

Piperine augments transcription inhibitory activity of rifampicin by severalfold in *Mycobacterium smegmatis*

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A 24 : 1 (w/w) mixture of antibiotic rifampicin and a plant product piperine show remarkable growth-inhibitory effect on *Mycobacterium smegmatis*, and this inhibition is higher than that of rifampicin alone. Interestingly, piperine alone, even at higher concentration, does not inhibit the growth of mycobacteria. As RNA polymerase is the site of action of rifampicin, the enzyme was purified from *M. smegmatis* and the mixture of rifampicin and piperine was found to abrogate non-specific transcription catalysed by *M. smegmatis* RNA polymerase. Here too the effect is higher than rifampicin alone and piperine shows no effect independently. When RNA polymerase was purified from a rifampicin-resistant strain of *M. smegmatis*, the enzymatic activity, otherwise resistant to rifampicin, significantly decreases in the presence of piperine along with rifampicin. Modelling studies have been carried out to explain this 'bioenhancing' behaviour of piperine.

THE mechanistic sophistication of the antibiotic rifampicin to inhibit bacterial transcription along with its therapeutic success to control mycobacterial infection has made it a subject of critical research since long. It is now well established that rifampicin inhibits transcription of DNA template exclusively by binding to the *b*-subunit of RNA polymerase^{1,2}. All the rifampicin-resistant mutants map in three different clusters in the *b*-subunit of *E. coli* RNA polymerase³ and it is expected to be not very different in mycobacterial transcription machinery.

In our attempts to find out natural products that could mimic the action of rifampicin in order to inhibit bacterial transcription, we have obtained piperine, a black pepper extract which exhibits diverse biological activities, many of which may have potential therapeutic use⁴⁻⁶. Interestingly, piperine alone does not show any noticeable activity to inhibit growth of *Mycobacterium smegmatis*; however, when added along with rifampicin, piperine has been found to augment the growth inhibition potential of rifampicin severalfold. We show here that piperine along with rifampicin abrogates transcrip-

tion catalysed by purified *M. smegmatis* RNA polymerase and the degree of inhibition is appreciably higher than that of rifampicin alone. This effect, termed as 'bioenhancing' effect by us, was observed even with RNA polymerase isolated from rifampicin-resistant *M. smegmatis*.

Materials and methods

Rifampicin was obtained from Sigma, USA and piperine (% purification > 96), purified from the black pepper⁴ was obtained as a gift from Regional Research Laboratory, Jammu, India. The structure of rifampicin and piperine are given in Figure 1. In all the following experiments we used either the pure rifampicin or a mixture of 24 : 1 (w/w) rifampicin : piperine and termed it as compound A.

Enzymes

M. smegmatis MC²155 was grown in Middlebrook 7H9 medium up to the mid-log phase and RNA polymerase was purified from approximately 10 g of wet weight cells following essentially the protocol of Burgess and Jendrisak⁷. In short, cells were treated with lysozyme and sodium deoxycholate and subsequently disrupted with continuous-flow French Press. The soluble fraction was precipitated with 0.35% polyethyleneimine. Active RNA polymerase was recovered from the polyethyleneimine pellet with TGED buffer (10 mM *Tris*-HCl, pH 8, 5% glycerol, 1 mM EDTA, 1 mM dithiothreitol) containing 1 M NaCl. The enzyme was precipitated with 35% ammonium sulphate and fractionated over Heparin-Sepharose (Pharmacia) column as well as Bio-gel 150 (Bio-Rad) column⁸. Peak fractions containing RNA polymerase were identified by optical density measurements and assayed as described below.

In vitro multiple round transcription assay

A quantitative assay to measure RNA polymerase activity was performed as described by Lowe *et al.*⁹, with

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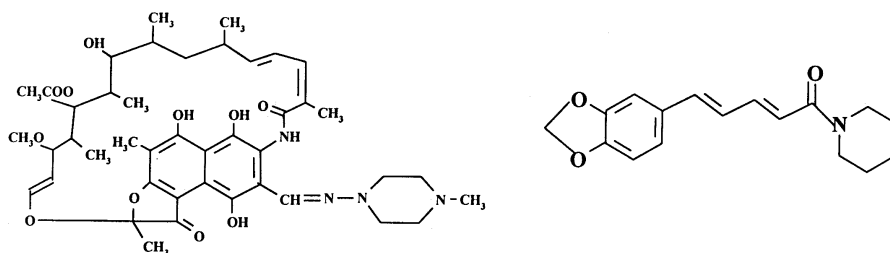


