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Effect of rifampicin & isoniazid on cytochrome P-450 in mycobacteria

Geetha Ramachandran & Prema Gurumurthy

Tuberculosis Research Centre (ICMR), Chennai, India

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Background & objectives: **Rifampicin and isoniazid are the most important first line drugs used in the treatment of tuberculosis. These drugs are also used in combination with other medications to treat co-infections. It, therefore, becomes important to study the effect of these drugs on the drug metabolizing system, namely, cytochrome P-450, not only in the host but also in the bacteria. We report the effect of rifampicin and isoniazid on the cytochrome P-450 activity in** *Mycobacterium smegmatis* **and** *M tuberculosis* **$H_{37}Rv$.**

Methods: **Subinhibitory concentrations of rifampicin and isoniazid were added to the organisms after they had attained the growth phase and cytochrome P-450 activity was estimated in the membranous fractions of the bacteria at different time points.**

Results: **Rifampicin was able to significantly enhance cytochrome P-450 in both** *M. smegmatis* **and** *M. tuberculosis* **$H_{37}Rv$. Isoniazid was found to inhibit cytochrome P-450 in** *M. tuberculosis* **$H_{37}Rv$, While there seemed to be no effect in** *M. smegmatis.*

Interpretation & conslusion: **We report here the effect of rifampicin and isoniazid on mycobacterial cytochrome P-450. These findings are similar to those found in eukaryotic organisms. The role of mycobacterial cytochrome P-450 in the metabolism of drugs within the bacteria needs to be elucidated.**

Key words Cytochrome P-450 - isoniazid - mycobacteria - rifampicin

Standard short course regimens for tuberculosis are used world-wide. Unfortunately, the emergence of multiple drug - resistant tuberculosis in many parts of the world is leading to a diversification of drug regimens and to the use of drugs that are more toxic *per se* and more likely to interact with other drugs. The mechanisms by which antimicrobial agents alter the biotransformation of other drugs is increasingly understood to reflect inhibition or induction of specific cytochrome P-450 enzymes[1]. The Cytochrome P-450 group of proteins function as terminal monooxygenases in the microsomal mixed function oxidase system and bring about biotransformation and detoxification of a wide variety of drugs.

Rifampicin (RMP) is usually administered for 4 to 6 months with other anti-tuberculosis drugs or medications from other classes. A potential for drug interactions often exists because RMP is a potent inducer of hepatic drug metabolism, as evidenced by a proliferation of smooth endoplasmic reticulum[2] and an increase in the Cytochrome P-450 content in the liver[3]. Rifampicin can therefore, reduce the efficacy of several therapeutically important drugs by accelerating systemic elimination or by increasing

hepatic metabolism. This may result in sub-therapeutic concentrations of the accompanying drugs and failure of therapy.

Apart from treating tuberculosis, the use of isoniazid (INH) has increased over the past years, largely as a result of the co-epidemic of HIV infection. Isoniazid is given to patients who are also prescribed other medications for critical illness. Although it has best been recognised as an inhibitor of drug metabolism. it has a biphasic effect of inhibition - induction on a particular cytochrome P-450 isozyme[4].

Most reports that have so far emerged on drug interactions involving RMP and INH and cytochrome P-450 are based on animal and human studies. However, the role of bacterial cytochrome P-450 should not be ignored as the drugs act directly on the bacterial protein. We, therefore studied the *in vitro* effect of RMP and INH on cytochrome P-450 in *M. tuberculosis* $H_{37}Rv$ and a nontuberculous mycobacterial species, *viz., M. smegmatis.*

Material & Methods

Chemicals : Rifampicin, isoniazid, lysozyme, ß mercaptoethanol and phenyl methyl sulphonyl fluoride (PMSF), from Sigma Chemical Co., USA, and sodium dithionite from Riedel - DeHaan Ag, Hannover, were used in this study. All other chemicals used were of analytical grade.

