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Rifabutin autoinduction is caused by involvement of cytochrome P450 and cholinesterase

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Received June 8, 2007, accepted July 3, 2007

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Pharmazie 63: 156–159 (2008) *doi: 10.1691/ph.2008.7660*

Rifabutin exhibits remarkable autoinduction of its elimination, but the mechanism behind it was not fully known. Our work showed that rifabutin administration increased the metabolism of rifabutin itself in both *in vivo* studies and liver perfusion, and the half-life was decreased by 71.08% and 12.74%, respectively. Further results showed that rifabutin administration increased CYP3A, CYP2D and cholinesterase levels by 87.2%, 57.3% and 65.14%, respectively. The autoinduction phenomenon of rifabutin may, therefore, be attributed to induction of cholinesterase and CYP450 isoenzymes, such as CYP3A and CYP2D.

1. Induction

Rifabutin is an antimicrobial agent which shows a broad spectrum of activity particularly against Gram-positive microorganisms and *Mycobacteria* (Domingo et al. 2005). In addition to being more potent than rifampicin against *Mycobacterium tuberculosis*, rifabutin is effective against *M. leprae* and against *M. avium* complex and other atypical mycobacteria, which are generally resistant to most antibacterial agents including traditional anti-tuberculosis drugs (Breda et al. 1999).

As observed for rifampicin in human volunteers and in patients with tuberculosis, the antibiotic rifabutin exhibits remarkable autoinduction of its elimination during clinical treatment regimens (Breda et al. 1999). Despite its being used as first-line treatment in areas of multidrug resistance, the mechanism behind the autoinduction of rifabutin is still not fully known. Rifabutin is metabolized mainly by CYP450 isoenzymes and cholinesterase (Eugenia et al. 1996). These enzymes are all inducible and their induction may result in rifabutin autoinduction. Alternative explanations for the decrease in rifabutin bioavailability during multiple-dose administration, apart from induction of hepatocellular activity, include induction of P-glycoprotein mediated intestinal exsorption or decreased permeability at the gut wall membrane. Our goal was to investigate the mechanism behind the autoinduction of rifabutin, which can be a guide for predicting the clinical consequences.

2. Investigations and results

2.1. Autoinduction metabolism of rifabutin in vivo

Linear regression equations (peak area ratios versus rifabutin concentration) were $y = (0.1552 \pm 0.0231)$ x-(0.1335 ± 0.0379) $(R^2 = 0.9985 \pm 0.0004)$ with a concentration range of 0–50 mg/ml. The retention times of rifabutin and

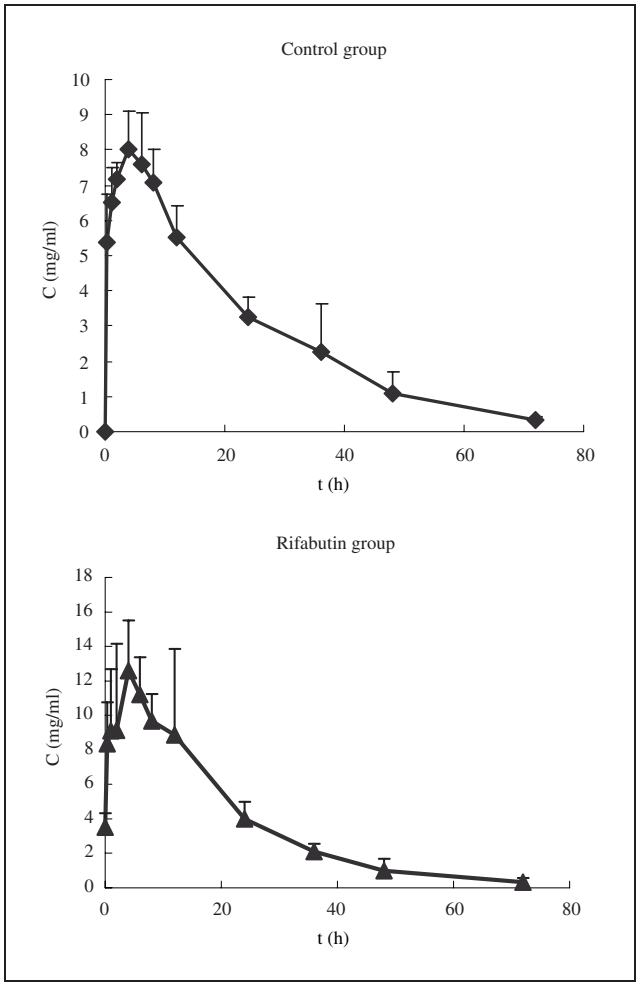

Fig. 1: Concentration-time curve of rifabutin in *in vivo* studies

Table 1: Metabolism parameters *in vivo* studies (n = 6, mean ± SD)

