Mechanisms of Resistance and Multi-resistance in Tuberculosis

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I. An Historical Overview of Tuberculosis

Tuberculosis is an old disease, probably as old as humanity, and has been featured in various works of art throughout history. In 1882, Robert KOCH discovered Mycobacterium tuberculosis. Since then, despite all the progress that has been made in tuberculosis research, in terms of vaccination and chemotherapy, tuberculosis remains a leading infectious disease. This situation is largely due to drug resistance.

Today, it is clear that economic and social factors affect the incidence of tuberculosis. Thus, even prior to the availability of chemotherapy, improved nutrition and sanitation conditions have already lowered the TB incidence.

The introduction of TB chemotherapy in 1950’s led to a significant decline in the incidence of the disease, particularly in developed countries. This reduction has prompted some public health professionals, mainly in the US, to claim that tuberculosis no longer poses a problem in developed countries. They have even eliminated many TB control programmes in the country. However, in the late 1980’s, a major outbreak of MDR-TB occurred in New York City, which has cost USD 1 billion to control. This outbreak has led to a renaissance in TB research.

Tuberculosis has remained a problem in developing countries all along. However, today we know that as long as the disease persists in one part of the world, it would quickly be transported to other parts. Today, tuberculosis is still ranked among the top three infectious killers, together with HIV and malaria.

II. The Epidemiology of Drug Resistant TB

Drug resistant TB has been a major problem impeding TB control, since the lengthy TB chemotherapy frequently selects drug resistance organisms. 50 million people are believed to be infected with MDR-TB, accounting for approximately 5% of all tuberculosis cases. According to WHO statistics, each year approximately 500,000 MDR-TB cases are identified, causing an estimated 110,000 deaths. Of these 500,000 cases, 40,000 XDR-TB are cases, leading to 25,000 deaths.

MDR-TB rates are rather high in the former Soviet Union and in China. The highest rates were observed in Azerbaijan, in which approximately 22% of new TB cases were due to MDR-TB. In the Inner Mongolia and Heilongjiang provinces of China, about 7% of the TB cases are MDR-TB cases. TB epidemiology has changed dramatically due to HIV-infection and drug resistance. Some people even call it ‘the new tuberculosis’.

In view of the lethal combination between HIV-infection and drug resistant tuberculosis, the WHO declared tuberculosis a global emergency in 1993. More recently, in 2005, following an outbreak of XDR-TB in Africa, tuberculosis was declared an emergency in that region.
The rapid progression and high fatality of the disease during that outbreak were somewhat unprecedented, and certainly alarming. As a reminder, XDR-TB is defined as MDR-TB, plus resistance to two major second line drugs, quinolone and aminoglycoside. XDR-TB cases have been reported in 45 countries worldwide.

III. Tuberculosis Chemotherapy

Significant progress has been made in TB chemotherapy. In the pre-antibiotic era, before 1940, there were no drugs against the disease. The tuberculosis treatment at the time consisted mainly of cod liver oils (which by the way, include vitamin D), bed rest and fresh air.

The first TB drug, Streptomycin, was discovered by Selman WAKSMAN and Albert SCHATZ in 1944. It was followed by PAS, which was discovered in 1946. Then, in 1952, two important first-line TB drugs were discovered, Isoniazid and Pyrazinamide. The last TB drug discovered was Rifampin, in 1963.

1. The DOTS Therapy

The best TB therapy available today is DOTS, a six month therapy with a 78%-96% cure rate. The treatment is divided into two phases. The initial phase consists of the daily use of four drugs, Isoniazid, Rifampin, Pyrazinamide, and Ethambutol, for a period of two months. It is then followed by a continuation phase, in which two drugs, Isoniazid and Rifampin, are taken three times a week for another four months. The major weakness of the DOTS therapy is that it is too long. The lengthy therapy causes patient compliance problems, which frequently lead to drug resistant TB.

2. Drug Combinations

It is noteworthy that the current TB therapy is based on the principle of drug combination. The first advantage of using a drug combination is that it reduces drug resistance. When TB drugs were tested individually, drug resistant bacteria were emerging. This issue was studied extensively by the British MRC, in a research conducted in 1944-1946, by Philip D’ARCY-HART. Clinical trials were conducted for Streptomycin, and revealed that adding PAS to Streptomycin can reduce the emergence of drug resistance.

