Emergent multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *Mycobacterium tuberculosis* are causing a crisis in efforts to treat patients with tuberculosis (TB). Such infections are highly lethal and on the rise, particularly in developing nations. In some cases, a single strain carries resistance to all available drugs for treating this pathogen.

Rup Lal of the University of Delhi dedicated 25 years of research to generating novel antibiotic candidates against TB, persevering through setbacks and disappointments. These efforts culminated in synthesis of novel rifamycin B analogs. A semisynthetic derivative of this analog is more potent than currently available drugs, including drug-resistant TB strains, he says. Meanwhile, his research group’s approach, which is based on genetic engineering to generate such analogs, is itself something of a breakthrough, one that will enable Lal and his collaborators to produce and evaluate many additional antimicrobial candidates in the future.

Drug-Resistant TB, a Crisis within the Global Tuberculosis Epidemic

TB is the second-leading cause of death worldwide due to a single infectious agent, second only to HIV/AIDS. TB is also responsible for 25% of HIV-related deaths, according to the World Health Organization (WHO). The death toll from TB in 2013 tallied 1.5 million, while 9 million individuals are infected globally.

Several classes of antibiotics are used to treat TB—typically, with drugs of several types used in combination. All these regimens are administered to patients for prolonged periods lasting from 6 months to 2 years, making it difficult for many individuals to complete. Poor adherence to these regimens, particularly for individuals living in developing nations where drug access can be challenging, contributes to rampant drug resistance among *M. tuberculosis* strains. “Drug-resistant TB is the manmade result of interrupted, erratic, or inadequate TB therapy, and its spread is undermining efforts to control the global TB epidemic,” notes the TB Alliance, a global nonprofit organization with offices in New York, Brussels, and Pretoria.

MDR TB, defined by resistance to both first-line antibiotics rifamycin and isoniazid, is a growing concern. Since its emergence in the 1980s, the number of MDR cases increased steadily, reaching an estimated 480,000 cases in 2013, according to WHO. In some regions, MDR-TB accounts for up to 20–30% of all TB cases.

Resistance against second-line treatments is also becoming more common, leaving patients...
and physicians with fewer and fewer options. These XDR strains, which are resistant to first- and second-line drugs, account for 9% of TB cases and are reported as occurring in more than 100 countries, according to WHO. Some totally resistant strains (TDR), which do not respond to any available antibiotics, are also being documented, officials note.

“TB has been a rampant health threat worldwide, and is most devastating in underdeveloped and developing countries,” Lal says. “It has been so for the last several decades, and the situation is worsening with the emergence of MDR and TDR strains. To combat this situation new rifamycin analogs are needed, as the drugs used currently are ineffective against these resistant strains.”

**Resistance Developed to Rifamycin and Several Analogs Critical for Treating TB**

Rifamycins are semisynthetic derivatives of the natural product rifamycin B, which is produced by the soil actinobacterium *Amycolatopsis mediterranei*. While rifamycin B itself has moderate antimicrobial activity, various semisynthetic analogs have improved potency as well as other properties that make them better drugs for treating TB. Discovered in the 1950s, rifamycins became a mainstay of TB treatment in the 1960s, significantly improving patient survival rates. However, resistance soon emerged as mutations in *M. tuberculosis* led to poorer binding of rifamycin to its target and, hence, reduced susceptibility.

Rifamycins target the bacterial RNA polymerase enzyme, blocking RNA chain elongation and downstream protein synthesis, which is essential for bacterial survival. Dozens of escape mutations have been described in clinical isolates of rifamycin-resistant *M. tuberculosis*, nearly all of them within an 81-bp region of the RNA polymerase gene. The vast majority of these alter at least one of three key codons that mediate interactions of the encoded enzyme with rifamycins.

One major approach to overcoming resistance to this and other antibiotics is to generate structural analogs of the parent antibiotic, allowing the analogs to circumvent the mutations that render the parent drugs inactive. However, rifamycin presents medicinal chemists with challenges that they could not readily surmount. The complicated structure of rifamycin makes them difficult to synthesize or to modify. “Structural complexity of rifamycin B limits the use of chemical tools to generate fundamentally different rifamycin analogs, such as modification of the backbone structure,” Lal says. “Only the naphthoquinone ring is sterically available for chemical modifications, while the rest of the chain is not amenable.”