Treatment	$T_{1/2}$/h	CL/L · h^{-1} · kg^{-1}	AUC/mg · L^{-1} · h^{-1}	T_{max}/h
Control	15.70 ± 2.94	1.51 ± 0.13	199.65 ± 18.80	5.33 ± 1.03
Rifabutin	11.16 ± 1.67*	1.09 ± 0.17	277.43 ± 38.45	3.33 ± 1.03

* $p < 0.05$ compared with control

IS were 7.8 min and 11.4 min, respectively. The concentration-time curves are shown in Fig. 1 for *in vivo* studies in the control and rifabutin groups. The metabolism parameters are shown in Table 1. Compared with the control group, the half-life of rifabutin metabolism was decreased by 71.08% in the rifabutin group.

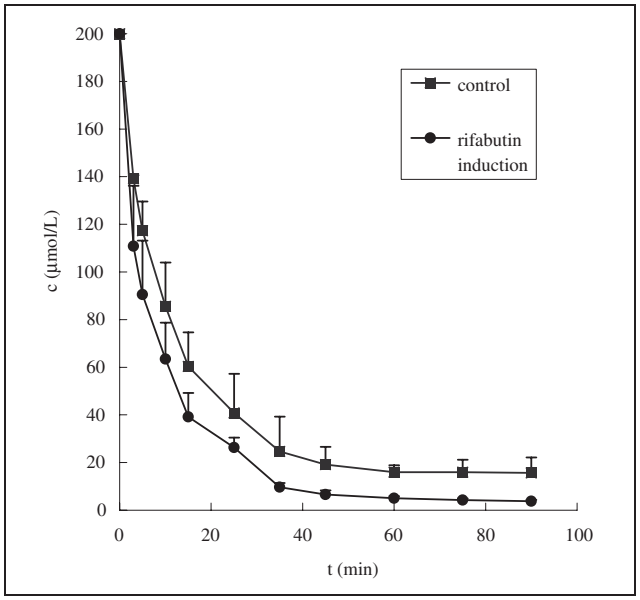

Fig. 2: Concentration-time curve of rifabutin in liver perfusion

Table 2: Metabolism parameters of liver perfusion *in situ* (n = 6, mean ± sd)

Treatment	$T_{1/2}$/min	CL/L · min^{-1} · kg^{-1}	AUC/mg · L^{-1} · min^{-1}
Control	22.64 ± 2.69	0.166 ± 0.034	4195.05 ± 714.46
Rifabutin	13.35 ± 3.29**	0.175 ± 0.059	2057.91 ± 319.63**

** $p < 0.01$ compared with control

Table 3: Microsome protein contents and P450 level in rat liver microsomes

Treatment (7 days, n = 6)	Microsome protein (mg/ml, means ± SD)	Cytochrome P450 level (nmol/mg protein, mean ± SD)
Control	7.67 ± 2.3	4.28 ± 1.16
Rifabutin	6.49 ± 0.51*	17.56 ± 1.79**

Daily treatment dosages equivalent to 300 mg/kg in rifabutin group. Saline used as control. * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control

2.2. Autoinduction metabolism of rifabutin in liver perfusion

The concentration-time curves in liver perfusion for the control and rifabutin groups are shown in Fig. 2. The metabolism parameters are shown in Table 2. The data showed that the half-life and AUC were decreased by 12.74% and 36.59%, respectively.

2.3. Effect of rifabutin on total CYP450 level and isoenzymes

Experimental results showed that treatment of rats with rifabutin (300 mg/kg for 7 days) increased the total CYP level by 410.3% (Table 3). Further results showed that rifabutin mainly increased CYP3A and CYP2D activity by 87.2% and 57.3% respectively at the above dosage, while it had no effect on the other main isoforms such as CYP1A, CYP2C and CYP2E (Table 4).

2.4. Effect of rifabutin on cholinesterase activity

Cholinesterase activities were increased by 65.14% in the rifabutin group, compared with the control group (Fig. 3).