A second advantage of drug combinations is that they can enhance the efficacy of the therapy. This point is illustrated by the Mitchison hypothesis, also referred to as the Special Bacterial Populations Theory. According to the theory, TB bacteria found in the lesions consist of four different sub-populations. Population A, which is actively growing, is killed by Isoniazid. In case of Isoniazid resistance, it is killed by Rifampicin, Streptomycin, or Ethambutol. Population B, which has a slower metabolism, is killed by Rifampicin. Population C, which resides in an acidic environment, is killed by PZA. Finally, population D is a dormant population, and there are currently no drugs that can effectively kill this population.

3. The Yin and Yang Model of Bacterial Life-Cycle

We have also developed the Yin and Yang model, which we believe can help explain the current practice of TB chemotherapy. Isoniazid and Rifampicin are used in both the initial and the continuation phase of TB therapy. Now, presumably, all the growing forms of the bacteria were already killed during the initial phase. Thus, Isoniazid should not be effective in the continuation phase.
Nevertheless, Isoniazid is effective in the continuation phase, and we believe its efficacy is due to reverters. In the Yin and Yang model, the Yang designates active metabolism bacteria, which are killed during the initial phase, while the Yin part designates the persisters. We believe that although PZA is a persister drug, it does not kill all the persisters. Thus, other persister drugs are required.

Following the initial two months of therapy, some reverters emerge from among the persisters. That phenomenon brings to mind ‘the urge of life’. Whenever the conditions are favourable, the bacteria wish to start growing again - to revert. Thus, Isoniazid and Rifampicin are used in the continuation phase to kill these reverters. The advantage of the model is that it is more dynamic.

IV. Drug Resistance Mechanisms

Drug resistance mechanisms can be divided into Genetic and Phenotypic types. In the TB case, the genetic drug resistance is caused by mutations in chromosomal genes, while plasmids and transposons play no role in drug resistance. Phenotypic drug resistance in persisters, on the other hand, causes major problems for lengthy TB chemotherapy treatments.

1. Virulence of Drug Resistance Tuberculosis

INH seems to be the only incidence where KatG mutations effect the virulence of the organism. Thus, KatG-negative INH-resistant strains with high-level resistance seem to be more attenuated, and less infectious for humans. Of course, resistance to other drugs is typically not associated with the attenuation of virulence. For example, the PZA-mono-resistant strain is still fully virulent, and is responsible for causing tuberculosis outbreaks.

2. The Drug Resistance Mechanisms of the Major TB Drugs

Significant progress has been made in understanding the drug resistance mechanisms to the major *M. tuberculosis* antibiotics.

- INH inhibits cell wall synthesis, and its prodrug should be activated by catalase-peroxidase. It then inhibits the target, InhA, which is the enoyl acyl carrier protein reductase. Mutations in KatG or InhA can thus cause INH resistance.

- Rifampicin inhibits RNA synthesis, and mutations in rpoB are involved in Rifampicin resistance.

- PZA inhibits membrane function, and mutations in PZase are involved in PZA resistance.

- EMB inhibits cell-wall component arabinogalactan synthesis, and mutations in embB are involved in EMB resistance.

- SM inhibits protein synthesis. The genes involved in SM resistance are rpsL and rrs. More recently, a third gene has been identified, gidB.

- Amikacin, Capreomycin, and Kanamycin inhibit protein synthesis. Mutations in rrs at position 1400, can cause cross-resistance to the three drugs.
- Quinolone drugs inhibit DNA synthesis. Mutations in gyrA account for 95% of quinolone resistance.

I would now like to focus on the resistance mechanisms of two drugs, INH and PZA.

