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Mini-Review

Overview of Small Molecules as Fluorescent Probes of *Mycobacterium tuberculosis*

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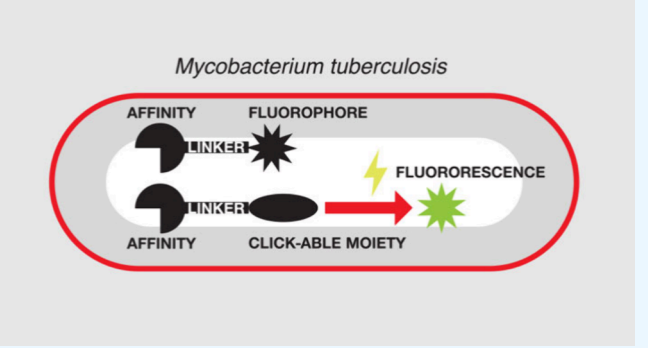
ABSTRACT: Tuberculosis (TB) remains one of the leading infectious causes of death worldwide. Detecting and precisely quantifying viable *Mycobacterium tuberculosis* (*M. tuberculosis*) is crucial for comprehending mycobacterial pathogenicity; the progression and outcomes of tuberculosis; and the action, efficacy, and resistance of drugs. Fluorescent probes have emerged as indispensable tools for studying the intricate structure and dynamic interactions of *M. tuberculosis* with its host environment. This minireview underscores the significance of small molecules as fluorescent probes in advancing our understanding of mycobacterial biology and highlights their potential for guiding the development of novel therapeutic interventions against tuberculosis.



INTRODUCTION

Tuberculosis (TB) is still a global public health concern despite World Health Organization (WHO) eradication efforts. In 2022, 7.5 million people had been diagnosed with tuberculosis and this is the highest number of people with TB ever diagnosed and treated in a year, since WHO began global TB monitoring in 1995.[1] Most of the TB infections are curable with early diagnosis and appropriate treatment, but drug-resistant TB is difficult to treat in immunocompetent and immunocompromised patients. The ability to monitor the metabolic status, growth, and division of *Mycobacterium tuberculosis* (*M. tuberculosis*; Mtb), the causative agent of TB, with precise temporal and spatial resolution, could significantly enhance the comprehension of host−pathogen interactions. This insight has the potential to drive both advancements in clinical detection and the exploration of innovative therapeutic agents. Tackling TB necessitates a comprehensive strategy that encompasses the creation of novel diagnostics and treatments and exploration into the intricate pathogenesis of the disease.[2] The recent rise of bioorthogonal chemistry (Staudinger ligation, as well as azide−alkyne, tetrazine−alkene, tetrazine−alkyne, and photoactivated tetrazole−alkene cycloadditions) has afforded new approaches to explore and study biological functionalities in vitro and in vivo.[3] In this minireview, we are interested in small molecules used in fluorescent and fluorogenic methods for Mtb imaging and antitubercular drug susceptibility testing.

A plethora of commercial tests are available for the diagnosis of TB in many settings. Many probes targeting specific mycobacterial components are available with a variety of applications ranging from diagnosis and drug screening to elucidation of Mtb microbiology. The most accurate method of mycobacteria detection is the bacteriological analysis via microscopic examination of a Ziehl−Neelsen stained culture growth. However, this old fashioned method is time-consuming as it requires weeks to grow the Mtb strain.[4] On the other hand, nucleic acid based diagnostic methods, such as GeneXpert, are high cost and technical skills required assays, difficult to be applied in resource-limited settings.[5] Various small molecules have been used for the design and synthesis of chemical probes to explore the structure of mycobacterial cells and elucidate Mtb−host interactions.[6] Fluorescent probes have gained significant popularity for imaging cells, with organic fluorophores being particularly favored. This preference stems from the wide range of available structures that offer diverse excitation and emission properties.

Trehalose Based Fluorescent Probes. Trehalose, a nonmammalian disaccharide, presents a crucial role in Mtb's metabolism and cellular function. Bertozzi's, Davis', Kiessling's, and Swarts' research groups have developed various trehalose based reporters to visualize mycobacteria rapid detection or cell division and antibiotic action on a mycobacterial cell wall.[3]