The effect of RMP and INH on the activity of cytochrome P-450 was analysed in *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$ under *in vitro* conditions. The organisms were grown in Sauton's liquid medium at 37°C in an incubator shaker. When the bacteria had attained the growth phase, subinhibitory concentrations of RMP (12 µg/ml for *M. smegmatis* and 0.125 and 0.25 µg/ml for *M.* tuberculosis $H_{37}Rv$) and INH (25 µg/ml for *M. smegmatis* and 0.05 and 0.10 µg/ml for *M. tuberculosis* $H_{37}Rv$) were added to separate flasks containing the medium with the organisms. Subinhibitory concentrations of the drugs were selected based on the minimum inhibitory concentration (MIC) of the drug against the two organisms. Stock solutions of the drugs were prepared under sterile conditions. Incubation of the flasks was continued at 37°C and the bacterial cells were harvested by centrifugation at the end of 1,3 and 6 h. Organisms from the flasks not containing the drugs were processed simultaneously and served as control for both the drugs, for each organism and at all 3 time points.

Preparation of membranous fraction of bacteria and estimation of cytochrome P-450 activity: As cytochrome P-450 is a membrane bound protein the membranous fraction of the bacteria was prepared and cytochrome P-450 activity was estimated according to the method standardized in our laboratory[5]. The activity of cytochrome P-450 was determined by suitably diluting the samples in 10 m *M* potassium phosphate buffer, *p* H 7.4 followed by addition of sodium dithionite. The reduced protein was made to bind with carbon monoxide and the spectrum was recorded between 500 and 300 nm in a dual beam spectrophotometer. The cytochrome P-450 concentration was calculated by taking the difference in the optical density between 450 and 490nm and using molar extinction coefficient of 9 1 mm/cm and expressed as nmol/mg protein. Studies with RMP and INH in both the bacteria were done 3 times, each in duplicate.

Student's t-test (unpaired and paired) was used to calculate the significance of the results and the level of significance was taken at 5 per cent.

Results

The cytochrome P-450 activity in *M. smegmatis* following the addition of subinhibitory concentrations of RMP and INH is shown in Table I. The cytochrome P-450 levels significantly *(P<0.05)* increased in the presence of 12.0 µg/ml of RMP at all 3 time points tested when compared with the corresponding control values. The difference in the cytochrome P-450 activity in the presence of RMP between first and third hour was significant *(P<0.*05). However, the activity increased only marginally at the sixth hour when compared to the third hour and this difference was not significant *(P=0.103).* In the case of INH, the cytochrome P-450 activity in *M. smegmatis* remained unaltered

even at 6 h. and was not significantly different from control values.

The cytochrome P-450 activity obtained in *M. tuberculosis* $H_{37}Rv$ following the addition of 0.125 and 0.25 µg/ml of RMP and 0.05 and 0.10 µg/ml INH is shown in Table 11. At both concentrations, RMP was able to significantly *(P<0.001)* induce cytochrome P-450 activity at the third and sixth hour, as compared to controls. However, the difference in the cytochrome P-450 level at first hour between the control and drug treated bacteria was not Significant *(P=0.202).* Induction of cytochrome P-450 activity appeared to increase with increase in the concentration of RMP.

Isoniazid (0.05 and 0.10 µg/ml) did not cause any significant change in the cytochrome P-450 activity in *M. tuberculosis* $H_{37}Rv$ at 1 and 3 h. However. at the sixth hour, both concentrations of the drug significantly *(P<0.05)* caused a lowering in the cytochrome P-450 levels, compared to controls. Increasing the concentration of INH did not cause any increase in the cytochrome P-450 activity.

Discussion

Drug interactions involving cytochrome P-450 generally result from one of two processes viz., enzyme induction or inhibition. The inhibition of drug metabolism by competition for the same enzyme may result in an undesirable elevation in plasma drug concentrations. Enzyme induction on the other hand may result in a reduction in pharmacological effect because of increased drug metabolism. Thus, induction / inhibition of cytochrome P-450 is of clinical importance for both therapeutic and toxicological reasons. In the safety evaluation of drugs and other chemicals. it is hence important to evaluate their possible inducing and inhibitory effects on the enzymes of drug metabolism.