Figure 1. Structures of rifampicin (left) and piperine (right).

minor modifications. A 40 μl assay mixture contained 40 mM Tris HCl, pH 8, 200 mM NaCl, 10 mM MgCl_2 , 1 mM EDTA, 14 mM *b*-mercaptoethanol, 200 μM each of ATP, CTP and GTP, 50 μM UTP, 1 μCi of [^3H] UTP and 1.5 μg of calf thymus DNA. The enzyme was incubated in the assay mix for 20 min and spotted directly onto DE-81 filters, which were presoaked with 5 mM EDTA. In one set of experiments, an incubation of different concentrations of antibiotic rifampicin with RNA polymerase at 37°C for 30 min was included before adding the substrates. Assays were also done with different concentrations of the compound A in the same way as for rifampicin. The dried filters were washed twice with 5% Na_2HPO_4 , thrice with water, and then once with ethanol and dried. Subsequently, the filters were placed into scintillation vials containing the toluene-based scintillation fluid and counted by scintillation spectrometry⁸.

Rifampicin-resistant RNA polymerase

M. smegmatis (MC² 155) cells were exposed to UV irradiation (UV lamp, Philips-30 watts/G30T8) for 30 s and plated on the MB7H9 agar with increasing concentration of rifampicin. Concentration of rifampicin ranged from 10 to 80 $\mu\text{g}/\text{ml}$ (MIC of rifampicin is ~ 50 $\mu\text{g}/\text{ml}$). Colonies were subsequently plated in the same way in higher concentrations of the antibiotic. RNA polymerase was purified from *M. smegmatis* growing at 200 $\mu\text{g}/\text{ml}$ of rifampicin following the protocol of Burgess and Jendrisak⁷.

Results and discussion

Figure 2a shows the effect of rifampicin and compound A on the growth of *M. smegmatis* on agar plate containing 7H9 medium with 2% glucose as the sole carbon source. It can be seen from the figure that rifampicin alone shows much less growth-inhibitory effect on *M. smegmatis* compared to that of compound A which has very little of piperine present along with rifampicin. In fact, a complete inhibition of growth was observed when 10 $\mu\text{g}/\text{ml}$ of compound A was added in the growth

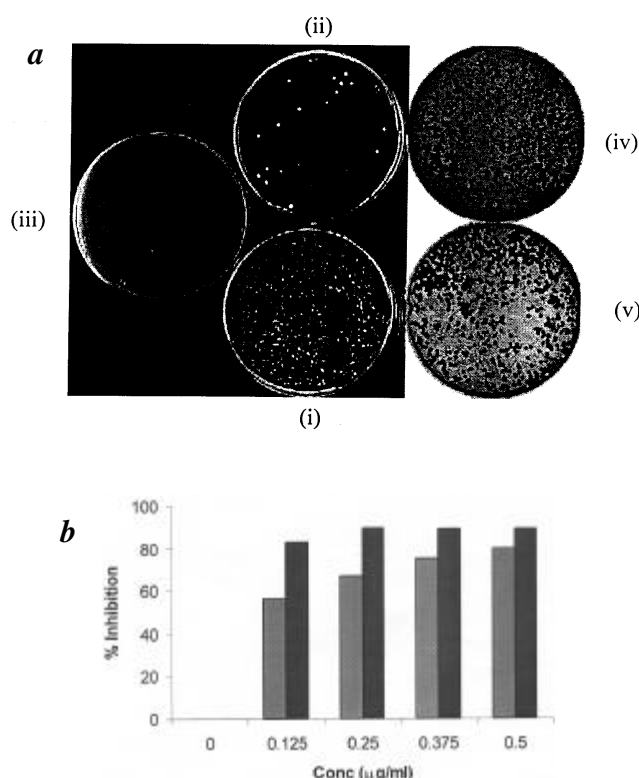


Figure 2. a, Effect of compound A and rifampicin on the growth of *M. smegmatis*. Concentrations of compound A – (i), 0.10 $\mu\text{g}/\text{ml}$; (ii), 5 $\mu\text{g}/\text{ml}$; (iii), 10 $\mu\text{g}/\text{ml}$ and rifampicin – (iv); 20 $\mu\text{g}/\text{ml}$; (v) 50 $\mu\text{g}/\text{ml}$; b, Effect of rifampicin () and compound A () on wild-type RNA polymerase from *M. smegmatis*.

medium, which contains 9.6 μg rifampicin and 0.4 μg piperine (Figure 2a). Rifampicin alone at the same concentration does not have such a dramatic effect. Piperine alone shows no inhibitory effect for the growth of *M. smegmatis* even at a higher concentration of 50 $\mu\text{g}/\text{ml}$ (not shown).