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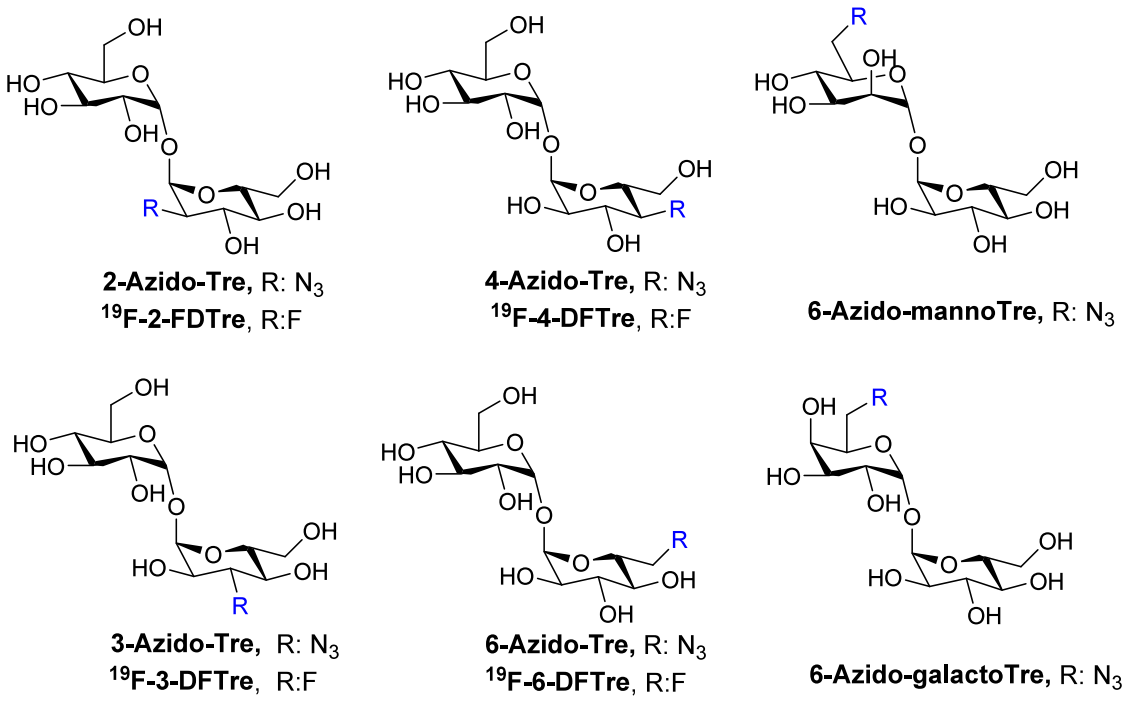

Figure 1. Azide and deoxyfluoro-D-trehalose derivatives.