In practice, chemists succeeded in producing only four clinically useful analogs of rifamycin B: rifampicin, rifaximin, rifabutin, and rifapentine, all modified on the C-3 or C-4 position of the naphthalene moiety. Resistance developed against all of them.

**Genetically Engineering for the Biosynthesis of Novel Rifamycin Analogs**

Despite these difficulties, medicinal chemists continued trying to generate additional rifamycin analogs but to no avail, according to Lal. “Further modification to produce effective analogs seemed untenable,” he says. Those failures from conventional efforts to generate additional analogs of rifamycin led Lal and his team to take a different approach. Setting aside organic chemistry for the moment, they turned instead to the microorganism that makes rifamycin B to see whether it could be changed to synthesize novel analogs of that natural product. Their strategy was to engineer *A. mediterranei* by modifying genes that encode some of the enzymes that synthesize rifamycin B. These changes then could yield novel structures that retain activity against *M. tuberculosis* but overcome resistance to older, semisynthetic members of this family of antibiotics.

This same approach was used to generate novel analogs of other antibiotics, including erythromycin and rapamycin. In both those cases, biosynthetic gene clusters within their respective producer strains, *Saccharopolyspora erythrae* and *Streptomyces hygroscopicus*, yielded novel analogs. Further, in both those cases and in the case of rifamycin B, key enzymes belong to the type I polyketide synthase (PKS) family, according to Lal.

“Type I PKSs, such as *ery*PKS, have been shown to be amendable to combinatorial biosynthetic modifications to give new analogs of antibiotics,” he says. “The genetic organization of the modules in the rifamycin biosynthetic pathway is collinear, similar to the erythromycin and rapamycin PKSs. This collinear architecture makes it a possible target for combinatorial biosynthesis.”

However, the approach was still a gamble. Other microbial PKS systems are difficult to
engineer due to the sheer number of genes and enzymatic modules involved. Additionally, downstream enzymes may not recognize or act on modified substrates being produced within genetically altered strains.

The biosynthetic gene cluster for rifamycin B contains dozens of genes, many with multiple enzymatic modules. These modules add various sidechains to intermediates along the pathway leading to rifamycin or rearrange the overall structure of specific intermediates. For example, starting with the relatively simple precursor molecule dihydroxybenzoic acid (AHBA), the rifA through rifE genes elongate its carbon backbone prior to cyclizing those downstream intermediates to close the polyketide ring.

Lal set his sights on modifying rifB, which encodes three acetyltransferase modules, each of which adds a 3-carbon propionate to the elongating chain. Through genetic recombination, Lal replaced one of these modules with an acetyltransferase-encoding module within a gene plucked from the rapamycin biosynthetic pathway, which instead adds 2-carbon acetate. This modification removes a carbon from the backbone at the C-24 position, which directly interacts with the target RNA polymerase. The final product following this specific modification in the pathway is 24-desmethylrifamycin B, a rifamycin analog that had never been made before, according to Lal. “This work focuses on the design strategy that gains access to rifamycin analogs in which modifications take place in the polyketide backbone,” he says. Other analogs made via medicinal chemistry before then were restricted to changes to the C-3 and C-4 of the naphthalene ring.

“It is conceivable that this is the most elegant of manipulations that have been done,” says David Rothstein, an industry consultant based near Boston, who worked on developing rifamycin analogs throughout much of his career. “It is quite elegant to put the module of one enzyme into another complex that is carrying out this reaction.”

**This Strategy for Modifying Rifamycin Took Decades To Implement**

Although an elegant concept, however, genetically manipulating *A. mediterranei*, proved far more difficult to implement than Lal anticipated. He and his collaborators spent more than 25 years to make the project work, dealing with numerous challenges and interim failures along the way.

