

Chapter 7

Drug-Drug Interaction

7.1 Introduction

Combination drug therapy and polypharmacy have introduced the concept of drug-drug interaction investigations in the drug discovery development program. Moreover, food-drug interactions are also an important aspect to be investigated.

Broadly, drug-drug interactions can be classified into pharmacodynamic and pharmacokinetic based interactions. Pharmacodynamic drug-drug interactions involve the synergistic and antagonistic aspect of individual drug's effects on the target receptor [1]. Pharmacokinetic interactions occur when one agent causes the blood level of another agent to be raised or lowered. Multiple mechanisms involving alterations in drug absorption, distribution, metabolism and excretion are the cause of pharmacokinetic based interactions [1]. Amongst them, metabolism based alteration is a predominant factor.

Metabolism based drug interactions involve inhibition and induction of drug metabolizing enzymes. However, clinically significant drug interactions involve CYPs. Hence, they are most discussed and investigated.

Section 7.2

Inhibition of CYPs

7.2 Inhibition of CYPs

Inhibition of drug metabolizing enzymes is the most common and important form of drug-drug interactions. Inhibition can be broadly classified into reversible and irreversible inhibition. It has been found that reversible inhibition is the common mechanism underlying inhibition of metabolism. Reversible inhibition has been categorized into competitive, noncompetitive and uncompetitive inhibition based on enzyme kinetics. Competitive inhibition involves competition between substrate and inhibitor for the same binding site of a CYP. In noncompetitive inhibition, the inhibitor and substrate bind to different sites of a CYP. Uncompetitive inhibition involves the binding of inhibitor to an enzyme that forms a complex with the substrate. Clinically significant interactions involving inhibition or induction have been illustrated by few case histories [2].

7.2.1 Interactions involving inhibition [2]

History

Amitriptyline (150 mg/day) was given to a 64 year old female to cure depression. However, there was no improvement in her mood. Hence, fluoxetine (40 mg/day) was included with amitriptyline. It was noticed that within three weeks, the patient's symptoms subsided. However, one week later, she collapsed at home and was found in a coma by a relative. The patient recovered consciousness two days later and made a full recovery.

Analysis

Fluoxetine was associated with the accumulation of amitriptyline. The accumulation was responsible for unconsciousness and could have been fatal had she not been discovered. It was assumed that fluoxetine must have prevented the clearance of amitriptyline.

History

Terfenadine was taken by a previously healthy 29 year old man to treat allergic rhinitis. He took it twice daily for one year. Besides he drank grape fruit juice two to three times weekly. One day he consumed two glasses of juice and then took his terfenadine dose. He fell ill within one hour, collapsed and died. The postmortem report of the individual

indicated 35 and 130 ng/mL of terfenadine and its metabolite in plasma, respectively. These levels are within range of previously noted arrhythmogenic levels of terfenadine. The individual had no evidence of impaired hepatic function.

Analysis

Grapefruit juice prevented the clearance of terfenadine. Hence, unusually high levels of terfenadine were present in the patient's plasma. This led to a fatal cardiac arrhythmia.

Thus, it can be concluded that fluoxetine and grapefruit were inhibiting the clearance of amitriptyline and terfenadine, respectively.

7.2.2 Experimental

7.2.2.1 Materials

HPLC-grade acetonitrile (ACN), tris (hydroxymethyl)aminomethane (tris base), KCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and a reduced form of β -nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA). Ammonium formate was purchased from Loba Chemie (Mumbai, Maharashtra, India). Male human liver microsome was procured from Sigma (St Louis, MO, USA). Piperaquine and Lumefantrine were procured from division of Pharmacokinetics and Metabolism.

7.2.2.2 Determination of IC_{50} of Lumefantrine and Piperaquine

CDRI 99/411 was incubated with Lumefantrine and Piperaquine to observe plausible drug- drug interactions. Varying concentrations of Lumefantrine (1 to 500 μM) and Piperaquine (1 to 250 μM) were incubated with CDRI 99/411 at $37 \pm 1^\circ\text{C}$. Table 1 summarizes the assay conditions.

