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EXPLORING THE INHIBITION POTENTIAL OF CYP2C9 ENZYME: IN VITRO ASSESSMENT AND PREDICTION OF GLIMEPIRIDE-SULFAMETHOXAZOLE INTERACTIONS.

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Abstract

CYP2C9 represents a genetically diverse enzyme that plays a crucial role in the oxidative transformation of nearly 15% of drugs subject to initial phase I metabolism. Given its integral involvement in drug metabolism, it becomes imperative to assess the dynamic attributes of CYP2C9 substrates, particularly in tandem with co-administered drugs that could potentially instigate inhibition. An example of a well-acknowledged drug interaction is the occurrence of hypoglycemia stemming from the concurrent use of sulfonylurea and sulfonamide drugs. Therefore, the focal point of this investigation encompassed a comprehensive exploration into the repercussions of CYP2C9 inhibition on the pharmacokinetics of glimepiride (GLM), employing sulfamethoxazole (SMZ) as a representative in vitro inhibitor of CYP2C9. This inquiry primarily utilized human liver microsomes (HLM) to shed light on the intricate interplay between GLM and SMZ by evaluating key kinetic parameters such as Km, Vmax, IC50, and Ki. The aspiration extended to predictive efforts aimed at deciphering potential in vivo drug interactions leveraging in vitro findings.

Within the concentration range of 30 to 1100 µMole, SMZ exhibited a discernible inhibitory effect specifically targeting CYP2C9-mediated GLM hydroxylation. Evident from an apparent IC50 (Ki) value of 400 µMole and an intrinsic Ki value of 290 µMole, this inhibition demonstrated a competitive pattern as showcased by the discernible increase in Km, while Vmax remained relatively stable. This outcome, consistent with predictions derived from Michaelis-Menten and LineweaverBurk plots, found further substantiation through the leftward positioning of the Ki value according to the Dixon plot, indicative of competitive inhibition. Importantly, the outcomes underscored that SMZ, when employed at concentrations below 500 µMole, could effectively serve as a specific CYP2C9 inhibitor for in vitro inquiries.

Insights garnered from the in vitro-in vivo correlation (IVIVC) analyses pointed towards a 1.5-fold escalation in the AUC of GLM when influenced by SMZ. This meticulous exploration delved into the inhibitory impact of SMZ on CYP2C9-mediated GLM metabolism, with the projected surge in GLM plasma concentrations warranting vigilance due to the associated risk of hypoglycemia upon concurrent administration of SMZ and GLM.

Keywords: Glimepiride; Sulfamethoxazole; Human liver microsomes; CYP2C9; IC50

Introduction

Accumulating scientific findings consistently underscore the pivotal role occupied by CYP2C9 as a significant player in the realm of human drug metabolism [1-4]. In light of CYP2C9's intricate engagement in the processing of pharmaceutical agents, a meticulous evaluation of the kinetic dynamics exhibited by substances acting as CYP2C9 substrates assumes critical importance, particularly when juxtaposed with the potential for inhibition induced by concurrent medications. A notable example of this intricate interplay is the propensity for hypoglycemic episodes arising from the co-administration of sulfonylurea compounds with sulfonamide drugs. Of note, oral hypoglycemic agents represent the cornerstone in the therapeutic management of Type 2 diabetes when conventional interventions such as diet, exercise, and weight reduction prove insufficient. Among the array of available oral hypoglycemic agents, the primary therapeutic focus gravitates towards sulfonylureas and biguanides, of which glimepiride (GLM) stands out prominently. The comprehensive metabolic transformation of GLM, either intravenously or orally administered, is primarily driven by oxidative processes. The consequential metabolites, notably the cyclohexyl hydroxymethyl derivative (M1) and carboxyl derivative (M2), underscore the integral involvement of Cytochrome P450 2C9 in the biotransformation of GLM. This metabolic trajectory, widely documented through diverse studies including those on Japanese subjects, establishes the foundation for the investigation at hand [5-8].