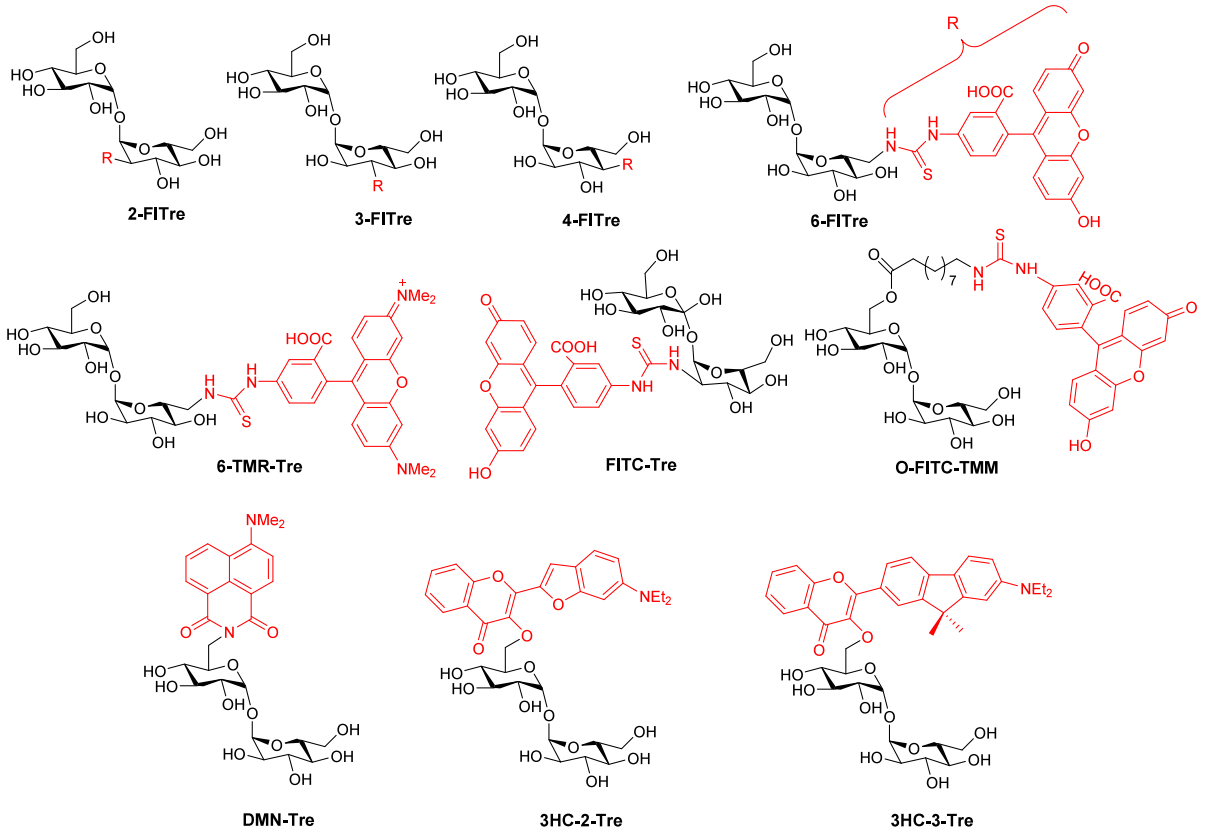

Figure 2. Fluorescent trehalose probes.

Bertozzi et al., for example, have used exogenous azido-modified trehalose molecules which were inserted into the mycobacterial cell wall and then were bioorthogonally ligated to alkyne-functionalized fluorescent probes (e.g., alk-AF488) via Cu(I)-catalyzed cycloaddition and fluorescently detected.[7] The nonradioactive deoxyfluoro-D-trehalose (^{19}FDTre) analogues were also implemented.[8] Moreover, 6-azido-modified asymmetric manno- and galactotrehalose were developed to resist degradation/isomerization by mycobacterial enzymes,[9] depicted in Figure 1.

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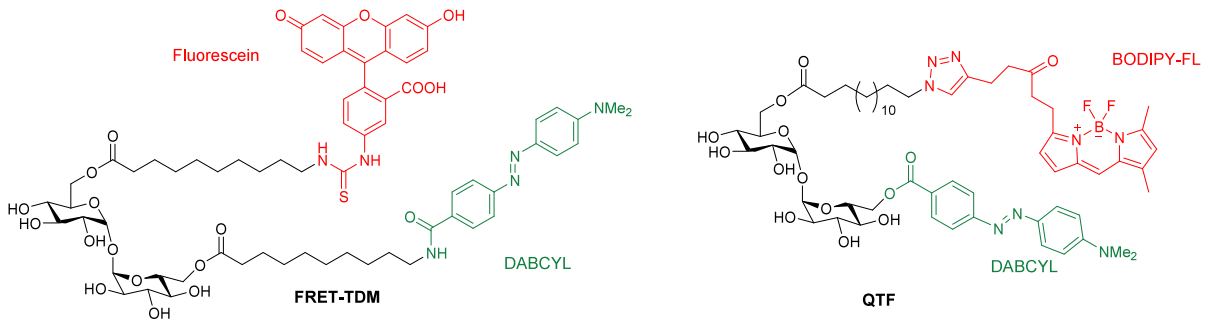

Figure 3. Turn-on fluorescent trehalose probes. The fluorophore is in red and the quencher is in green.

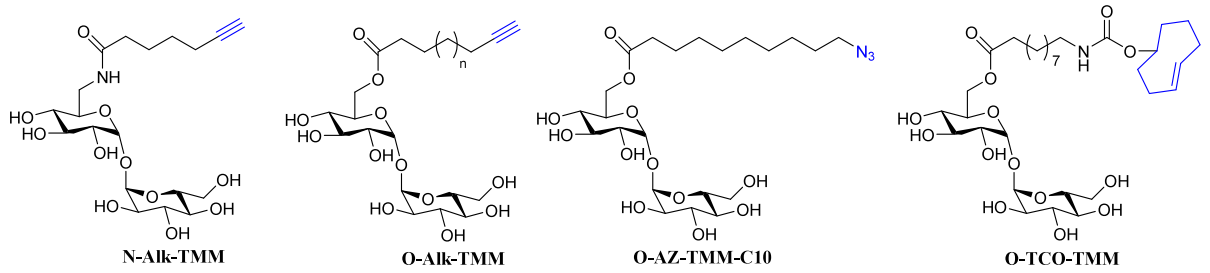

Figure 4. Click-able trehalose derivatives.

