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Prospects for New Anti-tuberculosis Drugs

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The discovery of streptomycin, over 50 years ago, heralded a new era in which it was anticipated that the scourge of tuberculosis (TB) would be conquered by drug treatment. The following two decades saw the introduction of more drugs, the most prominent of which were isoniazid and rifampicin, the mainstay of short-course chemotherapy today. Since then, there has been little in the way of further drug discovery and development. Synthetic analogues of rifampicin such as rifabutin, rifapentine, and most recently, KRM-1648, have been prepared, but none has yet had an impact on therapy. Other classes of broad-spectrum antibacterial agents such as the quinolones have recently been used to treat mycobacterial infections, but resistance to these has emerged.

Soon after the first drugs came into use in the clinic, it was discovered that monotherapy was of limited effectiveness because of the rapid emergence of drug-resistant strains of *Mycobacterium tuberculosis*. Further investigation revealed that this was due to selection of one or a few individuals in a large population of bacteria which had a pre-existing resistance to the agent. Thus, a combination of drugs was employed in therapy, in the knowledge that the likelihood of spontaneous mutations conferring resistance to two drugs occurring in the same organism is very remote indeed. This has been refined to the short-course chemotherapy regimen in use today—that is isoniazid and rifampicin taken over six months, supplemented with pyrazinamide for the first two months with ethambutol added when isoniazid resistance is suspected. Poor compliance with such a long, complex and unpleasant combination of drugs results in a significant treatment failure rate, with treatment having to be extended, sometimes to as long as two years, to fully cure the patient of infectious bacteria. Worse still, resistance may emerge to these first-line agents, and thereafter to a wide range of second-line antimycobacterials. Treating individuals infected with multi-drug resistant-TB (MDR-TB) is difficult, expensive and frequently unsuccessful. To overcome these problems, the World Health Organization (WHO) currently recommends following "DOTS" (directly observed therapy, short course) as the best clinical practice.

What is Needed?

The attributes of the perfect TB therapy would include, activity against all bacteria present during an infection, activity against all MDR strains, an orally active formulation, no toxic side-effects, long duration of action to make intermittent therapy possible, and ability to cure an infection in a very short space of time. Such a treatment in a single molecule may well be unattainable. Therefore, we can consider the next best option, which is to attack the distinct foci of infection, namely the rapidly-growing, extracellular bacteria, and the quiescent population of bacteria which survive within host professional phagocytes. The current generation of drugs exert their effect by targeting essential processes in the mycobacterial cell, such as biosynthesis of the cell wall polymers. New agents with similar rapid mycobactericidal properties, but with new and different modes of action, are urgently required to counter the threat posed by drug-resistant bacteria. In addition, we must explore targets in the interaction between the host and pathogen, factors essential for virulence of the bacterium. For instance, the means by which the organism escapes the killing mechanisms of the phagocytic cells in which it resides may be targeted in order that the bacteria may become susceptible to intracellular killing and destruction.

Probably the single most important advance would be to identify a means of reducing the overall duration of therapy. Shortening the drug course to three months or less would significantly increase compliance rates with a consequent improvement in the cure rate, and more importantly, a reduction in the number of active pulmonary cases, patients who are responsible for the spread of infection. However, to do this requires a leap forward in our fundamental understanding of how the organism functions in a quiescent, or dormant state, and of how it evades the host's immune system while in this form.

The question is often raised as to why the pharmaceutical industry has not developed existing classes of antibacterial agents into TB therapies. This is not as easy as it would at first appear. During development, modifications will have been made to the antibacterial agent which optimize its activity against a range of target pathogens, and so many will have little or no anti-TB activity. In contrast to treatment of acute infections where any side-effects may not be significant, moderate toxicity may preclude the use of a drug in long-term therapy. A related issue is that long-term use of a broad-spectrum agent may disturb the normal gut flora, and may lead to the emergence of resistance. TB-specific agents have their advantages, particularly in that the activity will be optimized for the very narrow mycobacterial spectrum, and they have a pharmacokinetic profile optimized for treating infection with a single pathogen. There is also no possibility of resistance transfer from other bacteria. The major drawback is the high risk—the full development costs must be recovered from use in TB only, and the market will not support a high price.

Targets for New Anti-mycobacterial Agents

Historically, the identity of essential targets in bacteria was revealed by genetic analysis. Such a search was facilitated in

organisms such as *Escherichia coli*, by the availability of conditional lethal mutants, that is, mutants which are able to grow under a particular set of conditions but which are unable to survive when those conditions are changed, such as when temperature is elevated. Genetic systems which permit analysis of such mutants, and methods for carrying out homologous recombination are available for *E. coli*, allowing the function of individual genes to be studied. No conditional lethal mutants of *M. tuberculosis* have been described and, until recently, no genetic tools were available for manipulation of mycobacteria. Despite the superb progress which has been made over the past decade, it is not yet possible to carry out these important experiments on a routine basis. The outlook for mycobacterial targets has been changed dramatically by the forthcoming availability of the complete DNA sequence of the mycobacterial genome. At the time of writing this paper, over a third of the sequence has been determined, and it is anticipated that it will be completed within the next year. By then, we will know every target there is in the bacterium—the challenge is to identify amongst them those which are of most immediate relevance.