Within this context, sulfamethoxazole (SMZ), recognized as a bacteriostatic antibiotic belonging to the sulfonamide category, emerges as a potent catalyst of CYP2C9 inhibition, a phenomenon firmly substantiated both in in vitro and in vivo settings. This attribute lends itself to the capacity of SMZ to significantly heighten the plasma concentrations and consequent effects of various drugs, thereby contributing to instances of hypoglycemia [9-11]. Nonetheless, the available literature presents a dearth of inquiries into the ramifications of enzyme inhibition vis-à-vis the metabolism of GLM in an in vitro setting utilizing human liver microsomes (HLM). Given the ability of SMZ to impede CYP2C9-mediated drug metabolism, coupled with the understanding that GLM stands as a substrate of CYP2C9 [5], the investigation becomes paramount to ascertain the potential influence of SMZ on GLM metabolism.

While the in vivo fate of GLM has been elucidated to a certain extent, a systematic endeavor to unravel the specific human cytochrome P450 (CYP) enzymes instrumental in the diverse pathways of GLM hydroxylation remains conspicuously absent. The crux of this study lies in characterizing the hydroxylation of GLM employing HLM and subsequently scrutinizing the potential impact of SMZ on GLM metabolism. The underpinning hypothesis posits that inhibitors of CYP2C9 would inevitably trigger an elevation in glimepiride plasma concentrations.

In the context of analytical techniques, LC/MS-based assays often necessitate the application of distinct ionization and ion detection modes due to the inherent structural diversity of CYP probe substrates. This complexity presents challenges in the development of LC/MS methodologies that facilitate simultaneous analysis. However, the majority of these compounds do exhibit ultraviolet (UV) light absorption properties. This insight permits the utilization of LC coupled with UV detection for the meticulous analysis of CYP probe substrates and their respective metabolites [12]. It's pertinent to acknowledge that UV detection does carry certain limitations with regards to sensitivity and selectivity. Notably, our preliminary findings offer encouraging indications,

affirming the adequacy of LC/UV sensitivity for the detection of GLM oxidative biotransformation within standard microsomal incubations, whether in the presence or absence of an inhibitory factor.

Methods

Chemicals and Reagents.

GLM was generously provided as a complimentary sample by Cadila Healthcare Ltd., situated in Ahmedabad, India. Correspondingly, SMZ was graciously offered as a sample by M/s Natco Pharma Ltd, located in Hyderabad, India. For the experimental procedures, Nicotinamide Adenine Dinucleotide Phosphate (NADPH), in its reduced tetrasodium salt form, alongside Magnesium chloride ($MgCl_2$), were procured from Himedia laboratories based in India. Ethylene diamine tetra acetic acid (EDTA), dipotassium hydrogenphosphate, and potassium dihydrogenphosphate were secured from S.d Fine-Chem Limited, also based in India. To ensure the precision and quality of the analytical procedures, HPLC-grade methanol and acetonitrile were sourced from Spectrochem India. Every other chemical and reagent utilized throughout the study adhered to the standards of analytical grade.

Microsomal Source

A collective reservoir comprising 50 Human Liver Microsomes (HLM), each possessing a concentration of 0.5 ml at 20 mg/ml, was procured from Xenotech LLC., USA. These HLM samples, encompassing a mixed gender distribution, were suspended within a medium containing 250 mM sucrose. To preserve their integrity and viability, these microsomes were preserved at a temperature of -80°C within a deep freezer. For utilization, the frozen microsomes underwent thawing via immersion of the vial in cold running water, subsequently being maintained within an ice water bath until ready for application. The quantitative parameters, encompassing the aggregate CYP450 content, protein concentrations, and the distinct activity levels attributed to each CYP450 isoform, were provided by the manufacturer itself.