3. INH

INH (Isoniazid) is an important front-line TB drug. It was first chemically synthesized in 1912 in Prague. However, its anti-tuberculosis activity was recognized only 40 years later, in 1952. At that time, it was discovered simultaneously by three pharmaceutical companies, Roche, Bayer, and Bristol Meyer Squibb. The discovery was based on two leads, Nicotinamide and sulfa-drugs. When INH was first used in clinical treatment, it had a remarkably efficient and rapid effect. The patients who used it felt better very quickly, and there was a euphoria atmosphere at hospital wards.

a. Mode of Action

INH is a prodrug. It enters the tuberculosis bacteria, where he is activated by catalase/peroxidase enzymes to reactive oxygen and organic species. These species then attack multiple targets in the tuberculosis bacteria. The principle target seems to be mycolic acid synthesis (inhA), but they also potentially damage DNA, as well as NAD metabolism.

b. Resistance

INH resistance was identified in 1952, soon after its discovery, by MIDDLEBROOK. He found out that INH-resistant TB strains often lose catalase/peroxidase enzymes. At the time, the association was not very clear, since not all INH-resistant strains lose the enzyme. However, In 1992, we cloned the catalase-peroxidase gene (KatG) and demonstrated that KatG deletion causes INH-resistance. A year later, in 1993, we demonstrated that inserting the KatG back into the KatG deleted strains restores not merely INH susceptibility, but also the catalase/peroxidase enzymes.

The loss of enzymes involved in drug activation is a rather newly discovered mechanism of drug resistance. Nevertheless, particularly in tuberculosis, it was shown to be a major one, since several important tuberculosis drugs, such as INH and PZA, require activation by bacterial enzymes.

The second gene involved in INH resistance is inhA, which was identified by a group headed by Bill JACOBS in 1994. KatG mutations are a more frequent cause of INH resistance, accounting for 60-90% of resistant strains, whereas inhA mutations account for merely 10-30% of the resistant strains. There are also some resistant strains without KatG or inhA mutations, suggesting that there are additional mechanisms of INH resistance. However, merely by detecting KatG and inhA mutations, it is possible to account for up to 90% of INH resistant strains.

c. Late Growth Effect

Last year, Salman SIDDIQUI observed that when a culture is left long enough during INH drug susceptibility testing, there is a late growth effect. This finding is disturbing, since it seems to suggest that the persisters that were not killed by the INH initially can later grow and acquire genetic resistance. It is noteworthy that this late growth effect has not been observed in Rifampicin drug susceptibility tests.
4. PZA

PZA is a highly paradoxical drug. In-vitro, in normal culture conditions, the drug has no activity against tuberculosis. It shows activity merely in acidic pH (5.5), and kills the bacteria extremely slowly. At the end of a two-week period, it kills merely 50-70% of the bacteria, which is not effective. Yet, in-vivo, PZA significantly shortens TB chemotherapy. It also appears to kill the non-growing, persister, form of tuberculosis bacteria.

Different studies have demonstrated that PZA kills old, dormant forms of the bacilli better more effectively than actively growing forms. The drug is more active in anaerobic conditions, which partly explains why it works well in-vivo. In the in-vivo lesions, the environment is hypoxic/anaerobic. In the mouse model, PZA has shown remarkable sterilizing activity against persister bacilli and is involved in shortening the therapy. When used alone, PZA does not show predictable activity in-vivo. However, when it is combined with either INH or Rifampicin, it demonstrated remarkable sterilizing activity.

a. The MCDERMOTT Experiment

The early pioneer work on the drug was conducted by Walsh MCDERMOTT, who established the acid pH requirement for PZA. He also demonstrated that PZA-resistant TB strains can lose Pzase activity, as well as the amazing sterilizing activity of the drug in the mouse model.

MCDERMOTT also conducted a critical experiment, which lay the foundation for short-term chemotherapy. He examined the CFUs in the spleen of TB-infected mice, treated with different drug combinations. The experiment included an untreated control group, and two groups treated with different drug combinations, over a period of 90 days. The first drug combination utilised, SPH, contained Streptomycin, PAS, and Isoniazid, and was considered the best drug combination available in the 1950s. MCDERMOTT demonstrated that the activity of SPH was highly efficient in the first 3-4 weeks of the treatment. However, the drug soon lost its efficacy.