The fluorescein-containing trehalose analogs were the first fluorescent probes (**FITC-Tre**) to be designed by Backus et al. to label Mtb macrophages both in vitro and in vivo.[10] A whole library of regioisomeric analogs of fluorescein−trehalose (**FITre**) were then synthesized by Rodriguez-Rivera et al.,[11] depicted in Figure 2. The latter derivatives exhibited higher labeling than **FITC-Tre** with 6-fluorescein−trehalose (**6-FITre**) being the more intense label. However, a limitation of these organic fluorophores is the high background fluorescence, which is diminished in 4-*N*,*N*-dimethylamino-1,8-naphthalimide-conjugated trehalose (**DMN-Tre**). This analog is used for the detection of Mtb from the sputum samples of TB patients but presents low brightness for adequate fluorescent signal. To overpass this limitation, trehalose was conjugated with 3-hydroxychromone and the new probe **3HC-3-Tre** was found to be 10 times more fluororescent than **DMN-Tre**. Another way of labeling mycobacterial cells is by using **O-FITC-TMM** which has shown high strain specificity. Furthermore, one more probe with labeling efficiency is the conjugated tetramethylrhodamine at the C-6 position on trehalose (**6-TMR-Tre**). Bertozzi et al. have also created solvatochromic dye-conjugated trehalose probes, based on 3-hydroxychromone moiety (3HC). In polar settings, these conjugated solvatochromic dyes (**3HC-2-Tre** and **3HC-3Tre**) do not fluoresce; however, in the lipophilic mycomembrane, that is not the case and fluorescence is observed. The color or the fluorescence intensity of the solvatochromic dyes depends on the polarity of the solvent. Moreover, theses dyes exhibit high quantum fluorescence yield and are red-shifted.[12]

A notable disadvantage of the organic fluorophores is photobleaching, which occurs when the fluorophore gradually loses its fluorescence ability particularly when exposed to continuous light irradiation. Fluorescent turn-on probes specifically are designed for the detection of mycobacteria to minimize this problem.[13] There is a long list of reported fluorescence turn-on probes that use trehalose. These probes use a variety of techniques, including enzyme activity, molecular rotor turn-on fluorophores, quencher−fluorophore systems, solvatochromic dyes, and fluorescence resonance energy transfer reactions (FRET). Trehalose probes do not interfere with mycobacterial functions or impede bacterial development. **FRET-TDM** is a fluorescence-quenched analogue of trehalose dimycolate (TDM) that fluoresces upon hydrolysis by TDMH (TDM hydrolase). TDM is generated by TMM which is formed with the help of the antigen 85 (Ag85) complex, which consists of several mycoloyltransferase isoforms that mediate the transfer of mycoloyl groups from trehalose monomycolate (TMM) to another. **FRET-TDM** consists of a simplified TDM scaffold attached to two linear 10 carbon acyl chains functionalized at their end with fluorescein as the fluorophore and DABCYL as the quencher,[14] illustrated in Figure 3.

The fluorophore and the quencher groups are close enough to each other to allow efficient fluorescence quenching while still being distal from the ester linkages, so as not to interfere with enzymatic cleavage. **FRET-TDM** is nonfluorescent in the absence of enzyme and then is efficiently activated by TDMH. Another study enhanced a selective fluorogenic probe that eliminates the need for washing and provides real-time insights into mycobacterial cell wall assembly in live bacteria. **QTF** (quencher−trehalose−fluorophore) is a fluorogenic probe that generates a fluorescent signal upon activation by mycolyltransferase. This fluorescent probe consists of a simplified TDM core in which the hydrophobic fluorescent dye BODIPY-FL is at the one end and the fluorescence quencher DABCYL is linked to the 6′-hydroxyl.[15] Real-time monitoring of mycolic acid membrane biosynthesis is achievable over multiple generations. Despite mycolyltransferases being highly secreted proteins, research indicates localized activity. **QTF** exhibits diagnostic utility as its processing by mycolyltransferases enables selective detection of mycobacteria.[16]

All of the above adducts are directly labeled with the use of fluorescent dyes. The following modified trehalose derivatives exploit the biosynthetic machinery of the cell wall to label mycolate-containing Mtb wall components. Various alkyne,

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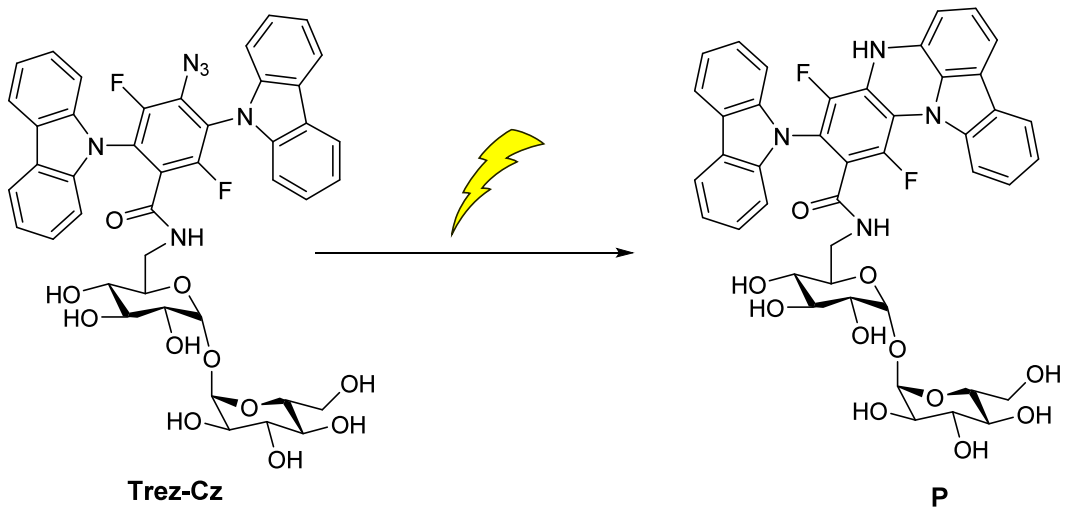

Figure 5. Photoactivation of fluorescent probe.