Analytical method for GLM *in vitro* metabolism

In brief, the incubation compositions encompassed 1M phosphate buffer, adjusted to a pH of 7.4, supplemented with 10mM of magnesium chloride ($MgCl_2$), 1mM of ethylenediaminetetraacetic acid (EDTA), and 10mM of Nicotinamide Adenine Dinucleotide Phosphate (NADPH). Additionally, the incubation medium incorporated 0.5mg/ml of HLM and 10µM of glimepiride. Duplicate tubes harboring the reaction mixture along with the NADPH solution (10mM) were allowed to establish equilibrium within a shaker incubator, operating at a speed of 150 revolutions per minute (rpm), for a duration of 5 minutes at a temperature of 37°C. Preliminary investigations, as documented earlier [5], corroborated that substrate consumption remained linear across a 30-minute timeframe, employing a liver microsomal protein concentration of 0.5mg/ml at 37°C. The reaction's commencement entailed the addition of pre-incubated NADPH, followed by its cessation using 100µl of acetonitrile maintained at icy temperatures. Subsequent to centrifugation at 10,000 rpm (4°C; 10 minutes), samples of the supernatant were directly introduced into an HPLC system. Whenever deemed necessary, volume adjustments were executed to attain a final volume of 200µl with buffer.

HPLC Conditions

An established HPLC technique complemented by UV detection was employed to quantify both glimepiride and its corresponding metabolite within the microsomal incubations, as previously validated [5,13]. Portions of the supernatants obtained from the centrifuged incubates were introduced into the HPLC system. This configuration involved a Shimadzu LC 20 AT pump, coupled with an SPD 20A UV detector, accompanied by a rheodyne 7725 fixed injector loop set at 20µl capacity. The chromatographic separation was orchestrated using a Thermo Scientific C18 Hypersil BDS column (250 x 4.6 mm) with a particle size of 5µ, supported by a Phenomenex C18

guard column (4x3mm). The mobile phase composition entailed a combination of 0.1% formic acid (adjusted to a pH of 3) and acetonitrile in a ratio of 55:45. Operating within ambient temperatures, the mobile phase was delivered at a consistent flow rate of 1ml/min. The quantification procedure relied on evaluating UV peak areas specifically at 228nm. This chromatographic setup enabled the elution of SMZ, GLM metabolite (M1), and GLM itself at 3.1 minutes, 3.6 minutes, and 9.3 minutes, respectively, as depicted in Figure 1.

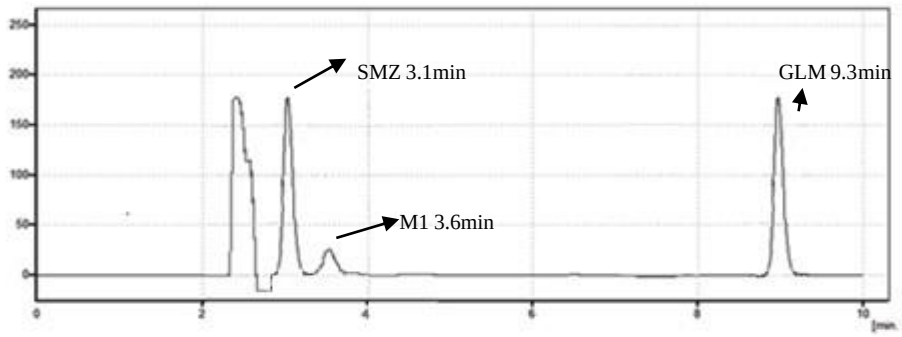

Figure1: HPLC chromatogram of SMZ(3.1 min), GLM metabolite (M_1, 3.6min) and GLM (9.3min)

IC50 Determination

The potential inhibitory influence exerted by SMZ on human CYP2C9 activity was assessed through the implementation of a designed model substrate reaction involving CYP2C9, specifically GLM in the form of its hydroxyglimepiride metabolite. The methodology employed proceeded as follows: A consistent concentration of the model substrate GLM (10 µM) was subjected to varying concentrations of SMZ (ranging from 0 to 110 mM). The reaction mixture, as detailed earlier, was introduced into the designated tubes and vigorously mixed for a duration of 5 seconds. Subsequently, the same protocol elucidated above was carried out. The quantification of SMZ's inhibitory effect on glimepiride metabolism was expressed as a percentage of the residual enzymatic activity, drawing a comparison with a control scenario devoid of any inhibitor. To ensure reliability, each assay was executed in duplicate.