Table 1. Cytochrome P-450 levels (nmol/mg protein) in *M. smegmatis* after addition of RMP and INH

Time after incubation (h)	RMP		INH	
	Control	Test (12.0 µg/ml)	Control	Test (25.0µg/ml)
I	0.39±0.06	0.57±0.10*	0.43±0.02	0.37±0.06
3	0.27±0.03	0.72±0.01*†	0.36±0.04	0.4±0.04
0	0.33±0.02	0.76±0.03*†	0.32±0.04	0.30±0.04

The values are mean±SD of 6 experiments. * *P<0.05* compared to control, †*P<0.05* compared to 1h. RMP, rifampicin INH, isoniazid

Table II. Cytochrome P-450 levels (nmol/mg protein) in *M. tuberculosis* $H_{37}Rv$ after addition of RMP and INH

Time after incubation (h)	RMP			INH		
	Control	Test l (0.125 µg/ml)	Test 2 (0.25 µg/ml)	Control	Test l (0.05 µg/ml)	Test 2 (0.10µg/ml)
l	0.58±0.02	0.64±0.09	0.42±0.03	0.25±0.02	0.37±0.06	0.37±0.06
3	0.55±0.04	0.94±0.02*	1.22±0.18*	0.42±0.06	0.42±0.06	0.40±0.06
6	0.58±0.01	0.84±0.09*	1.02±0.05*	0.27±0.06	0.18±0.05**	0.18±0.05**

The values are mean±SD of 6 experiments. * *P<0.001* compared to control, **P<0.05* compared to control, RMP, rifampicin; INH. isoniazid

Based on the results obtained in this study, it appears that RMP is able to significantly induce cytochrome P-450 activity in *M.* smegmatis and *M. tuberculosis* $H_{37}Rv$. This effect was felt at the third hour itself after exposure to the drug. It was further observed that RMP induction of cytochrome P-450 is dose dependent, a condition similar to that observed in other systems[6]. These results are in agreement with previous studies done in animals and humans[2,3,6-8]

It, thus, becomes clear that when RMP is given along with drugs that are metabolised through the cytochrome P-450 system, it should be borne in mind that the therapeutic efficacy of such drugs may be lowered due to enhanced metabolism and clearance. This observation gains significance with the use of antiretroviral drugs along with RMP. Protease inhibitors undergo metabo1ism through the cytochrome P-450 pathway and RMP is likely to lower the bioavailability of these drugs[9].

The results of this study also demonstrate that INH inhibited cytochrome P-450 of *M. tuberculosis* $H_{37}Rv$ *in vitro.* There are some reports that have shown INH as an inducer of the hepatic microsomal enzyme system[10]. It is quite possible that a single substance can act both as an inducer or as an inhibitor of the monoxygenase system depending on the dose used[11]. Oxidative metabolism represents a major route of elimination for many drugs and because many drugs can compete for the same enzyme. inhibition of cytochrome P-450 is one of the main reasons for drug interaction. The mechanism by which INH inhibits cytochrome P-450 could be due to the fact that the drug reaches a concentration that is critical for the formation of the catalytically inactive cytochrome P-450 - drug complex. Thus, when INH is given along with drugs that are metabolised through the cytochrome P-450 system, it is essential to periodically monitor for toxic side effects arising due to decreased metabolism of the accompanying drugs. Inhibition of cytochrome P-450 can also reduce clinical efficacy if the drug is a prodrug requiring metabolic activation to achieve its effect and activation.

Although RMP was able to significantly induce cytochorome P-450 in both *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$, INH could exert its inhibitory effect in only the latter. This selective inhibition could be due to poor penetration of the drug into *M. smegmatis.*

The effects of RMP and INH on cytochrome P-450 of eukaryotic origin has been extensively reported. However, the effect of these drugs on the bacterial protein has not been looked into. This seems to be the first report on the effect of RMP and INH on cytochrome P-450 in mycobacteria. Although 22 genes coding for different isoforms of cytochrome P-450 have been identified in the *M. tuberculosis* genome[12], the function of these genes have not been well understood. It is quite possible that the cytochrome P-450 present in *M. tuberculosis* also plays a role in bringing about metabolism of the drugs while they are within the bacteria. Studies at the molecular level are necessary to throw light on this aspect.

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Reprint requests : Dr Geetha Ramachandran, Department of Biochemistry, Tuberculosis Research Centre (ICMR), Mayor V.R. Ramanathan Road, Chetput, Chennai 600031, India