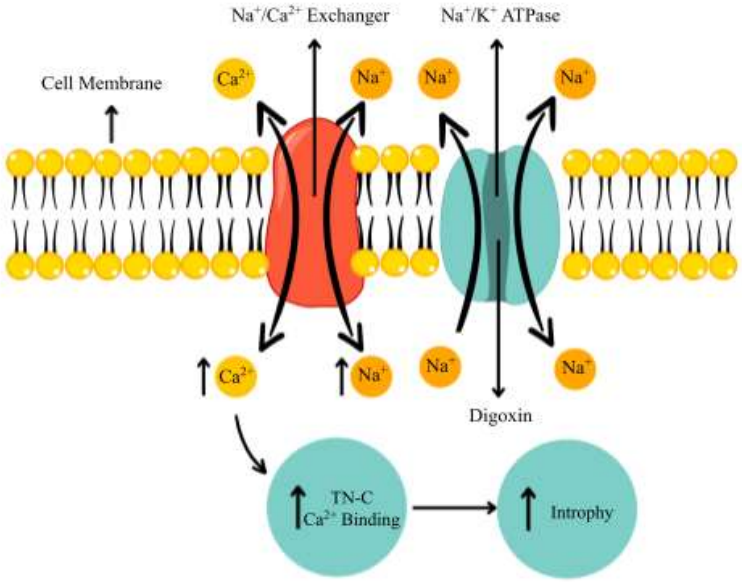

Figure 2. *Digoxin mechanism of action.*

Gene	Forward sequence	Reverse sequence	Forward position	Reverse position	Forward melting temp	Reverse melting temp	Product size	Annotation
16S rRNA	CAGCAGGGAAGAAATTCGAC	TTGAGCCCTCGGATTAGAGA	430	588	53.8	55	159	16S rRNA gene
Elen_2528	TGAGGAACCTGTGGTGATCG	CGCCGTGCCTGCTTCCTGCT	6	204	56.7	65.8	199	Cardiac glycoside reductase 1 (*cgr1*)
Elen_2529	TGCGCTGGTCGCAAGGTCTG	CGGCGCGCTTTTTCAGCGTT	425	658	63.0	62.3	234	Cardiac glycoside reductase 2 (*cgr2*)
Elen_2033	TCCTGAGGAGCTGCGAAAGCTG	CGCCCCTAATGCAGTCCCCG	24	141	61.5	63.2	118	LicD family protein
Elen_2416	ACGCACTCGTTCGTACGCGAAA	TCGCATTGCGTTATGAGCCGC	20	147	61.4	60.9	128	Hypothetical protein

Table 1. *E. lenta specific primers used for quantitative polymerase chain reaction (PCR).*

Digoxin is activated with the inhibition of Na^+/K^+ ATPase in cardiac myocytes [16] and cardiac ATP-sensitive K^+ channels (K_{ATP}) are heme-dependent. K_{ATP} channels regulate the excitability of cardiac ventricular myocytes, which is apparent in ischemic heart disease and myocardial infarction [18]. Therefore, *cgr1* (the hemebinding domain in *E. lenta*) modifies Na^+/K^+ ATPase, which leads to activation/deactivation of Na^+/K^+ ATPase, resulting in increased/decreased target affinity. In conclusion, for the cell to survive, *cgr1* is vital as it regulates cardiac ATP-sensitive K^+ channels. The regulation of these channels is necessary for dictating the microbial drug metabolism of digoxin. This information allows the prediction or manipulation of microbial drug metabolism.

2.2. GC Content

The GC content, otherwise known as the percentage of guanine and cytosine nucleotides in a genome provides valuable information about DNA stability or repair capabilities, dictate primer conditions for PCR experiments, have the potential to facilitates open chromatin and active transcription, influence chromosome structure and protein-coding sequences. The GC content is calculated as a percentage of nitrogenous bases on a DNA molecule, which is either guanine or cytosine following the equation,