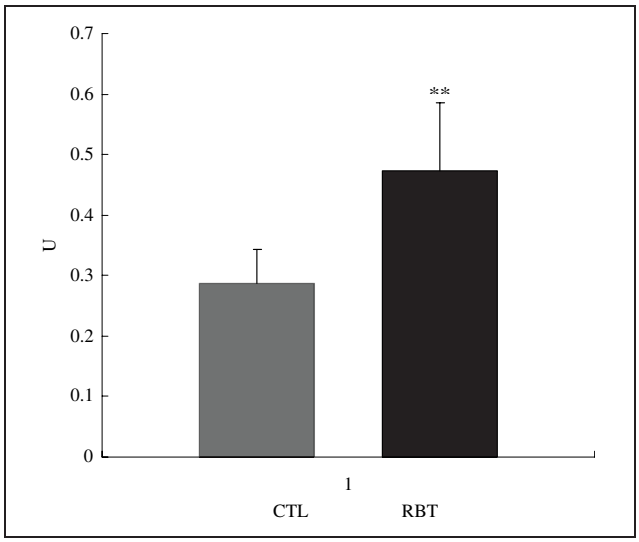

Fig. 3: Determination of cholinesterase activity
** $p < 0.01$ compared with control

Table 4: Effects of rifabutin on CYP isoforms CYP1A, CYP2C, CYP2D, CYP2E and CYP3A

Treatment (7 days, n = 6)	CYP activities (n mol/min.mg protein)				
	1A	2C	2D	2E	3A
Control	11940.2 ± 2560.5	5801.4 ± 415.5	82.9 ± 11.3	1490.7 ± 190.6	12.5 ± 4.0
Rifabutin	14540.1 ± 3589.4	6003.4 ± 502.4	129.0 ± 10.4*	1734.4 ± 300.0	23.4 ± 1.3**

Results represent means ± SD of six determinations. * $p < 0.05$ compared with control, ** $p < 0.01$ compared with control

3. Discussion

Multiple-dose oral administration of rifabutin can be utilised in the therapy of many diseases, such as pulmonary tuberculosis, *Mycobacterium avium* complex in HIV patients and *Helicobacter pylori*. However, multiple-dose oral administration of rifabutin increased the elimination of rifabutin, which is autoinduction of rifabutin. There were many explanations for the decrease in rifabutin bioavailability during multiple-dose administration, including induction of hepatocellular activity, induction of P-glycoprotein mediated intestinal exsorption or decreased permeability at the gut wall membrane. However, it is very important to know the exact mechanism in order to predict the clinical consequences.

Compared with the control group, the half-life of rifabutin was decreased by 71.08% in the rifabutin group. This meant that rifabutin exhibited remarkable autoinduction of its own elimination in *in vivo* studies. Further results also showed that it exhibited remarkable autoinduction in liver perfusion. The half-life and AUC of rifabutin in the rifabutin group were decreased by 12.74% and 36.59%, respectively. The above data clearly showed that rifabutin metabolism exhibited remarkable autoinduction both in *in vivo* studies and at the organic level. The organic metabolism in liver perfusion was not affected by induction of P-glycoprotein mediated intestinal exsorption or decreased permeability at the gut wall membrane, therefore, these data indicated they were not the correct explanations of the autoinduction phenomenon.

The liver contains a series of microsome hemoproteins called cytochromes P450 (CYP) that play an important role in the metabolic oxygenation of a variety of lipophilic chemicals including drugs, pesticides, food additives, and environmental pollutants. Many of the individual isoenzyme forms of cytochrome (particulary including CYP1A (13%), CYP2C (20%), CYP2D (2%), CYP2E (7%), and CYP3A (29%)) exhibit distinct substrate specificities that can be inhibited or induced by the compounds (Cedric et al. 2004). A previous report elucidated that rifabutin increased CYP3A activity while induction of other isoenzymes had not yet been studied in the case of rifabutin (Strolin 1995). Our work systematically evaluated the effect of rifabutin on CYP450 isoenzymes. In this paper, rifabutin administration increased the total CYP450 activity to 410.3% of the control in a 7-day period. CYP450 isoenzyme activities were then evaluated by the formation rate of specific metabolites. Our work showed firstly that rifabutin administration increased the CYP2D level by 57.3% in addition to increasing the CYP3A level by 87.2% which is in agreement with a previous report. It does not induce, or only to a very minor extent, CYP1A, CYP2C, or CYP2E. Therefore, the concomitant use of rifabutin and other drugs metabolized by CYP3A and CYP2D should be avoided if no interaction information is available.

Cholinesterase (EC 3.1.1.8) is synthesized mainly in hepatocytes and released into the blood (Ogunkeye and Roluga 2006). Serum cholinesterase activity is increased as a result of increased synthesis. Rifabutin administration increased the cholinesterase level of serum by 165.14%, which indicated that the synthesis of cholinesterase in liver was greatly increased after rifabutin administration. No previous paper has reported rifabutin administration increasing the cholinesterase level.