Table 1. Assay conditions for the determination of IC_{50} values of Lumefantrine and Piperaquine

Analytical conditions	
Analytical technique	LCMS/MS
Internal Standard	CDRI 99/357
Column	Phenomenex Luna C18 (50 × 2.00 mm, 5µm)
Flow rate	0.4 mL/min
Injection volume	10 µL
Mobile phase	ACN, 2mM Ammonium formate (pH 3.0)
Gradient , %B (min)	40 (0.01)- 40 (1)- 15 (6) – 5 (8) – 5 (14) – 40 (14.01) – 40 (18)
Mass Spectrometry conditions	
Polarity	Positive
Ion Spray Voltage	5500V
Temperature	400°C
Declustering Potential	30V
Collision Energy	20V
MRM transitions	CDRI 99/411 412.5-185 M1 & M2 428.5-201 CDRI 99/357 406.6-179.3
Incubation conditions	
Lumefantrine,	1 to 500 µM,
Piperaquine	1 to 250 µM
Rat liver microsomes,	0.4 mg/mL
Human liver microsomes	0.4 mg/mL
Incubation time	20 min (with rat liver microsomes) & 30 min (with human liver microsomes)
Sample Preparation	1:1 Protein precipitation with ice cold ACN

Calculation

The data was fit to the equation Sigmoidal-dose response (variable slope) and IC_{50} was determined by the following equation

$$Y = Bottom + (Top - Bottom) / [1 + 10^{\{(Log EC_{50} - X) * Hill Slope\}}]$$

Where, X is the logarithm of concentration. Y is the response and Y starts at *Bottom* and goes to *Top* with a sigmoid shape. EC_{50} is the concentration of substrate that gives a response half way between *Bottom* and *Top*.

7.2.3 Results and discussions

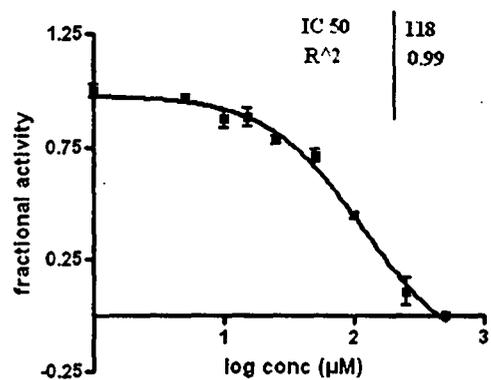
Monotherapy of artemisinin class of drugs leads to resistance in parasites. Hence, World Health Organization advocates artemisinin based combination therapy (ACT) as the first line of treatment. Moreover, ACT is considered best for malaria management. The best artemisinin based combinations are reported to be with longer acting drugs like Lumefantrine and Piperaquine. Consequently, antimalarial drug discovery and development program of Central Drug Research Institute, Lucknow, aims to develop suitable combinations of CDRI 99/411 with Lumefantrine or Piperaquine. Lumefantrine and Piperaquine are antimalarials with long plasma elimination half life.

IC_{50} values were determined (using product monitoring approach) by coincubating different concentrations of Lumefantrine and Piperaquine with CDRI 99/411. It was found that Lumefantrine inhibited the formation of regioisomeric metabolites and at non-linear regression ($r \geq 0.99$ and 0.97) IC_{50} values were $118 \mu\text{M}$ and $96 \mu\text{M}$ with rat liver microsomes (Figure 1). However, at $r \geq 0.93$ IC_{50} was $39 \mu\text{M}$ with human liver microsomes (Figure 2).

The coincubation of Piperaquine with CDRI 99/411 did not alter the rate of formation of regioisomeric metabolites in rat and human (Figure 3 & 4).

Figure 1. Determination of IC_{50} value with rat liver microsomes. Effect of Lumefantrine on the formation of regioisomer M1 (a) and M2 (b) in rat. Each bar represents the Mean \pm S.E. of the triplicate determination.