Lal and his wife Sukanya Lal began trying to manipulate the genes of *A. mediterranei* as post-doctoral fellows in Germany in 1988. During that early stage, it took them about a decade to develop and optimize cloning vectors and transformation protocols. Those efforts continued after
they returned to India, where he took a faculty position at the University of Delhi, and she joined Ramjas College at the same university, where she continues teaching undergraduate students. Moreover, Lal could not bring these genetic tools to bear on the rifamycin biosynthetic gene cluster prior to 1998 because those genes were still being characterized. Thus, it took another 15 years to accomplish the next steps of putting those tools to use.

Low transformation efficiencies and homologous recombination rates made for slow progress, with plenty of negative and false-positive results as well as funding challenges to confront. Despite these challenges and setbacks, Lal and his team persevered, motivated by the importance of their work to global health and for their homeland in particular. “TB claims 1 million lives per year globally, and 20–25% of these deaths are from India alone,” he says. Many MDR, XDR, and TDR strains, which have high mortality rates, circulate in India, he points out. “It was my stu-

Other Candidate Compounds with Activity against MDR-TB

Other research groups are seeking compounds with activity against multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*, including:

- One novel compound that also inhibits mycobacterial RNA polymerase, but is distinct from rifamycin, binds to the polymerase about 18 Å from the rifamycin binding site, making it effective against rifamycin-resistant MDR-TB strains, according to Vasu Nair and his colleagues at the University of Georgia, Athens. Further, it synergizes with PA-824, which inhibits *M. tuberculosis* cell wall synthesis, making a potent anti-MDR-TB cocktail. “The combination improved the MIC of compound 2 by eightfold, and that of PA-824 by fourfold,” he says.

- To circumvent resistance to isoniazid, another first-line treatment for TB, Ujjini Manjunatha at the Novartis Institute for Tropical Disease in Singapore and his collaborators are seeking direct inhibitors of the cell wall-synthesis enzyme. Isoniazid is a prodrug that depends on the enzyme encoded by KatG for activity, but mutations in this gene render it inactive against that enzyme. “Over the last two decades, efforts have yielded many potent structurally diverse direct InhA inhibitors, but so far with limited success in achieving an orally active candidate,” he says. However, a new class of compounds, the 4-hydroxy-2-pyridones, shows great promise. One of them, NITD-916, is five to eight times more potent than isoniazid itself, even against drug-resistant TB strains. “We propose that binding of 4-hydroxy-2-pyridones to the InhA-NADH complex inhibits the fatty acid elongation step, resulting in blocking the biosynthesis of mycolic acids, weakening of the cell wall… and ultimately lysing Mtb,” he says.

- The diamine SQ109 is active against drug-resistant clinical strains, and shows synergistic activity with the first-line drugs rifampicin and isoniazid in vitro and in vivo. according to Sequella in Rockville, Md. It is in a phase 2/3 pivotal trial in Russia. Meanwhile, SQ609, which contains a dipiperidine pharmacophore and targets the cell wall of this bacterial pathogen, shows additive or synergistic activity with all of the first-line TB drugs. Sequella also is developing Sutezolid, an oxazolidinone. Safe and well-tolerated, it showed significant early bactericidal activity and potential efficacy during a phase 2 clinical trial.

- Pretomanid, a nitroimidazole, belongs to a class of antibacterial agents with a novel mechanism of action against *M. tuberculosis*, according to the TB Alliance. This drug candidate, one of many being studied by the Alliance, is active in vitro against drug-resistant clinical isolates, and is a potent bactericidal agent when tested in mice. Moreover, it shows no significant cytochrome P450 interactions, and no significant activity against a broad range of gram-positive and gram-negative bacteria.

- Sirturo (Bedaquiline) a new drug for treating drug resistant tuberculosis was developed by Janssen, a part of Johnson & Johnson. In late 2012 it became the first new drug for TB to be approved by the Food and Drug Administration in 40 years. Its use is restricted to patients with MDR-TB. This novel orally administered TB drug is a diarylquinoline that inhibits mycobacterial ATP synthase but has no significant effect on ATP levels in human mitochondria.
Facing Further Challenges while Generating and Evaluating Rifamycin Analogs

In 2009, seeking support to continue this research, Lal applied for and received an ASM Indo-US Professorship (see box, p. 377). He also teamed up with Taifo Mahmud of Oregon State University, who is an expert in bioengineering and natural product chemistry, to analyze more than 100 candidate bacterial clones that were intended to produce rifamycin analogs.