Let us consider a way of ranking the importance of targets. The criteria that may be employed are: the target should be found uniquely in *M. tuberculosis*, be essential for the viability of *M. tuberculosis*, be unique to the immunopathology of *M. tuberculosis* infection, be amenable to study and be amenable to assay in relatively high throughput. The ideal scenario would be to identify targets which fall into one of the following classes, firstly, gene families which catalyse essential reactions, and secondly, processes which are essential to the survival of bacteria in more than one growth phase. An example of the former are the enzymes which assemble the arabinan portion of arabinogalactan, since each catalyse similar glycosyl transferase reactions and utilize the same substrate, decaprenyl-phosphate arabinose. The members of this enzyme family are very likely to be related in primary sequence and in the tertiary structure of their active sites. If a single molecule is able to interact with all the members of the family and can inhibit each enzyme, then it is likely that the lethal effect on the cell will not be reversed by a single mutation in any one of the target enzymes. This is analogous to the interaction of the β-lactam antibiotics with the penicillin binding proteins, which catalyse the transpeptidase reactions during the late stages of bacterial peptidoglycan assembly. In that case, resistance is mediated by enzymes which inactivate the drug. In searching for targets of the second ideal type mentioned above, studies of genes expressed under conditions of stress, such as at low pH or low oxygen tension may mimic the effect of the intracellular environment on growth, or the state of the cells when they are in a dormant phase. These may reveal targets that are essential for growth only in that state, but will also reveal which genes are essential in both stressed and normal growth phases.

The Action TB Initiative

The simultaneous recognition of the need for new anti-TB drugs and the lack of a fundamental understanding of both *M. tuberculosis* and the molecular pathogenesis of TB prompted Glaxo Wellcome to launch the Action TB Initiative in 1993. This is a wide ranging five-year research programme supported by a £10 million investment in academic research in centres in the UK, South Africa and Canada. The aims are to identify new drug targets and to gain a better understanding of the mechanisms by which immunity to TB develops, which may lead to development of new vaccines. A very diverse range of skills have been brought together in a highly interactive, open collaboration, a key feature of which is that academic researchers and Glaxo Wellcome scientists work in close partnership to ensure that discoveries are translated into potential new drugs as rapidly as possible. Regular meetings of everyone involved encourage exchange and sharing of information, ideas and materials.

From this programme, a number of potential drug targets have emerged. For example, researchers at the South African Institute for Medical Research in Johannesburg, working under the leadership of Professor Valerie Mizrahi, have been studying DNA polymerase I (Pol I) from *M. tuberculosis*. Pol I is important for repair of DNA damage, a process which is essential for the survival of all organisms, and may be particularly important for survival of *M. tuberculosis* in the DNA-damaging intracellular environment. They found that when the DNA sequence of the gene encoding this enzyme was determined, it was related to the sequences of similar proteins from other organisms, with the exception of a few key changes. At a particular position in the O helical region of the polymerase domain, a phenylalanine is commonly found, but the mycobacterial enzyme has a tyrosine at the corresponding location. This amino acid is situated in the active site of Pol I and this single change renders the mycobacterial enzyme unable to discriminate between natural deoxynucleotides and chain-terminating dideoxynucleotides. The mycobacterial Pol I incorporates the dideoxy-analogues with even greater efficiency than the non-discriminating DNA polymerase from bacteriophage T7. Thus it may be possible to exploit this difference to make a mycobacterial Pol I-specific inhibitor or to force the enzyme to incorporate an unnatural substrate into DNA, leading to further DNA damage.

A number of mycobacterial proteins have been shown to be glycosylated. Although glycoproteins are commonly found in eukaryotes, this post-translational modification is less frequently encountered in bacteria. Working at Imperial College School of Medicine at St Mary's, Professor Douglas Young's laboratory has studied glycosylation of the *M. tuberculosis* 19-kDa lipoprotein antigen as a model system. They showed that glycosylation takes place on two clusters of threonine residues located at the *N*-terminal region of the protein and went on to demonstrate that glycosylation protects the protein from proteolytic cleavage. Glycosylation may regulate conversion of a cell-associated to a soluble secreted form of the protein. Release of antigens from the cell may have important immunological consequences during infection. Study of this process has highlighted three potential types of target: enzymes which carry out the glycosylation reactions, protease which cleaves the antigen, and glycosidases which may remove the sugars.