$$\frac{Count\ (G+C)}{Count\ (A+T+G+C)} \times 100\%$$

The *cgr1* DNA length (Elen_2528) of 651bp showcased GC content of 64.208909% [19]. On the other hand, *cgr2* DNA length (Elen_2529) of 1683 bp demonstrated GC content of 63.874034% [19]. These calculations were performed by a contract research organization (CRO) Biologics International [20]. Recent studies have shown that genes that recombine have a greater GC content. Additionally, there seems to be an evolutionary force that is driving GC values to be higher in all bacterial species (with the exception of AT-rich genomes). Recombination has proven to enhance efficiency of selection by breaking linkage among sites [21]. In CRISPR Cas9, the targeting specificity depends on the 20 nucleotide sequence at the 5' end of the gRNA. Thereby, increasing the importance of the GC content when designing the 20 base pair guiding sequence of gRNA. The GC content should be in a 40%-80% range as it stabilizes the RNA:DNA duplex and also decreases off-target hybridization [22]. Thus with >63% GC content in both *cgr1* and *cgr2*, the *cgr* gene is a justified choice for finding suitable CRISPR editing sites.

2.3. Exclusivity

Since *cgr2* is exclusive to *E. lenta*, only *cgr1* is found in other organisms. The *cgr1* gene is found in the following bacteria and it plays a different role in each as shown in Table 2. Although the search output produced 1494 gene related results on the National Center for Biotechnology (NCBI) gene database, only eight included information about *cgr1* in related research as *cgr1* does not have significant function in those bacteria. Of the eight *cgr1* genes in various bacteria, three were found to be relevant to the human gut microbiota and the other five plant related. In *C. elegans* development, Ras-mediated signaling is necessary for the induction of vulval cell fates. *Cgr1* was identified by

Study	**Microorganism**	**Role of *cgr1***
[23]	*Saccharomyces cerevisiae*	Ribosome biogenesis
[24]	*Candida albicans*	Loss of function mutation
[23]	*C. elegans*	Ras-mediated signaling

Table 2. *Selected microorganisms discussing role of cgr1*

screening for suppressors of the vulval cell fates (caused by a gain-of-function mutation of the let-60 ras gene). *Cgr1* positively regulates induction of vulval cell fates by two *cgr1* loss-of-function mutations. It is necessary for larval viability so it also functions early in development [24]. It is suggested by Sun J et al. that cgr1 has a role in ribosome biogenesis for *S. cerevisiae* [25]. It encodes a conserved fungal protein that localizes to the nucleolus. Moy et al. tested to see if the localization reflected a role for cgr1 in ribosome biogenesis by examining two yeast cgr1 mutants. They examined for defects in ribosome synthesis and found that both of the strains had impaired growth rates [26].

2.4. Gene editing strategy

Digoxin, a drug derived from plant-derived cardiac glycoside *Digitalis lanata* has the ability to improve cardiac output and control heart rate in conditions of heart failure. Therefore it is imperative that the drug remains activated in the human body. The *cgr* operon must be mutated so that it no longer has the ability to reduce the drug. Since *cgr* is composed of *cgr1* and *cgr2*, either one or both of these genes can be mutated. Before editing the gene, it is crucial to understand the way that digoxin works and how it is inactivated. Digoxin takes effect indirectly when there is an inhibition of the Na+/K+ ATPase in cardiac myocytes, causing an efflux of NA+, and raising the intracellular Ca+ concentration. This drug has a very narrow therapeutic range, which is 0.5-2 ng/mL [16]. Some patients that take digoxin excrete dihydrodigoxin, which is the inactive digoxin metabolite where the lactone ring is reduced. This change in ring structure is what causes decreased target affinity due to shift in positioning within the binding pocket of the Na+/K+ ATPase. A study done by Haiser et al., 2014, concluded that the saturation of the lactone ring of digoxin is catalyzed by the drug microbiome causing drug inactivation after doing ex vivo incubation with rat and human fecal samples [15]. There is an increase in the excretion of reduced metabolites following the administration of prolonged release digoxin formulations. However, they also found that broad spectrum antimicrobial therapy blocks the formation of reduced digoxin metabolites with an increase of serum level in the drug. With this study along with others done over the years, it can be concluded that the gut microbiota is responsible for digoxin inactivation. Since the α,β-unsaturated lactone of digoxin and the unsaturated carboxylic acid of fumarate (*cgr2* exhibits structural homology to FAD-binding fumarate reductase enzymes) are structurally and electronically similar, digoxin is able to occupy the binding pocket of *cgr2*. This explains the shift in positioning within the binding pocket of Na+/K+ ATPase, causing inactivation of digoxin. Arginine is an amino acid used in the biosynthesis of proteins and it inhibits the reduction of digoxin. This is because arginine can repress *cgr* operon expression and thus it is able to inhibit conversion of digoxin to dihydrodigoxin. This has been proven by RNA-seq and qRT-PCR [16]. Interestingly, since arginine is the main source of nitrogen and carbon for *E. lenta*, it stimulates *E. lenta* cell growth at the same time. With the *cgr* gene is responsible for the inactivation of the cardiac drug digoxin, which is used to treat heart failure and heart rhythm problem, the key experiment that demonstrates that this gene is bad for health is when the researchers incubate low reducing fecal samples with the type (reducing) and FAA 1-3-56 (non-reducing) strains of *E. lenta*. They noticed that communities that were incubated with the type strain were able to reduce more digoxin than the type strain alone. This goes to show that co-culture of *E. lenta* with the fecal microbiome enhances the efficiency of digoxin reduction. This in turn increases the *cgr* ratio (the relative abundance of the *cgr* operon to the *E. lenta* 16S rRNA gene). Therefore, in the presence of a microbial community, the abundance of the *E. lenta* type strain is significantly increased so having a larger amount of the *cgr* gene in our gut means that digoxin will always be reduced more.

