Caught between two proteins: a mycobacterial inhibitor challenges the mold

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SUMMARY

Elucidating the target or mechanism of action of potential drugs in the discovery pipeline, is an integral component of most programs. For antibacterial compounds, generation of resistant mutants followed by whole genome sequencing has often been successful in uncovering the proteins involved in regulating compound activation, uptake, efflux and importantly, target processes. When this process succeeds, we are quick to declare a target. In a study reported by Sing et al (in press), the combination of resistant mutant generation, whole genome sequencing and recombineering to identify the target of a *Mycobacterium tuberculosis* growth inhibitor, pointed to a mechanism involving a scaffolding protein, Wag31, involved in polar elongation of mycobacterial cells. Time-lapse microscopy and electron microscopy confirmed the view that this inhibitor resulted in interruption of nascent cell wall biosynthesis. However, co-expression as well as regulated titration of the putative Wag31 target demonstrated that the wild-type allele was dominant and showed no synergy with the inhibitor. The most plausible explanation from their results was that this inhibitor interfered with the interaction of Wag31 with one of its interacting partners in the elongation complex.

Current clinically used antibacterials have all been discovered in phenotypic screens against whole cells using *in vitro* screening assays with the exception of pyrazinamide that was discovered in mouse infection studies(Zhang & Mitchison, 2003). The successes of whole cell screens contrasted to the failure of target-based screens to deliver leads to feed the drug development pipeline, have encouraged large-scale screening programs(VanderVen *et al.*, 2015) (Mak *et al.*, 2012) (Christophe *et al.*, 2009) (Grant *et al.*, 2013) (Stanley *et al.*, 2012) (Ananthan *et al.*, 2009) (Maddry *et al.*, 2009) (Ballell *et al.*, 2013) (Warrier *et al.*, 2015) (Tantry *et al.*, 2015) over the last decade in efforts to develop new drugs to combat the ever-prevalent scourge, tuberculosis.

Tuberculosis remains the leading killer from an infectious disease worldwide despite the availability of a chemotherapeutic regimen that can achieve greater than 90% cure rates under clinically optimal settings(Zhang & Mitchison, 2003). The current treatment regimen for drug sensitive tuberculosis entails 2 months of treatment with isoniazid, rifampicin, pyrazinamide and ethambutol followed by a 4-month continuation phase with isoniazid and rifampicin. Lack of patient adherence due to side effects associated with these drugs, the pill burden and socioeconomic factors have contributed to disease relapse and emergence of drug resistance. Development of new drugs with better safety indexes and/or new drugs that

will significantly shorten the duration of treatment would be predicted to lead to higher compliance and thus some hope of combatting this global threat. The defining features of a drug that would lead to treatment shortening are poorly understood. Conceptually it could be argued that a treatment shortening regimen would contain a drug that has a mechanism of action different from those engaged by current anti-tuberculars which comprise the current extended treatment regimen. In this respect, linezolid, a drug targeting the ribosome in a mechanism distinct from first and second line anti-tubercular agents, showed unexpected clinical efficacy(Lee *et al.*, 2015) considering its static activity in both *in vitro* and *in vivo* models. Its utility in shortening treatment duration remains to be established since its application is hampered by mitochondrial toxicity-related adverse events. Newer drugs such as bedaquiline and delamanid have novel mechanisms of action and have passed regulatory approval based on clinical efficacy but are currently restricted for use in drug-resistant tuberculosis(Gler *et al.*, 2012) (Diacon *et al.*, 2014). Activity against slowly- or non-replicating persisting mycobacteria has also been identified as an important hallmark of a drug that would lead to treatment shortening(Nathan & Barry, 2015) based on the hypothesis that the bacteria that persist in the face of months of chemotherapy are those that slowly replicate and/or persist in large caseous or necrotic lesions(Chen *et al.*, 2014). In the current treatment regimen, rifampicin and pyrazinamide were the two drugs that were key to the development of current short-course (6 month) chemotherapy(Fox *et al.*, 1999), with pyrazinamide showing selective activity against *M. tuberculosis* in various *in vitro* models of non-replicating persistence(Alnimr, 2015) with unproven clinical relevance. In contrast, drugs that target biosynthesis of the mycolyl-arabinogalactan component of the cell wall including isoniazid, ethionamide, ethambutol and thiacetazone, have limited efficacy under *in vitro* models of non-replicating persistence and thus are likely to have limited efficacy against the recalcitrant bacteria in caseous and necrotic granulomas. Despite this, biosynthesis of peptidoglycan remains an attractive drug target as evidenced by the cidality of meropenem/clavulanate against non-replicating *M. tuberculosis(Hugonnet et al., 2009).*

