Altered Gliclazide Metabolic Pathway and its Implications on Increased Therapeutic Response in CYP2C9*2: Molecular Dynamics simulation and Autodock Studies

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Abstract – *Among sulfonylureas, gliclazide is prescribed to 80% of the diabetic population and mainly metabolized by CYP2C9 in Caucasians. Our data shows that the orientation of the substrate is changed and therefore, the site of oxidation with respect to heme-Fe is altered in *2. This leads to an altered metabolic pathway in *2 and it is a rate limiting step in gliclazide metabolism. Our results also show that the position of 7-propionate side chain of ring A and 6-propionate side chain of ring D is flipped in *2 and thus, the stability of heme and oxidative potential of substrate in the active binding pocket are reduced. In summary, the altered pathway, and instability of heme and the substrate in the active site are contributing to decreased metabolic activity consistent with greater therapeutic response observed in patients carrying CYP2C9 *2 allele.*

Keywords: CYP2C9*2, gliclazide, therapeutic response, molecular dynamics, docking simulation, metabolic pathway

1 Introduction

Among sulfonylureas, gliclazide is dispensed almost 4 million prescriptions in UK [1] and 1.2 million prescriptions in Australia [2]. It is also given in combination with metformin to keep successful control of the disease [3, 4]. Comparing with other hypoglycemic agents, the incidence of hypoglycemia is relatively low in gliclazide and may have beneficial effects beyond reduction of blood glucose [5]. In Caucasians, gliclazide is extensively metabolized by CYP2C9. Pharmacokinetic clearance of gliclazide revealed the existence of two major metabolites due to the oxidation of methyl carbon of tolyl-group that constitutes ~60% of metabolites and hydroxylation at a specific site in the azabicyclo-octyl ring represent ~40% of metabolite observed in urine [6,7] as shown in Fig. 1.

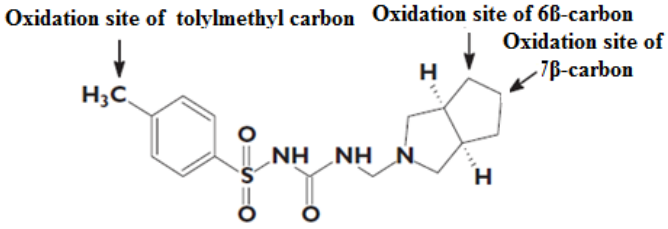

Fig. 1. Location of the hydroxylation sites in gliclazide

CYP2C9 is the major human enzyme of the cytochrome P450 2C subfamily and it is responsible for metabolism of ~10% of therapeutic drugs in the market. This gene is highly polymorphic [8,9] and so far twenty four alleles have been identified [10]. Two alleles, *2/*2 (R144C) and *3/*3 (I359L) genotype carriers had a lower gliclazide clearance, with reductions of 25 and 57%, respectively, relative to those carrying the wild type [11-13]. Crystallographic data confirmed that the I359L variation is located in proximity to the active center in the substrate recognition site (SRS) 5 and therefore, explain the loss of functional activity in the variant allele. However, the codon 144 amino acid substitution is located outside the active center and therefore, the loss of activity observed in this allele is not clear. Minor differences in frequencies of these genotypes between different ethnic subgroups of the Caucasians population have been reported and the variant CYP2C9*2 (*2) is almost absent in Africans and Asian population [13]. Pharmacogenetic study conducted in larger population of 1073 patients with type-2 diabetes recruited between 1992 and 2007 demonstrated that the loss-of-function alleles *2 are robustly associated with greater response to sulfonylureas and approximately 80% of the patients treated only with gliclazide in this study population [14]. The influence of CYP2C19 polymorphism in the pharmacokinetics of gliclazide has been reported in healthy Chinese population [15, 16]. This small discrepancy may be due to the ethnic differences and also due to the selection of smaller population for shorter periods of treatment.

