IJBPAS, August, 2015, 4(8), Special Issue: 245-256

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DRUG RESISTANCE OF *MYCOBACTERIUM TUBERCULOSIS* TO RIFAMPICIN AND ISONIAZID: A MOLECULAR APPROACH

AHMADISHOAR SHIVA[1], ROSHDIMALEKI MEHDI[2], HABIBOLLAHI MOHAMMAD-HOSSEIN[3], ABBASI MASOUMEH[2],

1, 2: Department of Microbiology, Malekan Branch, Islamic Azad University, Malekan, Iran

3: Department of Microbiology, Zahedan Medical Branch, Islamic Azad University, Zahedan, Iran

ABSTRACT

Tuberculosis (TB) is one of the important re-emerging infectiousdiseases. Due to its infectious nature, one third of the world'spopulation is latently infected with the causative agent *Mycobacteriumtuberculosis* and annual new cases of TB worldwide countsapproximately 9 million. This disease is associated with moredeaths than any other single infectious agents.The increasing emergence of multidrug-resistant tuberculosis (MDR-TB) in the era of HIV infection presents a major threat to effective control of tuberculosis. The Centers for Disease Control (CDC) defines MDR-TB as TB infectionthat is resistant to multiple anti-TB medications, includingat least two of the first line agents used to treat TB (isoniazidand rifampicin). Drug resistance in *Mycobacteriumtuberculosis* arises from spontaneous chromosomal mutations at low frequency. Clinical drug-resistant TB largely occurs as a result of man-made selection during disease treatment of these genetic alterations through erratic drug supply, suboptimal physician prescription and poor patient adherence. The relationship between drug resistance in *M. tuberculosis* strains and their virulence needs to be further investigated. Understanding the mechanisms of drug resistance in *M. tuberculosis* would enable the development of rapid molecular diagnostic tools and furnish possible insights into new drug development for the treatment of TB.

Keywords: Drug Resistance, Isoniazid, Rifampicin, Tuberculosis (TB)

INTRODUCTION

Drug-resistant TB is not a recent phenomenon. *M. tuberculosis* strains that were resistant to streptomycin (SM) appeared soon after the introduction of the drug for treatment of TB in 1943(1). Genetic resistance to an anti-tuberculosis drug is due to

spontaneous chromosomal mutations at a frequency of 10^{-6} to 10^{-8} mycobacterial replications. Amplification of the afore-mentioned genetic mutation through human error results in clinically drug resistant TB. These include 'monotherapy' due to irregular drug supply, inappropriate doctor prescription and, most importantly, poor patient adherence to treatment(2). Subsequent transmission of resistant *M. tuberculosis* strains from the index patient to others aggravates the problem. The MDR/XDR phenotype is caused by sequential accumulation of mutations in different genes involved in individual drug resistance. Although the definitions of 'acquired' and 'primary' drug resistance are conceptually relatively clear, in reality they are often subject to misclassification when previous treatment cannot be readily ascertained(3). The term 'initial' drug resistance is thus often preferred to 'primary' drug resistance to include 'unknown' or 'undisclosed' acquired drug resistance. The matter is currently further simplified by categorizing drug resistance in new cases and previously treated cases of TB. The latter refers to cases with treatment lasting for at least one month (4-6).

The emergence of drug-resistant strains has made the treatment of TB complex, costly, toxic, time-intensive, and less efficacious. Design of a treatment regimen for drug-resistant TB includes the administration of first-line drugs to which the strains remain susceptible together with second-line drugs. These second-line agents are more expensive, more difficult to administer (several require intravenous administration), and are often associated with severe toxicities, including hepatic and renal dysfunction (7,8).

Rifampicin

Rifampicin (RMP or RIF)is a bactericidal antibiotic drug of the rifamycin group and is an important first-line drug for the treatment of TB. Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase (1,5).Itis bactericidal for *M. tuberculosis*, with MICs ranging from 0.05 to 1 μg/ml on solid or liquid media, but the MIC is higher in egg media (MIC = 2.5–10 μg/ml). Strains with MICs < 1 μg/ml in liquid or agar medium or MICs < 40 μg/ml in Löwenstein-Jensen (LJ) medium are considered RMP-susceptible. RMP is active against both growing and stationary phase bacilli with low metabolic activity. The latter activity is related to its high sterilizing activity in vivo, correlating with its ability to shorten TB treatment from 12–18 months to 9 months.