In conclusion, the autoinduction of rifabutin was shown in both *in vivo* studies and liver perfusion, and rifabutin was found to induce not only CYP3A and CYP2D levels but also cholinesterase activity. The autoinduction phenomenon of rifabutin may, therefore, be mainly attributed to induction of cholinesterase and CYP450 isoenzymes.

4. Experimental

4.1. Chemicals and apparatus

Rifabutin (4-dexoxo-3,4,2-spiro-(*N*-isobutyl-4-piperidyl)-(1*H*)-imidazo(2,5-dihydro)-rifamycin S) was purchased from Chengdu Yuyang High-Tech Ltd (Chengdu City, China). Dextromethorphan (DEX), dextrorphan (DM), 3-methoxymorphian (3-MM), phenacetin (PHE), acetaminophen (ACE), tolbutamide (TOL), 4-hydroxy-tolbutamide (4-OH-TOL), chlorzoxazone (CHOL), 6-hydroxychlorzoxazone (6-OH-CHOL), glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH) and β-NADP ($NADP^+$) were all purchased from the Sigma Chemical Corp Ltd, USA. Methanol and acetonitrile were all purchased from Dikmapure Tech Inc, USA. All other reagents were purchased from domestic chemical companies.

Sample separations were performed on an Agilent-1100 HPLC with 1100 UV detector and 1100 auto injection (Agilent Corp Ltd, USA). Separation was achieved using an Agilent Symmetry ODS-C_{18} column (5 μ, ID 4.6×250mm) with a guard cartridge (4.6×10 mm). Other apparatus included a preparative ultracentrifuge (Hitachi-CP100MX, Japan), Masterflex (Barnant Company, model 77120-70, USA), UV-Vis Recording Spectrophotometer (Shimadzu, UV-2201, Japan), CentriVap Concentrator (Labconco company, USA) and Automatic Biochemical Analyzer (Hitachi-7180, Japan).

4.2. Animals

Male Wistar rats (200–250 g) were obtained from the Laboratory Animals Department of the Capital Medical University. Animals were kept on a 12 h light-12 h dark cycle, and had free access to food and water throughout the experiment. All animal handling procedures were performed in accordance with the principles of animal care outlined by the Chinese Academy of Medical Sciences.

4.3. Autoinduction metabolism of rifabutin in in vivo studies

Animals were divided into 2 groups at random, with 6 in each group, a rifabutin group (300 mg/kg by gastrogavage for 7 days, suspended in saline) and a control group. The control group received the same amount of normal saline for 7 days. The day before the pharmacokinetic study day, a cannula was implanted into the right jugular vein of each animal, under ether anesthesia. Venous blood was collected. Blood samples (0.25–0.3 ml) were obtained at time 0 (prior to drug administration), and 0.33, 1, 2, 4, 6, 8, 12, 24, 36, 48 and 72 h following a single administration of rifabutin (300 mg/kg). Blood samples were centrifuged immediately for the separation of plasma and were stored at −80 °C prior to drug assay.

Rifabutin concentrations in these samples were measured by a HPLC method previously reported with slight modification (Yau et al. 1996). The internal standard, diazepams was spiked into the samples and the mixture was vortexed for 60 s. All samples were then treated with dichloromethane at a ratio of 10 : 1. This was followed by centrifugation at 12000 rpm for 10 min. A volume of 0.8 ml of the supernatant was transferred to a clean 1.5 ml centrifuge tube and then concentrated by CentriVap Concentrator at 35 °C. The residue was reconstituted with 0.1 ml of mobile phase and an aliquot was analyzed using HPLC. The mobile phase employed for the plasma consisted of phosphate buffer (pH 4.1, 0.05 M)-acetonitrile (51.3 : 48.7, v/v). The flow rate of the mobile phase was set at 1.0 ml/min.

4.4. Autoinduction metabolism of rifabutin in liver perfusion

The treatment of the rats was the same as above. On the eighth day, rats were prepared for liver perfusion.

Livers of rats were perfused using the perfusion recirculating apparatus described by Thomas and Steffen (2007). The perfusion medium (Krebs-Henseleit buffer, final volume 50 ml) contained: 125 mM NaCl, 2.6 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 31 mM $NaHCO_3$ and 5.5 mM glucose. The perfusion medium was in equilibrium with $O_2 + CO_2$ (95 : 5), following which rifabutin was added to a final concentration of 0.2 mM. At different time periods after the addition of rifabutin, 0.4 ml samples of perfusate were taken successively (after 1, 3, 5, 10, 15, 25, 35, 45, 60, 75, 90 min) and then 100 μl 7% perchloric acid were added to precipitate protein. The supernatant of each sample was transferred to a plastic vial for HPLC analysis. The HPLC conditions for rifabutin were the same as above.

