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A Review on the Potential of Application of CRISPR/Cas9 Technology in CYP3A4 and CYP2D6 Genes: A Way to Move Forward in Identifying New Drug Targets

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The cytochrome P450 (CYP) enzymes are of a ubiquitous heme–thiolate proteins family that plays an important role within the biotransformation of endogenous molecules and xenobiotics as today's market is bio-transformed through CYP-mediated metabolism. Major CYP isoforms involved in most reactions are mainly CYP3A4 and CYP2D6. The CYP3A4 is found most extensively within the human liver and gut in which this CYP-isoform takes part in the phase I transformation of toxins, carcinogens, bile acids, and steroid hormones while CYP2D6 plays a central role in the oxidative metabolism of up to 25% of drugs in common clinical use, although it only accounts for 1-5% of the CYP liver content. With the help of vast gene editing technologies such as the CRISPR/Cas9 system, researchers have found its application to be useful and efficient in gene knockout studies involving genetic variants in association with drug metabolism due to its simplicity and affordability. Hence, this review aims to explore the potential use of CRISPR/Cas9 technology in gene editing in vitro studies particularly in knocking out the gene of interest, mainly drug metabolism enzymes. This article will explore how CRISPR/cas9 facilitates ex vivo assays of drug metabolism studies. The long-term goal for bio-editing is an in vivo treatment without the risk of causing harm to humans and providing a ready-to-be-used drug metabolism assay for ex vivo studies of drug development and pharmacokinetics study at a lower cost.

Keywords: CRISPR/Cas9; CYP3A4; CYP2D6; gene knockout; gene editing; hepatocyte cell lines

I. INTRODUCTION

Cytochrome (CYP) P450 is a superfamily of a heme-containing enzyme comprising over 2100 isoforms (Nelson, 2009). CYP P450 enzyme and other drug-metabolising enzymes are polymorphic which generate wide variations in the metabolic clearance of drugs (Dorr *et al.,* 2017). In general, it is estimated that polymorphism will affect around 20–25% of all drug therapies (Ingelman-Sundberg, 2004) to an extent that the therapy outcome is influenced. The CYP plays a critical role in the biotransformation of endogenous molecules and xenobiotics (Paine *et al.,* 2006) because these enzymes are vital in the early 80% of all phase I drug metabolism (Eichelbaum *et al.,* 2006).

CYP3A4 is one of the most extensive isoforms in the cytochrome P450 superfamily within the human liver and gut that involve the phase I transformation of toxins, carcinogens, bile acids, steroid hormones, and over 50% of the medication utilised in the clinic (Van Herwaarden *et. al.,* 2007; Martignoni *et al.,* 2006). Previous studies have stated that the CYP 3A4 is responsible for about 30% of the total CYP enzymes in the human liver and metabolises over 50% of clinically important drugs other than playing its crucial role in toxicity and carcinogenicity (Zanger & Schwab, 2013; Gonzalez, 2003; Scheer *et al.,* 2014). The CYP2D6 is another major member in the cytochrome P450 superfamily that

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plays a significant role in the oxidative metabolism of up to 25% of drugs in common clinical use even though it only accounts for 1-5% of the CYP liver content (Samer *et al.,* 2013).

Current reports have presented that the knockout of CYP2E1 (Wang *et al.,* 2016) or CYP3A1/2 (Lu *et al.,* 2017) done in vivo within rats using clustered regularly interspaced short palindromic repeats, CRISPR-associated protein 9 system (CRISPR/Cas9) as well as the knockout of CYP3A4 via Zinc Finger Nucleases (ZNF) could be of potential use in drug metabolism studies. However, the application using CRISPR/Cas9 in the attempt of knocking out CYP3A4 and CYP2D6 in a human hepatocyte cell line to further study the association of genetic variants with drug metabolism has not yet been reported. Thus, there is uncertainty about whether it is possible to knock out the CYP3A4 and CYP2D6 genes from the human hepatocyte cell line via the CRISPR/Cas9 technology.

The recent CRISPR/Cas9 system developed from *Streptococcus pyogenes* has greatly reduced the difficulties of genome editing in various species (Ma *et. al.,* 2014; Sternberg *et. al.,* 2014; Wang *et al.,* 2016). The CRISPR/Cas9 system consists of a non-specific nuclease, Cas9 protein, and one guide RNA (sgRNA) that directs Cas9 protein to the target sites using the principles of Watson-Crick base-pairing (Wang *et. al.,* 2016; Sternberg *et al.,* 2014). Compared with previous techniques, the CRISPR/Cas9 system shows distinct benefits in editing multiple genes simultaneously (Ma *et. al.,* 2014; Mali *et al.,* 2013). In addition, there is a newly established report of genomic CYP3A5 bioengineering in a human cancer cell line with drug metabolism analysis (Dorr *et al.,* 2017). Hence, it is hypothesised that this gene modification can also be applied in vitro to a human hepatocyte cell line containing functional CYP3A4 and CYP2D6 genes that can be knocked out with CRISPR/Cas9.

This review aims to explore the use of the CRISPR/Cas9 technology in gene editing in vitro studies, particularly gene knockout. The success in the knockout can contribute to the evaluation of the effects of genetic variants on drug metabolism, due to a single genetic variant being engineered into cell lines to produce an altered enzyme activity, gene regulation, or protein expression for drug transport or metabolism studies (Mali *et al.,* 2013).

II. METHODOLOGY

This narrative review is based on a series of literature searches that were done using multiple databases including PubMed, Google Scholar, The National Centre for Biotechnology Information (NCBI) Databases, and ISI Web of science. The reference lists of the identified research papers were inspected for further relevant literature (ancestry approach). The identified studies were thoroughly read and assessed according to the goals of the study.

III. DISCUSSION

A. The Cytochrome P450

Cytochrome P450 (CYP) is one of the largest drug enzymes from a protein family of heme-containing monooxygenases found in all tissue throughout the body except skeletal muscle and red blood cells (Guengerich, 2005). It plays a major role in drug pharmacokinetics and response variability (Zanger & Schwab, 2013). They are crucial for the metabolism of endogenous substances like steroid hormones, fatty acids, and most significantly xenobiotics together with medication and carcinogens. Over 50,000 CYP enzymes are described in most types of archaea, viruses, protists, bacteria, animals, plants, and fungi (Sigel *et al.,* 2007). Various CYP proteins have been discovered and extensively found throughout the body, showing significant contributions to chemical activation, deactivation, and carcinogenesis (Estabrook, 2003).

1. Occurrence and function

In mammals, CYP P450s are present in all tissues with the greatest concentrations that are found mainly in the endoplasmic reticulum of hepatic cells (liver and small intestines) (Pan *et al.,* 2017). They are involved in the processing and transport of protein, and Families 11, 24, and 27 are located in the mitochondrial inner membranes of steroidogenic tissues such as the ovary, breast adrenal cortex, testis, and placenta- all known as energy-producing cells (Guengerich *et al.,* 2016). The enzymes located in the endoplasmic reticulum normally metabolise exogenous compounds, mainly medications, environmental contaminants, and carcinogens, whereas enzymes found in mitochondria are typically involved in the synthesis and

conversion of endogenous lipophilic substances (steroids, bile acids, fatty acids, prostaglandins) into more polar (hydrophilic) products, that allow the elimination from the body through urine to avoid accumulation and toxicity.

Specific roles of CYPs in the brain have been identified based on previous research, including regulating endogenous $GABA_A$ receptor agonists, maintaining homeostasis of brain cholesterol, and removing retinoids (Guengerich *et al.,* 2016). It can therefore be concluded that CYPs play a key role in cellular metabolism and the maintenance of cellular homeostasis (Guengerich *et al.,* 2016).

2. Classification

The human genome consists of 57 CYP genes categorised by sequence homology into 18 different families and 44 subfamilies as shown in Figure 1. They are known as intrinsic membrane-bound proteins as there are 50 CYP genes in the endoplasmic reticulum while the other 7 are in the mitochondrial membrane (Guengerich *et al.,* 2016). Figure 1 summarises the enzymes in the families 1 to 3 (CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4) which are known to have the most active hepatic metabolism of xenobiotics involved in phase I drug metabolism. Table 1 includes the other CYP450 families according to sequence homology with a bigger role in endogenous functions (Manikandan & Nagini, 2018).