Ki Determination

The method predominantly serves as a convenient approach to promptly ascertain the inhibition constant, Ki. This involves plotting the reciprocal of the initial velocity (1/V) against an array of inhibitor concentrations [I], while keeping the substrate concentration, [S], constant. Upon executing this procedure for multiple [S] values, the resultant lines intersect at a specific juncture that corresponds to Ki. Importantly, the value of [I] at which this intersection transpires lies to the left of the ordinate in the context of competitive inhibition, specifically aligning with -Ki [14-15].

For the precise determination of inhibition constants (Ki), Dixon plots were generated by simultaneously incubating distinct concentrations of the inhibitor, SMZ (ranging from 0 to 50 mM), with GLM concentrations of 5 and 10µM. These reactions occurred within the presence of pooled HLM and an NADPH-generating system. All incubations strictly adhered to the established protocols as described earlier. Preceding the substrate's introduction, the incubation blend was subjected to a 5minute pre-incubation at 37°C, subsequently progressing for a span of 30 minutes before termination in a manner analogous to previous procedures. The calculation of the Ki value ensued through conventional graphical techniques.

Ki value was also estimated using the following formula:

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$$

where,

IC50 is half maximal inhibitory concentration **(**functional strength of the inhibitor**),** [S] is fixed substrate concentration, K_m is the concentration of substrate at which enzyme activity is at half maximal and Ki is apparent inhibition constant (binding affinity of the inhibitor).

Effect of SMZ on enzyme kinetics of Glimepiride:Nature of inhibition

To ascertain the apparent Michaelis-Menten constant (Km) and the maximum velocity of the reaction (Vmax), graphs depicting the relationship with substrate concentration were generated using GraphPad Prism 5 software. The evaluation of inhibition's characteristic nature, whether competitive or noncompetitive, was undertaken through the incorporation of SMZ as an inhibitor at a concentration of 500µM. This inhibitor was introduced to varying concentrations of GLM serving as the substrate, spanning a spectrum from 2 to 100□M.

Data analysis

Within this investigation, the vanishing of GLM within the medium, incubated at 37°C alongside HLM in the presence of NADPH, was quantified as a percentage in relation to the initial GLM quantity present in the unincubated medium. The outcomes derived from these measurements were presented as the percentage turnover rate whenever required. The pace of substrate disappearance was computed using the formula [(C0, initial - Cs, t min) / incubation time / CYP concentration], where C0, initial stands for the substrate concentration at the initiation point (0 minutes), and Cs, t min signifies the substrate concentration following a 30-minute incubation, involving a protein concentration of 0.5 mg/ml.

The determination of apparent kinetic parameters, encompassing Km and Vmax for the CYP2C9 catalyzed reaction within HLM, was executed through the utilization of GraphPad Prism 5 software. This endeavor was realized via a nonlinear least square regression analysis. Additionally, the assessment of IC50 values, denoting the inhibition of CYP2C9 activity, was conducted using Microsoft Excel 2007. All discerned outcomes were succinctly conveyed as the arithmetic mean along with their corresponding standard deviations (SD).