Pharmacokinetic studies show that 6ß- 7ß-, and tolymethyl- hydroxylation represents the rate-limiting pathway of gliclazide elimination [7]. Our previous molecular docking of gliclazide on *2 studies indicate that 6β- and 7β- carbon

atom is closer to heme-Fe [17]. Based on this, our hypothesis is that β-hydroxylation may be the preferred route of metabolism and this may lead to the reduced metabolic clearance of gliclazide observed in *2. Therefore, in this study we are proposing to use molecular dynamic simulation and automated molecular docking tools to better understand the altered substrate orientation, proton – heme distance, binding pocket, heme and gliclazide stabilization, and regioselectivity of metabolism in *2 allelic variant and thus it leads to the altered route of metabolism

2 Materials and methods

2.1 Computational methods of CYP2C9*1 & *2

With the X-ray crystal structure of human CYP2C9/flurbiprofen (PDB code 1R9O) [18] as a model, substrate free computational models of CYP2C9 *1 (wild, *1) and *2 (R144C) were constructed using the software tools VMD and NAMD [19, 20]. The missing amino acid residues 38 - 42 and 214 - 220 were also included in the computational models using Modeller [21]. The generated models were validated for their structural quality using Procheck [22, 23].

2.2 Molecular dynamics simulation

The generated computational models were further processed for MD simulation. The intermolecular hydrogen atoms were added and the complexes were solvated in a layer of TIP3 water molecules of 10Å radius, ionized at a physiological pH of 7 and subjected to energy minimization for 2000 steps. The minimized protein complex was simulated using NAMD for 600 picoseconds without any restrains at a constant temperature of 300 K. In each model, the lowest potential energy state was chosen for further analysis, whose stability was examined by calculating the root mean square deviation of the protein backbone.

2.3 Flexible docking

The simulated protein was further processed using the molecular modeling program CHIMERA [24] to remove water and ions, and add Gastegier charges and hydrogen atoms. Gliclazide was docked with the above models using the grid-based docking program AutoDock 4.2 [25-27], in which some of the key residues of the active site were kept flexible. The best ten clusters having the lowest energies and <2Å RMSD values were chosen for analysis.

3 Results and discussion

Modeller was used to generate *1 and *2 models and the Procheck program was used to check the stereochemical quality of a protein structure within the allowed Ramachandran region. The results show that 92% and 94% of residues in 3D structure of *1 and *2 lie in the most favored regions and 0.7% and 0.5% of residues lie in disallowed regions of the Ramachandran plot (Fig.1a & 1b). The docking results indicate that gliclazide is located nearby heme and surrounded by SRS residues. The location of SRS residues in *1 and *2 are in consistence with the results of the crystal structure of CYP2C9 [18] and confirms the validity of our docked models.

The automated molecular docking using Autodock was performed to further validate the reliability of the conformation of the SRS in *1 and *2 models (Fig. 2a & 2b).

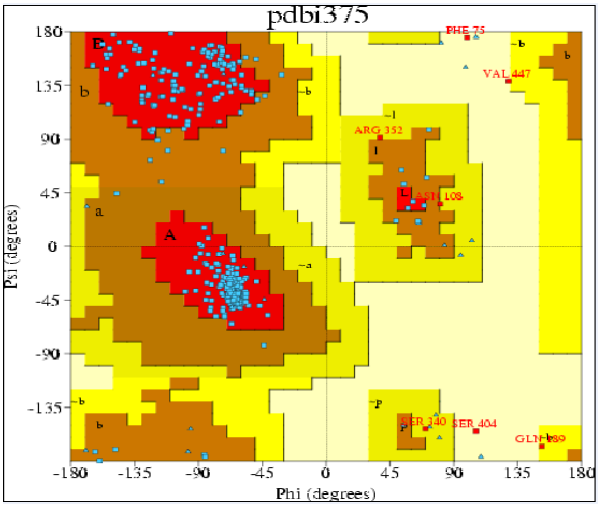

Fig. 1a. Ramachandran plot of *1[a]

[a] ***Ramachadran Plot statistics***

		No. of residues	%-tage
Most favoured regions	*[A,B,L]*	*373*	*92.1%*
Additional allowed regions	*[a,b,l,p]*	*25*	*6.2%*
Generously allowed regions	*[~a,~b,~l,~p]*	*4*	*1.0%*
Disallowed regions	*[XX]*	*3*	*0.7%*
Non-glycine and non-proline residues		*405*	*100.0%*
End-residues (excl. Gly and Pro)		*3*	
Glycine residues		*27*	
Proline residues		*31*	
Total number of residues		*466*	