The second drug combination used, HZ, consisted of Isoniazid and PZA. In the first four weeks of the experiment, HZ was significantly less effective than SPH, leaving a higher number of CFUs. However, its activity did not cease at the end of four weeks. Thus, although it had a slower start, its effect was longer, and it managed to remove all the bacilli from the spleen within two months.

However, three months after the treatment was stopped, one-third of the mice relapsed with tuberculosis. Furthermore, when immunosuppressant steroids were used, all mice relapsed. These relapses indicate that a persister population of the bacteria survived in the mice, although it did not grow or form colonies. Nevertheless, the experiment demonstrated the remarkable sterilizing activity of PZA, in combination with INH, and laid the foundation to short-term TB chemotherapy.

b. Resistance

Mutations in pncA gene are the major mechanism of PZA resistance. In an analysis of over 1 016 PZA-resistant strains, 857 of them (84%) exhibited pncA mutations, and we believe the real percentage might be even higher. For that reason, we believe that detecting pncA mutations would be a useful indicator for PZA resistance.
Mutations in the pncA gene are highly diverse, and seem to be scattered along the entire gene. Fortunately, since the pncA gene is rather small (560 bp), it is rather easy to sequence the pncA gene. We recommend doing so for all drug resistant TB strains, particularly since the current PZA susceptibility testing is time-consuming, unreliable and expensive. In fact, due to the many problems of the PZA susceptibility test, it is currently not conducted routinely by all clinical laboratories.

c. Mode of Action

The discovery of PZA was rather unconventional. In 1944, V. CHORINE, a French scientist, observed that Nicotinamide exhibits some activity against mycobacterium in animal models. Then, the Lederle Laboratory created analogs of Nicotinamide. PZA is also a Nicotinamide analog, and both compounds are converted by the pyrazinamidase/nicotinamidase enzyme into the active form.

PZA is a prodrug. It enters the TB bacteria, where it is converted by pyrazinamidase into the active form. Then, it shifts to the cell surface, where the acid pH plays a role. At neutral pH, the active form of the drug is a weak acid, and thus remains outside the cell. However, at an acid pH environment, a portion of the POA forms a conjugate acid, which permeates through the membrane. Then, it gets dissociated again. The cycle continues as long the extra-cellular pH is acidic, and the intracellular pH is neutral. This process disrupts the membrane energy, which is presumably extremely important for the persister forms of the bacteria.

Based on the model, we predicted that energy inhibitors would enhance PZA activity. Indeed, we found that by using energy inhibitors such as DCCD, PZA activity can be briefly enhanced. We then tried to identify DCCD analogues, which could serve as a tuberculosis drug. A pharmaceutical company developed the J compound, Diarylquinoline, which is identical to DCCD, with the same target, F1F0 H-ATPase. Remarkably, the J compound showed a synergy with PZA in-vivo. It is currently a drug candidate, and is undergoing clinical tests.

V. Drug Resistance Detection

Timely detection of drug resistance is important for the outcome of tuberculosis treatment. It is thus recommended that all drug-resistant TB strains be tested for drug susceptibility. However, TB drug resistant testing is rather problematic, since the lengthy chemotherapy frequently selects drug resistant organisms, and the bacteria grows rather slowly.

1. Molecular Tests

Different molecular tests are used for detecting mutations in drug resistance genes. They can be roughly divided into tests that detect known mutations versus those that detect unknown mutations in resistance genes. The tests that detect unknown mutations use DNA sequencing, as well as a microarray. The front-runner molecular tests are currently line probe assay, real-time PCR, and the LAMP assay.

The reason molecular tests are able to detect drug resistance rapidly is the high correlation between mutations and drug resistance. It is thus possible to detect most of the drug resistant strains based on the mutations in merely a few genes. For INH resistance, these genes include KatG315 and inhA; for Rifampicin resistance, rpoB; for PZA resistance, pncA; for
Fluoroquinolone resistance: gyrA; for SM resistance: RpsL43/88 and rrs; and for Amikacin, Kanamycin, and Capreomycin: rrs 1400A.

