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journal homepage: <http://ees.elsevier.com/apjtm>Original research <http://dx.doi.org/10.1016/j.apjtm.2015.07.013>Rifabutin reduces systemic exposure of an antimalarial drug 97/78 upon co-administration in rats: An *in-vivo* & *in-vitro* analysisYeshwant Singh¹, Mahendra Kumar Hidau^{1,2,3}, Shio Kumar Singh^{1*}¹Pharmacokinetics & Metabolism Division, CSIR – Central Drug Research Institute, Lucknow 226031, India²Academy of Scientific and Innovative Research, New Delhi, India

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ABSTRACT

Objective: To determine the potential drug–drug interactions between anti-malarial candidate 97/78 and anti-tubercular drug rifabutin *in-vivo* in rats followed by *in-vitro* investigation of the underlying mechanisms of drug interaction.

Methods: Single oral dose study was conducted in male and female rats at 40 mg/kg and 70 mg/kg for 97/78 and rifabutin respectively.

Results: It was reported that rifabutin co-administration altered pharmacokinetics of 97/63 (active metabolite of 97/78). A significant decrease was reported in the systemic exposure of 97/63 by a factor of 3–4. The AUC_{0-12h} values were (4.03 ± 0.60) and $(5.44 \pm 1.15) \mu\text{g h mL}^{-1}$ upon 97/78 administration alone, while the values were decreased to (1.13 ± 0.10) and $(1.23 \pm 1.13) \mu\text{g h mL}^{-1}$ upon rifabutin co-administration in male and female rats respectively. Statistically significant differences were also reported in C_{max} and T_{max} values upon rifabutin co-administration. *In-vitro* drug metabolism study in rat liver microsomes has shown that the metabolism of 97/63 was increased by 10%–12% upon rifabutin co-incubation. The extent of plasma protein binding of 97/63 was found to be decreased from 54%–55% to 6%–8% upon rifabutin addition.

Conclusions: It was concluded that rifabutin co-administration altered PK parameters of 97/63 in SD rats. However, no intersex influences were reported in the interaction pattern. The results obtained in the *in-vivo* study were well correlated with the *in-vitro* findings and can further be applied to explore other aspects of potential drug interactions between these two drugs.

1. Introduction

Tuberculosis and malaria co-epidemics are very prevalent in most of the tropical and sub-tropical areas of the world that creates serious damage to socio-economic status besides public health concerns. More than six million people are killed by the co-infection of these two diseases with AIDS annually [1–3]. Patients are supposed to take co-medications for malaria and

tuberculosis simultaneously, which may give rise to serious drug–drug interactions (DDIs). It has been reported that the greater the number of drugs is given to a patient, the more the risk of potential drug interactions. Incidences of such drug interactions raises up to 7% in those patients taking 6–10 drugs and 40% in those taking 16–20 drugs daily [4].

It is important to explore drug metabolism and pharmacokinetic (DMPK) properties of drug candidate in early stages of drug discovery and development. Besides DMPK, DDIs are routinely incorporated in the early phases of drug development pathway from a safety point of views. For this, preclinical studies are often carried out in experimental animals. From the preclinical data, the allometric scaling approaches that consider important anatomical and physiochemical variables in higher species as a power function of the body weight across species may be used to predict human pharmacokinetics (PK) aspects [5–8]. There are a number of gender-dependent and gender-specific

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characteristics that are known to influence the PK of a drug. Physiological and anatomical differences between the genders of a species are the responsible factors influencing PK of a drug differently [9,10].

In the present study, *in-vivo* drug interactions were investigated between a novel trioxane anti-malarial candidate 97/78 (phase I clinical trials completed) and antitubercular drug rifabutin (interacting drug). Single oral dose drug interaction studies were performed in male and female Sprague Dawley (SD) rats to determine the changes in PK and intersexual interaction pattern of 97/78 upon rifabutin co-administration. The results of single dose interaction study were analyzed and *in-vitro* studies were performed to find out the mechanism behind the existence of these *in-vivo* interactions.

