Chapter 4

Probing the interaction of the diarylquinoline TMC207 with its target mycobacterial ATP synthase

Abstract

Infections with *Mycobacterium tuberculosis* are substantially increasing on a worldwide scale and new antibiotics are urgently needed to combat concomitantly emerging drug-resistant mycobacterial strains. The diarylquinoline TMC207 is a highly promising drug candidate for treatment of tuberculosis. This compound kills *M. tuberculosis* by binding to a new target, mycobacterial ATP synthase. In this study we used biochemical assays and binding studies to characterize the interaction between TMC207 and ATP synthase. We show that TMC207 acts independent of the proton motive force and does not compete with protons for a common binding site. The drug is active on mycobacterial ATP synthesis at neutral and acidic pH with no significant change in affinity between pH 5.25 and pH 7.5, indicating that the protonated form of TMC207 is the active drug entity. The interaction of TMC207 with ATP synthase can be explained by a one-site binding mechanism, the drug molecule thus binds to a defined binding site on ATP synthase. TMC207 affinity for its target decreases with increasing ionic strength, suggesting that electrostatic forces play a significant role in drug binding.

Our results are consistent with previous docking studies and provide experimental support for a predicted function of TMC207 in mimicking key residues in the proton transfer chain and blocking rotary movement of subunit c during catalysis. Furthermore, the high affinity of TMC207 at low proton motive force and low pH values may in part explain the exceptional ability of this compound to efficiently kill mycobacteria in different microenvironments.

4.1 Introduction

Tuberculosis causes approximately 2 million deaths per year and an estimated 1/3 of the world population harbors *Mycobacterium tuberculosis* in a dormant or latent form [Dye & Williams, 2010, Russel et al., 2010]. Infections with multidrug-resistant and extensively drug-resistant mycobacterial strains as well as co-infection with HIV pose a global health challenge [Gandhi et al., 2010, Mandavilli, 2007, World Health Organization, 2010]. Existing drug regimens need to be administered for at least 6 months, and up to 24 months in case of drug-resistant tuberculosis [Gandhi et al., 2010,Koul et al., 2011]. To counteract development of drug-resistant strains and to shorten tuberculosis treatment the discovery of new drugs, validation of new target proteins, and understanding of drug/target interactions are essential [Bald & Koul, 2010,Koul et al., 2011, Ma
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Figure 4.1: (A) Structure formula of TMC207. (B) ATP synthase subunit composition with subunit c in grey. A homology model of a subunit c monomer from Mycobacterium tuberculosis is shown enlarged. The acidic residue Glu61, essential for proton transport, is depicted in red. Point mutations that influence mycobacterial sensitivity for TMC207 are indicated in colour.

et al., 2010].

Energy metabolism has emerged as a new target-pathway for development of new anti-tubercular drugs [Bald & Koul, 2010, Zhang & Mitchison, 2003]. The diarylquinoline TMC207 (Fig. 4.1A) is a highly promising candidate for treatment of drug-resistant tuberculosis and for shortening of tuberculosis treatment [Andries et al., 2005, Ibrahim et al., 2007, Lounis et al., 2006]. TMC207 acts on a novel target, mycobacterial ATP synthase [Koul et al., 2007] and is highly active on replicating as well as on dormant mycobacteria [Koul et al., 2008, Rao et al., 2008]. In phase II clinical trials addition of TMC207 to standard therapy antibacterial regimens strongly accelerated conversion to a negative sputum culture as compared to placebo [Diacon et al., 2009]. TMC207 acts in a highly selective manner, with only minimal effect on human ATP synthase and only minor side effects in human patients [Andries et al., 2005, Diacon et al., 2009, Haagsma et al., 2009].