“To our utter dismay, we found out that all the mutants that we had generated produced only rifamycin B and not the expected (novel) compound,” Lal recalls. Still they did not give up. “Although it was a very frustrating experience, we discussed how to proceed further and got a new strain of *Amycolatopsis mediterranei* S699 from Taifo Mahmud that had some additional features to make our selection of mutants more accurate and easy.”

This strain made a huge difference and, in 2011, Lal and his collaborators at last struck gold, he says. Aeshna Nigam, one of Lal’s graduate students, generated fresh mutants that again were sent to Mahmud for analysis. Three strains in this new set produced the long-sought novel rifamycin analog, 24-desmethylrifamycin B. The expertise of Mahmud and his group proved critical, Lal says.

This biochemical success was worth celebrating on its own, but whether the novel analog had any antimicrobial activity was yet to be determined. Even if it did, it might not be effective against drug-resistant strains of *M. tuberculosis*. Many MDR-TB strains are cross-resistant against multiple rifamycin analogs because escape mutations tend to allow RNA polymerase to evade structural similarities across this class of antibiotics.

To evaluate the activity of the new compound, Lal turned to his collaborator Yogendra Singh, recipient of the 2013 ASM Moselio Schaechter Distinguished Service Award (see box, p. 377), at the Institute of Genomics and Integrative Biology (CSIR-IGIB), Delhi. After converting the bacterial product into its semisynthetic derivative, 24-desmethyrlrifamycin, the novel analog performed in vitro comparatively to, or better than, several clinically used members of the rifampicin family of antibiotics against various pathogenic bacteria, including *Staphylococcus aureus*, *M. tuberculosis*, and even rifampicin-resistant *M. tuberculosis* strains, according to Singh and his collaborators.

“While we anticipated that altering the functional group at this particular position would be directly associated with the change in the antibiotic potential, we were not very sure if the analog would be biologically effective or not, and the work with CSIR-IGIB helped us in proving its effectiveness,” Lal says. “Its better antibiotic potential has been a fortunate discovery.” He postulates that the activity against rifampicin-resistant strains may be due to increased flexibility of the demethylated compound, allowing it to bind to mutated RNA polymerases while older analogs of rifampicin cannot. The analog might be further improved by chemically modifying its C-3 and C-4 positions, he adds.

Resistance Possibilities Loom Even for Novel Analogs

The success of these new compounds could be short-lived, warns Rothstein. Resistance tends to develop quickly against rifamycin antibiotics because of the abundance of RNA polymerase escape mutations, he says.

Lal agrees and anticipates producing many different novel analogs with which to keep ahead of such resistance. “Now with the proof of concept in hand we are trying to develop a library of rifamycin analogs, with modifications targeted at other domains and modules of the rifamycin PKS gene cluster,” he says. “The combined genetic and synthetic approach holds great potential to generate a wide variety of rifamycin analogs that may be effective against the global threat of MDR-TB... and other life-threatening pathogens.”

“This [technique] is a real breakthrough,” says Rothstein. “That’s not to say that the compounds produced will be necessarily better. That has to be tested. It’s a roulette wheel if what comes out will be any better.” New analogs might have improved—or worse—toxicity profiles or fewer drug-drug interactions, which could give them an edge in or keep them out of the clinic. Rifamycins are notorious for inducing cytochrome P450 drug-metabolizing enzymes, which degrade other drugs and undermines overall efficacy in...
treating TB patients, especially those who are also being treated for HIV/AIDS. A novel rifamycin analog without these issues would be ideal for clinical development.

Lal’s approach might also be applied more broadly to generate analogs of other antibiotics, as has been done with erythromycin and rapamycin. However, Lal warns that the lengthy process of developing tools for genetically engineering antibiotic-producing microbial species can be cumbersome. “It would require extensive standardization of protocols as the technique is organism- and antibiotic-specific,” he says. “Despite these limitations, the scope of diversifying natural products through mechanisms as this is endless. Misadventures with strong lessons . . . are not failures [but] part of the march to success.”

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