2.5. Specificity

The gRNA can bind to either of the two DNA strands, however, it should perfectly complement the region that needs to be targeted. For target binding to occur, the PAM (protospacer adjacent motif) has to be positioned right after the target. The sequence of the PAM is necessary for target binding and depends on the Cas9 species, ie, the GC-content. Specificity can also be modified prior to Cas9 being introduced. The architecture of the gRNA can be changed or even the Cas9 protein itself. To narrow down the gRNA target sites, there are some rules that can be followed for greater specificity. For example, target sites that code for amino acids near the 3' terminus of the protein should be avoided so that the cell is less able to use an alternative ATG downstream of the start codon. Another factor would be to avoid target sites that code for amino acids close to the 5' terminus of the protein in order to increase the likelihood of creating non-functional allele. An important factor to consider is the homology directed repair where new information goes into the DNA. For successful gene knockout, single cell cloning and subsequent cloning is required and thus editing the DNA with the right gRNA is vital. Before using Homologous-Directed Repair (HDR), it is advised to create indels and conduct a mismatch cleavage assay (e.g. Surveyor) to make sure that gRNA is efficiently cutting the target sequence. Additionally, it is important to keep in mind that efficiency goes down drastically if the cut site is >30 nucleotides from the proximal ends of the repair template and therefore the choices for gRNAs is further limited. Another aspect to consider is off-target binding. Off target-effects of the CRISPR Cas9 system can be due to mismatch tolerance between the gRNA and target site [27].

3. Conclusion

Our approach proposes a method for insertion of arginine into *cgr2* so that *E. lenta* is able to grow, blocking its ability to reduce digoxin. Although it has been shown that high arginine

levels are able to block digoxin metabolism, the methodology remains unclear. Further research via computational analysis of transcription factor binding sites or screening *E. lenta* genomic libraries for transcription factors can help uncover why arginine prevents digoxin from being metabolized. Interactions of *cgr* could be tested with arginine, to get heterologous expression of the *cgr* operon and subsequent purification of the encoded proteins.These gene-editing methods could further be tested ex-vivo and in-vivo in mice. Other factors that limit use of CRISPR-Cas9 technologies are delivery mechanisms, off-target effects and specificity. Due to the large size of the associated Cas9 protein, traditional methods of delivery are not as effective to reach target cells or tissues [28]. Off-target effects are caused due to modifications in the genome caused by CRISPR-Cas9 outside of the target cell or tissue, this can be harmful as it can lead to alteration in cellular function [29]. Future work focusing on optimizing CRISPR-Cas9 protocols for enhancing efficiency and reliable modifications supported by innovative bioinformatic tools can further advance precision medicine [30] [31] [32].

While this study elaborated on the *cgr* operon function with respect to CRISPR-Cas9, there are numerous gene applications to be explored. These applications can have various effects on point of care diagnostics, hydrogel microneedle applications and lab-on-chip technologies [33] [34] [35]. A hydrogel based matrix can be useful for targeted transdermal delivery, proving to be a useful alternative to traditional deployment techniques, improving efficiency of gene editing over time [36] [37] [38]. Furthermore, capacitive micromachined ultrasonic transducers (CMUT) based technology can be utilized to detect and monitor CRISPR-Cas9 activity [39].