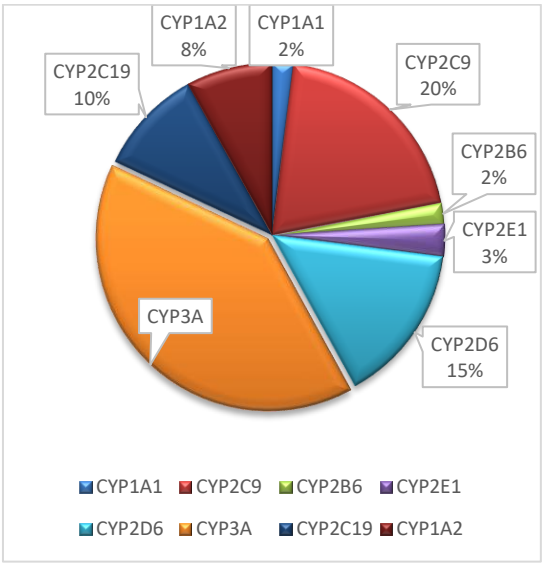

Figure 1. Contributions of individual CYP450s to the metabolism of drugs marketed

Cytochrome P450 pathways are also classified by similar gene sequences within which they are given a family number such as CYP1, CYP2 and a subfamily letter such as CYP1A, CYP2D and are then differentiated by a number for the isoform or individual enzymes such as CYP1A1 and CYP2D6. In humans, almost 80% of oxidative metabolism and approximately 50% of the overall elimination of common clinical drugs can be attributed to one or more of the various CYPs, from the CYP families (Zhao *et al.,* 2021).

Mainly, after being ingested, drugs will go through biotransformation. There will be changes in therapeutic efficacy which explains greater consideration during the development and discovery of drugs related to the metabolism mediated by these three isoforms (Wen *et. al.,* 2008; Dockens *et al.,* 2006). They are mainly expressed in the liver, and also occur in other organs such as the small intestine (reducing drug bioavailability), lungs, placenta, and kidneys (Lynch & Price, 2007). Factors and mechanisms that are involved in each CYP expression are affected by a few factors including genetic polymorphisms, cytokines, xenobiotics activation, hormones and diseases regulation, age, gender, and others (Zanger & Schwab, 2013). P450s can also be classified by the nature of the substrates they oxidise, particularly in mammals, as shown below in Table 2 (Guengerich *et. al.,* 2016; Guengerich, 2006; Nelson *et al.,* 2006).

Table 2. Classification of CYP450 according to major substrate class

Substrate	**P450 Subfamilies**
Sterols	1B1, 7A1, 7B1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 27A1, 39A1
Xenobiotics	1A1, 1A2, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 3A4, 3A5, 3A7
Fatty acids	2J2, 4A11, 4B1, 4F12
Eicosanoids	4F1, 4F3, 4F8, 5A1, 8A1
Vitamins	2R1, 24, 26A1, 26B1, 26C1, 27B1
Unknown	2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F11, 4F22, 4V2, 4X1, 4Z1, 20A1, 27C1

Table 1. Classification of CYP450 according to sequence homology

Family	Subfamily	Genes	Pseudogenes	Function
CYP1	CYP1A	1A1,1A2		Drug and steroid metabolism
	CYP1B	1B1		
	CYP1D		1D1P	
CYP2	CYP2A	2A6, 2A7, 2A13	2AB1P, 2AC1P	Drug metabolism and synthesis of cholesterol, steroids, and other lipids
	CYP2B	2B6	2B7P	
	CYP2C	2C8, 2C9, 2C18, 2C19	2C23P, 2C58P, 2C59P, 2C60P	
	CYP2D	2D6, 2D7	2D8P	
	CYP2E	2E1		
	CYP2F	2F1	2F2P	
CYP3	CYP3A	3A4, 3A5, 3A73A43	3A51P, 3A52P, 3A54P, 3A137P	Drug and steroid metabolism
CYP4	CYP4A	4A11, 4A22,	4A26P, 4A27P, 4A44P	Arachidonic acid and fatty acid metabolism
	CYP4B	41B1		
	CYP4F	4F2, 4F3, 4F8, 4F9, 4F22, 4F11, 4F12	4F9P, 4F10P, 4F23P, 4F24P, 4F25P	
CYP5	CYP5A	5A1		Thromboxane A synthase
CYP7	CYP7A	7A1		Bile acid biosynthesis 7-alpha hydroxylase of the steroid nucleus
	CYP7B	7B1		
CYP8	CYP8A	8A1		Varied (bile acid biosynthesis prostacyclin synthase)
	CYP8B	8B1		
CYP11	CYP11A	11A1		Steroid biosynthesis
	CYP11B	11B1, 11B2		
CYP17	CYP17A	17A1		Steroid biosynthesis, 17-alpha hydroxylase
CYP19	CYP11A	19A1		Steroid biosynthesis
CYP20	CYP20A	20A1		
CYP21	CYP21A	21A2		Steroid biosynthesis
CYP24	CYP24A	24A1		Vitamin D degeneration
CYP26	CYP26A	26A1		Retinoic acid hydroxylase
	CYP26B	26B1		
	CYP26C	26C1		
CYP27	CYP27A	27A1		Various biosynthesis function
	CYP27B	27B1		
	CYP27C	27C1		
CYP39	CYP39A	39A1		7-alpha hydroxylation of 24-hydroxycholesterol
CYP46	CYP46A	46A1		Cholesterol 24-hydroxylase
CYP51	CYP51A	51A1		Cholesterol biosynthesis

3. Role of cytochrome P450 in drug metabolism

In a normal drug metabolism, most of the orally administered drugs are lipid soluble and nonpolar rather than hydrophilic and polar (for pharmacokinetic profile). Lipophilic drugs need to be in a hydrophilic state for the elimination process (Del Tredici *et al.,* 2018).

All lipophilic drugs will undergo two-phase liver biotransformation after absorption which are:

- Phase I (Catabolic): Oxidation, Reduction or Hydrolysis: Conversion of lipophilic drugs to active or inactive metabolites (most important phase I reactions are catalysed by cytochrome P450 [CYP 450] microsomal enzymes).
- Phase II (Anabolic): Conjugation: Many drugs (and active metabolites) undergo second biotransformation to render them polar and hydrophilic to allow for aqueous solubility for excretion in urine or faeces (not affected by CYP enzymes).

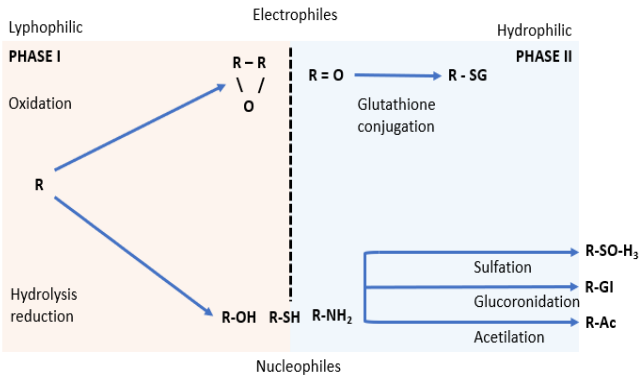

Figure 2. Phase I of drug metabolism involves CYP P450 and Phase II involves other enzymes

However, escape of reactive intermediates from phase II detoxification systems which detoxify electrophilic metabolites into non-toxic substances may have an interaction with cellular macromolecules (DNA, RNA, and proteins) which result in adverse drug reactions (ADRs) that will eventually contribute to major obstacles in drug treatment and drug development. The expression and functions of CYPs influence physiological factors such as age, sex, hormones, environment, genetic polymorphisms, and pathological factors such as cancer, inflammation, and cholestasis. These factors and the drug-drug interactions have contributed to the rise of ADRs of clinical manifestations due to the consequences of CYP-catalysed reactions. Major mechanisms underlying the interaction between drugs can indeed imitate genetic defects (CYP inhibitors) or increased metabolism (CYP inducers).