2. Materials and methods

2.1. Chemicals

Rifabutin was obtained as a gift sample from Lupin (Pune), India. Reference standards (purity > 98%) of 97/78, 97/63 (Figure 1a) and internal standard (IS) arteether (Figure 1b) were obtained from the medicinal chemistry division, CDRI, Lucknow, India. Blank plasma was collected from drug free healthy male and female SD rats procured from the Laboratory Animal Services Division, CDRI. All other chemicals and reagents were of analytical liquid chromatographic (LC) grade. Dextran coated charcoal for plasma protein binding study was obtained from Sigma chemicals USA. Dulbecco's Phosphate buffered saline (Ca^{2+} and Mg^{2+} free) was purchased from Hi media Lab. Pvt. Ltd., Mumbai. For metabolism stability studies, Tris (hydroxymethyl) aminomethane (tris base), KCl, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, bovine serum albumin and a reduced form of β -nicotinamide adenine dinucleotide phosphate were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). 1-aminobenzotriazole, Bradford reagent and diethyl ether were procured from Qualigens (Mumbai), Loba Chemie (Mumbai) and TKM Pharma (Hyderabad, AP, India), respectively. Rat liver microsomes were prepared by using the methods adapted from literature [11].

2.2. Subjects and study design

Healthy male and female SD rats (250–270 g) free from any symptoms of pathophysiology were obtained from Laboratory

Animal Services Division, CDRI (approval no. IAEC/2012/91Ns) were allowed to acclimatize for 3 d in ventilated polypropylene cages at standard laboratory conditions [12 h light–dark cycle, $(22 \pm 2)^\circ\text{C}$ temperature and $(55 \pm 5)\%$ relative humidity] before the commencement of study. Subjects were fasted for about 10–12 h before and 2 h following drug administration with water *ab libitum*. Animals were cared in accordance with principles of the guide for care and use of laboratory animals [Department of health education and welfare, number (NIH) 85-32].

2.2.1. In-vivo studies

We conducted a parallel design, single dose/single dose pharmacokinetic interaction study that comprised of two groups of animals. One group of subjects was orally administered with 97/78 at 40 mg/kg, while other group received 97/78 followed by rifabutin at 40 mg/kg dose. Sparse sampling approach was used to collect blood samples through cardiac puncture (first sample) and inferior vena cava (terminal sample). Blood samples were collected before dosing (0 time point) and up to 96 h post dose. Blood samples were stored in ice until centrifugation. Plasma was separated by centrifugation at 2200 g for 5 min and stored at -60°C until analysis. Analysis was performed within a month of sample collection. All experimental procedures were approved and performed in accordance with the guidelines of the Institutional Animal Experimentation Ethics Committee (IAEC/CPCSEA/Regulatory Agencies).

The formulations used in the study were aqueous suspension of 97/78 and rifabutin in 0.25% carboxy methyl cellulose. Both the formulations were subjected to quality control checks to ensure strength and content uniformity. The strength of the formulations for 97/78 and rifabutin was 20 and 35 mg/mL respectively.

2.2.2. In-vitro studies

In-vitro studies were then conducted to find out the mechanism behind the *in-vivo* study observations. *In-vitro* plasma protein binding studies were conducted to find out the changes in the plasma protein bindings of 97/78, while *in-vitro* drug metabolism studies were conducted to find out the interactions at the level of metabolism.

Protein binding was estimated using modified charcoal adsorption method adopted from literature [12]. The study was conducted in three replicates each at 100 and 1000 ng/mL