(a)



(b)

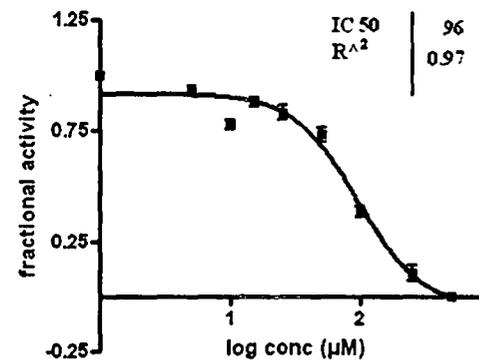


Figure 2. Determination of IC_{50} value with human liver microsomes. Effect of Lumefantrine on the formation of regioisomer M2 in human. Each bar represents the Mean \pm S.E. of the triplicate determination.

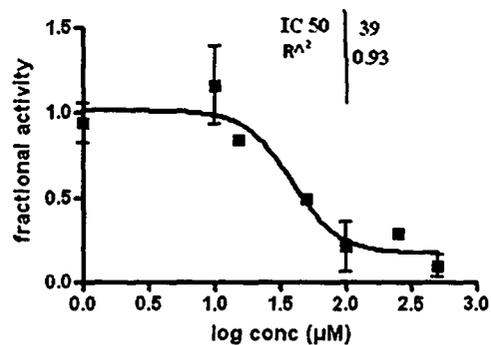
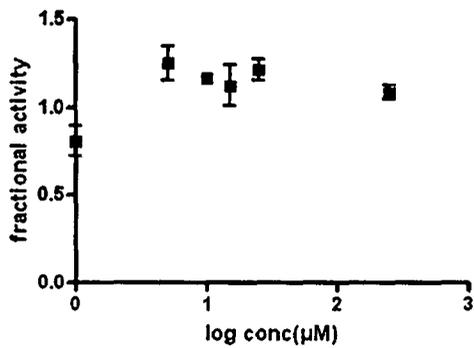


Figure 3. Effect of Piperazine on the formation of regioisomer M1 (a) and M2 (b) in rat. Each bar represents the Mean \pm S.E. of the triplicate determination.



Figure 4. Effect of Piperazine on the formation of regioisomer M2 in human. Each bar represents the Mean \pm S.E. of the triplicate determination.



7.2.4 Conclusion

CDRI 99/411 was incubated with different concentrations of Lumefantrine and Piperaquine. It was found that Piperaquine did not inhibit the formation of metabolites with rat and human liver microsomes and therefore may exhibit better anti-malarial efficacy in combination than Lumefantrine.

Section 7.3

Induction of CYPs

7.3 Introduction

The assessment of induction of CYPs by candidate drugs is prerequisite in the preclinical evaluation of toxicity and drug-drug interactions [3]. It is well known that the mechanism underlying inductions are the increased rate of synthesis or the attenuation of rate of degradation of CYPs [4]. The increased rate in the synthesis of CYPs involves receptor mediated mechanism.

The study of induction includes transcription (mRNA) and translation (protein) steps of the central dogma and measurement of enzyme activities. Hepatic induction of CYPs can be investigated by *in vitro*, *ex vivo*, and *in vivo* methods. Conventional approaches for preclinical evaluation of induction involves daily dosing of the drug for few days or a week in a preclinical animal model (rat, mice) and analyzing it by Western blot and enzyme activity [3]. However, the approach is time consuming and labor intensive.

With the advent of sensitive and dynamic technology like Real Time PCR, the screening of CYP inducing compounds in the early drug discovery stage has been enhanced. Real Time PCR has the ability to quantify induction of CYPs or other drug metabolizing genes at early time points (0.5 days) [3]. Thus, the sensitivity of Real Time PCR has enabled characterization of induction at low doses and early time points in *in vivo* models [3, 5].