4. CYP inducers and CYP inhibitors

Both CYP induction and inhibition are major mechanisms that underlie drug-drug interactions (Gopisankar, 2018). Increased or decreased CYP enzymes expression will lead to serious toxicological consequences due to an increase in drug metabolism that mediates drug-drug interaction, bioactivation of xenobiotics to carcinogenic or toxic metabolites, and endocrine disruption (Kalra, 2007; Tompkins & Wallace, 2007). CYP induction of various active parent drugs has been reported to increase the metabolism and elimination of the drug thereby reducing its pharmacological effects (Manikandan & Nagini, 2018). Furthermore, it may also activate prodrugs to electrophilic, active metabolite forms, thus contributing to its pharmacodynamic effects (Manikandan & Nagini, 2018).

CYP enzyme inhibition is involved in the competition between drugs for the same binding site (Manikandan & Nagini, 2018). This can be seen from a previous study that shows desipramine which is metabolised by CYP2D6 being strongly inhibited by the binding of fluoxetine to the same isoenzyme (Manikandan & Nagini, 2018). Enzyme inhibition reduces the biotransformation or clearance of drugs including several anti-cancer agents resulting in increasing drug plasma levels of drugs, potentially leading to clinical toxicity or diminished therapeutic effect (Manikandan & Nagini, 2018). If the drug is a prodrug, then the effect is decreased. Therefore, the pharmacokinetic drug-drug interactions caused by inhibition mechanisms can be observed and eventually help in the development of safer anti-cancer regimens (Manikandan & Nagini, 2018).

From the previous study, it is reported that inhibition of CYP2D6 mediated metabolic conversion of dextromethorphan is caused by Quinidine thus, systemic bioavailability will increase and in the usage of pseudobulbar effect, there will be fewer adverse reactions (Schoedel *et al.,* 2014). Examples of drugs that are potent inducers include phenobarbital, phenytoin, and rifampicin. Many glucocorticoids in clinical use also induce CYP3A4. Some organochlorine pesticides such as dichlorodiphenyltrichloroethane and endrin also induce CYP3A4 (Hakkola *et al,* 2018). On the other hand, reversible inhibition might happen because of competition between CYP3A4 substrates. For instance, oestrogen and antidepressants interact when the menstrual cycle is in the late luteal phase (Soldin, *et al.,* 2011).

5. CYP3A4

Cytochrome P450 3A4 which is abbreviated as CYP3A4 is a crucial enzyme within the body, chiefly found within the liver and gut. It oxidises xenobiotics which is a tiny foreign organic molecule-like toxins or medicine so they would be expelled from the body. While several medicines are deactivated by CYP3A4, some drugs are activated by the enzyme. Some substances such as grapefruit juice and a few medicines can interfere with the CYP3A4 action. These interactions can result in either amplification or weakening of the action of medicine that is modified by CYP3A4 as it is a member of the

cytochrome P450 family of oxidising enzymes. Many other members of this family are involved in drug metabolism with the CYP3A4 being the most common and the most versatile one. In humans, the CYP3A4 protein is encoded by the CYP3A4 gene (Pelkonen *et al.,* 2008) as its sequence is a part of the cluster of cytochromes P450 genes on chromosome 7q22.1 (Hashimoto *et al.,* 1993).

The human CYP3 family consists solely of 1 subfamily- the CYP3A, which is found on chromosome 7q22.1 and features a size of 231 kb. It contains the four CYP genes which are 3A4, 3A5, 3A7, and 3A43. The CYP3A4 is the most expressed P450 in intestinal enterocytes, with levels dissimilar to the liver, and contributes significantly to the first-pass metabolism of orally administered medication (Inoue *et al.,* 1992). In different extrahepatic tissues as well as the tract, brain, lung, and kidney, CYP3A5 expression seems to be predominant or the same as CYP3A4 (Ding & Kaminsky, 2003; Dutheil *et al.,* 2008).

Multiple signalling pathways contribute to the complex regulation of the CYP3A genes. Moreover, the CYP3A4 shows vital activity and expression variations in females versus males (Dvorak *et. al.,* 2003; Wolbold, 2003; Cotreau *et el.,* 2005). The CYP3A subfamily enzymes play a significant role in the metabolism of nearly 30% of clinically used medication from the majority of therapeutic classes (Lamba *et. al.,* 2010; Bu, 2006; Liu *et al.,* 2007). The site of CYP3A4 is large and flexible and may accommodate and metabolise several preferentially lipophilic compounds with relatively large structures (Zanger *et. al.,* 2008; Scott & Halpert, 2005).

Typically, large substrates are mostly immunosuppressants such as cyclosporin A and tacrolimus, macrolide antibiotics like erythromycin, and anti-cancer medication such as Taxol. CYP3A4 is also an efficient steroid hydroxylase with a vital role in the catabolism of many endogenous steroids together with androgen, progesterone, androstenedione, cortisol, and bile acids.

5.1 Classes and clinical impact of CYP3A4 polymorphism

According to New Zealand Medicines and Medical Devices Safety Authority, the population variability of CYP3A4 activity is greater than 100-fold making it significantly high. Some of the variability can lead to allelic variation. A study involving a single nucleotide polymorphism (CYP3A4*22) appears to be linked with a reduced expression and activity by 1.7 to 5-fold lesser. Even so, this variant frequency is around 2% of the population which limits its involvement in the overall CYP3A4 variability. Another identified polymorphism is CYP3A4*1B, appearing in around 2–9% of some populations, but this variant has yet to establish its functional effect (MEDSAFE, 2014).

5.2 CYP3A4 role in drug metabolism

Given that it is expressed relatively highly in the liver and gut, CYP3A4 is frequently regarded as the most significant drug-metabolising enzyme. Undoubtedly, CYP3A4 makes up between 15 to 20 percent of the hepatic CYP content, making it one of the most prevalent CYP enzymes in the liver and the CYP3A4 expression varies greatly between individuals, up to 100 times. Intense intra- and inter-individual variations in CYP3A4 expression and function contribute to unpredictable drug response and toxicity. It is known that a wide range of environmental, genetic, and physiological variables affect the expression and function of CYP3A4 (Klein & Zanger., 2013). CYP3A4 can metabolise up to 50% of all medications, and this subclass of pharmaceuticals is present in practically all therapeutic drug classes. Although there is a substantial environmental factor e.g., diet, concomitant drugs, and diseases, that influences the expression of the enzyme, the variation in CYP3A4 activity is unimodal, and genetics is still thought to have a large role in interindividual variation in CYP3A4-mediated drug metabolism. The CYP3A4 gene's intron 6 polymorphisms (CYP3A4*22), which are linked to altered plasma drug levels and decreased hepatic CYP3A4 expression, help to explain some of this heritability (Tirona & Kim, 2017).

Eight CYP3A5 splice variants have been identified thus far. An important variant, CYP3A5*3 is defined by the presence of SNP 6986A>G (rs776746) in intron 3 and produces a non-functional CYP3A5 protein in homozygous carriers (CYP3A5*3/*3). Asians and Caucasians are more likely to have this faulty variation. As a result, depending on the person's ethnicity, CYP3A5 is expressed in about 10–25 percent of people. When expressed, CYP3A5 can equal CYP3A4 activity by making up roughly 50% of the total hepatic CYP3A content (Reviewed in Saiz-Rodriguez., *et al.,* 2020).

6. *CYP2D6*

There are 16 full-length genes in the CYP2 family, which all have 9 exons and 8 introns (Zanger & Schwab, 2013). One of the largest gene clusters is a cluster of CYP2D on chromosome 22q13.2 with the only CYP2D6 functional (protein-coding) gene (Zanger & Schwab, 2013). Cytochrome P450 (P450) 2D6 is a major drug-metabolising enzyme for approximately 25% of common clinical marketed drugs and is expressed in the liver and extrahepatic organs (such as the brain and intestine) (Pan *et al.,* 2017).