Author Contributions

TN, KD, SV- investigation, resources, formal analysis, writing – original draft, writing – review & editing. KD, SV- conceptualization.

Conflicts of Interest

The authors declare no competing financial interests or conflicts of interest.

References

[1] Y. Ishino, M. Krupovic, and P. Forterre, “History of CRISPR-Cas from Encounter with a Mysterious Repeated Sequence to Genome Editing Technology,” Journal of bacteriology, vol. 200, no. 7, pp. e00580-17, Apr. 2018, doi: https://doi.org/10.1128/JB.00580-17.

[2] Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in Escherichia coli, and identification of the gene product. J Bacteriol. 1987 Dec;169(12):5429-33. doi: 10.1128/jb.169.12.5429-5433.1987.

[3] I. Gostimskaya, “CRISPR–Cas9: A History of Its Discovery and Ethical Considerations of Its Use in Genome Editing,” Biochemistry (Moscow), vol. 87, no. 8, pp. 777–788, Aug. 2022, doi: https://doi.org/10.1134/s0006297922080090.

[4] M. Redman, A. King, C. Watson, and D. King, “What Is CRISPR/Cas9?,” Archives of Disease in Childhood - Education & Practice Edition, vol. 101, no. 4, pp. 213–215, Apr. 2016, doi: https://doi.org/10.1136/archdischild-2016-310459.

[5] Periketi, P.; Kaur, K. .; Naseer Vaid, F. .; Sree M, Y. .; Madhu, M. .; Verma, S. .; Dhingra, K. . Blood Brain Barrier-on-a-Chip Permeation to Model Neurological Diseases Using Microfluidic Biosensors. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 78-93. https://doi.org/10.60087/jklst.v3.n4.p78.

[6] P. Liang et al., “CRISPR/Cas9-mediated Gene Editing in Human Tripronuclear Zygotes,” Protein & Cell, vol. 6, no. 5, pp. 363–372, Apr. 2015, doi: https://doi.org/10.1007/s13238-015-0153-5.

[7] G. J. Knott and J. A. Doudna, “CRISPR-Cas guides the future of genetic engineering,” Science, vol. 361, no. 6405, pp. 866–869, Aug. 2018, doi: https://doi.org/10.1126/science.aat5011.

[8] M. L. Nuccio, H. Claeys, and K. S. Heyndrickx, “CRISPR-Cas technology in corn: a new key to unlock genetic knowledge and create novel products,” Molecular Breeding, vol. 41, no. 2, Feb. 2021, doi: https://doi.org/10.1007/s11032-021-01200-9.

[9] M. Tiruneh G/Medhin, E. Chekol Abebe, T. Sisay, N. Berhane, T. Bekele, and T. Asmamaw Dejenie, “Current Applications and Future Perspectives of CRISPR-Cas9 for the Treatment of Lung Cancer,” Biologics: Targets and Therapy, vol. Volume 15, no. 15, pp. 199–204, May 2021, doi: https://doi.org/10.2147/btt.s310312.

[10] Pundlik, A.; Verma, S.; Dhingra, K. Neural Pathways Involved in Emotional Regulation and Emotional Intelligence. J. Knowl. Learn. Sci. Technol. 2024, 3 (3), 165-192. https://doi.org/10.60087/jklst.vol3.n3.p.165-192.

[11] Gupte, P.; Dhingra, K.; Saloni , V. Precision Gene Editing Strategies With CRISPR-Cas9 for Advancing Cancer Immunotherapy and Alzheimer’s Disease. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 11-21. https://doi.org/10.60087/jklst.v3.n4.p11.

[12] S. Altschul, "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs," Nucleic Acids Research, vol. 25, no. 17, pp. 3389–3402, Sep. 2018, doi: https://doi.org/10.1093/nar/25.17.3389.

[13] J. Soding, A. Biegert, and A. N. Lupas, "The HHpred interactive server for protein homology detection and structure prediction," Nucleic Acids Research, vol. 33, no. Web Server, pp. W244–W248, Jul. 2005, doi: https://doi.org/10.1093/nar/gki408.

[14] Kazim, I.; Gande, T.; Reyher, E. .; Gyatsho Bhutia, K. .; Dhingra, K.; Verma, S. Advancements in Sequencing technologies:: From Genomic Revolution to Single-Cell Insights in Precision Medicine. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 108-124. https://doi.org/10.60087/jklst.v3.n4.p108.