7.3.1 Experimental

7.3.1.1 Materials

LightCycler 480 SYBR Green 1 Master (Roche, Mumbai, Maharashtra, India), LightCycler 480 multiwell plate 96 with sealing foil (Roche, Mumbai, Maharashtra, India), LightCycler 480 Real-Time PCR System (Roche, Mannheim, Germany) cDNA synthesis kit, BioRad (RNA quantification), cuvette, H₂O₂ (Loba Chemie, Mumbai, Maharashtra, India), Chloroform (Sisco Research Laboratory, Mumbai, Maharashtra, India), IPA (Sisco Research Laboratory, Mumbai, Maharashtra, India), Tri reagent

(Sigma, St. Louis, Mo., USA), Absolute alcohol (Merck, Mumbai, Maharashtra, India), ethidium bromide, agarose (Genei, Bangalore, Karnataka, India), Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA), bromophenol blue, Tris base, glacial acetic acid, RNA ladder, nuclease free water, Diethylpyrocarbonate and dexamethasone (Sigma, St. Louis, Mo., USA), Primers

7.3.1.2 Primer designing

Primers are oligonucleotides with known sequence specific for the gene of interest. Primers for Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) were designed using Roche software (Table 2). The product size with each primer was between 70 to 140 base pairs. GAPDH (house keeping gene) was taken as internal control and was coamplified with the gene of interest. Thus, it was used in the normalization of the polymerase chain reaction.

Lyophilized primers were reconstituted in nuclease free water. The concentration of each mother stock was 100 μ M. Working stocks (10 μ M) were prepared from mother stocks. Stock solutions were stored at -20°C.

Table 2. Forward and reverse primer sequences of rat liver drug metabolizing genes (specific for qRT-PCR)

S. No	Rat CYP P450	Primers	Gene Accession Number
1	CYP1A2	Forward 5'-TCC TAC AAC TCT GCC AGT CTC C -3' Reverse 5'-CCT CTC AAC ACC CAG AAC ACT-3'	NM_012541.3
2	CYP3A2	Forward 5'-ACC CGT CTG GAT TCT AAG CA -3' Reverse 5'-TGG AAT TAT TAT GAG CGT TCA GC-3'	U09742.1
3	CYP2E1	Forward 5'-CAG TCA CTG GAC ATC AAC TGC -3' Reverse 5'-GGG TTC TTG GCT GTG TTT TT-3'	NM_031543.1
4.	CYP2C11	Forward 5'- TCC GAC TTT TCT TAA CTT GAT GC-3' Reverse 5'- AAT GGC AGG GAA AGT ATT GC-3'	NM_019184.2
6.	FMO1	Forward 5'-TGT GCC AAA TTC TCT GTT CCT-3' Reverse 5'-GGG CGT TTT GTT ATA CTG CAC -3'	M84719.1
7.	GAPDH	Forward 5'-TGGGAAGCTGGTCATCAAC -3' Reverse 5'-GCATCACCCATTGATGTT -3'	NM_017008.3

7.3.1.3 Animals and treatment

Institutionally bred male Sprague Dawley rats (225±25 g) housed in an environmentally controlled room (12 hours light and 12 hours dark with ambient temperature and humidity) were procured and kept for overnight fasting and water was given *ad libitum*. Animals were handled in compliance with the Institutional Animals Ethics Committee of CDRI.

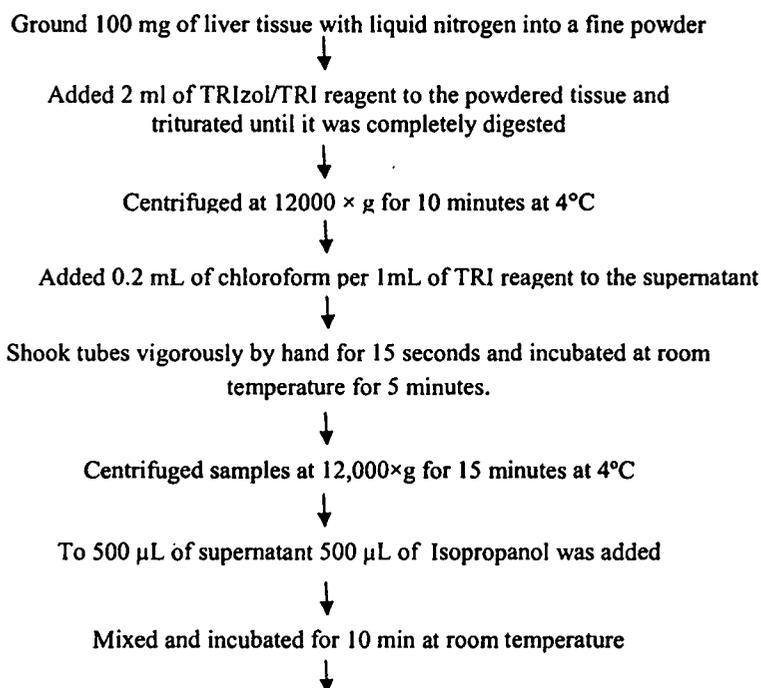
Male Sprague Dawley rats (180±20 gm) were grouped in a set of three as test (CDRI 99/411 treated) control and positive control (dexamethasone treated). A dose of 12 mg/kg in *Arachis* oil (as decided for pharmacokinetic study in an adjusted dose strength such that the volume factor remained 1 mL/kg) was orally administered with a soft gavage to animals in the experimental set and only *Arachis* oil (vehicle) to animals in