ATP synthase is a ubiquitous key enzyme in energy metabolism of virtually all cells that utilizes the energy stored in a trans-membrane electrochemical
potential difference of a coupling ion for production of ATP [Junge et al., 2009]. In mycobacteria, ATP synthase has been proven essential for growth on both fermentable as well as non-fermentable carbon sources [Tran & Cook, 2005]. Bacterial ATP synthase is composed of a membrane-embedded $F_0$ sector with the subunit composition $a_1b_2c_{10-15}$ and a hydrophilic $F_1$ part, consisting of subunits $a_3\beta_3\gamma\delta\varepsilon$ (Fig. 4.1B). Proton flow through $F_0$ triggers rotation of the oligomeric subunit $c$ ring that is coupled to rotation of the $\gamma$ subunit within the $(\alpha\beta)_3$ hexamer of $F_1$ and finally drives synthesis of ATP [Boyer, 1993, Diez et al., 2004, Noji et al., 1997]. A significant step in proton transport is proton binding to an essential acidic residue in the central, trans-membrane part of subunit $c$ [Pogoryelov et al., 2010]. TMC207 binds to purified mycobacterial subunit $c$ [Koul et al., 2007] and mycobacterial sensitivity for TMC207 is influenced by point mutations located in the vicinity of the acidic residue in subunit $c$ (Glu61 in M. tuberculosis, Fig. 4.1B) [Andries et al., 2005, Huitric et al., 2007, Huitric et al., 2010, Koul et al., 2007, Petrella et al., 2006]. These findings suggest that TMC207 may bind in that central, mostly hydrophobic part of subunit $c$. Based on docking studies it has been proposed that TMC207 binds at the interface of subunits $c$ and subunit $a$ [de Jonge et al., 2007]. The drug is predicted to mimic a conserved basic residue in the proton transfer chain, arginine186, subsequently interfering with the rotary movement of subunit $c$. [de Jonge et al., 2007]. However, no high-resolution structure is available for mycobacterial ATP synthase or its subunits. Moreover, biochemical data on TMC207/target interaction to test the predictions from the docking studies are scarce.

In the present report we used biochemical assays and binding studies to investigate the mode of binding between TMC207 and mycobacterial ATP synthase. We study factors potentially influencing drug/target interaction, such as the proton motive force, the pH value and buffer ionic strength. The results are correlated with proposed models for the TMC207 binding site and discussed in view of TMC207 being active in different microenvironments.

### 4.2 Materials and methods

#### 4.2.1 Bacterial strains and growth conditions

*Mycobacterium smegmatis* mc²155 was kindly provided by B.J. Appelmelk, Department of Molecular Cell Biology & Immunology, VU University Medical Center Amsterdam, the Netherlands. Replicating cultures of *M. smegmatis* were grown in Middlebrook 7H9 broth (Difco) with 10% Middlebrook albumin
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Dextrose catalase enrichment (BBL) and 0.05% Tween-80 at 37°C to the late exponential phase.

4.2.2 Preparation of inverted membrane vesicles

Inverted membrane vesicles (IMVs) of *M. smegmatis* were prepared as described previously [Koul et al., 2007]. Briefly, cells were pelleted by centrifugation at 5,000 g for 20 min and washed once with Phosphate-buffered saline (PBS, pH 7.4). Five grams of cells (wet weight) were resuspended in 10 mL of 50 mM MOPS-KOH (pH 7.5), 2 mM MgCl$_2$ including protease inhibitors (complete, EDTA free; protease inhibitor cocktail tablets from Roche). Lysozyme (1.2 mg mL$^{-1}$), 1500 units of deoxyribonuclease I (Invitrogen) and 13 mM MgCl$_2$ were added and cells were incubated with stirring at room temperature for 45 min. The cells were broken by three passages through a pre-cooled French pressure cell at 20,000 psi (Thermo Electron, 40K). The lysate was centrifuged at 5,000 g and 4°C for 20 min to remove unbroken cells. The supernatant was centrifuged at 370,000 g and 4°C for 1 h and the pellet of IMVs was washed with 50 mM MOPS-KOH (pH 7.5), 2 mM MgCl$_2$. After the second centrifugation step, the inverted membrane fraction was resuspended in an appropriate volume of 50 mM MOPS-KOH (pH 7.5), 2 mM MgCl$_2$.