Rifamycins are critical to sterilization and are the key drugs associated with the development of modern short-course therapy for human TB. Currently, three rifamcyins are used for the treatment of TB: RIF, rifapentine, and rifabutin (1,2). These agents differ primarily

in their human pharmacology, but with respect to their antimycobacterial mechanisms of action and

resistance they are similar (5).

The principal target of rifamycins is the β subunit of RNA polymerase (RNAP), which is encoded by the gene *rpoB* (Rv0667 in *M. tuberculosis* H37Rv or MT0695 in *M. tuberculosis* CDC1551). Binding of a rifamycin to the RpoB protein inhibits the activity of RNAP, thereby preventing transcription. Because a basal level of transcription occurs in stationary growth phases, rifamycins are active against mycobacterial quiescent states. It is this activity that is

thought to underpin the sterilizing activity that has effectively shortened the duration of TB chemotherapy from 18 months to 9 months with the introduction of RIF(3,5).

Mechanism of action

Rifampicin inhibits bacterial DNA-dependent RNA synthesis by inhibiting bacterial DNA-dependent RNA polymerase.RMP has long been known to have its impact after RNAP binds to DNA and initiates transcription. Studies have shown that rather than inhibiting RNAP initiation, RIF prevents the elongation of RNA when the transcript becomes 2 to 3 nucleotides in length. In 1999 Darst and colleagues completed the first crystal structure of an RNAP, namely, that for *Thermusaquaticus* and in 2001 the structure of RIF-bound RNAP was reported (3). These structures showed that RIF binds deep within the DNA/RNA channel of RNAP but more than 12 A ˚ away from the active site (5).

More recent studies using the crystal structure of *Escherichia coli* RNAP have revealed a yet more complex, multistep mechanism of RNAP action: (a) RNAP binds to promoter DNA, producing an RNAP-promoter closed complex where the DNA remains double-stranded and is not loaded into the RNAP active-center cleft; (b) RNAP loads DNA into, and unwinds DNA in, the RNAP active-center cleft, yielding an RNAP-promoter open complex (RPoc); (c) RNAP synthesizes the first ∼10 nucleotides of RNA using a "scrunching" mechanism whereby RNAP remains stationary on promoter DNA, pulling downstream DNA into the cleft (calledthe RNAP promoter initial transcribing complex [RPitc]); and (d) RNAP escapes the promoter and synthesizes the rest of the RNA using a "stepping" mechanism, in which RNAP translocates relative to DNA, as a transcription elongation complex. RIF has been shown to exert its action by preventing step "c", namely, the formation of the RNAP initial transcribing

complexRPitc. These properties led to the proposal that rifamycins inhibit the formation of RPitc through a steric-occlusion mechanism, whereby RIF binds adjacent to the RNAP active center, along the path of the

RNA product, and physically prevents synthesis or retention of RNA products >2 to 3 nucleotides in length (4,5,6).

RMP bound within its β subunit pocket is 12 A ˚ away from the active site of RNAP, where ribonucleotides complementary to the DNA template strand are added to the growing RNA chain. However, the RIF-binding pocket within the β subunit is situated in the main DNA/RNA channel of RNAP. The binding of RIF to its cognate pocket obstructs this channel, preventing the elongation of RNA molecules beyond 2 to 3 nucleotides in length.

Mechanism of resistance

RNAP in *M. tuberculosis*, as in most bacterial species, is comprised of four polypeptides: α, β, β′, and σ, arranged in a five-subunit enzyme, α2ββ′σ. Single genes (*rpoA*, *rpoB*, and *rpoC*) encode the α, β, and β′ subunits, respectively, while 13 sigma factors may serve as the promoter-recognition subunits (7). The mechanism by which these mutations confer RIF resistance has been inferred from X-ray crystallography studies of RNAP from other bacterial species, in particular *Thermusaquaticus* and *E. coli*. Greater than 95% of RIF-resistant strains of *M. tuberculosis* harbor nonsynonymous mutations in the *rpoB* gene encoding the β subunit of RNAP (8). These mutations cluster in an 81-base-pair region of the *rpoB* gene known as the RIF resistance-determining region (RRDR) (5,8,9).