The estimated half-life of human CYP2D6 / ranges from 46.6 hours to 51 hours (Del Tredici *et al.,* 2018). Any factors involved in the stability changes and degradation of human CYPs will give impact their half-life and thus affect the drug metabolism (Del Tredici *et al.,* 2018). The CYP2D6 locus on chromosome 22q13.1 also harbours two non-functional pseudogenes which are CYP2D7 and CYP2D8P (Zanger & Schwab, 2013). CYP2D7 is expressed in the liver as mRNA, but the introduction of an insertion in the first exon will result in a shift of the reading frame, thus, disrupting protein expression. In contrast, CYP2D8P is a true pseudogene with several gene-disrupting mutation accumulations (Zanger & Schwab, 2013). Meanwhile, no CYP2D6 gene knockout has been reported so far.

6.1 Classes of CYP2D6 metabolisers

Hepatic CYP2D6 protein content varies in the individual mainly due to genetic polymorphism (Zanger & Schwab, 2013). From the CYP allele database, CYP2D6 is stated to have the highest number of variant alleles, 63 of which may affect the gene products' function or activity (Hendrychová *et al.,* 2011). CYP2D6 is the only non-inducible enzyme that has resulted in a significant contribution of genetic variation to interindividual enzyme activity which strongly depends on ethnicity and is not significantly influenced by gender, smoking, or alcohol consumption among all cytochrome P450s drug metabolisers (Zanger & Schwab, 2013). It is the first P450 to demonstrate its monogenic distribution and genetic polymorphism that significantly affects the metabolism of approximately 50% of clinically used drugs (Guengerich *et al.,* 2016).

The roles of the CYPs 2A6, 2B6, 2D6, 2C19, 2C9, and 3A5 are influenced by polymorphisms of multiallelic genetics that highly depend on ethnicity. Thus, this explains the variable CYP2D6 activities among individuals (Ingelman-Sundberg *et al.,* 2007). This led the population to be categorised according to distinct pharmacogenetic phenotypes and termed as poor, intermediate, extensive, and ultra-rapid metabolisers (Hendrychová *et al.,* 2011).

In a recent study conducted in The United States, CYP2D6 activity levels that are predicted based on the number of functional alleles distinguish the CYP2D6 phenotype to 2% of poor metaboliser (PM), 3% of intermediate metaboliser (IM), 92% of extensive metaboliser (EM) and 5% of ultra-rapid metaboliser (UM) (Del Tredici *et al.,* 2018). It is also reported that PMs are found mainly in Europe, while UMs are mainly found primarily in North Africa and Oceania. Due to the high Asian prevalence of the *CYP2D6*10* allele, IMs are located to a great extent in Asia (Ingelman-Sundberg *et al.,* 2007).

The phenotypic variations of PMs refer to alleles carriers of compound heterozygous or homozygous with a complete lack of function (null allele) due to defective or deleted genes; EMs refer to the "normal" phenotype, that typically represents the major proportion of the population carrying 2 functional genes; IMs carry only one normal or functionally defective allele, lead to impaired drug oxidation capacity; and the UMs phenotype emerges from gain-of-function variants with more than 2 active genes encoding a certain P450 (Zanger & Schwab, 2013).

6.2 Clinical impact of CYP2D6 polymorphism

In its pharmacological sense, the consideration of the pharmacogenetics of drug metabolising enzymes is important. Variants of loss-of-function will result in clearance reduction and increased plasma concentrations. In contrast, variants of gain-of-function will lead to increased clearance and lower concentrations of the drug. This results in increased and decreased drug impact, respectively, and possibly drug-related toxicity due to overdose if the medication is pharmacologically active. However, the contrary is to be expected in metabolically activated (prodrug), resulting in consideration of the metabolite's pharmacological activity or toxicity (Zanger & Schwab, 2013).

There are many clinical impacts of CYP2D6 polymorphisms on drug therapy that have been reported so far. In active parent drugs, the UMs will face a lack of efficacy while toxicity

will be suffered in PMs (Del Tredici *et al.,* 2018). For example, in UMs, there will be a loss of antidepressant therapeutic efficacy, while in several psychotropics (desipramine, venlafaxine, amitriptyline, haloperidol), PMs suffer an increased risk of toxicity (Hendrychová *et al.,* 2011).

It also affects the analgesic response to prodrug opioids such as codeine, tramadol, and oxycodone (Zanger & Schwab, 2013). Reduction of analgesic effects was observed in PMs for CYP2D6, while life-threatening toxicity cases with tramadol and codeine were reported in Ums (Hendrychová *et al.,* 2011). An increased risk of metoprolol, timolol, carvedilol, and propafenone toxicity was also correlated with CYP2D6 PM phenotype (Hendrychová *et al.,* 2011).

6.3 CYP2D6 role in drug metabolism

There is a large number of drugs mainly metabolised by CYP2D6 compared to its relatively low liver expression. It constitutes the metabolism of 25% of all clinically used drugs from virtually all therapeutic groups. The enzyme is influenced by polymorphism to an extent that therapy outcome is impaired and the CYP plays a critical role since these enzymes are responsible for about 80% of all drug metabolism in phase I (Hendrychová *et al.,* 2011).

CYP2D6 is the predominant pathway for the bioactivation or elimination of many centrally acting drugs, such as tricyclic and other second-generation antidepressants (amitriptyline, paroxetine), serotonin-selective reuptake inhibitors (SSRI), opioids analgesics (codeine, tramadol), β-blockers (bufuralol, metroprolol), antipsychotic (aripiprazole, risperidone), and anti-cancer drugs, in particular, the selective oestrogen receptor modifier (SERM) tamoxifen, and many others (Zanger & Schwab, 2013).

From previous studies, it is reported that the role of the CYP2D6 is important in abuse drug metabolism, and has been shown that several procarcinogens and neurotoxins are bioactivated (Zanger & Schwab, 2013). It acts as a CYP inhibitor which is prone to inhibition by numerous compounds that need not be substrates but bind with high affinity to the enzyme such as quinidine or methadone (Zanger & Schwab, 2013). Some of these inhibitors are strong enough to introduce a phenomenon known as phenocopying which will alter the patient's apparent phenotype (Zanger & Schwab, 2013). Due to its vital role in humans, more effort should be put into the study of CYP2D6 properties mainly in physiology and pathology.

In this review, we analyse the effectiveness of gene editing in vitro by using CRISPR/Cas9 technology, especially on the CYP2D6 and the CYP3A4 genes.

B. Gene Editing Technology

The basis of gene editing is the endogenous cellular repair mechanism that causes DNA double-strand breaks (DSBs) (Scheer *et. al.,* 2012; Maeder & Gersbach, 2016). DNA breaks are repaired through either of these 2 major pathways: homology-directed repair (HDR) or non-homologous end joining (NHEJ). Precise introduction of a targeted DSB is the. most critical issues when implementing two of the pathways.

There are currently three well-defined gene editing tools, which are based on nucleases enzyme activity and are used to induce the site-specific DSBs; they are ZFNs, Transcription Activator-Like Effector Nucleases (TALENs), and Clustered Regularly Interspaced Palindromic Repeats (CRISPRs) with CRISPR-associated (Cas) nucleases (Karlgren *et al.,* 2018). All three techniques have enabled targeted genetic modifications with high precision not only in cultured cells but also in animals and plants as well (Kotagama *et al.,* 2019).

They act by inserting, deleting, modifying, or replacing the DNA in the genome of a living organism. Each of these systems is characterised by a dual-strand cleavage nuclease domain and an adaptable sequence-specific DNA binding domain (Gupta *et al.,* 2019).