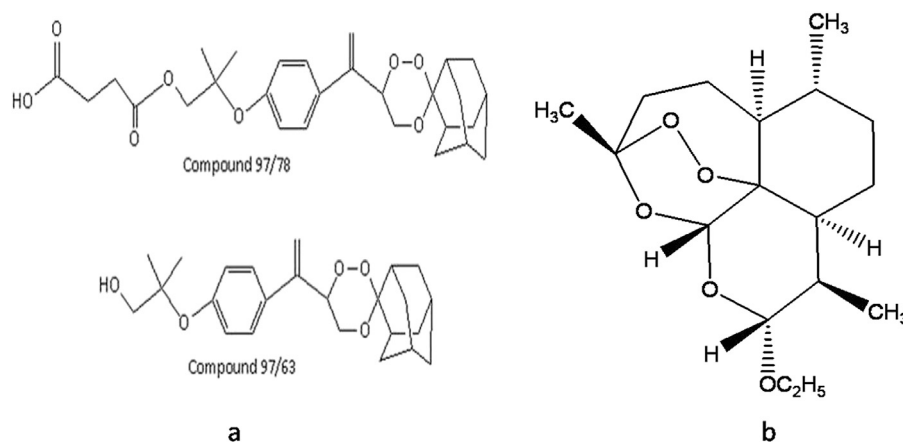


Figure 1. Chemical structure of 97/78, 97/63 (a) and IS arteether (b).

spiked plasma concentrations. The reactions were carried out in two sets, one set involving spiked 97/63 only, while other set spiked with 97/63 and rifabutin. The spiked plasma was allowed to equilibrate for 20 min before the commencement of study. Serial samples (200 μ L) were withdrawn at 0, 5, 10 and 15 min in polypropylene centrifuge tubes and centrifuged at 10000 g for 4 min at 37 $^{\circ}$ C. Supernatant was collected and stored at -70 $^{\circ}$ C till LC-MS/MS analysis.

97/63 alone, 97/63 with rifabutin and testosterone (as a control) were incubated separately in a shaking water bath in an incubation mixture of 0.1 M Tris buffer, 5 mM $MgCl_2 \cdot 6H_2O$ and 0.4 mg/mL of rat microsomes at [(37 \pm 1) $^{\circ}$ C]. The concentrations used for incubation were 2 mM, for 97/63, 5 mM for rifampicin and 2 mM for testosterone. β -nicotinamide adenine dinucleotide phosphate (2 mM) was pre-incubation for 4 min before the commencement of the study. Two sets of control were used in the study. In the first set, β -nicotinamide adenine dinucleotide phosphate was substituted with an equal volume of Tris buffer, while other set of controls was incubated consisting of all incubation components except microsomes. The reactions were quenched with ice cold ACN spiked with 4 μ M of IS at different time points ranging from 0 to 60 min. Samples were processed and supernatant was subjected to UFLC analysis. Before starting these experiments, non-specific binding of drug 97/63 with microsomal proteins was determined by incubating 97/63 in an optimized incubation milieu (0.4 mg/mL of rat liver microsomes at [(37 \pm 1) $^{\circ}$ C] in a shaking water bath at varying concentrations ranging from 0.2 to 30 μ M for 5 min.

2.3. Pharmacokinetic sample processing and bioanalysis

All study samples were quantitatively estimated for the determination of 97/63 (active metabolite of 97/78). A partially validated method in Q-trap 5500 LC-MS/MS mass spectrometer (Applied Biosystems, MDS Sciex, USA) with Analyst 1.6 software was used to determine plasma concentration of 97/63. The assay was linear over the range 0.625–80 ng/mL 10 μ L of IS was spiked into each study sample followed by a single step protein precipitation method using acetonitrile. Mass spectrometry was performed in positive mode electro spray ionization with capillary voltage 5.5 KV. The MS/MS transitions of m/z 418.2 \rightarrow 119.1 and 330.3 \rightarrow 267.4 were used to quantify 97/63 and IS respectively. HPLC elution was carried out in isocratic mode with mobile phase comprising of 80:20 (v/v) ACN (0.1% formic acid) and ammonium acetate buffer (10 mM, pH 4.0) using a Phenomenex C 18 Columbus (50 mm \times 2 mm, 5 μ particle size) equipped with a security guard cartridge. Separation was achieved at 450 μ L/min flow rate with injection volume of 10 μ L [13,14]. Processed sample were maintained in auto sampler at 4 $^{\circ}$ C.