Studies exploring the mechanism of action of hits from conventional whole cell screens do not allow researchers to pre-select their targets of choice. The process of taking a hit from a screen to a pre-clinical candidate is long and arduous with chemistry efforts often surpassing biological studies aimed at understanding the mechanism of action of the hits that have passed initial formal hit assessment assays. Thus the biology often lags behind as seen with pretomanid (Mukherjee & Boshoff, 2011)and SQ109(Sacksteder *et al.*, 2012) where a lead was identified prior to the deciphering of the mechanism of action of these candidates. Although target identification is not absolutely essential in the drug development pipeline, it remains an important milestone. The identification of a target can assist rational drug design strategies, it enables identification of alternative scaffolds that may overcome medicinal chemistry liabilities encountered with the original scaffold, enables in-depth understanding of potential obstacles such as mechanisms of resistance and it allows target validation in the appropriate *in vivo* models while hits or leads are optimized for the desired ADME-T properties. Only favorable pharmacokinetic and pharmacodynamic properties allow an informed decision to be made about the lead in question based on results from *in vivo* efficacy studies. The typical process of drug target identification involves generation of resistant mutants(Ioerger *et al.*, 2013) wherein mutations in the target have allowed the

identification of the mechanism of action of drugs such as bedaquiline(Andries *et al.*, 2005), Q203(Pethe *et al.*, 2013) and the benzothiazinones(Makarov *et al.*, 2009). For agents such as griselimycin, target amplification in the genome confirmed the target of the natural product(Kling *et al.*, 2015). Similarly, resistance to an indazole sulfonamide scaffold was only observed in mutants that resulted in gene amplification, with subsequent structural studies providing an explanation: the inhibitor's interactions were almost exclusively made with the cofactor in the active site of the target, inosine monophosphate dehydrogenase(Park *et al.*, 2016). In some cases, the target is refractory to mutations that confer resistance. For anti-tubercular compounds such as cyclomarin, the ClpC1 protease component was identified by a chemical proteomics approach wherein the proteins that bound the inhibitor were identified using cyclomarin-linked beads(Schmitt *et al.*, 2011). Intriguingly, resistance-conferring mutations in *clpC1* were identified for another natural product, lassomycin, that targets the ClpC1P1P2 protease(Gavrish *et al.*, 2014), emphasizing the importance of the *nature* of association of an inhibitor with its target in the ability of target mutation to confer resistance. Resistance mutations could not be identified for SQ109 and its mechanism of action was deciphered by macromolecular incorporation assays and analyses of trehalose mycolate lipid profiles that suggested the MmpL3 transporter as the target. This was further supported by *mmpL3* mutants raised to other putative MmpL3 inhibitors, although direct inhibition or engagement of MmpL3 was never demonstrated(Tahlan *et al.*, 2012).

The challenges in drug target identification are highlighted in the elegant study by Singh & Dhar and colleagues (in press). An amino-pyrimidine-sulfonamide (APYS) inhibitor of *M. tuberculosis* growth was identified in a whole cell screen. The pipeline of drug target identification through generation of resistant mutants according to typical drug target identification programs showed that all resistant mutants had amino acid substitutions in the C-terminal coiled coil domain of the Wag31 protein with their role in conferring resistance confirmed by recombineering. Wag31, the mycobacterial homolog of DivIVA, is an essential scaffolding protein that is localized at the bacterial poles and that orchestrates polar growth in actinobacteria through its interaction with proteins involved in DNA segregation and cell wall synthesis(Donovan & Bramkamp, 2014). This protein is also found in other gram-positive bacteria but, in bacteria such as *Bacillus subtilis,* interacts with other proteins involved in cytokinesis. Thus, polar growth does not occur in all bacteria that express this protein(Donovan & Bramkamp, 2014). The phosphorylation state of Wag31 in mycobacteria, and its subsequent interaction with cognate binding partners, is regulated by the essential serine/threonine kinases PknA and PknB involved in regulating cell division, thereby controlling the rate of polar peptidoglycan biosynthesis(Jani *et al.*, 2010). Not only is the identification of a mechanism of action centered around Wag31 function a novel drug target, but its central role in coordinating cell division and new peptidoglycan biosynthesis is particularly attractive. Accordingly, treatment of cells with the APYS inhibitor resulted in cell lysis, reminiscent of that seen during inhibition of peptidoglycan biosynthesis (Fig. 1). If a picture paints a thousand words, the insightful use of time-lapse microscopy provided visual support of a mechanism of action centered around Wag31. This technique revealed that APYS inhibitor treatment resulted in swelling primarily at the old pole but that this was followed by division, after which both the daughter cell containing the old pole as well as the sibling containing the new pole, lyzed. Survivors always emerged from daughter cells

containing the new pole. In contrast, down-regulation of Wag31 expression resulted in swelling of the new pole, likely because the old pole already contains pre-assembled Wag31.