Both ZFN and TALENs played a vital role in editing their targeted gene, but the application is limited due to their complexity, difficulty, and expensiveness. Thus, a simple, reliable, efficient, and affordable approach for precise genome modification known as CRISPR/Cas9 system has been widely used in gene editing studies as no engineering of novel proteins for each DNA target site is involved (Porteus, 2015). The CRISPR/Cas9 system is an RNA-mediated adaptive immune system found in bacteria and archaea (Horvath & Barrangou, 2010) that protect against virus and plasmids according to its characteristics of recognising and binding target DNA sequences. Its precise site-specific shifts are facilitated by programmable guide RNA that recognises and guides the nuclease to the target DNA sequence and a restrictive enzyme complex known as Cas9 nuclease for

target DNA sequence cleavage resulting in a highly efficient gene-editing tool (Doudna & Charpentier, 2014).

Widely utilised in prokaryotic (Barrangou *et al.,* 2007), mammalian, and plant systems, it is concluded that these newer tools can provide wider applications of gene therapy ranging from medications to crop improvement. However, although the usage of the CRISPR/Cas9 system is ubiquitous, its use is restricted only to a few genetic disorders (Scheer *et al.,* 2012).

1. Common gene editing technology that uses nuclease enzyme

1.1 Zinc-Finger Nucleases (ZFNs)

Restrictions endonuclease FokI is responsible for catalytic cleavage of the target DNA (Gupta & Musunuru, 2014), which then generates a DBS with cohesive overhangs to extract the gene function information. Effective and versatile gene-editing ZFNs work by separating the DNA-binding consisting of eukaryotic transcription factors and zinc finger(s) and DNA-cleaving domains (Carroll, 2011).

The cleavage of the site-specific genome is induced considering that it can only identify a restricted number of bases by manipulating the ZFN complex to recognise two sequences located on either side of the target site (Carroll, 2011). After the identification of the relevant site, the *FokI* restriction enzyme will regulate the genome cleavage, thereby forming a DSB in the genome that can be manipulated as required (Carroll, 2011).

1.2 Transcription Activator-Like Effector Nucleases (TALENs)

Although the functions are similar to ZFNs, TALENs have different origins. It contains two domains which are an activator for N-terminal transcription like an effector (TALE) DNA-binding domain and the catalytic C-terminal domain of restriction endonuclease *FokI*.

TALENs are a group of proteins produced by pathogenic plant bacteria to help in their infection cycle (Gupta & Musunuru, 2014). These bacteria develop a protein with an approximate sequence of 33 to 35 amino acids. A spacer sequence is common between 13 and 28 amino acids (Gupta *et al.,* 2019). The TALEN pairs usually have a target sequence of 30–40 bp in length.

Repeat outcomes polymorphism is used to create repeat-variable diresidues (RVDs) that are of high-level nucleotide binding preference. This will create proteins that can identify bases from the base of genome sequences (Bogdanove & Voytas, 2011). As TALENs have a similar use as ZFNs, these protein structures can result in binding with a *FokI* to form a DSB within the genome. Although these 2 techniques of gene editing tools are relatively well developed, they are quite expensive, time-consuming, and require a lot of specific proteins to model in accordance with the requirement, resulting in a comparatively inefficient gene-editing tool (Gupta & Musunuru, 2014). Due to the disadvantages, researchers have come out with a more simple, reliable, efficient, and affordable approach to precisely modify the genome known as the CRISPR/Cas9 system.

1.3 CRISPR/Cas9 system

A newer genome editing tool known as the CRISPR/Cas9 system is modified to be implemented as a versatile, adaptable, and target-specific genome editing tool by employing Cas proteins guided by gRNA to cleave the target DNA sequence. In the CRISPR/Cas9 system, two vital components involved are gRNA and an endonuclease (Cas9). The ease and efficiency at which the Cas9 endonuclease targets and disrupts the specific genes are simply by changing the gRNA sequence, resulting in the generation of genome-wide CRISPR-knockout libraries both for animal model and human cells (Koike-Yusa *et. al.,* 2014; Shalem *et. al.,* 2014; Wang *et al.,* 2014). Table 3 provides ZFNs, TALENs, and CRISPR/Cas9 comparisons with the key properties of the genome editing tools.

1.3.1 Classification of CRISPR/Cas9 system

The CRISPR-Cas system is divided into 3 major types, namely Type I, Type II, and Type III according to sequence, locus organisation, contents, and phylogeny. The Six types identified in the type I system are categorised by signature protein presence with both helicase and DNase domains intended for target degradation. Type II systems have been classified into two subtypes II-A and II- B which encode Cas1 and Cas2, the Cas9 signature protein, and Cas4.

Cas9 aid in adaptation, engage in CRISPR RNA (crRNA) processing, and cleaves the target DNA assisted in

assemblage by crRNA and an additional RNA called trans-activating CRISPR RNA (tracrRNA).

Table 3. Comparison of major properties of the genome editing tools between ZFNs, TALENs, and CRISPR/Cas9

Properties	**ZFN**	**TALENs**	**CRISPR/Cas 9**
Designing parameter	Protein	Protein	RNA
Component involved in sequence recognition	Protein-DNA	Protein-DNA	RNA-DNA
Targeting efficiency	Less specific and efficient	Moderately specific and efficient	Highly specificity and efficiency
Viral delivery	Easy	Moderate	Moderate
Off-target mutagenesis	Variable	Low	Moderate
Cost-effectiveness	Very expensive	Expensive	Cheap

Type I and II systems target the DNA while Type III systems target DNA and/or RNA containing the signature protein Cas10. The Cas proteins consisting of approximately 45 Cas gene families found in a wide range of CRISPR subtypes are mostly designed for the Type III-A(Csm) or Type III-B(Cmr) complexes. Cas 1 and Cas 2 are universal in all CRISPR loci, whereas Cas3, Cas9, and Cas10 are specific for type I, II, and III CRISPR-Cas systems, respectively. Among all of the CRISPR-Cas types, the type II system has received more attention than the rest because it can induce DSBs in the target DNA (Gupta et, al., 2019).

1.3.2 The mechanism of the CRISPR/Cas9 system

The CRISPR/Cas9 system is an RNA-guided endonuclease DNA system, made up of endonuclease Cas9 and single-guide RNA (sgRNA) (Gupta *et al.,* 2019). The sgRNA with 18–20-nucleotide sequence complementing a target sequence directly before a protospacer adjacent motif (PAM). Cas9 is an RNA-guided DNA nuclease enzyme that introduced targeted loss-of-function mutations at specific genome sites through the generation of DNA-DSBs at specific genomic loci (Gupta *et al.,* 2019). Activated Cas-9 nuclease makes double-stranded breaks at a site 3 base pair upstream to PAM. Then the double-stranded break is repaired by either non-homologous end joining or homology-directed repair cellular mechanisms. Once the complementary region and the target region are properly paired, then the RuvC nuclease and HNH nuclease domain will cut the target DNA by cleaving the complementary strand and non-complementary strand, respectively.

DSBs can be repaired by two different mechanisms which are the NHEJ-DNA repair pathway and the HDR pathway. NHEJ is error-prone, producing inserts/deletions (indels) at the DSB site that lead to functions loss due to premature stop codon or frame-shifts (Karlgren *et al.,* 2018) while HDR will find the homologous DNA sequence presence and on finding one brings about homologous recombination and it is less error-prone. Since Cas9 acts as a general endonuclease, only sgRNA is needed to synthesise chemically, transcribed in vitro, or cellularly expressed to provide specificity, thus resulting in knockout or desired mutation introduction into the target gene, respectively. Alternatively, the DSB can be repaired by HDR in the presence of a donor DNA template to the targeted region, which allows specific nucleotide(s) replacement, the introduction of mutations, or the insertion of sequences in the targeted region (Karlgren *et al.,* 2018).

It is envisaged that the generated knockout hepatocytes cell lines of the CYP3A4 and CYP2D6 genes will lead us to a better understanding of their role in drug metabolism and its potential functions under physiological and pathological conditions.

Gene knockout is a genetically altering technique with genes either completely deleted or being inactivated due to mutation. Knockout is widely used to determine the targeted

gene's function and detect the protein products as well as related diseases that might occur when their functions are inhibited. Until now, however, neither CYP3A4 nor CYP2D6 gene knockout, either in an animal model or cell line, has been generated.