2.4. Statistical analysis

The study endpoints examined were area under the curve (AUC_{0-last}), maximum plasma concentration (C_{max}), time to attain C_{max} (T_{max}), elimination half life ($T_{1/2}$) and mean residence time (MRT) and the relative bioavailability (RB). The PK parameters were calculated by non-compartmental analysis using Winnonlin software (Version 1.5, Pharsight Corporation). All results were expressed as mean \pm SEM.

RB was calculated as:

$$\% RB = \frac{AUC_{co-admin}}{AUC_{control}} \times 100 \quad (1)$$

The results of the *in-vitro* metabolic study were expressed as the % drug remaining, which was calculated as:

$$\% \text{ Drug remaining} = \frac{\text{drug conc. after incubation}}{\text{drug conc. at } t=0} \times 100 \quad (2)$$

The % plasma protein binding was calculated as,

$$\% \text{ Plasma protein binding} = \frac{\text{Drug conc. analyzed at } t=0}{\text{Spiked plasma conc.}} \times 100 \quad (3)$$

It has been reported that 97/78 (prodrug) converts into 97/63 (active metabolite) within a few minutes in biological matrices like plasma [15], thus samples were analyzed for the determination of 97/63, post 97/78 oral dose administration alone and with rifabutin in male and female rats. All the results are expressed as mean values \pm SEM, $n = 4$. The values of various PK parameters for 97/78 alone and co-administration with rifabutin were statistically compared with '2-tailed Students *t*-test'. The values were considered statistically significantly different for $P < 0.05$.

3. Results

3.1. Pharmacokinetic drug interaction studies

3.1.1. Male rats

Table 1 summarizes PK parameters of 97/63 post 97/78 administration alone and with rifabutin in male rats. The value of C_{max} was (0.56 \pm 0.18) μ g/mL at corresponding T_{max} of (5.33 \pm 0.76) h when 97/78 was given alone. While upon co-administration with rifabutin these values were found (0.13 \pm 0.03) μ g/mL and (1.83 \pm 1.25) h for C_{max} and T_{max} respectively. Figure 2 represents plasma concentration-time profile of 97/63, when 97/78 was given alone and in combination with rifabutin in male rats.

3.1.2. Female rats

Table 1 summarizes PK parameters of 97/63 post 97/78 administration alone and with rifabutin in female rats. The value of C_{max} was (0.79 \pm 0.10) μ g/mL at corresponding T_{max} of (6.00 \pm 0.00) h when 97/78 was given alone. While upon co-

Table 1

Comparative PK parameters of 97/63 after 97/78 alone and co-administration with rifabutin in male and female rats (mean \pm SEM, $n = 3$).

PK parameters	97/78 alone		97/78 + Rifabutin	
	Male rats	Female rats	Male rats	Female rats
C_{max} (μ g/mL)	0.56 \pm 0.18	0.79 \pm 0.10	0.13 \pm 0.03 [#]	0.17 \pm 0.03 [#]
T_{max} (h)	5.33 \pm 0.76	6.00 \pm 0.00	2.25 \pm 0.86 [#]	1.83 \pm 1.25 [#]
AUC_{0-last} (μ g h mL ⁻¹)	4.03 \pm 0.60	5.44 \pm 1.15	1.13 \pm 0.10 [#]	1.23 \pm 1.13 [#]
$T_{1/2}$ (h)	6.49 \pm 1.64	6.09 \pm 2.40	4.30 \pm 1.13	6.23 \pm 1.09
MRT (h)	11.58 \pm 1.55	11.65 \pm 1.36	10.17 \pm 0.82	10.12 \pm 1.30

[#]Statistically significantly different ($P < 0.05$), when compared to base line PK profile i.e., 97/78 administration alone.