RIF binds between two structural domains of the RNAP β subunit. The mutations in the RRDR all occur in residues in or near this pocket. All of these mutations prevent effective binding of RIF within the β subunit pocket. Approximately half of the mutations accounting for RIF resistance occur in amino acids within this binding pocket that are in close enoughproximity to directly interact with RIF when it is complexed with RNAP. Other residues known to accountfor RIF resistance occur one layer removed from thebinding pocket and can lead to distortions of the bindingpocket that also prevent RIF binding (3,5).

Competence of RIF-resistant mutants and compensatory mutations

Mutations in the RRDR prevent binding of RIF to RNAP and at the same time permit effective function of RNAP. When *M. tuberculosis* is grown in the presence of RIF, mutants with classic mutations in the RRDR are easily selected. Such laboratory-derived *M. tuberculosis rpoB* mutant strains show a variable reduction in fitness as measured by their in vitro growth rate. In contrast, some clinical isolates that are RIF resistantwith the same RRDR mutation displayed fewer fitness costs (using in vitro growth rates) when compared with their wild-type counterparts, raising the possibility of compensatory mechanisms to correct for loss of fitness

accrued by the original RIF resistance-conferring mutation (11).

Recent studies have compared laboratory-derived *rpoB* RIF-resistant strains, passaged in vitro to select for compensatory mutations, with collections of MDR clinical isolates. These studies have shown that RIFresistant strains with classic mutations in the RRDR also contain other mutations that are associated with a reduction in the fitness cost of the RIF resistanceconferringmutation. These putative fitness-compensatory mutations localized to the *rpoA* gene encoding the α subunit of RNAP and to the *rpoC* gene encoding the β′ subunit of RNAP (12). Although functional analysis of these mutations has not been completed in *M. tuberculosis*, studies in other RIF-resistant organisms have confirmed that secondary mutations in *rpoA* and *rpoC*do act in a compensatory manner. Some also confer low-level resistance to RIF (13). Hence, it appears that mutations occurring in other subunits of the multisubunit RNAP enzyme can compensate for the relative fitness loss of mutations in the *rpoB* RRDR that confer high-level RIF resistance.

Isoniazid (INH)

Isoniazid is another first line agent for the treatment of TBinfection.INH is the most widely used first-line anti-tuberculosis drug. Since its discovery in 1952, INH has been the cornerstone of all effective regimens for the treatment of TB disease and latent infection. *M. tuberculosis* is highly susceptible to INH (minimum inhibitory concentration[MIC] 0.02–0.2 μg/ml). INH is only active against growing tubercle bacilli, and is not activeagainst non-replicating bacilli or under anaerobic conditions. INH resistance is the most common form of antituberculosis drug resistance encountered, whether inisolation or in combination with other drugs (5). INHmonoresistantTB is relatively easy to treat.INH is a first-line drug for TB that has rapid bactericidal activity. During combination therapy of drugsusceptible TB infection, INH is the drug principally responsible for the 1 to 2 log decline of bacterial load in sputum seen within the first 14 days of treatment (14). INH is also widely used as a treatment for latent infection of *M. tuberculosis*. While its structure of a pyridine ring and hydrazide group is simple, the mechanism of action of INH is incredibly complex.

Mechanism of action

Isoniazid is a prodrug and must be activated by a bacterial catalase-peroxidase enzyme that in *M. tuberculosis* is called KatG. It has long been postulated that INH inhibits the biosynthesis of mycolic acids (15), resulting in accumulation of long-chain fatty acids (16) and cell death (17). However, the details of this complex process have only recently been elucidated. INH is a prodrug that is activated

by the mycobacterial catalase-peroxidase enzyme, *katG*, encoded by katG (Rv1908c, MT1959) (18). Following activation by KatG, INH forms an adduct with NAD (19). This INH-NAD adduct then binds and inhibits a mycobacterial protein, InhA (20). *inhA* (Rv1484, MT1531) encodes InhA, an NADH-dependent enoyl-acyl carrier protein (ACP) reductase (21,22), which is part of the fatty acid elongation system, fatty acid synthase type II (FASII), responsible for mycolic acid biosynthesis (23). Inhibition of InhA by the INH-NAD adduct results in intracellular accumulation of long-chain fatty acids, decreased mycolic acid biosynthesis, and eventual
cell death.