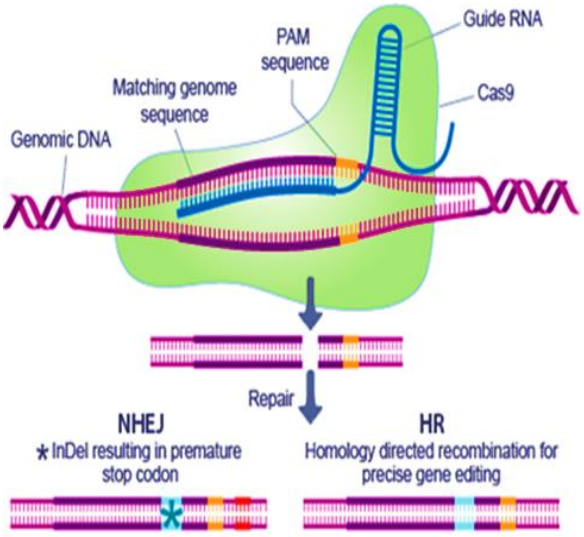

Figure 4. Mechanism of CRISPR/Cas9 system (Source: www.transomic.com)

1.3.3 Delivery Systems of CRISPR/Cas9

Efficient CRISPR/Cas protein tool delivery to the target cells (Liu *et al.,* 2017) will ensure that the tool reaches the desired cell or tissue and is one of the major requirements in minimising off-target effects within the gene (Roy *et al.,* 2018). Several physical and viral systems were utilised for the CRISPR/Cas9 delivery to the target site. The physical system comprises of gold nanoparticles, electroporation, mechanical cell deformation, cell-penetrating peptide, lipid-mediated transfection, DNA nanoclews, hydrodynamic delivery, microinjection, and induced transduction by osmocytosis and propanebetaine (Liu *et al.,* 2017). It serves a few advantages including safer usage compared to viral vectors with no size limitation for transgenic DNA. CRISPR/Cas9 physical delivery is efficient in the development of knockout in both cell lines and animal models, but a recent report has reported relatively poor delivery efficacy in in vivo applications (Liu *et al.,* 2017).

Viral delivery systems are the most efficient systems in delivering plasmid-based nucleic acids to mammalian cells whether in vitro or in vivo. Hence, the plasmid is delivered using CRISPR/Cas9 to mammalian cells (Liu *et al.,* 2017). In gene transduction, two types of viral systems frequently used are lentivirus and adeno-associated virus (AAV) (Grimm *et al.,* 2008).

Lentivirus-mediated CRISPRCas9 has achieved successful results in both in vitro and in vivo systems (Zufferey *et al.,* 1998). The benefit of high infection efficiency even in non-diving cells brings an important role in gene modification of cells like the liver and brain (Liu *et al.,* 2017).

The AAVs are non-pathogenic and mild immunogenic with serotype specificity. It can infect both dividing and nondividing cells. However, the limitation of AAV-mediated CRISPR/Cas9 (Liu *et al.,* 2017) can be overcome by attempting dual AAVs that can separately deliver Cas-9 encoding DNA and sgRNA (Zetsche *et al.,* 2015). The injection of two AAVs into one target cell is also challenging.

1.3.4 Challenges of CRISPR/Cas9 system

Despite the CRISPR/Cas9 technology's great potential as a genome-editing tool, many challenges need to be addressed (Roy *et al.,* 2018). Lack of security and effective delivery mechanisms, off-target effects, and ethical issues are a few of the major barriers to the CRISPR/Cas9 system in clinical applications (Liu *et al.,* 2017).

It is vital that off-target mutations are not induced in the genome following the use of CRISPR gene targeting technology. Detecting these off-target mutations is found to be more difficult than detecting mutations on-target.

This is due to the unknown position and number of off-target mutations. Comparing CRISPR/Cas9 to ZFNs and TALENs, CRISPR/Cas9 is more likely to produce off-target changes. This is because Cas9 acts as a monomer while ZFNs and TALENs have dimeric assemblies. Therefore, Cas9 is limited to recognising shorter target sequences.

Additionally, a certain level of mismatch mutations is tolerated by the sgRNA. In bacterial cells, the CRISPR system is reported to exhibit high specificity. However, in mammalian cells, the system has shown a notably high frequency of non-specific nuclease activity that causes mutagenesis of regions other than the specific targets (Fu *et al.,* 2013).

The off-target mutations are the effect of sgRNA binding to DNA sites with less than-perfect complementarity (Van

Campenhout *et al.,* 2019). Although off-target events can be scarce, they must not be overlooked as there is a tendency that another gene might be mutated, inflicting an effect or phenotype that might be confused with the one expected from the on-target mutation.

However, because it is possible to pre-select cell lines that carry the desired genotype without unwanted off-target mutations (Heckl *et al.,* 2014), many other ways are currently under study to decrease the chance of off-target effects like improved algorithms to design gRNAs and engineering Cas enzymes with higher fidelity and specificity (Heckl *et al.,* 2014). In addition, this drawback might be tackled by using multiple targeting strategies against the same product to make sure that they manufacture a similar phenotype. Table 4 below shows a list of various sgRNA designing bioinformatics tools for the CRISPR/Cas9 system to reduce the off-target effects during the hybridisation of sgRNA onto the target sequences.

One of the main challenges with CRISPR/Cas9-based therapy is HDR's low efficiency (Roy *et al.,* 2018). In DSB repairing mechanisms, NHEJ is more efficient than HDR and thus, is suitable for generating indels to knockout mutations.

In order to overcome this limitation, a mutated Cas9 domain was constructed (loss of RuvC and HNH domain function by D10A and H840A mutations, respectively) to produce a single-strand break nickase in the target DNA instead of DSB (Karlgren *et al.,* 2018). It provides more specific and efficient activity in reducing the number of off-target effects considerably (Ran *et al.,* 2013). Both single and paired strands nickase can be used. Paired nickases in cooperative genome engineering produce a long overhang on each of the cleaved ends instead of blunt ends providing even bigger control over precise gene integration and insertion thus overcoming the non-specificity of the wild-type CRISPR-Cas system. An increase in target length will decrease the risk of the same sequence present in other regions of the genome. Since a double-stranded break would be formed only if both targets match in proximity, this will lower the probability of off-target DSBs.

The single nick is repaired by the high-fidelity Base Excision Repair mechanism, thereby off-target mutagenesis is reduced (Ran *et. al.,* 2013a; Ran *et al.,* 2013b). On the other hand, in the paired nickase system, the target site is selected where the presence of one PAM on each strand is at a distance from each other (Gupta *et al.,* 2019). Thus, the probable PAM nearest to the originally cloned target site is searched for in the complementary strand. A minimal distance of about 40-50 nucleotides is kept between the two cleavage sites (Gupta *et al.,* 2019).

Knocking down CYP3As and CYP2D6 might be challenging due to their polymorphic nature and also the existence of pseudogenes might complicate the process of finding a specific gRNA.

Table 4. Various single guide RNA (sgRNA) designing bioinformatics tools for the CRISPR/Cas9 system to minimise off-target effects

Tool Name	Description and function	Reference
CRISPR-DO	Specific for both coding and non-coding targets provides information regarding off-targeted sites and their functional conservation	Ma *et al.* (2016)
CRISPRpred	Efficient designing of sgRNA based on target in silico prediction	Rahman *et al.* (2017)
CRISPR-P 2.0	Predict on-target scores, analyse, and detect guide sequence	Liu *et al.* (2017)
sgRNA Scorer 2.0	Design sgRNA for several PAM sites	Chari *et al.* (2017)
CRISPR-Local	Design sgRNA for non-reference cultivars, predict sgRNA that can target multiple genes	Sun *et al.* (2018)

CRISPRlnc	Design sgRNA for lncRNAs, works for all species	Cheng *et al.* (2019)

C. Ethical Issues

Since preclinical or clinical trials, ethical issues involved in applying the CRISPR/Cas9 system have arisen (Brokowski & Adli, 2019) mainly concerning the potential and technological limitations of the CRISPR technology (Caplan *et al.,* 2015). The ethics of gene editing remains the major obstacle, although the International Summit on Human Gene Editing has already published the acceptability in research involving basic DNA sequences using CRISPR.