4.2.3 Assay of ATP synthesis

ATP synthesis activity was measured as described previously [Haagsma et al., 2009] with the modifications described below. IMVs (1 mg mL$^{-1}$) were incubated in either 50 mM MES-KOH (pH 5.25) or 50 mM MOPS-KOH (pH 6.0-7.5) containing 2 mM MgCl$_2$, 2 mM ADP, 20 mM KH$_2$PO$_4$, 100 µM P$^1,P^5$-di(adenosine-5′) pentaphosphate (Ap5A), 25.4 mM glucose, 11.8 U mL$^{-1}$ hexokinase (Sigma) and protease inhibitors (complete, EDTA-free; protease inhibitor cocktail tablets from Roche). To manipulate the proton motive force samples were supplemented with varying concentrations of uncoupler SF6847. Samples (0.25 mL) were incubated with vigorous stirring in 18-mL flasks at 37°C. The concentration of NADH to initiate the reaction was varied between 5-15 mM. After 1 h, each reaction was stopped with 25 mM EDTA, followed by transfer to ice. Samples were transferred to Eppendorf tubes, boiled for 5 min and centrifuged (10,000 g, 20 min) to remove denatured protein. In the supernatants, the synthesized glucose-6-phosphate was oxidized by 2.5 mM NADP in the presence of 3 U mL$^{-1}$ of glucose-6-phosphate dehydrogenase (Roche). NADPH formation was monitored using a spectrophotometer at 340 nm. The
50% inhibitory concentrations (IC\textsubscript{50}s) were determined using GraphPad Prism version 5.00 for Macintosh, GraphPad Software, San Diego California USA. The data were fitted with a model describing a one-site binding hyperbola.

4.2.4 BIAcore binding studies

Binding studies using Surface Plasmon Resonance technology were carried out using a BIAcore 2000 machine with a carboxymethyl (CM-5) analytical chip. An amine-analog of TMC207, which carries an amino group instead of the bromine [Koul et al., 2007], was bound to the chip at 25°C as follows. 30 µL of an equimolar mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) was used to activate the carboxymethyl surface of the chip. Subsequently, 30 µL of the TMC207 amine analogue (50 µM) in 10 mM Hepes-KOH (pH 7.5), 2 mM MgCl\textsubscript{2}, 150 mM NaCl was bound to the activated chip at a flow rate of 2 µL min\textsuperscript{-1}. Non-reacted activated EDC/NHS on the chip surface was blocked by the infusion of 50 µL of 1 M ethanolamine.

Subunit c from Mycobacterium tuberculosis was purified as described in previously [Koul et al., 2007]. The purified subunit c (13 µM) was injected onto the compound-linked Biacore chip at a flow rate of 30 µL min\textsuperscript{-1} in 10 mM Hepes-KOH (pH 7.5), 2 mM MgCl\textsubscript{2}, 0.5% Triton X-100 containing 50 mM, 150 mM or 300 mM NaCl (37°C). Association, dissociation and equilibrium dissociation constants were determined using GraphPad Prism version 5.00 for Macintosh, GraphPad Software, San Diego California USA.

4.3 Results and discussion

4.3.1 TMC207 does not compete with protons for a common binding site

We investigated the effect of the proton motive force on ATP synthesis inhibition by TMC207. TMC207 may interfere with ATP synthesis by competing with protons for the same binding site on ATP synthase. A high proton motive force may then outcompete TMC207 from its binding site, leading to reduced drug/target binding. Conversely, a decreased proton motive force then would lead to increased TMC207 binding. Moreover, the proton motive force not only supplies the energy required for synthesis of ATP, but also constitutes an important factor regulating the conformation of ATP synthase (for review...
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A Substrate Uncoupler

0 5 10 15 20

0 25 50 75 100

0.1% EtOH

0.001 µM SF6847

0.01 µM SF6847

0.03 µM SF6847

0.07 µM SF6847

0.1 µM SF6847

1 µM SF6847

Time (min)

Fluorescence intensity (a.u.)

B

0 nM TMC207

2.5 nM TMC207

5 nM TMC207

7.5 nM TMC207

0 nM TMC207 D32V

7.5 nM TMC207 D32V

Uncoupler (µM)

Relative activity (%)

Figure 4.2: ATP synthesis and inhibition by TMC207 at low proton motive force. (A) Inverted membrane vesicles from Mycobacterium smegmatis were diluted to 0.18 mg mL\(^{-1}\) in buffer containing 2 µM ACMA. To detect the proton motive force, quenching of ACMA fluorescence was investigated after addition of 5 mM succinate in the presence of increasing concentrations of the uncoupler SF6847. At the indicated time point, 1 µM of uncoupler SF6847 was added as control to collapse the proton gradient. (B) ATP synthesis by membrane vesicles of M. smegmatis (1 mg mL\(^{-1}\)) was measured in the presence of TMC207 and varying concentrations of uncoupler SF6847 to modulate the proton motive force. Samples were incubated at 37°C for 1 h in the presence of an ADP-regenerating system, and produced ATP was quantified spectrophotometrically by monitoring oxidation of glucose-6-phosphate with NADP\(^+\). As a control, 100 µM DCCD was added.