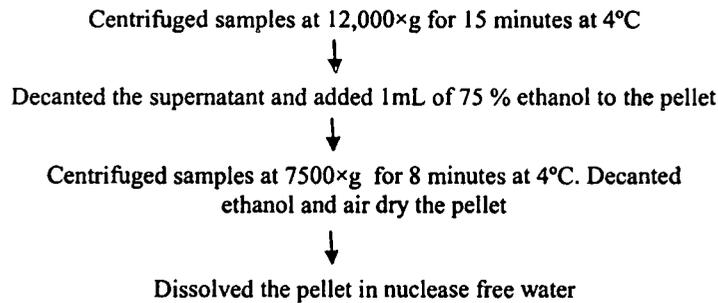
the control set [6]. Dexamethasone was administered subcutaneously at a dose of 0.8 mg/kg [7]. Liver was excised from treated rats (vehicle, CDRI 99/411 and dexamethasone) at T_{max} .

7.3.1.4 Isolation of total RNA

Fresh liver tissue was excised from male Sprague Dawley rat under sterile conditions. The tissue was finely powdered using liquid nitrogen and digested using TRI reagent. The lysate was centrifuged at 12000×g for 10 minutes at 4°C to sediment cell debris, etc. and phase separation step was performed with the supernatant. Phase separation involved treatment of supernatant with chloroform, incubation and centrifugation at 12000×g for 15 minutes at 4°C. Phase separation was succeeded by RNA precipitation. RNA precipitation was achieved by treating aqueous layer obtained in phase separation with isopropanol. Precipitated RNA was centrifuged at 12000×g for 15 minutes at 4°C for sedimentation. The RNA pellet was washed with 75% ethanol, dried and dissolved in nuclease free water.

Scheme 1. Isolation of total RNA from rat liver





7.3.1.5 Quantification of Total isolated RNA

Quantification was performed by diluting the total isolated RNA with nuclease free water in 1:60 ratio. Absorbance was recorded at 260 and 280 nm using spectrophotometer (BioRad Spectrophotometer). The concentration (expressed in $\mu\text{g}/\mu\text{L}$) of RNA and purity was determined by the spectrophotometer.

7.3.1.6 Agarose gel electrophoresis

The quality of isolated total RNA was assessed by using agarose gel electrophoresis.

Preparation of Reagents

- **0.5M EDTA(pH8.0) for 100ml**

Na ₂ EDTA	18.6 gm
Triple distilled water	70 mL

Adjust pH to 8.0 with NaOH. Make up the final volume up to 100 mL. Autoclave and store at room temperature.

- **50X TAE (for 100 mL)**

Tris base	24.2 gm
Glacial acetic acid	5.71 mL
0.5M EDTA (pH 8)	2.0 mL

Autoclave and store at 4°C.