The risk of incomplete editing, off-target results, and limited efficiency have restricted CRISPR's use in clinical applications (Brokowski & Adli, 2019). In addition, the long-term effects are still unclear whether the modified species might be permanently affected and whether the correction will be inherited (Mulvihill *et al.,* 2017). There is also no information on how to mediate the adverse effects generated by these genetic alterations.

The CRISPR application is still limited due to a lack of understanding of both genetic makeup and biological phenotypes (Brokowski & Adli, 2019). Since its release in 2012, the CRISPR/Cas9 as a DNA modification tool has progressed substantially.

Besides that, the gene introduction into living organisms may transform previously non-invasive species into highly invasive species that, in a matter of decades, could diminish the native species. It is therefore important to realise that, given the enormous benefits of the tool, proper understanding, and use of the CRISPR/Cas9 tool need to be established to optimise the benefit with minimal conflict. Discussions on ethical implications cannot be accomplished without taking into consideration the increasing concern about human germline alteration.

D. Approach of Gene Targeting Technology

Gene targeting is the process in which the genetic locus or a specific sequence in a living cell has been modified or altered deliberately, whether by introducing the point of mutation in the gene sequence, removing the exons, or adding or by deleting a gene. This process requires DNA that holds the part of the gene to be targeted, a reporter gene, and a selectable marker (dominant) assembled in bacteria. This technology contributes to a significant application of the gene therapy process which involves correcting a pre-existing mutated allele of a gene return to its wild-type (a "knock-in") to improve the pathological phenotype related to the mutation. The second application is the inactivation of genes ("knockouts"), a process in which the two wild-type alleles of a gene are disrupted so that the loss-of-function phenotype associated with that particular gene can be further investigated and determined. A gene knockout abbreviated by KO is a genetic technique that causes one of the organism's genes to be made defective and functionless. This is known as "knocking out" the organisms. Knockout organisms or knockouts are used to further investigate the function of the gene, commonly by studying the consequence or result of gene loss.

Although these two processes are theoretically different from each other, they are similar in each application and require a form of DNA DSB repair (DSBR) termed homologous recombination (HR). DSBR has two mechanisms. NHEJ links end together, often creating indels in the process. In genome editing, this can be used to knock out gene function. HDR is the most common form is homologous recombination, which fixes DSBs using DNA with a similar sequence. Providing cells with external homologous donor DNA introduces edits via HDR. Many genome-editing systems work by activating DSB repair at specific sites using engineered ZFNs, TALENs, or Mega-nucleases (Bogdanove *et al.,* 2018). Currently, the dominant genome-editing method is CRISPR/Cas9 (Komor *et. al.,* 2017; Paschon *et al.,* 2019) and as seen in Table 5 is the list of papers that used CRISPR/Cas9 as a gene editing tool in knocking out and knocking in few subtypes of CYP450 (Karlgren *et al.,* 2018). With the use of this application, theoretically, the knocking out of certain genes that are involved in expressing the CYP3A4 enzymes would render it to be functionless. Hence, it could be used to investigate in the future, discoveries in pharmacogenomics involving CYP3A4. In previous studies, researchers have made inferences from

the difference between the KO organism and normal individuals (Hall *et al.,* 2009). They infer that the KO technique is different from a gene knock-in. Knocking out two genes at once in an organism is known as a double knockout (DKO). Likewise, the terms triple knockout (TKO) and quadruple knockouts (QKO) indicate three or four knocked-out genes, respectively. However, one needs to distinguish between heterozygous and homozygous KOs. In heterozygous knockout, only one of two gene copies (alleles) is knocked out meanwhile homozygous knockout indicates that both gene copies are knocked out.

E. Cell Lines

Genetic knockout in animal models particularly mice has been widely used to study the clinically relevant P450 metabolism since the mid-1990s. However, the usage of mouse models has 2 major drawbacks. First, mice have relatively small plasma and tissue volumes therefore, more advanced analytical instruments and methods are needed. Second, there are large intraspecies differences between mice and humans specifically in toxicological and pharmacokinetic studies. However, P450 genetic knockouts in rat models also have their disadvantages although their size is large and have closer physiologic characteristics to humans. Rat models of embryonic stem cell lines are not stable thus the genetic knockout is much more difficult than in the mouse (Wei *et al.,* 2018). In vitro liver cell culture models are gaining importance in pharmacological and toxicological research.

Table 5. Overview of Cytochrome P450s genes edited using CRISPR/Cas9

Gene/Protein	**Cell line or Organism**	**Modification**	**Reference**
CYP1A1/ CYP1A1	Induced pluripotent stem cells	Knock-in of a Luciferase reporter cassette at the transcription start site	Smith *et al.* (2016)
Cyp2b9/10/13/ CYP2B9/10/13	C57BL/6 mice	Knockout of *Cyp2b9*, *Cyp2b10* and *Cyp2b13*	Kumar *et al.* (2017)
Cyp2c11/ CYP2C11	Sprague-Dawley rats	Knockout of *Cyp2c11*	Wei *et al.* (2018)
Cyp2d1/2/3/4/5/ CYP2D1/2/3/4/5	Wistar rats	Knockout of the *Cyp2d* cluster	Yoshimi *et al.* (2016)
Cyp2d1/2/3/4/5/ CYP2D1/2/3/4/5	Wistar rats	Knockout of the rat *Cyp2d* cluster and knock-in of human *CYP2D6*	Yoshimi *et al.* (2016)
Cyp2e1/ CYP2E1	Sprague-Dawley rats	Knockout of *Cyp2e1*	Wang *et al.* (2016)
cyp26a1/ CYP26A1	*Silurus meridionalis*	Knockout of *cyp26a1*	Li *et al.* (2016)
Cyp3a1/2/ CYP3A1/2	Sprague-Dawley rats	Knockout of *Cyp3a1* and *Cyp3a2*	Lu *et al.* (2017)
CYP3A5/ CYP3A5	Human hepatocyte cell line HuH-7	Editing of the *CYP3A5*3* locus to generate *CYP3A5*1*	Dorr *et al.* (2017)
CYP90B1/ CYP90B1 (PtoDWF4)	*Populus tomentosa*	Knockout of *CYP90B1*	Shen *et al.* (2018)
CYP734A4/ CYP734A4	Japonica rice cultivar Zhonghua11	Knockout of *CYP734A4*	Qian *et al.* (2017)

The supply of cells used is crucial for the relevancy and predictive value of such models. Primary human hepatocytes (PHH) are presently thought of to be the gold standard for hepatic in vitro culture models since they directly reflect the particular metabolism and functionality of the human liver; but the insufficiency and difficult logistics of PHH have driven researchers to explore different cell sources, as well as liver cell lines and pluripotent stem cells.

Liver cell lines generated from hepatomas or by genetic manipulation are widely used because of their good accessibility, however, they are generally altered in certain metabolic functions. For the past few years, adult and pluripotent stem cells are attracting increasing attention, due to their ability to proliferate and differentiate into hepatocyte-like cells in vitro.

Therefore, in vitro liver cell line is suitable to be used for CYP2D6 gene knockout. It provides many benefits, including being cost-effective, and convenient, in unlimited materials supply, and bypassing ethical issues of animal and human tissue usage. Cell lines also provide a valuable pure population of cells, which provides a consistent sample and reproducible results. Cell line models are reported to be genetically identical with the exception of any specific altered genetic variant (Dorr *et al.,* 2017).

F. Human Liver Cell Line

Human hepatic cell lines generated from tumour tissue or by the genetic engineering of primary human liver cells are being used extensively in in vitro culture models because they are easily accessible. Having a high proliferation capacity and stable metabolism of the cells makes them a convenient tool to be used in in vitro studies under standardised and reproducible conditions. Nevertheless, this also causes them to have a high proliferation potential of transformed cell lines which is associated with a loss of differentiated functions. This results in some deficiencies in functional performance. Therefore, any study involving the use of liver cell lines in vitro research must consider the specific functional properties of the cell line used.