4.5. Effect of rifabutin on total CYP450 level and isoenzymes

Animals were divided into 2 groups at random, with 6 in each group, a rifabutin group (300 mg/kg by gastrogavage for 7 days, dissolved in saline)

Table 5: CYP probe substrate, enzyme reaction and metabolites

Isoenzyme	Probe substrate	CYP-catalysis	Metabolite
CYP1A2	Phenacetin	O-deethylase	Acetaminophen
CYP2C9	Tolbutamide	Methylhydroxylation	4-Hydroxytolbutamide
CYP2D6	Dextromethorphan	O-Demethylation	Dextrorphan
CYP2E1	Chlorzoxazone	Hydroxylation	6-Hydroxychlorzoxazone
CYP3A	Dextromethorphan	N-Demethylation	3-Methoxymorphinan

and a control group. The control group received the same amount of normal saline for 7 days. On the eighth day, the rats were all prepared for liver microsome. The method of liver microsome preparation was as described by Yuri et al. (2007).

Total CYP content was measured by the method of Omura and Sato (1964). The activity was determined with a Shimadzu UV-2201 UV-Vis recording spectrophotometer. Microsome protein was determined by the method of Lowry et al. (1951), using Bovine Serum Albumin as standard solution.

In this paper, some special substrates were incubated and their metabolites were measured. The rate of formation of these metabolites can be used to evaluate the activities of the CYP450 isoenzymes. The effect of rifabutin on CYP450 isoenzymes was evaluated by comparing the rate of formation of these metabolites in the rifabutin and control groups. Suitable CYP isoform-selective substrates have been identified and are commonly used as probes for the role of specific CYP enzymes in drug metabolism (Table 5). PHE, TOL and CHOL are specific substrates for CYP1A, CYP2C and CYP2E, respectively (Tian et al. 2002; Corinne et al. 2002). DEX is reduced rapidly to DM after oral absorption by the polymorphic CYP2D. In a parallel pathway, it is N-demethylated to 3 MM, which is catalyzed by CYP3A (Minno et al. 2004). To ensure the selectively of these markers and represent the concentration of the drug *in vitro*, a substrate concentration near its K_m value was utilized in all incubation samples in the assay. Liver microsome from the rifabutin group or the control group was used. The incubation volume was 0.5 ml, containing 1.0 mg protein per tube, 100 mM potassium dihydrogen phosphate buffer and a NADPH-generating system. The samples were preincubated for 10 min prior to the addition of G-6-PDH, following 3 min incubation at 37 °C in a shaking water bath. The sample preparation was the same as in the above section. The difference in isoform activity between the rifabutin and control groups was determined by the quantity of metabolite formed.

The HPLC conditions were determined according to Jingcheng Tang et al. (2006): For PHE and ACE (CYP1A) the mobile phase was 0.1% triethylamine solution (pH 3.85) acetonitrile (80 : 20, v/v). UV detection was at 254 nm and the retention times were 14.8 min and 3.6 min, respectively. For TOL and 4-OH-TOL (CYP2C) the mobile phase was 0.1% triethylamine solution (pH 3.85) acetonitrile (60 : 40, v/v). UV detection was at 230 nm and the retention times were 12.5 min and 4.3 min respectively. For DEX, DM and 3MM (CYP2D and CYP3A) the mobile phase was 0.1% triethylamine solution (pH 4.85) acetonitrile (76 : 24, v/v). UV detection was at 277 nm and the retention times were 13.1 min, 12.3 min and 4.4 min respectively. For CHOL and 6-OH-CHOL (CYP2E) the mobile phase was water with acetic acid (pH 3.18) acetonitrile (67 : 33, v/v). UV detection was at 287 nm and the retention times were 10.7 min and 4.4 min respectively.

4.6. Effect of rifabutin on cholinesterase activity

The blood samples from the rifabutin group and the control group were utilized for measuring cholinesterase activities. The activities were determined with an Automatic Biochemical Analyzer (Hitachi-7180, Japan).

4.7. Pharmacokinetic and statistical analysis

The pharmacokinetic parameters were analyzed using the 3P87 program (recommended by the Chinese Pharmacological Society) by non-compartmental analysis. All data are presented as means ± SD. Statistical comparisons were made by SPSS11.5 software. Values of $p < 0.05$ were regarded as significant.

Acknowledgements: This work was jointly supported by the National Natural Science Foundation of China (29973005) and the Beijing Municipal Commission of Education (KM200410025007).

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