[15] H. J. Haiser, K. L. Seim, E. P. Balskus, and P. J. Turnbaugh, "Mechanistic insight into digoxin inactivation byEggerthella lentaaugments our understanding of its pharmacokinetics," Gut Microbes, vol. 5, no. 2, pp. 233–238, Jan. 2014, doi: https://doi.org/10.4161/gmic.27915.

[16] J. Lindenbaum, D. Rund, V. P. Butler, D. Tse-Eng, and J. R. Saha, "Inactivation of Digoxin by the Gut Flora: Reversal by Antibiotic Therapy," The New England Journal of Medicine, vol. 305, no. 14, pp. 789–794, Oct. 1981, doi: https://doi.org/10.1056/nejm198110013051403.

[17] E. Saunders et al., "Complete genome sequence of Eggerthella lenta type strain (IPP VPI 0255T)," Standards in genomic sciences, vol. 1, no. (2), pp. 174–182, Sep. 2009, doi: https://doi.org/10.4056/sigs.33592.

[18] M. J. Burton et al., "A heme-binding domain controls regulation of ATP-dependent potassium channels," Proceedings of the National Academy of Sciences, vol. 113, no. 14, pp. 3785–3790, Mar. 2016, doi: https://doi.org/10.1073/pnas.1600211113.

[19] "Eggerthella lenta DSM 2243, complete sequence," NCBI Nucleotide, Mar. 2024, Accessed: Jul. 05, 2024.https://www.ncbi.nlm.nih.gov/nuccore/NC_013204.1?from=2957948&to=2958598&report=genbank&strand=true

[20] "About Us - Introduction of BiologicsCorp," BiologicsCorp. https://www.biologicscorp.com/about-us/#.Wgo8QRNSxQI

[21] F. Lassalle, S. Périan, T. Bataillon, X. Nesme, L. Duret, and V. Daubin, "GC-Content Evolution in Bacterial Genomes: The Biased Gene Conversion Hypothesis Expands," PLOS Genetics, vol. 11, no. 2, p. e1004941, Feb. 2015, doi: https://doi.org/10.1371/journal.pgen.1004941.

[22] D. Yang et al., "GC-Content Dependence of Elastic and Overstretching Properties of DNA:RNA Hybrid Duplexes," Biophysical Journal, vol. 119, no. 4, pp. 852–861, Aug. 2020, doi: https://doi.org/10.1016/j.bpj.2020.06.034.

[23] T. I. Moy, D. Boettner, J. C. Rhodes, P. A. Silver, and D. S. Askew, "Identification of a role for Saccharomyces cerevisiae Cgr1p in pre-rRNA processing and 60S ribosome subunit synthesis," Microbiology, vol. 148, no. 4, pp. 1081–1090, Apr. 2002, doi: https://doi.org/10.1099/00221287-148-4-1081.

[24] J. L. Goldstein, D. Glossip, S. Nayak, and K. Kornfeld, "The CRAL/TRIO and GOLD Domain Protein CGR-1 Promotes Induction of Vulval Cell Fates in Caenorhabditis elegans and Interacts Genetically With the Ras Signaling Pathway," Genetics, vol. 172, no. 2, pp. 929–942, Feb. 2006, doi: https://doi.org/10.1534/genetics.104.035550.

[25] J. Sun, M. McFarland, D. Boettner, J. Panepinto, J. C. Rhodes, and D. S. Askew, "Cgr1p, a Novel Nucleolar Protein Encoded by Saccharomyces cerevisiae Orf YGL0292w," Current Microbiology, vol. 42, no. 1, pp. 65–69, Jan. 2001, doi: https://doi.org/10.1007/s002840010180.

[26] T. I. Moy, D. Boettner, J. C. Rhodes, P. A. Silver, and D. S. Askew, "Identification of a role for Saccharomyces cerevisiae Cgr1p in pre-rRNA processing and 60S ribosome subunit synthesis," Microbiology, vol. 148, no. 4, pp. 1081–1090, Apr. 2002, doi: https://doi.org/10.1099/00221287-148-4-1081.

[27] Dongre, A. .; Nale, T. . .; Ramavajhala, A.; Mahanta, D. .; Sharma, . O. .; Wadhwa, H. H. .; Dhingra, K. .; Verma, S. . The Evolution of Transdermal Drug Delivery: From Patches to Smart Microneedle-Biosensor Systems. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 160-168. https://doi.org/10.60087/jklst.vol3.n4.p160.