1. Applications of immortalised human hepatocyte

In recent years, both adult and foetal human hepatic cell lines have been explored for research purposes. Several immortalised human hepatocytes, including PH5CH, TPH1, NKNT-3, and NeHepLxHT cells, have indeed been successfully used as tools in research focused on hepatitis C virus or hepatitis Bvirus (HBV) (Wei *et. al.,* 2018; Kato *et. al.,* 1996; Ikeda *et. al.,* 2010; Raychoudhuri *et. al.,* 2010; Raychoudhuri *et al.,* 2011). A murine model of HBV viremia based on a human hepatocyte-derived cell line transfected with HBV DNA has been described and offers opportunities for in vivo HBV research (Kato *et al.,* 1996). Human hepatic cell lines have also been applied as cellular models to investigate the processes of hepatocarcinogenesis and steatosis (Ikeda *et. al.,* 2010; Raychoudhuri *et al.,* 2010). The HHL cell line proved useful during the development of adeno-associated viral vectors for liver-directed gene therapy (Wei *et. al.,* 2018; Kato *et. al.,* 1996; Ikeda *et al.,* 2010). Besides their application in fundamental research, different hepatic cell lines are equally addressed as suitable in vitro tools for screening and safety testing of drug candidates. In this regard, Hc3716-hTERT immortalised hepatocytes constitute an appropriate in vitro model for predicting the side effects of telomere-targeting drugs (Raychoudhuri *et al.,* 2010). Furthermore, Fa2N4 cells may be used as a routine screening system for pregnane X receptor-mediated CYP3A4 induction (Raychoudhuri *et al.,* 2011). Similarly, the hepatic THLE cell line and THLE-CYP sublines have been reported as promising models for the investigation of CYP- mediated drug metabolism and liver toxicity (Heckl, *et. al.,* 2014; Raychoudhuri *et al.,* 2011). However, NKNT-3 cells appeared to be less suitable than the hepatoma cell line HCC1.2 for the development of improved in vitro genotoxicity test systems (Heckl *et al.,* 2014).

2. THLE-2 cell line as parental cell line

THLE-2 cell line (ATCC® CRL-2706™) is derived from primary normal liver/left lobe epithelial cells by infection with SV40 large T antigen derived from adult human (Brown *et al.,* 2000). Albumin and cytokeratin 18 were expressed in early passages, reflecting the hepatocytes and non-parenchymal cells' expression features.

Retained albumin secretion by hepatocytes is equal to the normal primary human hepatocyte. Once the retroviral vector containing the *Bgl*I-*Hpa*I fragment of SV40 T antigen is introduced into the amphotropic packaging cell line PA317, the virus is generated. THLE-2 cells metabolise benzo[a]pyrene, N-nitrosodimethylamine, and aflatoxin B1 to their ultimate carcinogenic metabolites that adduct DNA, indicating the functional cytochrome P450 pathways (Shiraha *et al.,* 2013).

It is reported that these immortalised human liver cells constitute an in vitro model for pharmacological and toxicological studies and the aetiology and pathogenesis of human hepatocellular carcinoma investigation (Brown *et al.,* 2000). In a recent study of cell lines involved in hepatocyte-specific functions, enzymes activities containing cytochrome P-450 reductase, catalase, nicotinamide adenine dinucleotide phosphate, superoxide dismutase, glutathione S-transferase, and epoxide hydrolase were maintained. Immortalised cell lines also exist from various sources either in chromosomal abnormality cells known as tumorous cells that continuously divide, or cells that have been engineered artificially to proliferate indefinitely and can therefore be cultured for long periods of time. Due to the continuous division of immortalised cells, it will fill the dish or flask in which they develop. Thus, a fraction of the multiplying cells will be moved to new dishes by passaging (also known as splitting) to provide room for continued proliferation.

Derivation of the human cell line THLE-2 from healthy liver cells is a sustainable option to identify the presence of an alternative pathway after the knocking out of gene Cyp2D6. Recent reports show that the knockout of CYP2E1 (Wang *et al.,* 2016) or CYP3A1/2 (Lu *et al.,* 2017) in rats using the CRISPR/Cas9 system could be used in drug metabolism studies; however, the use of CRISPR/Cas9 on modified human cell lines to observe the presence of protein expression after knocking out genes involved in drug metabolism has not been reported. Therefore, we hypothesise that human liver cell lines can be engineered with CRISPR/Cas9 to determine the presence of alternative pathway on drug metabolism.

IV. CONCLUSION

Gene editing technology such as gene knockout has been used in vast studies to study mainly pharmacogenomics. Studies have shown a high rate of success in using gene editing tools such as CRISPR/Cas9 in knocking out genes of interest. Based on this review, the importance of the CYP2D6 gene has been widely explained in drug metabolism as it consists of the predominant pathway for the elimination or bioactivation of many centrally acting drugs. As 25% of most drug therapies are estimated to be influenced by polymorphism, the study of this gene is crucial in determining the best treatment for the patients. CYP3A4 takes part in the phase I transformation of toxins, carcinogens, bile acids, and steroid hormones.

A versatile gene manipulating tool of the CRISPR/Cas9 system with sgRNAs and a DNA splicing protein allows inducing DSB at selected sites that are determined by the guide RNA modification as required. Immense potential related to genomic medicine arises as treatment outcomes associated with polymorphism genes can be rectified at the level of the genome itself. Editing the genes using CRISPR/Cas9 aims at finding drug metabolism affected by the gene and enhancing the therapy outcome. Despite limitations in using CRISPR/Cas9 being present such as off-target effects, these can be solved by specifically targeting the gene sequence involved in expressing the CYP3A4 and CYP2D6, by using gene editing tool software to easily design guide RNAs.

Even with the availability of other gene editing tools, CRISPR/Cas9 has made it much easier to achieve this goal at a much faster pace largely due to its simplicity of use and its high versatility. However, while on this journey of uncovering information, we should be aware of the limitations. Thus, it should be our goal to attain this knowledge base without undermining the future use of this genetic tool in some way.

THLE2 cells express phenotypic characteristics of normal adult liver epithelial cells and retain phase I and II enzyme activities that include the ability to metabolise carcinogens to their ultimate carcinogenic metabolites capable of binding DNA, hence the cell line is suggested as a cheaper alternative for drug metabolism studies.

In conclusion, the CRISPR/Cas9 invention has undeniably influenced the field of biomedical sciences monumentally. The available technologies will bring rapid and precise

alterations to the genome easily and subsequently, an abundance of knowledge that will certainly benefit future generations. Further development and responsible use of knowledge avenues depend greatly on the safe navigation of ethical matters and the avoidance of any unsustainable utilisation to which this tool may be subjected. In summary, previous studies showed a possibility of success in knocking out CYP3A4 and CYP2D6 using CRISPR/Cas9 technology, however, due to limited studies in using cell lines, the need to carry out lab-based study is crucial to reconfirm this conclusion. As this review serves a vast amount of information on the informative and innovative application of the CRISPR/Cas9 technology, the first report of CYP26D and CYP3A4 gene knockout in a human cell line using this technology is possible.

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VI. AUTHOR CONTRIBUTIONS

AAO: lead in Writing – Original Draft; lead in Investigation; equal in Conceptualisation; equal in Methodology; equal in Writing – Review and Editing; supporting in Visualisation

FH: lead in Conceptualisation; lead in Funding; lead in Visualisation; equal in Writing – Review and Editing; equal in Investigation; equal in Methodology

RMY: equal in Writing – Review and Editing; equal in Methodology; supporting in Writing – Review and Editing

SNNA: equal in Investigation; supporting in Writing – Original Draft; supporting in Visualisation

AZAH: equal in Investigation; supporting in Writing – Original Draft; supporting in Visualisation.

VII. CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

VIII. REFERENCES

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