We have mentioned earlier that the *b*-subunit of bacterial RNA polymerase is the target of rifampicin and thus we decided to check the consequence of the addition of compound A in a typical *in vitro* transcription assay catalysed by purified *M. smegmatis* RNA polymerase over non-specific template. It can be seen from Figure 2b that the difference in the inhibitory potential

of rifampicin and compound A is most pronounced at a lower concentration. Again, piperine alone does not have any effect on the *in vitro* transcription assay catalysed by *M. smegmatis* RNA polymerase (not shown).

The results presented above prompted us to ask whether compound A would show its effect on the transcription activity of RNA polymerase isolated from a strain of *M. smegmatis*, which exhibits rifampicin resistant phenotype. Rifampicin-resistant *M. smegmatis* was generated in the usual way with increasing concentration of rifampicin as described in the previous section. RNA polymerase from such strains was isolated following the same protocol as that of wild type enzyme. Figure 3a shows that even at a high concentration of 10 $\mu\text{g/ml}$ of rifampicin, RNA polymerase isolated from rifampicin-resistant strain shows only 15% inhibition of its activity, whereas wild-type RNA polymerase was sensitive to even much lower concentration of rifampicin as expected. On the contrary, compound A having only a small concentration of piperine present along with rifampicin completely abolishes the transcriptional activity of rifampicin resistant RNA polymerase (Figure 3b). However, the effect of piperine to inhibit the growth of rifampicin-resistant mutant of *M. smegmatis* is noticeable only at a higher concentration.

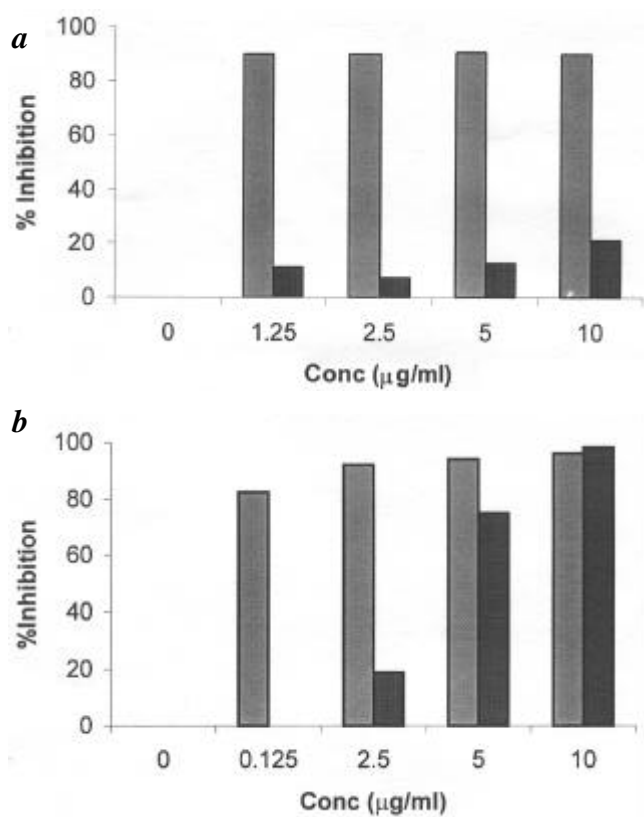


Figure 3. *a*, Effect of rifampicin on wild-type () and rifampicin-resistant mutant () RNA polymerase from *M. smegmatis*; *b*, Effect of compound A on wild-type () and rifampicin-resistant () RNA polymerase from *M. smegmatis*.