- **1.0% agarose gel in a conical flask (for 40 ml)**

Agarose	0.4 gm
50X TAE buffer	0.8 mL (so that concentration becomes 1X in 40ml)
Autoclaved (TDW)	39.2 mL

Casting of agarose gel

0.5 μL of 10 mg/mL of ethidium bromide was added to the molten agarose (cooled to 65°C). The molten agarose was then poured in the casting apparatus preset with comb (to form wells) and was allowed to form gel. The comb is then removed after gel is formed.

Preparation of electrophoresis buffer (1X TAE)

1X TAE was prepared by diluting 50 X TAE with autoclaved TDW. It was poured in the electrophoresis apparatus.

Sample preparation

Samples for loading were prepared as follows:

- RNA 1 μg
- 6X Loading Dye (Bromophenol blue) 2 μL
- Autoclaved TDW X μL (to make total volume to 12 μL)
- Total volume 12 μL

Agarose gel was then placed in the electrophoresis apparatus containing 1X electrophoresis buffer. 10 μL of the each sample was loaded in individual wells. Electrophoretic pack was set at 70 V and electrophoresis was performed. The gel was then visualized using UV trans-illuminator or Gel documentation system.

7.3.1.7 First strand cDNA synthesis

cDNA was synthesized using RevertAid First Strand cDNA synthesis kit. It was synthesized as instructed in the manual. Briefly, it involved the following:

Reagents were added into a sterile eppendorf tube placed on ice in the described order 0.5 μg RNA, 1 μL random hexamer primer and nuclease free water to make the total volume to 12 μL

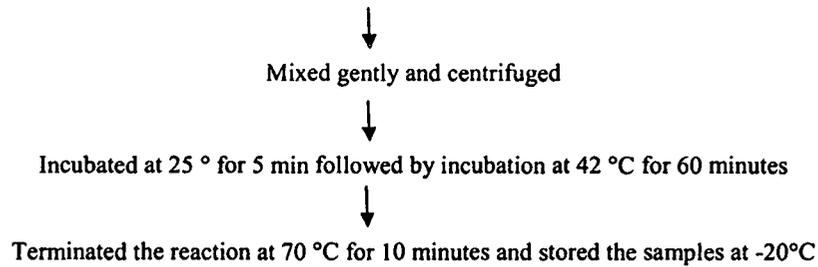


Incubated at 65°C for 5 min



Chilled the tube on ice and added the reagents in the described order 4 μL of 5X Reaction buffer, 1 μL of Ribolock RNaseTM Inhibitor (20 U/ μL), 2 μL of 10 mM dNTP mix and 1 μL of RevertAidTM M-MuLV Reverse Transcriptase (200 U/ μL). The total volume of the reaction was 20 μL .





7.3.1.8 Quantitative Real time PCR (qRT-PCR)

qRT PCR (LightCycler 480) was performed using the LightCycler® 480 DNA SYBR Green I kit. Briefly, the reaction was composed of 10 µL of SyBr Green, 1 µL each of 10 µM of forward and reverse primer (specific for each gene), 7 µL of PCR grade water and 1 µL of cDNA. Total volume of the reaction was 20 µL. All samples were run in quadruplicate.

The qRT-PCR reaction consisted of preincubation at 94°C for 10 minutes, denaturation 95°C for 05 seconds annealing at a primer specific temperature for 30 seconds and elongation at 72°C. The number of amplification cycles was set to 45.

Calculation

Delta delta C_t method

$$\text{Fold change} = 2^{-\Delta\Delta C_T}$$

Where ,

$$\Delta\Delta C_T = \Delta C_{T(\text{treated})} - \Delta C_{T(\text{control})}$$

Treated $\Delta C_{T(\text{treated})} = C_{T(\text{CYP or FMO})} - C_{T(\text{GAPDH})}$