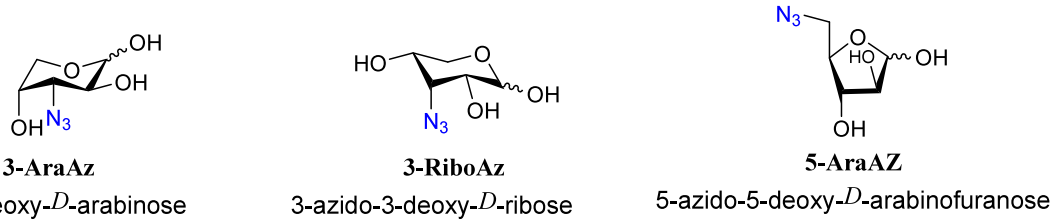

Figure 6. Click-able modified pentoses.

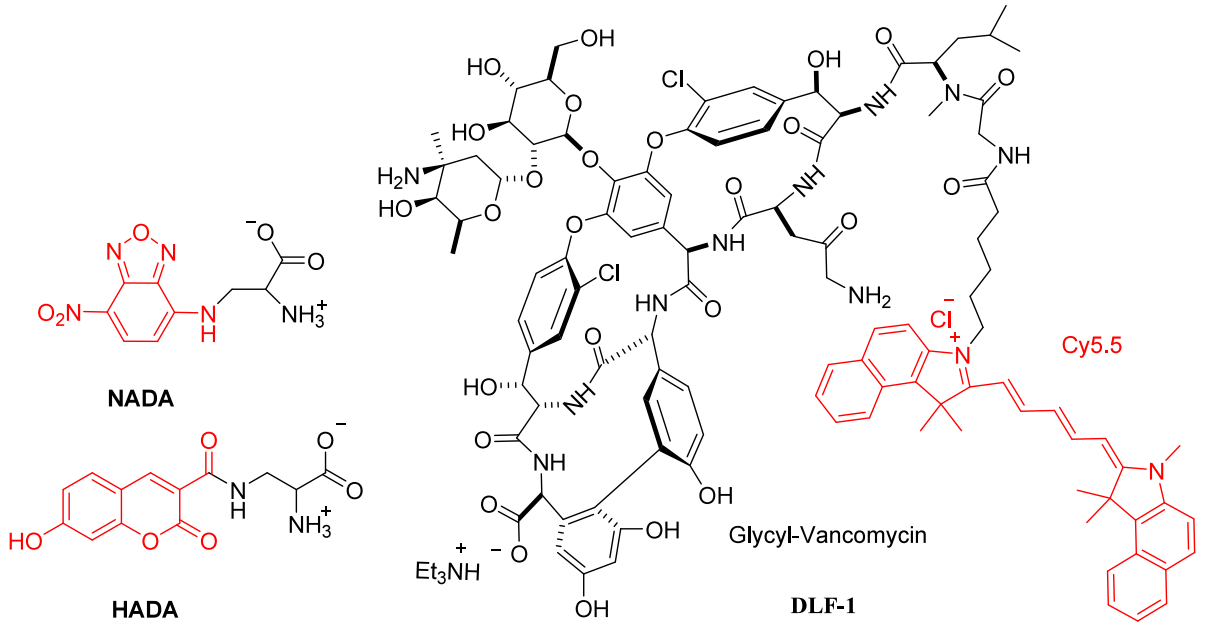

Figure 7. Amino acid chemical probes for peptidoglycan labeling in Mtb.

azide, and octenyl adducts (e.g., N-Alk-TMM, O-Alk-TMM, O-AZ-TMM-C10, and O-TCO-TMM, respectively) undergo "Cu-free" click chemistry inside living cells, to be fluorescently labeled and detected,[17] as depicted in Figure 4.