see [Feniouk & Yoshida, 2008]. Consequently, the affinity of several known ATP synthase inhibitors depends significantly on the proton motive force [Bald et al., 1998, Bald et al., 1999, Syroeshkin et al., 1995].

The proton motive force across inverted membrane vesicles (IMVs) of Mycobacterium smegmatis was monitored with the ACMA quenching method as in [Haagsma et al., 2010] and modulated using an uncoupler, SF6847 (Fig. 4.2A). As expected, with increasing uncoupler concentration the ATP synthesis activity decreased in a dose-dependent manner, with < 10 % residual activity in the presence of 10 µM uncoupler (Fig. 4.2B). We then tested three selected concentrations of TMC207 (2.5 nM, 5 nM and 7.5 nM), which in the absence of uncoupler decreased ATP synthesis activity by respectively 25 %, 50 % and 67 %. As depicted in Fig. 1B the inhibitory effect of TMC207 did not significantly change at lower proton motive force. The drug concentrations for half-maximal
inhibition ($IC_{50}$) values were determined to 5.0-7.5 nM TMC207 for all uncoupler concentrations investigated. As a control, for membrane vesicles carrying the resistance mutation D32V in subunit c [Andries et al., 2005], no inhibition by 7.5 nM TMC207 was detected (Fig. 4.2B). As a further control, inhibition by sodium azide (10 mM), an inhibitor known to act in a proton motive force dependent manner [Syroeshkin et al., 1995; Bald et al., 1998], increased from < 5% in the absence of the uncoupler to > 50% in the presence of the highest uncoupler concentration (data not shown). Thus, the proton motive force does not significantly influence the target’s ability for binding of TMC207, neither by inducing conformational changes in ATP synthase, nor by outcompeting TMC207 from its binding site. These data strongly suggest that TMC207 does not directly compete with protons for a common binding site.

### 4.3.2 TMC207 inhibits ATP synthesis at low and neutral pH values

Next, we investigated if (de-) protonation of TMC207 or of mycobacterial ATP synthase affects drug/target interaction. The membrane vesicles from *M. smegmatis* were capable of detectable ATP synthesis activity over the whole pH range investigated (pH 5.25-pH 7.5). As shown in Fig. 4.3, the external pH did not significantly influence the inhibitory action of TMC207 between pH 5.25 – 7.5, with $IC_{50}$ values determined to 5.0 – 7.5 nM for all pH values tested. Thus, neither (de-) protonation of TMC207 nor (de-) protonation of the target in the pH range investigated significantly changed the drug’s ability to interact with its target. The dimethyl-amino group of TMC207 can take up a proton, which can be observed by a peak shift from 1.87 ppm to 2.18 ppm in the $^1$H NMR spectrum (data not shown). A pKa value of 9.0 – 10.0 in aqueous solution is predicted, although this value may be lower in a hydrophobic membrane environment. Most of the inhibitor molecules will be protonated at neutral or acidic pH and the concentration of protonated TMC207 will not change significantly during a titration between pH 5.25 and 7.5 (< 1.1-fold increase according to the Henderson-Hasselbalch equation, assuming a pKa of 9.5). The concentration of unprotonated TMC207 is expected to decrease strongly from pH 7.5 to pH 5.25 (> 100-fold according to the Henderson-Hasselbalch equation). The lack of pH dependency observed in our experiments thus suggests that the protonated form of TMC207 is the active drug entity.
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Figure 4.3: Effect of TMC207 on mycobacterial ATP synthesis at low pH. ATP synthesis in the presence of TMC207 and varying external pH values was measured for Mycobacterium smegmatis inverted membrane vesicles (1 mg mL\(^{-1}\)). Samples were incubated at 37 °C for 1 h in the presence of an ADP-regenerating system, and produced ATP was quantified spectrophotometrically by monitoring oxidation of glucose-6-phosphate with NADP\(^+\). As a control, 100 µM DCCD was added.