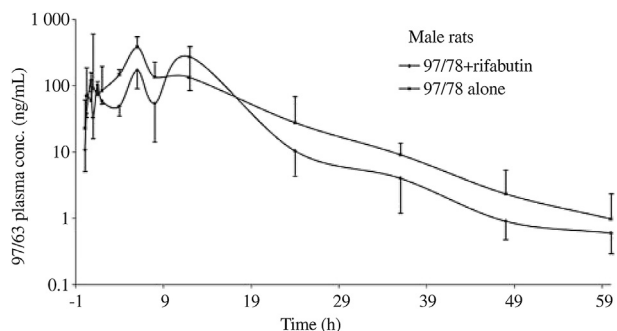


Figure 2. Plasma concentration–time profile of 97/63, upon 97/78 alone and co-administration with rifabutin in male rats ($n = 3$).

administration with rifabutin these values were found (0.17 ± 0.03) $\mu\text{g/mL}$ and (1.83 ± 1.25) h for C_{max} and T_{max} respectively. Figure 3 represents plasma concentration–time profile of 97/63, when 97/78 was given alone and in combination with rifabutin in female rats.

3.2. In-vitro drug interaction studies

3.2.1. In-vitro metabolism study

The experiment was carried out to determine the non-specific binding of 97/63 with microsomal proteins. Non-specific binding was predicted from the disappearance of 97/63, upon its incubation in an optimized incubation milieu of rat liver microsomes at various concentrations ranging from 0.1 to 20 μM for 5, 10, 15 min. It revealed that there were non-significant protein-ligand interactions (data not shown). Figure 4 represents the % drug remaining upon incubation of 97/63 alone and

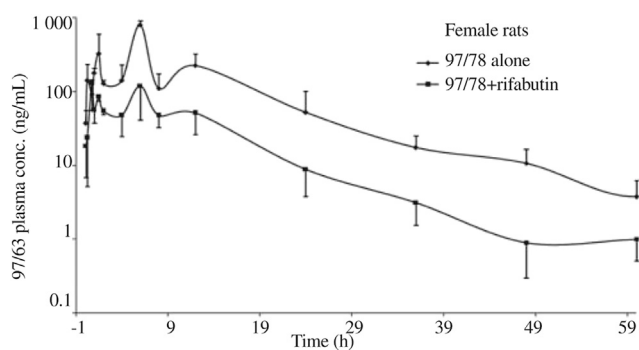


Figure 3. Plasma concentration–time profile of 97/63, upon 97/78 alone and co-administration with rifabutin in female rats ($n = 3$).

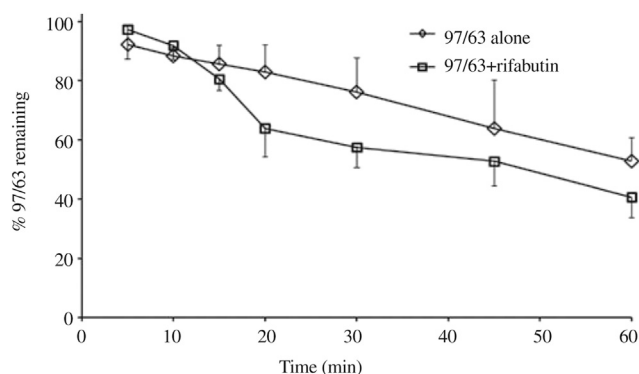


Figure 4. Percent drug remaining in the incubation mixture containing 97/63 alone and 97/63 with rifabutin.

Table 2

Percent plasma protein binding of 97/63, when spiked alone and with rifabutin in SD rat plasma.

Spiked plasma conc.(ng/mL)	% Plasma protein binding (97/63 spiked alone)	% Plasma protein binding (97/63 + Rifabutin)	P-value
100	55.33 \pm 15.30	6.00 \pm 1.00	0.03 [#]
1000	54.66 \pm 3.51	8.46 \pm 1.20	0.02 [#]

[#]Statistically significantly different ($P < 0.05$).

97/63 with rifabutin in optimized microsomal mixture at various time intervals.

3.2.2. In-vitro plasma protein binding study

Table 2 represents % plasma protein binding of 97/63, when spiked alone and with rifabutin in SD rat plasma. It was found that there is slight increase in 97/63 metabolism when 97/63 was incubated with rifabutin, compared to metabolism of 97/63 upon alone incubation.