In addition to all of the above, a trehalose-derivatized carbazole (**Tre-Cz**) is a fluorescent turn-on probe for mycobacteria imaging that has been developed. There is hardly much fluorescence present in the azido carbazole itself. However, when the aryl azide is photoactivated, a singlet nitrene is created. This nitrene then interacts with the nearby aryl ring to produce the fluorescent product **P**, as illustrated in Figure 5. **Tre-Cz** is efficiently taken up by metabolically active mycobacteria. In addition to successfully enabling imaging of mycobacteria in the presence of other Gram-positive and Gram-negative bacteria as well as mycobacteria present in sputum, **Tre-Cz** exhibits great selectivity toward mycobacteria.[13]

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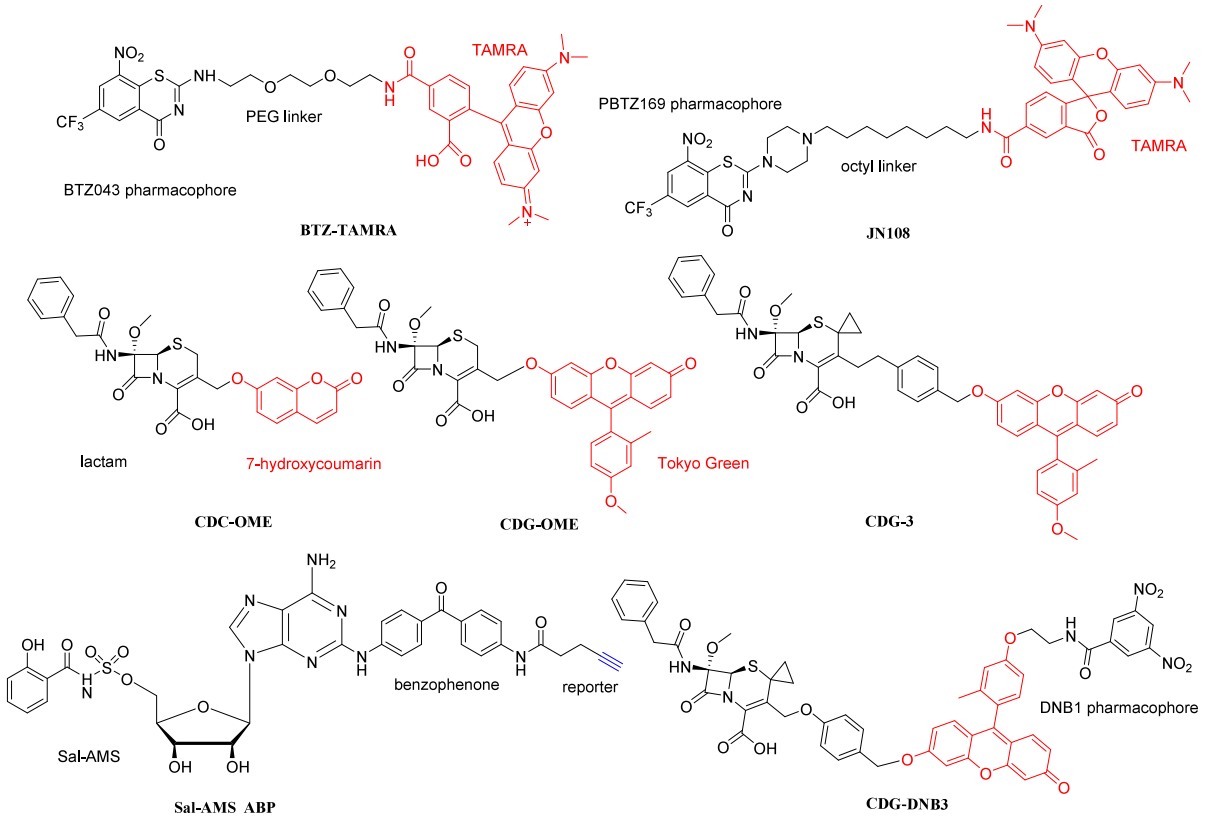

Figure 8. Activity based fluorescent probes and reporter.

Leveraging mycobacterial cell wall metabolism, D-arabinose-modified probes can also selectively label the Mtb cell wall. Once these azido moieties, depicted in Figure 6, are incorporated into the mycobacterial cell envelope, they get fluorescent modification by using the cyclooctyne derivative dibenzocyclooctyne (DIBO)-AlexaFluor 488. The fluorescently labeled bacteria are subsequently analyzed by flow cytometry.[18]

Amino Acid Based Fluorescent Probes. The development of chemical probes for monitoring the peptidoglycan assembly in mycobacterial cell wall offers the identification of new targets and elucidates the mode of action of current antimycobacterial drugs. As D-alanine is a major component in the biosynthesis of peptidoglycans, fluorescent 3-amino-D-alanine derivatives coupled to 7-nitrobenzofurazan (**NADA**) and to 7-hydroxycoumarin-3-carboxylic acid (**HADA**), respectively, have been used for spatiotemporal dynamic studies.[19] Another fluorescent imaging probe, **DLF-1**, operates at a long wavelength and exhibits significant quantitative capabilities. This probe consists of vancomycin along with the fluorescent dye Cy5.5 and presents high binding affinity to the terminal dipeptide of D-Ala-D-Ala in Mtb peptidoglycan. It identifies *M. tuberculosis* in both actively replicating and nonreplicating dormant conditions in a laboratory setting. Moreover, **DLF-1** enables the quantification of labeled Mtb present in cells.[16] However, at its current stage, **DLF-1** cannot be directly employed in TB diagnostic assays due to its lack of specificity for mycobacteria. This imaging technique will be applied in quantifying the response to anti-TB therapy. These probes are presented in Figure 7.