VI. Bacterial Persisters

Bacterial persistence is an enormous problem for TB chemotherapy. It is demonstrated by the Cornell model, which was also developed by Walsh MCDERMOTT. The model is based on a study, which was already described earlier. In the study, mice infected with TB were treated with INH and PZA for three months. At the end of the period, no bacilli were found in the infected organs. However, when the treatment was stopped, within three months one-third of the mice relapsed with TB. If immuno-suppressing steroids were used, all the mice relapsed.

When MCDERMOTT observed the results, he expressed his frustration by making the following comment: “When one realizes that, even though the bacilli vanish, and there is…truly latent infection, the bacilli are, nevertheless, still there…drug susceptible, I think you will agree with me that it shows that ‘you can’t win.’”. Due to the great interest in the persistence problem, some progress has been made. However, the detailed mechanism is not clear yet.

VII. Development of New Drugs

I would like to overview several drugs that are currently undergoing clinical trials. First, there seems to be a renewed interest in Rifapentine. Jacque GROSSET and Eric NUERMBERGER found that a higher dose of Rifapentine in combination with PZA and moxifloxacin can shorten TB therapy from six months to three months in mice. The new drug combination is called PMZ (Rifapentine, moxifloxacin, and PZA).

There are also several drugs under clinical development.

- New fluoroquinolones drugs, such as moxifloxacin and gatifloxacin show promise to shorten the clinical therapy to four months. They are currently in Phase II trials.
- Ethambutol analog, SQ-109, is currently is Phase I trials.
- Nitroimidazoles: nitroimidazolepyran-PA-824, is in Phase I trials.
- OPC-67683, developed by Otsuka, is currently in Phase II trials.
- Diarylquinoline drugs, or the J compound, which I have discussed earlier, are also in Phase II clinical trials.

Thus, there is significant promise for the development of a short-term chemotherapy in the coming years. A key problem is that we do not quite know how to identify persister drugs, since there is no efficient in-vitro correlate of their sterilizing activity in-vivo. It is also important to remember that persistent bacteria are merely one reason the TB therapy is so long. Another reasons, which clinicians often do not like to admit, is that the drugs are simply not efficient enough.

VIII. Conclusion

TB remains a leading infectious disease worldwide, with approximately nine million new cases, and two million deaths per year. The BCG vaccine is not extremely efficient. The TB
chemotherapy is able to cure the disease, but is too long, and sub-optimal. Several factors, such as latent infection (persister bacteria), HIV and drug resistant TB make the problem more difficult. On the TB-control strategy side, we still use microscopy in a culture, which is a hundred year old technology; and the BCG vaccine, which is eighty-year old.

Thus, to better control TB, we require more efficient and rapid diagnostic tools, a better vaccine, drugs that can address the TB latent infection problem, and above all, better TB chemotherapy, which would enable us to shorten the therapy treatment from three months to a few weeks. I believe the fight against TB requires a political commitment. Socio-economic factors are also extremely important, and can positively or negatively impact the situation.

IX. Question and Answer Session

Participant

Is there cross-resistance between different drugs, such as Rifampicin and Rifapentine?

Ying ZHANG

Rifampicin and Rifapentine are known to have a high degree of cross-resistance. Thus, if a strain is resistant to Rifampicin, using Rifapentine would often not be effective. Similarly, if a strain is resistant to one quinolone drug, it would typically show cross-resistance to other quinolone drugs.

Steffen STENGER

When we send clinical isolates for drug resistance testing, we sometimes obtain a differentiation in terms of INH resistance, depending on the MIC. That is, sometimes the indication is low-level INH resistance, and sometimes high-level resistance. Is there a genetic difference associated with low-level or high-level resistance? In addition, we are often advised to prescribe INH even when low-level resistance is detected, since it is such an important drug during the first weeks of tuberculosis therapy. Do you feel believe that course of action makes sense?

Ying ZHANG

Low-level resistance to INH could be caused by InhA promoter mutations, while KatG mutations are often associated with a high-level resistance. We have also found a few INH low-level resistance strains that do not exhibit mutations in either genes, but we do not know the mechanism involved in this cases.

It is true that since low-level resistance is sometimes unstable, some clinicians advise to use INH anyways. Some mouse model studies do demonstrate that low-level INH resistant strains still respond to INH treatment.