[a] *Based on an analysis of **118** structures of resolution of at least **2.0** Angstroms and R-factor no greater than **20.0** a good quality model would be expected to have over **90%** in the most favoured regions [A,B,L]*

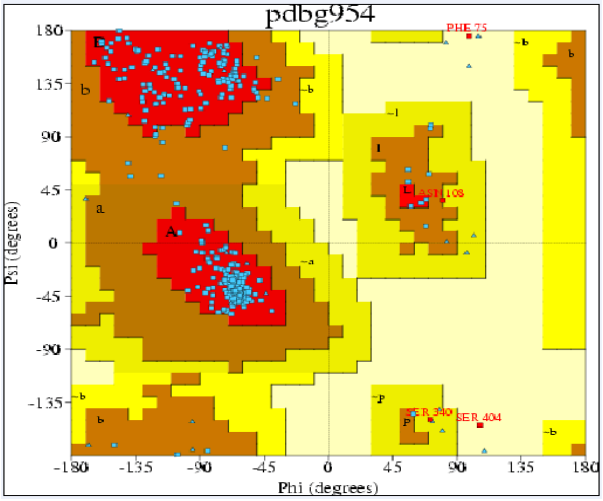

Fig. 1b. Ramachandran plot of *2[a]

[a]***Ramachadran Plot statistics***

		No. of residues	*%-tage*
Most favoured regions	*[A,B,L]*	*380*	*93.8%*
Additional allowed regions	*[a,b,l,p]*	*21*	*5.2%*
Generously allowed regions	*[~a,~b,~l,~p]*	*2*	*0.5%*
Disallowed regions	*[XX]*	*2*	*0.5%*
Non-glycine and non-proline residues		*405*	*100.0%*
End-residues (excl. Gly and Pro)		*3*	
Glycine residues		*27*	
Proline residues		*31*	
Total number of residues		*466*	

[a]*Based on an analysis of **118** structures of resolution of at least **2.0** Angstroms and R-factor no greater than **20.0** a good quality model would be expected to have over **90%** in the most favoured regions [A,B,L]*