Mechanism of resistance:

The two main molecular mechanisms of INH resistance are (i) loss of INH activation by katG (24) and (ii) increases in *inhA* expression or modification of the InhA target. Approximately 75 to 90% of INH resistance can be attributed to polymorphisms in the *katG* gene or the *inhA* promoter and gene (25). The majority of clinical isolates that are resistant to INH harbor mutations in *katG* (26, 27). A broad range of polymorphisms has been reported worldwideincluding missense and nonsense mutations, insertions, deletions, truncation, and rarely, whole gene deletions (24, 28, 29). Mutations in *katG* result in a reduced ability to form the INH-NAD adduct (30) and a high level of INH resistance. The most common mutation occurring in clinical isolates in *katG* is S315T (27, 31–35).

Polymorphisms in *katG* may result in diminished or loss of catalase and peroxidase activity. Because these enzymatic activities are critical for *M. tuberculosis* defense against reactive oxygen species and virulence in vivo (36–38), mutations in *katG* can lead to a loss of fitness to the bacterium. However, unlike other polymorphisms, the S315T mutation renders *katG* unable to activate INH while still retaining some peroxidase and catalase activities, reducing the fitness cost to this particular mutation. It is likely that this retention of catalase-peroxidase activity and fitness with the S315T *katG* is responsible for the predominance of this mutation among drug-resistant isolates (38).

Mechanism of resistance

Overexpression or target modification: *inhA*

While the majority of INH-resistant clinical strains contain *katG* mutations, a significant portion of INHresistant clinical isolates have a wild-type *katG*, suggesting the existence of additional resistance mechanisms. Other such mechanisms involve changes in *inhA* expression and modification of the InhA target.Association with INH resistance has been well documented for polymorphisms within the *inhA* promoter at the −15T and −8A loci. Mutations at this site result in

overexpression of *inhA*, which confers low-level resistance to INH and cross-resistance to ethionamide (ETH) (39, 40). The prevalence of *inhA* promoter mutations varies geographically, but these mutations rarelyaccount for greater than 20% of INH resistance (40, 42). While structural mutations in InhA are rare, the S94A mutation reduces affinity for the NADH cofactor and confers low-level resistance (22). Approximately 10% of INH resistance is unexplained by mutations in *katG* and *inhA* (25). Other genes including *kasA*, *ahpC*, *ndh*, and the *ahpC-oxyR* intragenic region have been associated with resistance to INH, but their impact on resistance among clinical isolates remains unclear (43,44).

CONCLUSION

Despite having 100% coverage of directly observed treatment short-course, multi drug-resistant (MDR) tuberculosis (TB) is still increasing inmajority countries. Early detection of MDR-TB by rapid molecular test andearly initiation of treatment will effectively stop this trend.

To develop rapid diagnostic tools, molecular characterization of genes conferring Mycobacterium tuberculosis resistance to rifampicin (RIF) and isoniazid (INH) will be required. Drug resistance in *Mycobacteriumtuberculosis* arises from spontaneous chromosomal mutations at low frequency.

Greater than 95% of RIF-resistant strains of *M. tuberculosis* harbor non synonymous mutations in the *rpoB* gene encoding the β subunit of RNAP. These mutations cluster in an 81-base-pair region of the *rpoB* gene known as the Rifampicin resistance-determining region (RRDR).Unlike RIF, Isoniazid resistance is associated with mutations inseveral genes such as *katG*, *inhA*, *kasA*, *ahpC* and *oxyR*.

Majority of INH resistant strains carried mutations at codon315 of the *katG* gene and 20-35% strains contained mutation at the *inhA* regulatory region.

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