4.3.3 Electrostatic interactions are important for binding of TMC207

Docking studies predict that electrostatic interactions play an important role in binding of TMC207 to ATP synthase [de Jonge et al., 2007, Upadhayaya et al., 2009].

To test this prediction, we determined the effect of buffer ionic strength on TMC207 action. ATP synthesis by M. smegmatis membrane vesicles was susceptible to TMC207 at all ionic strengths conditions investigated (0 mM, 300 mM and 600 mM NaCl) (Fig. 4.4A). However, sensitivity was clearly lower at high ionic strength, with IC\(_{50}\) values increasing from 3.9 nM at 50 mM NaCl, 5.0 nM (300 mM NaCl) to 12.9 nM (600 mM NaCl).

To support this finding, we used Surface Plasmon Resonance Sensing to characterize the interaction between TMC207 and purified ATP synthase subunit c. For these experiments we used an analog of TMC207, which carries an amino group instead of a bromine group [Koul et al., 2007] linked onto the chip. Subsequently, we tested the influence of various salt concentrations (50 – 300 mM) in the subunit c sample and in the running buffer on drug/target interac-
Figure 4.4: Electrostatic interactions are important for binding of TMC207. (A) ATP synthesis in the presence of TMC207 and increasing sodium chloride concentrations was measured for inverted membrane vesicles of *Mycobacterium smegmatis* (1 mg mL\(^{-1}\)). Samples were incubated at 37 °C for 1 h in the presence of an ADP-regenerating system, and produced ATP was quantified spectrophotometrically by monitoring oxidation of glucose-6-phosphate with NADP\(^+\). As a control, 100 µM DCCD was added. (B) BIAcore binding studies. Purified subunit c from wild-type *Mycobacterium tuberculosis* was injected onto a chip with immobilized amine analog of TMC207 in the presence of 50, 150 and 300 mM NaCl at 37 °C.

Our results suggest that electrostatic forces are an important factor for binding of TMC207 to ATP synthase, more specifically to its subunit c.
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4.3.4 TMC207 binds to a distinct binding site in ATP synthase

ATP synthase is a complex membrane protein and in particular its membrane spanning regions may provide multiple binding sites for a predominantly hydrophobic molecule, such as TMC207. Therefore, we investigated if the inhibition of synthesis by TMC207 can be explained by binding of a single molecule TMC207 per ATP synthase complex. As depicted in Fig. 4.5A, the dose-dependent inhibition of the ATP synthesis by TMC207 could be fitted accurately ($R^2 > 0.99$) with a simple one-site saturation-binding curve. This indicates that interaction of TMC207 with a distinct binding site in ATP synthase is responsible for inhibition of ATP synthesis.

In order to corroborate this result, we also determined if binding of TMC207 to purified subunit c from *M. tuberculosis* or to the purified ATP synthase holoenzyme from *Bacillus PS3* is consistent with a one-site binding mechanism. The Surface Plasmon Resonance studies showed that both *M. tuberculosis* subunit c and *Bacillus PS3* ATP synthase bound to the TMC207 amine-analog immobilized on a BIAcore chip (Fig. 4.5B and C). In both cases the obtained binding curves could be fitted well ($R^2 > 0.99$) with a simple mono-exponential model, indicating only one type of binding site (Fig. 4.5B and C). Taken together, our results suggest that TMC207 binds to a distinct drug-binding site, most likely one molecule TMC207 is sufficient to block the target enzymes activity.

4.3.5 Mechanism of TMC207/target interaction

Insight in the mode of binding of antibacterial drugs to their target proteins is an important step in understanding the mechanism of drug action. Moreover, new drug derivates may be designed based on knowledge of drug/target interaction [Upadhayaya *et al.*, 2009]. Previously, docking studies based on free energy minimization predicted a binding niche for TMC207 in mycobacterial ATP synthase [de Jonge *et al.*, 2007, Upadhayaya *et al.*, 2009]. This site is mainly made up by subunit c, supplemented with residues from subunit a. More specifically, TMC207 in an extended conformation [Gaurrand *et al.*, 2006] has been proposed to interact via its protonated basic amino group with the carboxyl group of glutamate61 in subunit c [de Jonge *et al.*, 2007, Upadhayaya *et al.*, 2009].