Figure 5 represents plasma concentration–time profile of 97/63, when 97/78 was given alone in male and female rats.

On the other hand, when 97/78 was co-administered with rifabutin in male rats, statistically significant differences were reported in PK parameters of 97/63. It was observed that rifabutin co-administration reduced systemic exposure of 97/63 by a factor of 3–4. In terms of $\text{AUC}_{0-\text{last}}$, the value was (4.03 ± 0.60) $\mu\text{g h mL}^{-1}$ when 97/78 was given alone, while it was decreased to (1.13 ± 0.10) $\mu\text{g h mL}^{-1}$ upon co-administration of rifabutin (Figure 6).

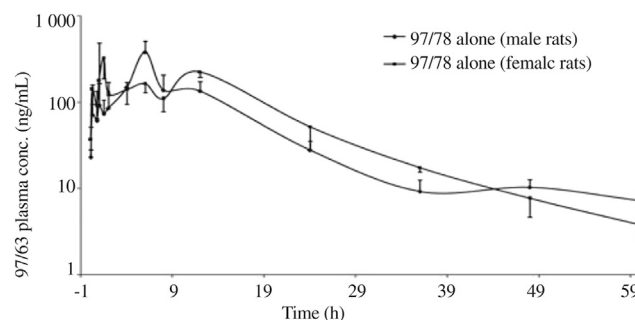


Figure 5. Plasma concentration–time profile of 97/63, when 97/78 was given alone in male and female rats (mean \pm SEM, $n = 3$).

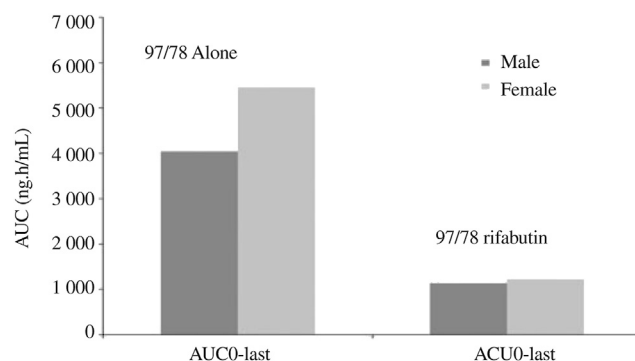


Figure 6. Comparative AUCs of 97/63 upon 97/78 alone and co-administration with rifabutin in male and female rats (mean \pm SEM, $n = 3$).

4. Discussion

It is important to assess drug's safety and effectiveness during the drug development phase in terms of drug interactions between investigational new drug and other drugs, so as to define existence of any potential DDIs that in turn indicates the need for additional therapeutics monitoring, dose adjustments and/or contraindication to concurrent use [8]. Generally, the more the number of drugs is given simultaneously the greater is the potential of existence of DDIs. Keeping this in view, the study was designed to determine the influence of rifabutin co-administration on PK of 97/63 in male and female SD rats to assess co-administered and intersexual differences.

It was found that 97/63 exhibited irregular plasma concentration–time profile in both the genders when 97/78 was either given alone or with rifabutin. However, when 97/78 was given alone, there were no intersexual differences in PK parameters of 97/63 i.e., both the genders exhibited similar PK profile as evident from the data in our study. Though, the value of C_{max} in female rats was found to be greater than male rats, but the difference was statistically non-significant in terms of $P > 0.05$. All other PK parameters were comparable for both the genders.

On the other hand, when 97/78 was co-administered with rifabutin in male rats, statistically significant differences were reported in PK parameters of 97/63. It was observed that rifabutin co-administration reduced systemic exposure of 97/63 by a factor of 3–4. In the similar manner, 3–4 fold decrease was observed in C_{max} which is explicitly indicated by the reduced maximum plasma concentration of 97/63 from (0.56 ± 0.18) to (0.13 ± 0.03) $\mu\text{g/mL}$ upon rifabutin co-administration. Statistically significant difference was also observed for T_{max} values. However, $T_{1/2}$ and MRT values were comparable in both the cases i.e. no significant differences were observed.