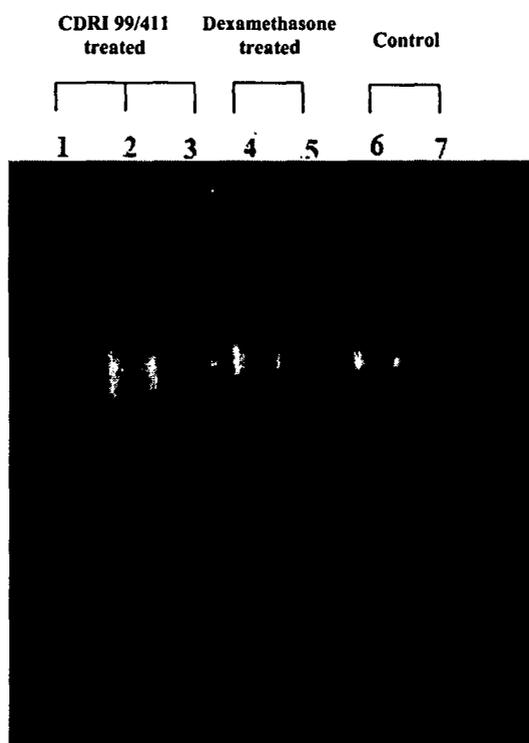
Control rats $\Delta C_{T(\text{control})} = C_{T(\text{CYP or FMO})} - C_{T(\text{GAPDH})}$

7.3.2 Results and discussions

7.3.2.1 Quantification of Total RNA

The isolated total RNA was quantified and purity was assessed by recording absorbance at 260 and 280 nm. It was found that the ratio of A_{260}/A_{280} was close to 2 (purity index). Quality was assessed using 1% agarose gel electrophoresis (Figure 5). Two bands were visible. 28S rRNA migrated at a slow rate compared to 18S rRNA.

Figure 5. Total RNA isolated from CDRI 99/411, control and dexamethasone treated rat liver. The gel shows bands of 28S and 18S rRNA.



7.3.2.2 Quantification of gene expression changes in post dosed male Sprague Dawley rats (application of qRT-PCR)

CYP and FMO gene expression changes were observed at pharmacokinetic dose and time point (T_{max}). 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 (Figure 6). The fold change in the expression of genes in CDRI 99/411 treated rats is shown in Figure 6.

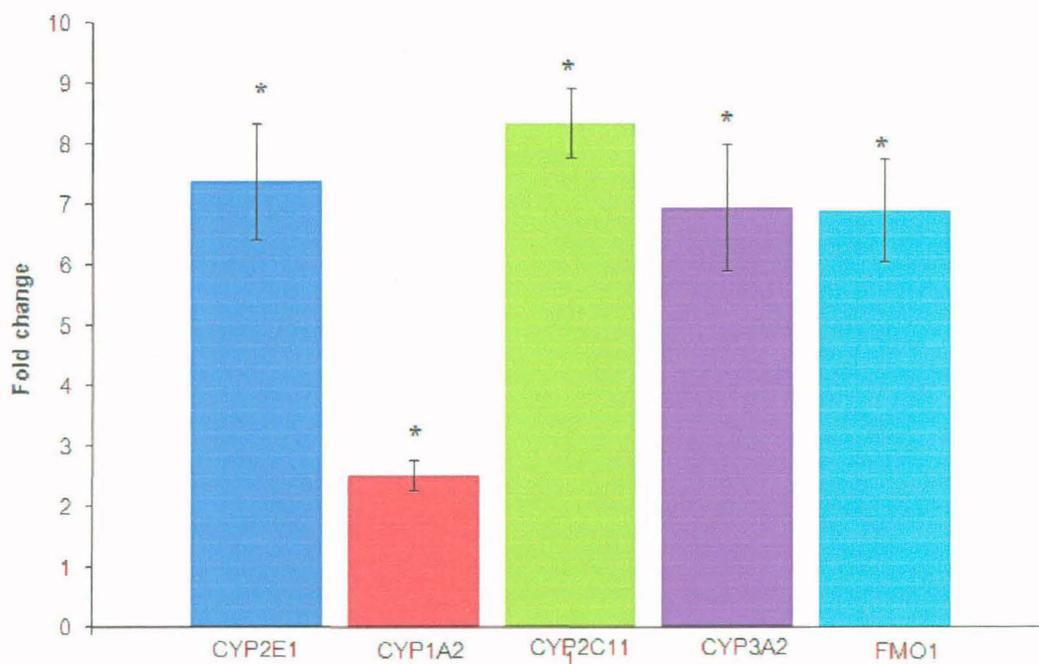
Dexamethasone is a model inducer of CYP 3A2 isoform in rodents [8]. Hence, dexamethasone treated rats were taken as positive control. Statistically significant changes (*t-Test: Two-Sample Assuming Equal Variances*, $p < 0.05$) in the expression of 2C11, 2E1, 3A2 and FMO1 were found between dexamethasone treated and control rats (Figure 7). The fold change in the expression of genes in dexamethasone treated rats is shown in Figure 7. Statistically insignificant ($p > 0.05$) change was observed with the expression of CYP1A2 isoform. The fold change in the expression of CYP1A2 was 1.1 (Figure 7).