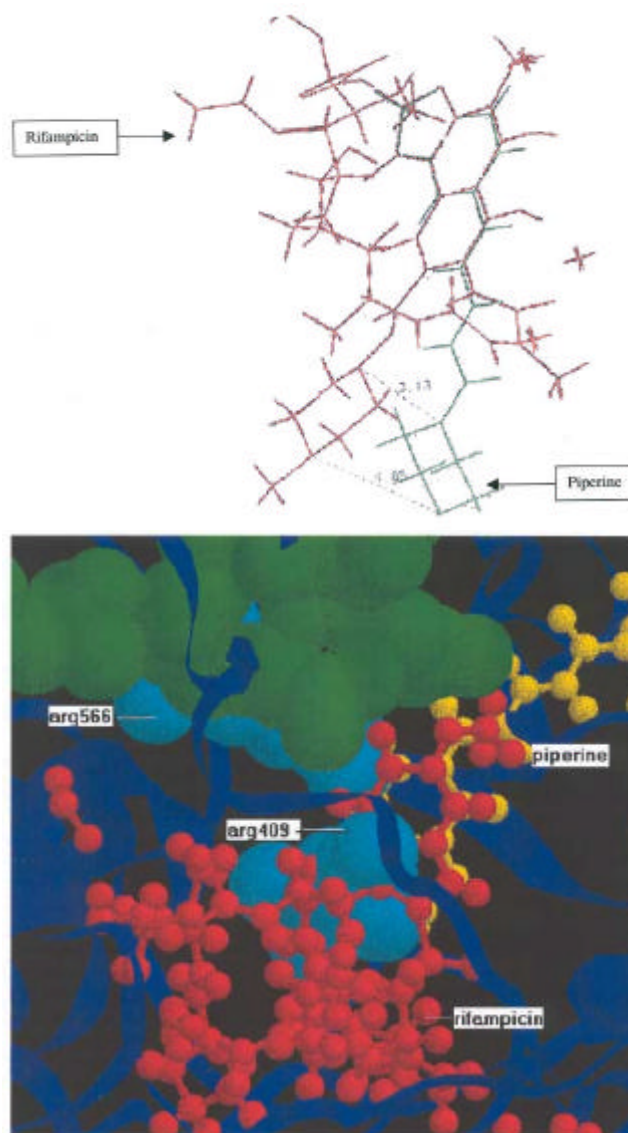


Figure 4. *a*, Superimposition of the structures of rifampicin and piperine; *b*, Molecular modelling of rifampicin and piperine using Insight II package. Red, rifampicin; yellow, piperine; Arg 566 and Arg 409 are shown in the light blue colour. Rest of the moieties (green and dark blue) is due to the *b*-subunit backbone.

This result was surprising to us, particularly in the light of the observation that piperine alone has no effect on mycobacterial growth or transcriptional activity of its RNA polymerase. As *b*-subunit of RNA polymerase is the only target of rifampicin, we hypothesized that piperine enhances the binding ability of rifampicin to RNA polymerase. The inhibition of growth of mycobacteria in the presence of compound A perhaps is due to its effect on RNA polymerase, although other modes of inhibition cannot be ruled out. However, inhibition of transcription of the purified enzyme in the presence of both rifampicin and compound A suggests that they may

have the same site of action over the enzyme. Figure 4 *a* depicts superimposition of the structures of rifampicin and piperine, which indicates stacking interaction between the aromatic residues of both the compounds. We attempted to model the binding of both rifampicin and piperine together on RNA polymerase with the help of Insight II package of molecular modelling. The crystal structure of *Thermus aquaticus* RNA polymerase is now known¹⁰ and we have used the coordinates of this enzyme to model the rifampicin and compound A binding site. Although there are substantial homologies among different subunits of RNA polymerase from *T. aquaticus* and *M. smegmatis*, it should be kept in mind that they are from different species and Figure 4 *b*, at its best, represents only the binding of compound A to *T. aquaticus* RNA polymerase.

The location of rifampicin in the **b**-subunit of *E. coli* RNA polymerase, which has significant homology with that of *T. aquaticus* RNA polymerase has been worked out in great detail over the last several years^{3,10,11}. There are three clusters of amino acids at the **b**-subunit of RNA polymerase which undergo mutations in rifampicin resistant strain of *E. coli*. Two amino acids R-566 and R-409 are known to be participating directly in the rifampicin pocket. Figure 4 *b* shows that both rifampicin and piperine could be accommodated in the pocket through stacking interaction between them. This may help to augment the transcription inhibitory activity of rifampicin. We have no explanation to offer on how compound A at higher concentration can inhibit tran-

scription catalysed by rifampicin-resistant RNA polymerase, particularly when piperine alone has no effect. More structural data are necessary to understand this point.

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ACKNOWLEDGEMENTS. We wish to express our deep sense of gratitude to Regional Research Laboratory, Jammu for piperine samples and Council of Scientific and Industrial Research for financial support.

Received 19 January 2001; revised accepted 2 March 2001