Results

Determination of K_m and V_{max} for GLM metabolism by nonlinear and linear transformations

The metabolism of GLM, conducted in the presence of HLM, conformed to the principles of Michaelis-Menten kinetics. The application of a nonlinear least squares regression methodology rendered Km and Vmax values of 28.90 ± 2.97 µMole and 0.559 ± 0.017 µMole/min/mg protein, correspondingly. Parallelly, through a Lineweaver-Burk plot analysis, the Km and Vmax values were deduced as 29.411 ± 1.25 µMole and 0.571 ± 0.020 µMole/min/mg protein, as depicted in Figure 2 and 3. This dual approach highlighted a remarkable congruence between the values derived from both the nonlinear and linear transformations of the data. It is noteworthy that each data point presented in this study is representative of a mean derived from no fewer than two parallel incubations.

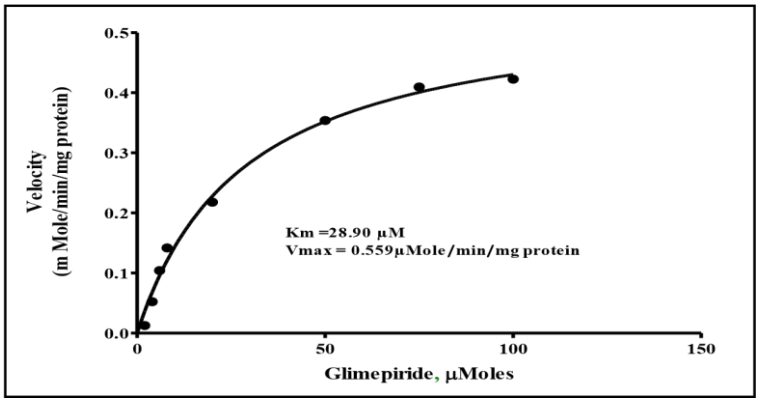

Figure 2. Michaelis Menten plot for GLM oxidative biotransformation in HLM.

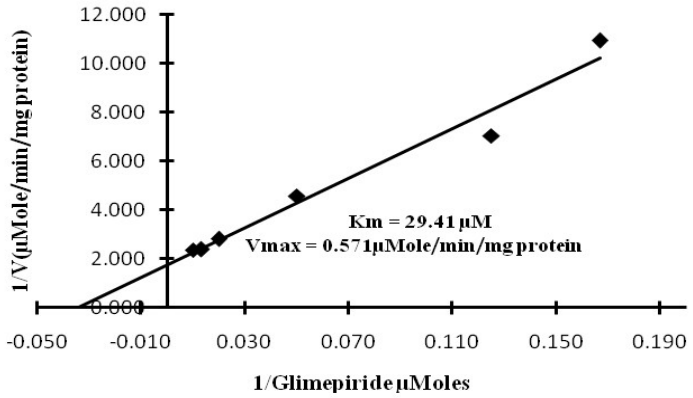

Figure 3. Lineweaver Burk plot for GLM oxidative biotransformation inHLM.

IC50 Determination

For an in-depth assessment of SMZ's influence on CYP2C9 activity, the hydroxylation of GLM was executed, incorporating liver microsomes both with and without the presence of the inhibitor. The impact of SMZ on the CYP2C9-catalyzed GLM hydroxylation within liver microsomes is visually depicted in Figure 4. Notably, spanning concentrations from 50 to 500 µMole, SMZ unveiled a discernible selective inhibition of CYP2C9-mediated glimepiride metabolism, with an apparent IC50 value calculated at 400 µMole.