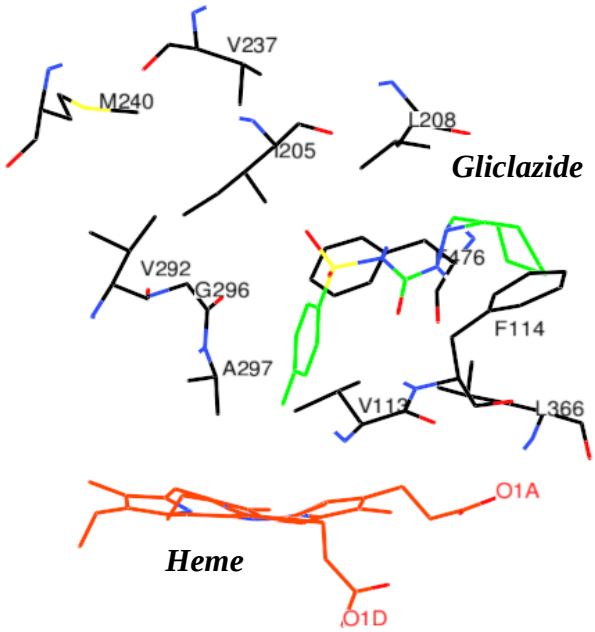

Fig. 2a. Substrate recognition site of *1

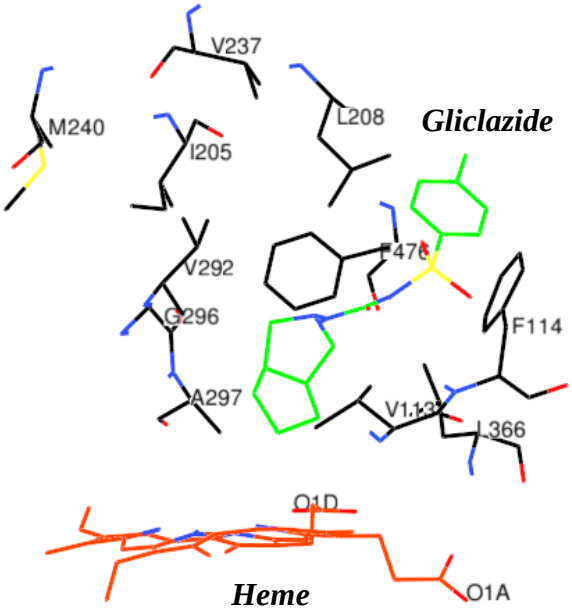

Fig. 2b. Substrate recognition site of *2

In CYP2C9 crystal structure study, the heme and active binding pocket are buried deep into the protein molecule and the substrate should access the binding pocket for the occurrence of catalysis. Since the substrate access channel and the binding pocket near the heme-Fe play an important role in the determination of the orientation of the substrate towards heme-Fe, we have examined these factors in this study. Our previous study shows that the number of amino acids forming the hydrophobic cage is not changed in *2 but the size of the substrate access channel is reduced from 10.3Å (*1) to 9.3Å (*1) [17], and this may change the orientation of the substrate entering into the binding pocket and thus alter the position of the substrate in the binding pocket.. Since the size of the substrate access channel is smaller in *2, azabicyclo- group may enter first rather than the bulky methyl-phenyl group. We believe that this resulted in the complete change in the orientation of gliclazide in the binding pocket (Fig. 3a & 3b).

