SUMMARY

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Drug metabolism and pharmacokinetics have co-evolved as a vital discipline in drug discovery and development program to counteract increasing attrition related to poor absorption, distribution, metabolism and excretion properties of new chemical entities (undesirable ADME due to sub-optimal physicochemical properties of new chemical entities). Moreover, drug metabolism has emerged as an interfacial discipline in the multidisciplinary drug discovery and development program.

CDRI 99/411 is a potent 1,2,4-trioxane anti-malarial candidate compound of the Central Drug Research Institute, India. The 1,2,4-trioxane anti-malarial derivative has been extensively investigated with respect to pharmacodynamics, pharmacokinetics and preclinical safety and toxicity. However, drug metabolism aspect of CDRI 99/411 remained unexplored and undisclosed. Hence, it was aimed to conduct metabolic investigations of CDRI 99/411 to corroborate its preclinical investigations. Metabolic investigations on artemisinin and its derivatives revealed that CYPs are the principal Phase I drug metabolizing enzymes. Moreover, it has been reported that CYPs are involved in the metabolism of spiroozonide OZ277. Hence, it was hypothesized that CYPs are likely to be involved in the metabolism of CDRI 99/411.

The study was executed using *in vitro* and *in vivo* methods. Preliminary investigations revealed that CDRI 99/411 was undergoing metabolism with rat S9 fraction. Thus, prospective *in vitro* metabolic investigations of CDRI 99/411 were designed with male rat and human liver microsomes. Howsoever, *in vivo* investigations were limited to male rats.

Briefly, the study involved the preparation of liver microsomes, determination of *in vitro* metabolic half-life ($t_{1/2}$) and *in vitro* hepatic intrinsic clearance (Cl_{int}), metabolite identification and structural elucidation, enzyme kinetics, CYP phenotyping and drug-drug interactions.

The study commenced with the preparation of rat liver homogenate (microsomes). Protein content was estimated using Bradford reagent with BSA as the standard and was adjusted to 10 mg/mL. The quality of microsomes was assessed using Phenacetin-o-deethylase activity of CYP 1A2 using liquid chromatography-tandem mass

spectrometry (LCMS/MS). The in-house prepared rat liver microsomes were used for metabolic investigations of CDRI 99/411.

Preliminary *in vitro* metabolic investigations were performed to assess the *in vitro* metabolic $t_{1/2}$ and *in vitro* hepatic Cl_{int} of CDRI 99/411 in male Sprague–Dawley rat and human liver microsomes using validated high performance liquid chromatography with photodiode array detector (HPLC-PDA).

Chromatographic conditions were optimized by the selection of appropriate column and mobile phase composition. It was observed that 90% ACN and 10% of triple distilled water was suitable (peak shape and resolution) for the elution of CDRI 99/411 and IS. Sample preparation was performed using 1:1 protein precipitation method. Ice cold acetonitrile was used as the protein precipitating solvent. The Calibration Curve was found to be linear for the range 0.1-5.0 μg/mL. The assay was validated and intra-day and inter-day accuracy and precision was determined at low (200 ng/mL), medium (1000 ng/mL) and high (4000 ng/mL) quality control (QC). Precision and accuracy on both occasions were found within accepted variable limits.

The validated method was used for the determination of *in vitro* metabolic half-life ($t_{1/2}$) and *in vitro* hepatic intrinsic clearance (Cl_{int}) associated with metabolic stability. The observed *in vitro* $t_{1/2}$ of the compound with rat and human liver microsomes was 13 min with *in vitro* Cl_{int} 130.7 ± 25.0 μL/min/mg and 19 min with *in vitro* Cl_{int} 89.3 ± 17.40 μL/min/mg, respectively. These observations suggested moderate metabolic degradation and *in vitro* Cl_{int} with insignificant difference ($p > 0.05$) in the metabolic stability profile in rat and human.

Furthermore, it was endeavored to identify metabolites of CDRI 99/411 in rat and humans. A gradient time program with a run time of 35 minutes was developed using HPLC-PDA. On incubation with rat liver microsomes, two metabolites were visible at 247 nm. The formation of metabolites was inhibited by 1-ABT (suicidal inhibitor). Hence, it was concluded that CYPs are the principal enzymes involved in the metabolism of CDRI 99/411. The formation of metabolites was increased by scaling the incubation composition. Metabolites were isolated and purified by HPLC. Structural elucidation of metabolites was performed with mass spectrometry. Different scan types were performed and mass spectra of the parent and metabolites were compared. Fragment interpretation

was performed and putative structures were drawn. Based on mass spectrometry data it was concluded that the modification (hydroxylation) was occurring at benzene ring. Moreover, it was hypothesized that the benzene ring oxidation and regioisomeric metabolite formation proceeded *via* epoxidation and NIH shift (1, 2 hydride shift intramolecular rearrangement). It was also found that M1 and M2 were the circulating metabolites of CDRI 99/411 in male rats. M1 and M2 were also formed with male human liver microsomes. However, M2 was the major metabolite with male human liver microsomes.

The identification of metabolites was a gateway for prospective metabolic studies involving enzyme kinetics, reaction phenotyping and determination of IC_{50} values of Lumefantrine and Piperaquine using product monitoring approach.

Enzyme kinetics of CDRI 99/411 was performed using both substrate depletion and product monitoring approach (monitoring the rate of formation of M1 and M2). It was concluded that enzyme-substrate interaction was sigmoidal with rat and human liver microsomes. One substrate-two binding site theory was proposed for the sigmoidal kinetics of CDRI 99/411.

CYP reaction phenotyping with chemical inhibitors revealed that CYP 3A is the principal isoform involved in the metabolism of CDRI 99/411 in rats and humans. *In silico* docking studies were performed to understand the interaction of CDRI 99/411 and its metabolites with CYP 3A4 isoenzyme. It was found that testosterone and CDRI 99/411 were binding at the same site and in a similar fashion. Thus, they exhibit Hill kinetics. Moreover, M1 and M2 were also binding to the active site of CYP 3A4. Hence, M1 and M2 are potential substrates for CYP 3A isoform.

Drug combinations of CDRI 99/411 with long acting antimalarials like Lumefantrine and Piperaquine were investigated. Moreover, WHO advocates combinations of artemisinin class of drugs with longer acting drugs like Lumefantrine and Piperaquine. Hence, different concentrations of Lumefantrine and Piperaquine were incubated with CDRI 99/411 and IC_{50} values were determined. It was found that Piperaquine did not alter the formation of metabolites with rat and human liver microsomes.

Furthermore, induction of CYPs was assessed in post dosed male Sprague Dawley rats at pharmacokinetic dose and time point T_{max}. Induction was evaluated at transcriptional (mRNA) level. Quantification of mRNA was performed using quantitative Real Time PCR. Statistically significant changes (*t-Test: Two-Sample Assuming Equal Variances, $p< 0.05$*) in the expression of CYP 1A2, 2C11, 2E1, 3A2 and FMO1 were found between CDRI 99/411 treated and control rats. Transcriptional activation, receptor cross-talk and stabilization of transcripts (mRNA) may be the probable mechanisms involved in the induction process.