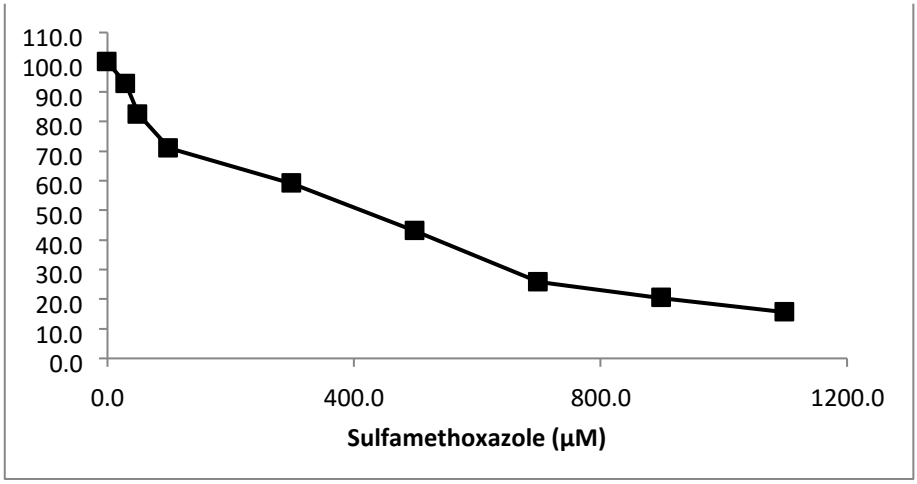

Figure 4: The inhibitory effect SMZ on GLM hydroxylation

Ki Determination

Exact inhibition constants (*K*i) were meticulously derived from Dixon plots (as depicted in Figure 3). These plots emerged from co-incubations that involved varied concentrations of SMZ (ranging from 0 to 50 mM), in combination with GLM at concentrations of 5 and 10µM. This experiment transpired in the presence of pooled HLM and a system facilitating NADPH generation. Through an analysis of the Dixon plot achieved with HLM, the Ki value pertaining to SMZ's influence on GLM hydroxylation was ascertained at 290µMole. The derivation of this value was rooted in plotting the reciprocal of the initial velocity (1/V) against a series of inhibitor concentrations [I], while keeping the substrate concentration constant at 5 and 10 µMole. This Ki value of 290µMole, situated to the left of the ordinate as induced by SMZ's inhibitory effect, signaled competitive inhibition. This positioning corresponded to -Ki, as evidenced in Figure 5.

Interestingly, when employing the formula Ki=IC50/(1+[S]/Km), and substituting the pertinent values (IC50=400 µMole, [S]= 10µMole, Km= 28.90µMole), the Ki value was computed at

297.17µMole. Notably, these two methods of Ki determination, namely the graphical approach and the equation-based approach, yielded results that closely aligned with each other.

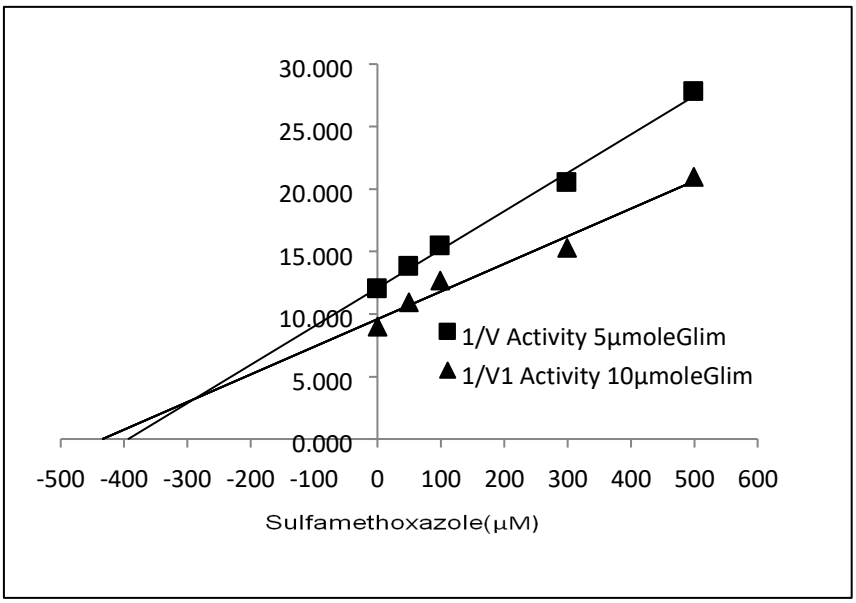

Figure 5: Dixon plots showing effect of SMZ (0, 5,10, 30 and 50 mM) when coincubated with 5 µM and 10µM GLM in the presence of pooled HLM.