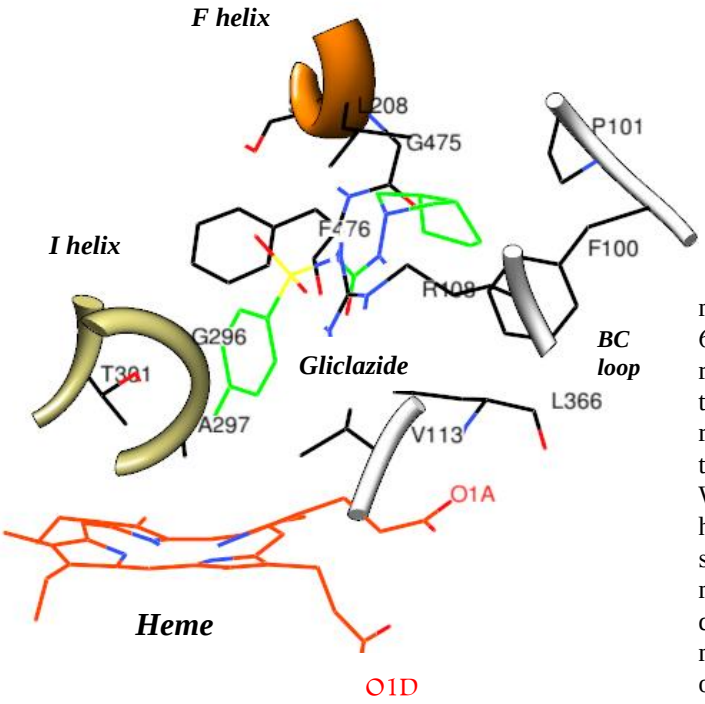

Fig. 3a. Binding pocket of *1 after docking gliclazide

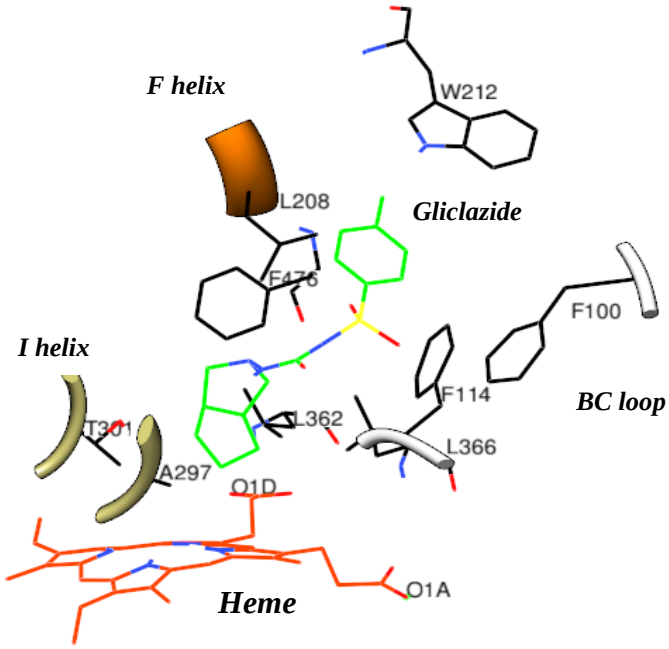

Fig. 3b. Binding pocket of *2 after docking gliclazide

Our previous studies show that the distance between tolyl methyl carbon atom of gliclazide and the heme is 4.1Å and 6β-carbon atom and the heme is 10.3Å in *1 [17]. These results correlate with the pharmacokinetic data which shows that the tolylmethyl hydroxylation is the major pathway responsible for metabolic clearance of gliclazide in *1 [6] and this constitutes ~60% of the metabolites detected in urine. While in *2, tolylmethyl carbon atom is located at 14.7Å & hydroxyl group is located at 4.5Å from heme and this suggests that β-hydroxylation may be favored route of metabolic clearance of gliclazide. According to pharmokinetic data, this route of metabolism constitutes only ~40% of the metabolite and therefore, it may explain the reduced activity observed in diabetic patients carrying *2 allele. Previous study [28] shows that the differences in the distance between substrate proton to heme-Fe play a key role in the observed differences in catalytic activity. NMR derived T_1-relaxation studies conducted with the probe substrate flurbiprofen and co-incubation of flurbiprofen with dapsone show that the movement of flurbiprofen protons closer to the heme iron partially explains heteractivation observed in CYP2C9 allelic variants [28,29].

Amino acids in the binding pocket of both *1 and *2 are similar, except BC loop amino acids V113 and R108 are not present in *2 (Fig. 3a & 3b). R108 stabilizes the gliclazide by binding to the acidic group of gliclazide and formation of hydrogen bonds (Table 1). R108 itself is stabilized by the formation of hydrogen bond with D293, thus gliclazide positions itself in proximity with heme prosthetic group for subsequent oxidation in *1 as represented in Table 1. While

gliclazide and R108 stabilization by the hydrogen bonding are lacking in *2 (Table 1). These results are consistent with the proposed catalysis model for P450 [30, 31]. Hydophobic amino acids, G296 and G475, that stabilizes the binding pocket are absent in *2 (Fig. 3b).

Table 1: Gliclazide stabilization in *1 compared to *2 by hydrogen bond formation

Donor	Acceptor	Distance (Å)[a]		Angle (°)[b]	
		*1	*2	*1	*2
R108	Gliclazide Carboxyl O1	2.0	5.6	153	116
R108	Gliclazide Carboxyl O1	1.9	6.5	160	102
R108	D293 OD2	2	4.4	124	124

The donor and acceptor distance is <3.5 Å[a] [30] and 180°±45° [b][31], a hydrogen bond is defined to be formed

Many factors are known to affect the heme redox potential, including proximal heme ligand and propionate and substrate orientations and interactions with the immediate protein environment. In *1 complex, the A ring propionate is stabilized by the formation of hydrogen bonding with S365, L366, and R97, whereas the D ring is stabilized by R124, R433, and W120 by the formation of hydrogen bonds (Fig. 4a ; Table 2).