Activity Based Fluorescent Probes. The identification of potential drug targets and the discovery of novel small molecules is related to the development of activity-based and affinity-based probes, known as ABP and AfBP, respectively. ABPs are designed to rely on certain aspects of the enzyme's catalytic mechanism, ensuring detection only when the enzyme is catalytically active. ABPs' distinguished feature is that they form a covalent bond with the enzyme's active site, thereby inhibiting subsequent catalytic activity, similar to a "suicide substrate" or irreversible inhibitor. On the other hand, an affinity-based probe (AfBP) achieves labeling by binding to a specific site on a protein, not necessarily an enzyme's active site, and then undergoing a nonspecific covalent bonding event, such as photochemical cross-linking or the spontaneous trapping of a nearby (noncatalytic) functional group of a protein. A carefully designed AfBP can be transformed into a functional ABP. The crucial difference between these two lies in the requirement for a catalytically active enzyme.[20]

One of the most well-known targets in anti-TB drug discovery is decaprenylphosphoryl-β-D-ribofuranose-2′-epimerase 1 (DprE1), which is an essential enzyme for mycobacterial wall integrity. Many chemical scaffolds have been found to inhibit this enzyme, among them being the benzothiazinones BTZ043 and PBTZ169 in clinical trials. These two benzothiazinones have been used to form the potent fluorophore tagged analogs **BTZ-TAMRA** and **JN108.**[21]

Regarding another enzymic target, MtbA is one of many adenylating enzymes of *M.tuberculosis*, which is labeled by a clickable inhibitor, **Sal-AMS ABP**. The latter consists of the MtbA inhibitor Sal-AMS, which bears at C-2 of the adenosine ring a photoreactive benzophenone, followed by a clickable alkyne as the reporter motif. When, a rhodamine−azide (TAMRA-N_3) conjugates with the terminal alkyne the whole adduct is visualized by fluorescence scanning.[22]

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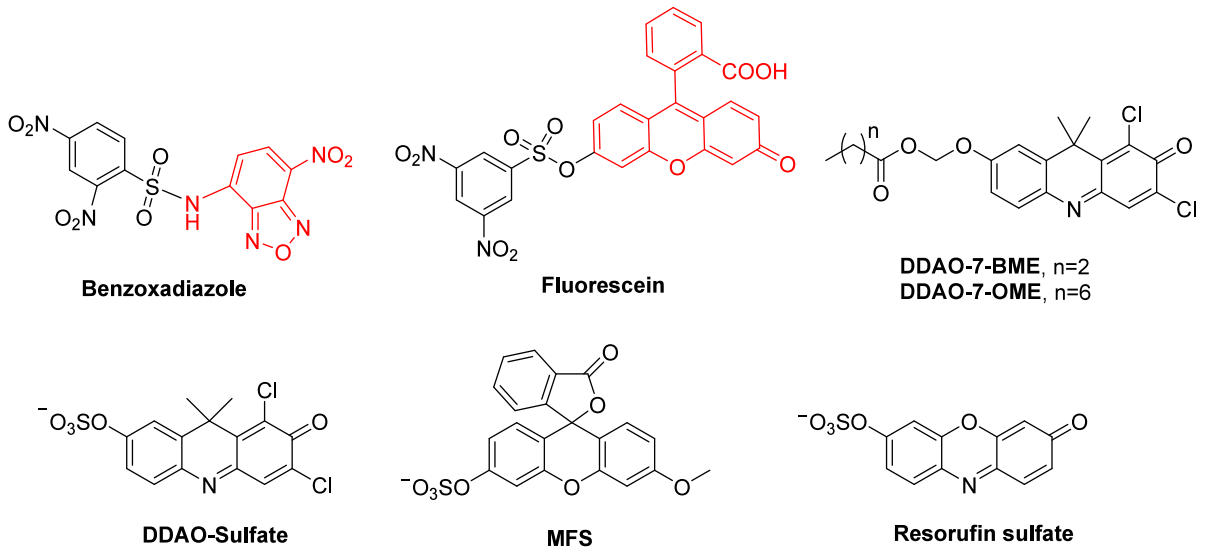

Figure 9. Enzyme activated fluorogenic probes.