Effect of SMZ on enzyme kinetics of Glimepiride:Nature of inhibition

As indicated in Figure 2, the outcomes drawn from in vitro experimentation strongly suggest the competitive manner of inhibition, as reflected by the increased Km value (32.26 µMole), while the Vmax (0.526 µMole/min/mg protein) remained nearly unchanged.

Additionally, the dataset pertaining to reaction velocities was subjected to analysis by plotting the reciprocal of the initial velocity (1/V) against the reciprocal of an array of substrate concentrations (1/GLM). This was conducted at a consistent concentration of the inhibitor [500 µM SMZ], drawing comparison with the control conditions where the inhibitor was absent (utilizing the Lineweaver-Burk equation). Illustrated in Figure 5, the points of intersection were ascertained through graphical assessment facilitated by Microsoft Excel 2007. Upon a Lineweaver-Burk plot analysis, it was observed that the Km value exhibited an augmentation, transitioning from 29.411 to 32.258 µMole, while the Vmax remained relatively unaffected, holding at 0.525 µMole/min/mg protein in the presence of 500µM SMZ as the inhibitor (Table 1). Notably, a commendable consistency emerged between the values derived from both the nonlinear and linear data transformations.

Table 1: Michaelis-Menten Kinetics Best-fit values (in presence of 500 µM SMZ)

Michaelis-Menten Kinetics Best-fit values	
Vmax (µM/min/mg protein)	0.526
Km(µM)	32.26
SD	
Vmax (µM/min/mg protein)	±0.031
Km(µM)	±4.31
Goodness of Fit	
Degrees of Freedom	6
R^2	0.9905
Absolute Sum of Squares	0.001796
Analyzed	8

Prediction of Increase in AUC of Glimepiride from *In Vitro* Metabolic Data: *IVIVC*

With the increasing accessibility of fractions of human tissues like HLM and human hepatocytes for in vitro investigations, endeavors have been made to quantitatively anticipate the consequences of drug metabolism and interactions in vivo through in vitro data. For scenarios involving competitive or noncompetitive inhibition of drug metabolism, the extent of potential in vivo interactions can be evaluated by assessing the ratio [I]u / Ki, wherein [I]u signifies the concentration of the inhibitor in its unbound state around the enzyme, and Ki denotes the in vitro inhibition constant of the inhibitor. However, it's noteworthy that measuring [I]u directly in humans is often unfeasible.

In instances where drugs undergo passive diffusion into the liver, [I]u can be approximated as equivalent to the unbound concentration present in the liver sinusoid at a steady state. To mitigate the possibility of generating false-negative predictions attributed to underestimating [I]u, the highest unbound concentration at the point of entry into the liver, where the blood from the hepatic artery and portal vein converge ([I]in,max,u), is considered, effectively using it as the maximum value of [I]u [16-17]. This methodology has been introduced as a means to provide insight into predicting the utmost extent of inhibition.

According to the perfusion model, the peak inhibitor concentration at the liver's inlet ([I]in,max) can be calculated using the formula:

$$[I]in,max = [I]max + ka \times Dose \times Fa / Qh \quad (1)$$

where ka is the absorption rate constant, Dose is the amount of inhibitor administered, Fa is the fraction absorbed from gut to the portal vein, and Qh is the hepatic blood flow rate. The reported literature [I]max value of 217 and ka x Dose x Fa / Qh value of 244 for SMZ as inhibitor was used for the calculations[11]. Hence [I]in,max was found to be 461.