The researchers, however, hit a road-block when trying to establish that Wag31 was the target for this inhibitor. Expression of a wild-type allele in a resistant mutant conferred drug susceptibility and, conversely, expression of a *wag31*^{Q201R} resistant allele in *trans* in wild-type cells did not confer resistance, showing that the wild-type allele was dominant. Regulated expression of Wag31 using a tetracycline-regulated promoter driving the native copy of *wag31* also showed no synergy between protein down-regulation and APYS treatment and also did not shift the minimum inhibitory concentration of the inhibitor. The finding that none of the *wag31* mutants showed a defect in growth argues against changes in Wag31 function affecting activity of downstream targets. The only conclusion from these results was that the APYS inhibitor affects interaction of Wag31 with one of its many binding partners. The finding that all amino acid substitutions mapped to the hydrophobic side of the C-terminal helix of Wag31 supports this notion. Unfortunately, the lack of titration of Wag31 expression with APYS susceptibility complicates interpretation of the nature of this protein-protein interaction. There are many binding partners for Wag31 of which only a few have been identified to date. For example, AccA3, an essential acetyl-CoA carboxylase involved in lipid biosynthesis for both cytoplasmic membrane and cell wall production, has been shown to interact with Wag31(Xu *et al.*, 2014), yet APYS did not affect lipid biosynthesis, arguing against a role for AccA3 in the mechanism of action of this inhibitor. Other interacting partners for Wag31 include the penicillin-binding protein FtsI, CwsA and PknB(Mukherjee *et al.*, 2009) (Plocinski *et al.*, 2012) although, besides the phosphorylation of Thr73 by PknB, the exact nature of these interactions is not clearly defined.

What emerges from this work is the strong message that "discovering a drug target" carries a far deeper significance than what is reported from studies where the events unfold following their logical, anticipated course. Singh & Dhar et al (in press) used approaches that are consistently used in the traditional framework of drug discovery and combined these with time-lapse imaging studies which confirmed the interpretation that their hit inhibited the Wag31 component of the mycobacterial elongation complex. However, an analysis of protein level titration indicated that this inhibitor had a more complex effect of the protein machinery of the macromolecular protein machinery that directs elongation during cell growth. This study only emphasizes the complexities of the biological studies that support the drug discovery pipeline and challenges the framework of experiments designed to identify mechanisms of action in the "one inhibitor-one protein target" model that has been the mold for so long.

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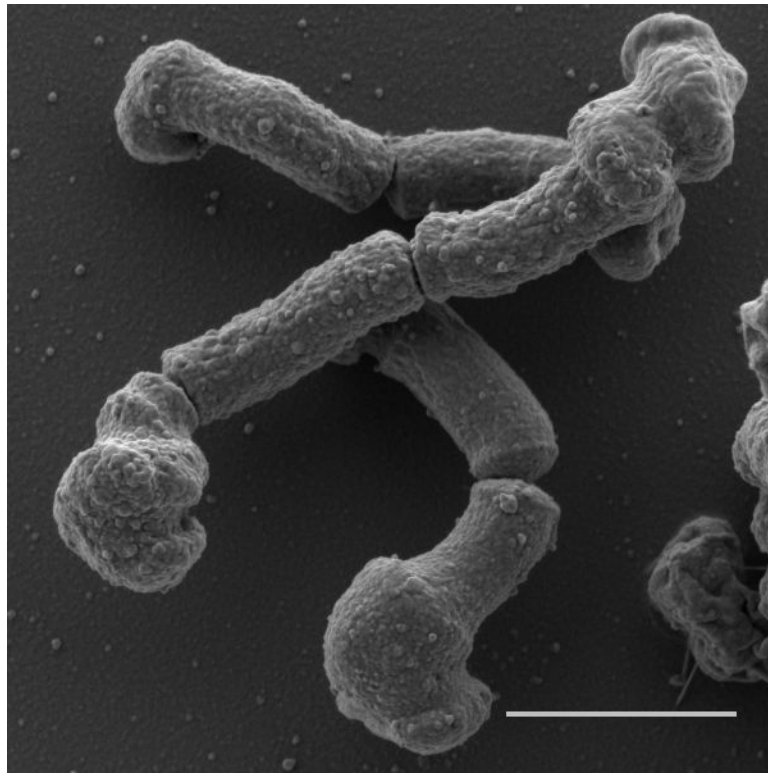
Figure 1.
Scanning electron microscopy image showing the polar deformation of *M. tuberculosis* cells prior to cell lysis after 24 hours of exposure to the APYS inhibitor. The polar swelling is particularly prominent at the old pole. Scale bar is 1μM. Imagine courtesy of Gaëlle S. Kolly.