It has been reported that the induction of CYP 1A, CYP2B, CYP3A and CYP4A are mediated by aryl hydrocarbon receptor (Ahr), constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor alpha (PPAR α), respectively. PXR and CAR receptors were initially considered to be independent regulators for CYP3A and CYP2B genes, respectively. Moreover, the xenobiotic response elements of PXR and CAR are also known to be independent. However, significant cross regulation caused by the adaptive recognition of the opposing elements has been observed. PXR and CAR targets are known to induce large number of overlapping set of genes. PXR is also known to regulate genes of Phase I enzymes including CYP2C, CYP2A, CYP1A, CYP1B, CYP4F, FMOs, monoamine oxidase, etc. and Phase II enzymes. It has also been documented that CAR is also responsible in the regulation of FMO [9].

Mechanism of induction of CYP2E1 is mediated by transcriptional activation, increased mRNA translatability, stabilization of transcripts (mRNA) and decreased rate of degradation of transcripts [10].

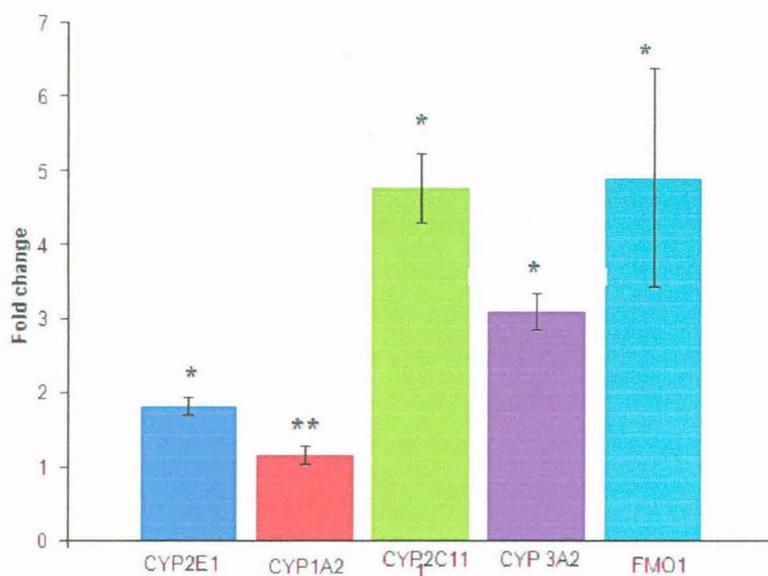
Hence, it may be hypothesized that the above mentioned mechanisms may be involved in the induction of CYPs and FMO1 by CDRI 99/411.

Figure 6. Fold change in the expression of mRNA of hepatic CYPs and FMO1 in male Sprague Dawley rats treated with CDRI 99/411 (mean \pm SD, N=3).



* $p < 0.05$

Figure 7. Fold change in the expression of mRNA of hepatic CYPs and FMO1 in male Sprague Dawley rats treated with Dexamethasone (mean \pm SD, N=3).



* $p < 0.05$, ** $p=0.2$

7.3.3 Conclusions

CDRI 99/411 was found to induce hepatic CYPs and FMO1 in male Sprague Dawley rats at pharmacokinetic dose and time point (T_{max}). Transcriptional activation, receptor cross-talk and stabilization of transcripts (mRNA) may be the probable mechanisms involved in the induction process.

Reference

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