[28] Chandna, R. .; Bansal, A.; Kumar, A.; Hardia, S.; Daramola, O.; Sahu, A.; Verma, K.; Dhingra, K.; Verma, S. Skin Disease Classification Using Two Path Deep Transfer Learning Models. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 169-187. https://doi.org/10.60087/jklst.v3.n4.p169.

[29] M. A. Mengstie et al., "Recent Advancements in Reducing the Off-Target Effect of CRISPR-Cas9 Genome Editing," Biologics: Targets and Therapy, vol. 18, pp. 21–28, Jan. 2024, doi: https://doi.org/10.2147/BTT.S429411.

[30] Chilmakuri, L.; Mishra, A. K.; Shokeen, D. .; Gupta, P. .; Wadhwa, H. H.; Dhingra, K. .; Verma, S. A Wearable EMG Sensor for Continuous Wrist Neuromuscular Activity for Monitoring. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 148-159. https://doi.org/10.60087/jklst.v3.n4.p148.

[31] Mehta, A.; Alaiashy, O.; Kumar, P.; Tamilinian, V.; Besong, S.; Balpande, S.; Verma, S.; Dhingra, K. Advancing Model-Based Systems Engineering in Biomedical and Aerospace research:: A Comprehensive Review and Future Directions. J. Knowl. Learn. Sci. Technol. 2024, 3 (4), 133-147. https://doi.org/10.60087/jklst.v3.n4.p133.

[32] Ruthwik Guntupalli, Saloni Verma and Karan Dhingra 2024. Impact of Healthcare Digitization: Systems Approach for Integrating Biosensor Devices and Electronic Health with Artificial Intelligence. American Scientific Research Journal for Engineering, Technology, and Sciences. 98, 1 (Aug. 2024), 246–257, https://asrjetsjournal.org/index.php/American_Scientific_Journal/article/view/10786/2789.

[33] H. J. Pandya et al., "Label-free electrical sensing of bacteria in eye wash samples: A step towards point-of-care detection of pathogens in patients with infectious keratitis," Biosensors and Bioelectronics, vol. 91, pp. 32–39, May 2017, doi: https://doi.org/10.1016/j.bios.2016.12.035.

[34] H. J. Pandya et al., "A microfluidic platform for drug screening in a 3D cancer microenvironment," Biosensors and Bioelectronics, vol. 94, pp. 632–642, Aug. 2017, doi: https://doi.org/10.1016/j.bios.2017.03.054.

[35] M. Safavieh et al., "Paper microchip with a graphene-modified silver nano-composite electrode for electrical sensing of microbial pathogens," Nanoscale, vol. 9, no. 5, pp. 1852–1861, 2017, doi: https://doi.org/10.1039/c6nr06417e.

[36] GhavamiNejad P, GhavamiNejad A, Zheng H, Dhingra K, Samarikhalaj M, Poudineh M., "A Conductive Hydrogel Microneedle-Based Assay Integrating PEDOT:PSS and Ag-Pt Nanoparticles for Real-Time, Enzyme-Less, and Electrochemical Sensing of Glucose," Advanced Healthcare Materials, vol. 12, no. 1, Oct. 2022, doi: https://doi.org/10.1002/adhm.202202362.

[37] S. Odinotski et al., "A Conductive Hydrogel-Based Microneedle Platform for Real-Time pH Measurement in Live Animals," Small, vol. 18, no. 45, Sep. 2022, doi: https://doi.org/10.1002/smll.202200201.

[38] GhavamiNejad P, GhavamiNejad A, Zheng H, Dhingra K, Samarikhalaj M, Poudineh M., "A Conductive Hydrogel Mi-croneedle-Based Assay Integrating PEDOT:PSS and Ag-Pt Nanoparticles for Real-Time, Enzyme-Less, and Electro-chemical Sensing of Glucose," Advanced Healthcare Materials, vol. 12, no. 1, Oct. 2022, doi: https://doi.org/10.1002/adhm.202202362.

[39] Kulkarni S, Dhingra K, Verma S., "Applications of CMUT Technology in Medical Diagnostics: From Photoacoustic to Ultrasonic Imaging", International Journal of Science and Research (IJSR), Volume 13 Issue 6, June 2024, pp. 1264-1269, https://www.ijsr.net/archive/v13i6/SR24619062609.pdf.