Similarly, in female rats statistically significant differences were reported in PK parameters of 97/63 when 97/78 was co-administered with rifabutin. It was observed that rifabutin co-administration reduced systemic exposure of 97/63 by a factor of 3–4 like male rats. In terms of AUC_{0-last} , the value was (5.44 ± 1.15) $\mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$ when 97/78 was given alone, while it was decreased to (1.23 ± 1.13) $\mu\text{g} \cdot \text{h} \cdot \text{mL}^{-1}$ upon co-administration of rifabutin. Similar to male rats, maximum plasma concentration of 97/63 was reduced from (0.79 ± 0.10) to (0.17 ± 0.03) $\mu\text{g/mL}$ upon rifabutin co-administration in females too, indicating 3–4 fold decrease in C_{max} . Statistically significant difference was observed for T_{max} values, while $T_{1/2}$ and MRT values were comparable in both the cases i.e. no significant differences were observed.

As discussed earlier, no significant differences (in terms of P -value) were observed in PK parameters of 97/63 when 97/78 was given alone in male and female rats i.e., no intersexual influences were reported. In the similar way, when rifabutin was co-administered with 97/78, no intersex differences were observed in the interaction pattern. In both the genders, 3–4 fold decrease was reported in the systemic exposure of 97/63 upon rifabutin co-administration with 97/78.

Overall, *in-vivo* studies have shown that there was a significant decrease in the systemic exposure of 97/63 upon rifabutin co-administration in both the sexes. However, we hypothesized that there could be multi-fold decrease in the systemic exposure of 97/63, when 97/78 would have been co-administered with rifabutin for long duration, since rifabutin is a moderate inducer of hepatic CYP 3A. Thus, a multiple dose study may be required to estimate and explore these aspects of interactions. The results

of our study thus provide a basic information about the existence of drug interactions between rifabutin and 97/78, which can be used to conduct a multiple dose drug interaction study.

Further, we exercised to find out the mechanism behind the existence of interaction between 97/78 and rifabutin *in-vitro*. *In-vitro* metabolism studies in rat liver microsome revealed that upon rifabutin co-incubation with 97/63, there was 10%–12% increase in the metabolism of 97/63 compared to 97/63 incubation alone. It was found that about 48% of 97/63 get metabolized upon alone incubation, compared to about 60% metabolism of 97/63 upon rifabutin co-incubation. Thus, it could be reasoned that the decreased systemic exposure of 97/63 upon rifabutin co-administration can be in-part attributed to the increased metabolism of 97/63 in presence of rifabutin. However, others factors like interference in the absorption may be one among the reasons responsible for decreased systemic exposure of 97/63.

In-vitro plasma protein binding studies have shown that there was a very strong displacement of 97/63 from plasma proteins when 97/63 was added with rifabutin into rat plasma. The extent of plasma protein binding of 97/63 was found to be decreased from 54% to 55% to a very low value of 6%–8% upon rifabutin addition. It could be speculated from the results of plasma protein bindings that upon displacement of 97/63 from plasma proteins, the increased free drug concentration of 97/63 is more susceptible to extraction by hepatocytes and hence more liable to get metabolized by hepatic CYPs, thus contributing to the decrease in the systemic exposure of 97/63.

From the above discussion, it could be concluded that rifabutin co-administration altered PK parameters of 97/63 in SD rats. A significant decrease was reported in the systemic exposure of 97/63, which indicates that the combination of these two drugs may require some dose adjustments. However, no intersexual influences were reported in the interaction pattern. The results of *in-vivo* interaction studies were well correlated with *in-vitro* studies and attempts were made to explain the mechanism behind the existence of drug interaction. Though this was a single dose interaction study in SD rats, multiple dose drug interaction study can be conducted on the basis of the informations obtained in this study. Further, clinical studies are required to determine the potential of these drug interactions in humans. The results obtained in this study can be extrapolated to humans using proper simulation models keeping in view all the gender and species differences in relation to physio-chemical variations.

Conflict of interest statement

We declare that we have no conflict of interest.

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