Mtb β-lactamase (BlaC), which hydrolyses the β-lactam ring of the corresponding antibiotics, is the target of cephalosporin based fluorogenic substrates. Examples of the latter are the conjugated lactam with 7-hydroxyccoumarin (**CDC-OME**) or the more sensitive and specific **CDG-OME**, wherein the lactam ring is coupled with fluophore Tokyo Green. One more probe, **CDG-3**, bears on the lactam backbone a cyclopropyl group. This modification was based on the BlaC tolerance of a bulkier substitution on its substrate. **CDG-3** is able to detect Mtb in clinical samples.[23] These probes were then evolved to dual targeting of both BlaC and DprE1 in order to further enhance the Mtb specificity. An example of this dual targeting probes is **CDG-DNB3**, depicted in Figure 8. BlaC hydrolyzes the lactam ring to activate the fluorophore, and DprE1 covalently retains and immobilizes the fluorescent probe. Bacteria without BlaC and/or DprE1 activity would not be able to be fluorescently labeled due to the lack of fluorescent activation (no BlaC) or signal retention (no DprE1) in cells.[23]

Mtb L,D-transpeptidase (LdtMt2), is also a promising antitubercular target which is involved in cell-wall biosynthesis. Up-to-day, there is not yet an efficient method for testing the inhibition of this series of enzymes. However, a fluorescence-based assay for LdtMt2 is suitable for high-throughput screening. Two cysteine-selective fluorogenic probes, the benzoxadiazole and the fluorescein adducts, depicted in Figure 9, release a fluorophore upon reaction with LdtMt2, allowing to β-lactam antibiotics to assess the catalytic site of the enzyme.[24]

Sulfatases are a class of enzymes that are conserved across various tubercular mycobacteria. 7-Hydroxy-9*H*-1,3-dichloro-9,9-dimethylacridin-2-one sulfate (**DDAO-sulfate**), 3-*O*-methylfluorescein sulfate (**MFS**) and resorufin sulfate (**RS**), all of which give blue-shifted hydrolysis products as compared to DDAO, are used to detect sulfatase activity in protein gel-resolved mycobacterial lysates.[25] This assay revealed that mycobacteria have distinct sulfatase activity patterns, or "fingerprints". The above fluorophore molecules excite and emit in the middle of the visible range and are detected on most plate readers, gel imagers, and standard fluorescence microscopes.

There are also various serine hydrolases which are detected in replicating dormant and reactivation conditions of Mtb. Acyloxymethyl ether masked fluorogenic probes, similar to the aforementioned probes, have been developed and used for detecting and tracking esterase and lipase activities in a wide range of assay formats. The butanoylmethyl ether (**BME**)-masked and the octanoylmethyl ether (**OME**)-masked **DDAO** are effective probes for detecting Mtb esterases that are active during infections.[26]

CONCLUSION AND FUTURE OUTLOOK

The mycobacterial biology offers new challenges as well as opportunities for drug discovery and the development of chemical probes. The utilization of fluorescent probes has significantly advanced the understanding of mycobacterial cells' structure and their interactions with host environments, particularly in the context of Mtb infection. The insights gained from these studies not only enhanced our understanding of the intricate mechanisms underlying tuberculosis pathogenesis but also offered potential targets for therapeutic intervention. Artificial intelligence (AI) has recently shown great promise in the development of fluorescent probes, which are vital for visualizing and tracking biological processes within cells and tissue. Even though, the development of fluorescent probes traditionally relies on researchers' experiential understanding and intuition, an interpretable explanatory approach has been introduced which utilizes sophisticated artificial intelligence techniques to predict fluorescent probes targeting specific organelles. This framework offers a deeper understanding of targeting mechanisms and enables rapid evaluation, facilitating more rational design processes.[27] The AI approach for antitubercular drug discovery or Mtb-targeted fluorescent probes faces challenges stemming from insufficient high-quality data sets, the intricate nature of Mtb biology and its disease progression, as well as ethical and social considerations. Beyond concerns like data privacy and algorithmic bias, addressing human supervision and societal fairness, AI models should accurately represent the dynamic interplay between Mtb and the host immune system. Achieving success in AI-driven Mtb drug discovery necessitates interdisciplinary cooperation, transparent data sharing practices, and robust

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ethical oversight. Novel challenges in fluorescent probe technology, such as AI embracement in the probe advance, have the potential to revolutionize the field of bioimaging and biomedical research and deepen our knowledge of mycobacterial biology and ultimately aid in the development of more effective interventions to combat tuberculosis. By providing real-time visualization and molecular-level insights, fluorescent probes pave the way for the development of innovative diagnostic tools and therapeutic strategies tailored to combat Mtb infection. In this minireview, we have highlighted the use of small molecules as fluorescent probes leveraging the mycobacterial biology.

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Notes

The authors declare no competing financial interest.

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