Our results show that TMC207 binds to a single drug-binding site within ATP synthase with electrostatic interactions playing an important role in drug binding. Most likely, the protonated form of TMC207 is the active molecule.
Figure 4.5: TMC207 binds to one defined binding site in ATP synthase. (A) The dose-dependency of ATP synthesis inhibition by TMC207 in inverted membrane vesicles of *Mycobacterium smegmatis* was fitted with a one-site binding hyperbola \(y = \frac{104.92}{6.3 + x}, \quad R^2 > 0.99\). (B) Binding of purified ATP synthase subunit c from *Mycobacterium tuberculosis* to an amine analog of TMC207 linked onto a BIAcore chip was fitted using mono-exponential binding models (Association = \(R_{eq} (1 - e^{-537.37x})\) and Dissociation = \(165.654e^{-0.002295(x-45)}\), \(R^2 > 0.99\)), and (C) Binding of purified ATP synthase from *Bacillus PS3* to an amine analog of TMC207 linked onto a BIAcore chip was fitted using mono-exponential binding models (Association = \(R_{eq} (1 - e^{-153.7x})\) and Dissociation = \(8575.97e^{-0.0001030(x-1187)}\), \(R^2 > 0.99\)).
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These results are consistent with the model proposed by [de Jonge et al., 2007], as the predicted interaction of the protonated amino group of TMC207 with Glu61 predominantly is electrostatic in character and contributes significantly to efficient drug binding. These electrostatic interactions are expected to be accompanied by hydrophobic and stacking interactions between aromatic rings of TMC207 and aromatic side chains in subunit c [Upadhayaya et al., 2009], which may explain why in our experiments even at high salt concentrations still significant binding was observed. The observed lack of competition between TMC207 and protons for a common binding site suggests that protonated TMC207 may interfere with conformational changes in ATP synthase, e.g. block the rotary motion of subunit c. This result is consistent with the hypothesis that TMC207 prevents rotation of subunit c by mimicking the function of arginine186 in subunit a [de Jonge et al., 2007], a conserved basic residue in the proton transfer chain [Valiyaveetil & Fillingame, 1997].

Taken together, our results are consistent with previous predictions based on docking studies. TMC207, bound in a defined niche at the interface of subunits c and subunit a, may interfere with proton transfer and subsequently block conformational changes associated with ATP synthase activity.

4.3.6 TMC207 can be active in a broad range of physiological microenvironments

Mycobacteria can persist in a mammalian host in “low energy” environments due to exceptional metabolic flexibility [Berney & Cook, 2010], e.g. in poorly aerated parts of the lung, within encapsulated lesions or within the endosome system of host macrophages [Flynn & Chan, 2003]. Bacteria in these microenvironments are notoriously difficult to kill with antibacterials, such as isoniazid or ethionamide [Gengenbacher et al., 2010, Gomez & McKinney, 2004]. Previously, it was demonstrated that mycobacteria cultivated in vitro in low oxygen tension model systems were efficiently killed by TMC207 [Koul et al., 2008, Rao et al., 2008]. However, in addition to low oxygen tension, mycobacterial microenvironments can display nutrient limitation, which may allow for only a low proton motive force across the cytoplasmatic membrane [Huang et al., 2007]. Moreover, mammalian granuloma can be acidic due to active inflammation, with pH values as low as 5.0 [Zhang & Mitchison, 2003]. The high affinity of TMC207 for its target at both low proton motive force and low pH values may contribute to the drug’s ability to render infected tissue culture-negative in mice faster than current first- and second-line antibiotics [Andries et al., 2005, Ibrahim et al., 2007, Lounis et al., 2006].
4.4 Conclusion

Our results show that TMC207 binds to a distinct drug-binding site in its target and we provide experimental support for a binding model previously proposed based on docking studies [de Jonge et al., 2007]. The drug most likely interferes with proton transfer and blocks conformational changes associated with proton flow.

TMC207 efficiently interacts with its target independent of environmental conditions such as the local pH and the proton motive force. These properties, combined with the essentiality of the target, may explain how TMC207 can act as a highly potent antibacterial drug.

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References


References


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