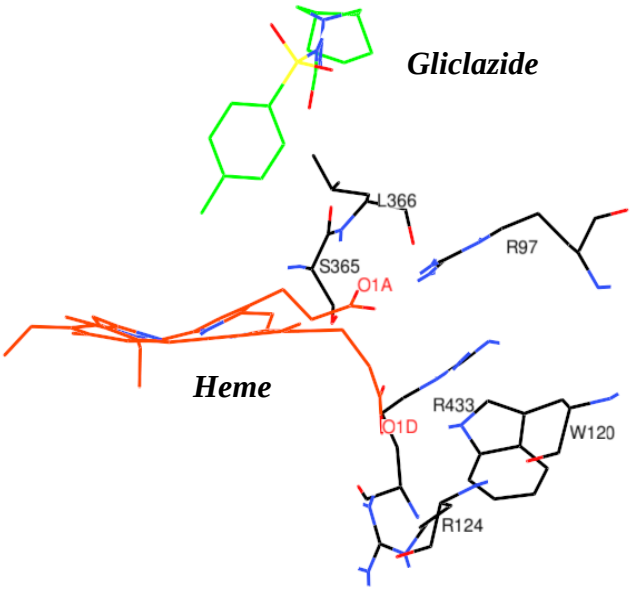

Fig. 4a. Heme stabilizing amino acids of *1

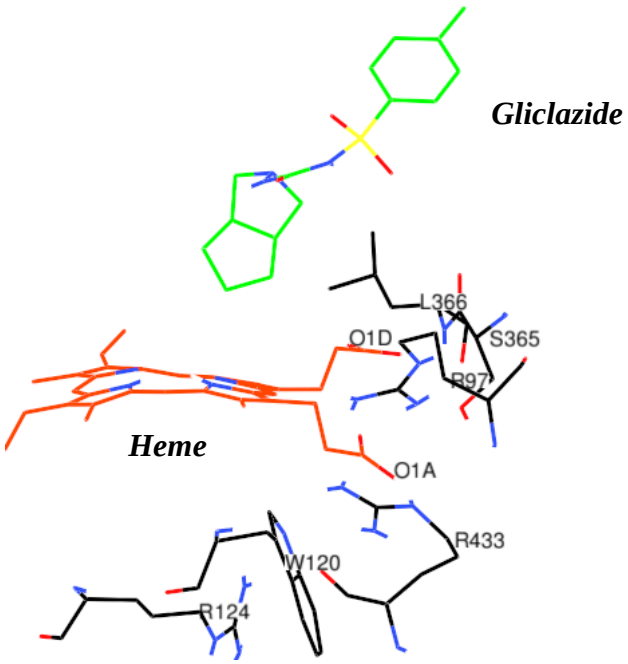

Fig. 4b. Heme stabilizing amino acids of *2

Table 2 : Heme stabilization in *1 compared to *2 by hydrogen bond formation

Donor	Acceptor	Distance (Å)[a]		Angle (°)[b]	
		*1	*2	*1	*2
W120 NE1	Heme O1D	1.9	15.7	168	158
R124 NH1	Heme O1D	1.8	14.6	162	77
S365 NG1	Heme O1A	1.6	4.1	164	108
L366 NH	Heme O1A	1.9	4.4	157	149
R433 NH1	Heme O2D	2.4	14.2	143	58

The donor and acceptor distance is <3.5 Å[a] [30] and 180°±45° [b][31], a hydrogen bond is defined to be formed.

This structure is consistent with the closed form of 2C enzyme reported earlier [32]. In contrast to this conformation, the position of rings A and D are flipped in *2 and the stabilization of both propionate rings are lacking (Fig. 4b; Table 2). These alterations in heme coordination may affect the heme redox potential. Mutagenesis and structural studies indicate the importance of the movement of the ring A towards substrate for the occurrence of oxidation [33]. Replacement of the D-ring resulted in the loss of enzyme activity and confirms the importance of this propionate in catalytic activity [33].

4 Conclusions

In summary, our present study shows that the orientation of gliclazide is altered significantly and changes the nature of the functional group located closer to heme-Fe and therefore, the site of oxidation is changed in *2. Since tolyl-methyl group is closer to heme-Fe, tolylmethyl-hydroxylation of gliclazide is the preferred route of metabolism in *1. While

azabicyclo-octyl ring is closer to heme-Fe and therefore, 6β- and 7β-hydroxylation is the preferred route of metabolism in *2. The reduced catalytic activity in *2 is consistent with pharmacokinetic data where the detection of 6β-hydroxylation metabolite is only ~40%. The position of SRS amino acid residues are not altered but in the binding pocket, B-C loop amino acid residues are missing in *2. In addition, we show that the substrate access channel and a significant change in the structural link between substrate binding and the binding of redox partners would also partly explain the reduced catalytic efficiency.

5 Acknowledements

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