In our own laboratories, working in collaboration with Dr Michael McNeil at Colorado State University, we have studied the early steps in formation of the galactose residues in the cell wall arabinogalactan. In this polymer, galactose is present in the thermodynamically-unfavourable five-membered furanose conformation, rarely found in nature, and so agents active against the enzymes responsible for its synthesis are likely to

possess very high specificity of action. We investigated the enzymes of the Leloir pathway in *Mycobacterium smegmatis* to find that both UDPglucose epimerase and galactose-1-phosphate uridyl transferase are present. The epimerase interconverts UDPglucose and UDPgalactose – the galactose moiety of product is exclusively in the pyranose form. Uridyl transferase forms UDPgalactose from UDPglucose and galactose-1-phosphate. With galactopyranose-1-phosphate as substrate, again the galactose moiety of product is exclusively pyranose; galactofuranose-1-phosphate was neither a substrate nor an inhibitor. We concluded that furanose ring formation occurs later so we investigated interconversion of UDPgalactopyranose and UDPgalactofuranose, firstly by analysing the product of a reaction catalysed by the *E. coli glf* gene product, the enzyme UDPgalactopyranose mutase. The equilibrium point of the reaction results in only approximately 6% conversion to the furanose form of the sugar nucleotide. It was not possible to show a similar conversion in the pyranose to furanose direction in extracts from *M. smegmatis*, presumably because of this unfavourable equilibrium position. However, using the *E. coli* enzyme to catalyse the forward conversion, we were able to purify a small quantity of UDPgalactofuranose which we used to demonstrate the interconversion in the furanose to pyranose direction, proving that the enzyme is present in mycobacteria.

From Target to Lead

Once a target has been identified and validated, the next step in drug discovery is to find a lead molecule with activity against it. Normally, further modification of the structure will be necessary to generate a drug candidate having all the properties desired in the final drug. There are two complementary approaches to lead identification, namely rational design and screening. It is appropriate to employ the former when there is some specific knowledge of the target, such as the nature of an enzyme's substrate or its reaction mechanism, or in the relatively rare event that the complete three-dimensional structure of the protein is known. Considerable effort may be required to obtain this information, an investment in resources that is only warranted when one is very confident of the validity of the target. In contrast, random screening is very effective in situations in which there is not an obvious rational route to an inhibitor, for example, a DNA-binding protein interaction or a receptor ligand interaction, or where finding an inhibitor will in itself help to validate the target. More importantly, screening allows a very diverse range of structural types to be tested against the target, and by testing as many samples as possible, the chances of finding an agent are improved. In practice, a combination of both approaches is employed.

The factors which influence the success of high-throughput screening are the assays employed, and the diversity and number of samples which are tested. An assay must be based on an output that in some way reflects the way the target normally behaves, yet it must be sufficiently robust that it can accept samples containing low concentrations of organic solvents and salts. Traditionally, most companies have relied upon two sources of samples for testing, namely compound banks and natural products. A compound bank is built up over many years from in-house medicinal chemistry projects or by obtaining material from academic and other laboratories. Greater diversity is obtained by testing natural products from the widest possible range of sources. These natural products include molecules which cannot easily be made synthetically and so would not otherwise be available for testing. *Actinomyces*, and *Streptomyces* species in particular, have been a particularly rich source of drugs, many of which are in common use. Extracts prepared from other bacteria, from fungi, from lower eukaryotes, and from plants are also tested.

Much attention has been focused in recent years on the science of combinatorial chemistry, which facilitates the synthesis, using robotics, of very large numbers of molecules from a relatively small number of components. To illustrate the power of this approach, if one set out to generate one million different molecules in a traditional, non-combinatorial manor, one would require 1 000 001 reagents. However, by using combinatorial chemistry the same number of molecules can be generated with only 300 reagents ($100 \times 100 \times 100$). In order to be able to screen this number of compounds, advanced robotics have been utilized extensively in this part of the screening process. With an assay that can be made to work in a format that is compatible with these systems, it is currently possible to test 50 000 sample points in one week, a task which would have taken many months to complete in a fully manual operation only a few years ago.

At Glaxo Wellcome, we have developed a simple screen to find agents with whole-cell antimycobacterial activity and have now used this to identify a number of lead compounds. Safety and time considerations preclude the use of *M. tuberculosis* in whole-cell screening, so we searched for a rapidly growing saprophytic mycobacterium with comparable drug sensitivities, and we selected a strain of *Mycobacterium aurum*. In collaboration with Professor Peter Andrew of Leicester University, we have cloned the *luxA* and *luxB* genes from *Vibrio harveyi* to yield a strain which produces light (bioluminesces) in the presence of a specific substrate, decanal, but only when the cells are viable. Thus, following incubation of this organism in the presence of test samples, compounds with antimycobacterial activity can be readily identified as those which eliminate the bioluminescence.

Conclusion

Our understanding of *M. tuberculosis* and its complex interactions with the host are becoming ever more clear. The anticipated completion of the DNA sequence of the *M. tuberculosis* genome will provide a complete description of every target in the bacillus. Advances in molecular genetics provide the tools to analyse the sequence and it will soon be possible to identify all those targets which are essential to survival of the organism under a variety of growth conditions. Advances in combinatorial chemistry and in robotic systems for screening very large numbers of samples make it likely that we will soon be able to identify novel compounds with activity against *M. tuberculosis*. The prospects for developing new drugs which are active against today's MDR strains, with novel modes of action, and which have improved pharmacological properties are better today than at any time in the past.