Using the unbound fraction in the blood (fu) which is reported to be 0.35 for SMZ[11], [I]in,max,u was obtained as

$$[I]in,max,u = fu \times [I]in,max \quad (2)$$

which yielded value of 161.35.In clinical situations, the substrate concentration is usually much lower than Km and hence maximum degree of interaction (R = area under the curve [AUC] (+ inhibitor) / AUC (control)) is expressed as

$$R = 1 + [I]u / Ki \quad (3)$$

A 1.542 fold increase in AUC was observed by substituting the respective values(1+ 161.35/290) where *K*i is the dissociation constant for the enzyme–inhibitor complex obtained from the Dixon plot analysis, which was found to be 290µMole (Figure 3). The AUC of GLM was predicted to increase about 1.5-fold by co administration of SMZ, suggesting the risk of hypoglycemia.

Discussion

Within a concentration spectrum spanning from 30 to 1100 µMole, SMZ exhibited a discernible discriminatory inhibition of CYP2C9-mediated GLM hydroxylation. This was underscored by an apparent IC50 (Ki) value of 400 µMole and a Ki value of 290 µMole. The pattern of inhibition was conclusively determined as competitive, evident through the augmentation in Km value and the relatively unaffected Vmax, corroborated by analyses through both Michaelis-Menten and Lineweaver-Burk plots. Furthermore, the Ki value acquired via Dixon plot, positioned to the left of the ordinate (-290 µMole), further confirmed the competitive nature of inhibition. These outcomes collectively affirm that SMZ, particularly at concentrations lower than 500 µMole, stands as a promising selective inhibitor for CYP2C9 in in vitro investigations. Additionally, even at concentrations reaching 1000 µMole, SMZ sustains its high selectivity as an inhibitor of CYP2C9. Given the acknowledged drug interaction resulting in hypoglycemia from the concomitant use of sulfonylurea and sulfonamides, it's noteworthy that the structural similarities between sulfonamides

and sulfonylureas render the former capable of augmenting insulin release, particularly in susceptible individuals. The potential of sulfonamides to enhance the hypoglycemic effects of sulfonylurea agents is well-recognized when administered together.
Inferences drawn from in vitro to in vivo correlation (IVIVC) analyses underscore that SMZ imparts a considerable impact on GLM's area under the curve (AUC), resulting in an increase exceeding 1.5fold. This predicted rise in GLM's plasma concentration is noteworthy, as it suggests a noteworthy risk of hypoglycemia when SMZ is co-administered alongside GLM.

Conclusions

In summation, the pivotal role of CYP2C9 in facilitating GLM hydroxylation is evident. Within this study, a methodical exploration was conducted to assess the inhibitory influences of sulfonamides on CYP2C9-mediated GLM metabolism. It is noteworthy that this assessment can be extended to encompass other drugs that share a reliance on CYP2C9 for their metabolism. However, it is prudent to exercise caution when extrapolating the inhibitory effects observed in vitro to real-world in vivo scenarios. Consequently, a vigilant approach is recommended for the coadministration of sulfamethoxazole alongside glimepiride, particularly to mitigate the risk of hypoglycemic episodes. Furthermore, this cautious consideration extends to CYP2C9 substrates characterized by narrow therapeutic windows, emphasizing the necessity for vigilant monitoring in such cases.

List of abbreviations

Glimepiride GLM
Sulfamethoxazole SMZ
Human liver microsomes HLM
Nicotinamide Adenine Dinucleotide Phosphate, reduced tetra sodium salt NADPH
Ethylene diamine tetra acetic acid EDTA
Magnesium chloride $MgCl_2$
Cytochrome P450 CYP450
Liquid chromatography with ultraviolet LC/UV
Michaelis-Menten constant K_m
Maximal velocity of the reaction V_{max}
Inhibition constant Ki
Half maximal inhibitory concentrationIC50
Invitro invivo correlation *IVIVC*
Area under curve AUC

Competing interests

The author(s) declare that they have no competing interests.

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