

# **Cholesterol degradation: a promising novel drug target against Tuberculosis**

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## Abstract

One third of the world population is infected by *Mycobacterium tuberculosis*. Although for most patients, the disease is in its latent phase, this still poses a major threat for global health. Especially as drug resistant strains of this bacterium arise, effectively treating tuberculosis becomes increasingly difficult. New drugs against tuberculosis are constantly being developed, but the pipeline may be too narrow and multiple strategies have to be invoked to keep ahead of this disease. Alongside improvement of existing drugs and high throughput screening, target based drug design is one of the viable strategies. An interesting target is the cholesterol catabolism of *Mtb* as intervention in this pathway results in both accumulation of toxic intermediates and deprivation from an important energy and carbon source. Therefore successful inhibition of one or several of the key enzymes for this pathway, may be a decent way to combat *Mtb*. All known enzymes are discussed in this thesis on their potential to become a viable drug target, and for what enzymes further characterisation is necessary. Although not the entire pathway has yet been fully understood, several candidate enzymes for drug development arise, and their inhibitors may be leads for future clinical trials.

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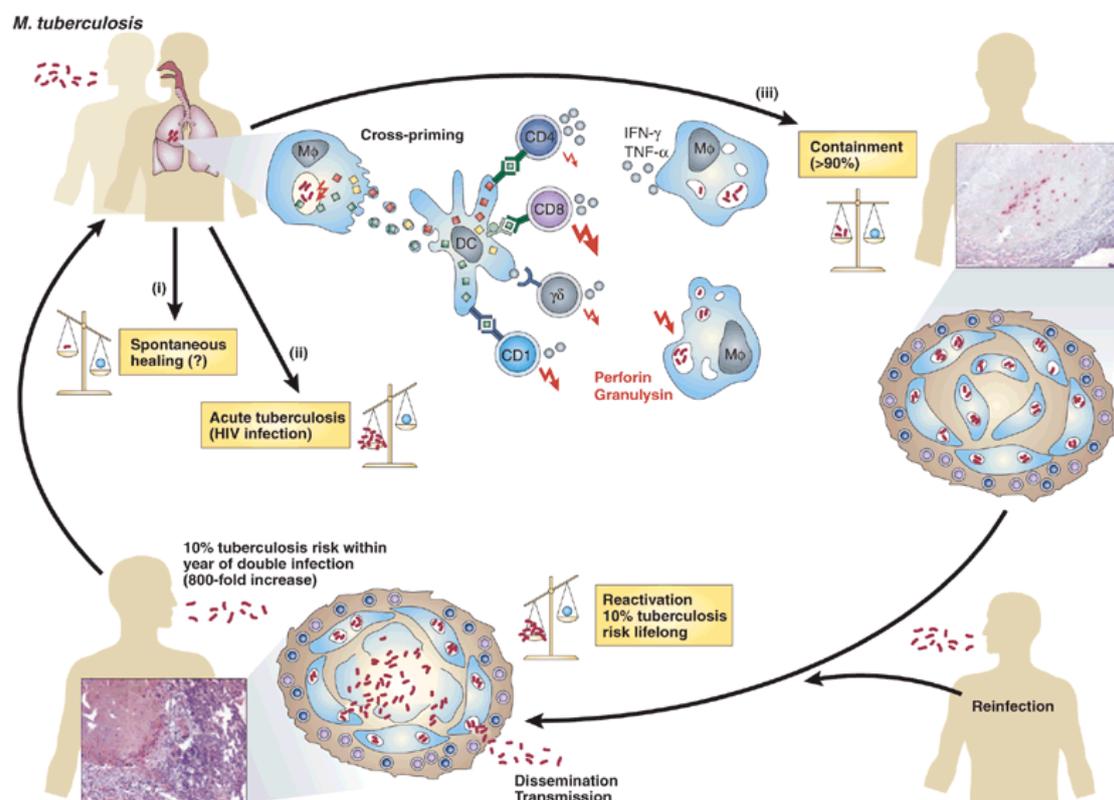
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## 1. Introduction

Two billion people are estimated to be infected with Tuberculosis (TB) caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*), which equals one third of the world population (WHO, 2015). The vast majority of these people (90-95 %) is not aware of their infection. They exhibit no symptoms and TB is in its latent phase. When the host immune system is weakened by for instance AIDS or ageing, latent TB infection can develop into active TB, causing symptoms and patients becoming infectious.

*Mtb* is transmitted via aerosols, tiny droplets containing *Mtb* bacteria spread by coughs of infected patients, and infection can occur after inhaling *Mtb* bacteria. This pathogen is well-adapted, as *Mtb* is able to avoid the mucus in the trachea, and enter into the deeper parts of the lungs. Alveolar macrophages await there to devour invading bacteria, but these are deceived by *Mtb*. After phagocytosis, *Mtb* produces a protein that prevents fusion of the phagosome with the lysosome that would otherwise result in lysis of this bacterium (Kaufmann and McMichael, 2005).

While residing in the macrophages, *Mtb* proliferates and causes an infection called primary TB. The immune system responds to this infection by creating a granuloma causing caseous necrosis. This results in fibrosis and calcification, and the pathogens are either completely destroyed, or can persist in a dormant stage, latent TB. After reactivation, *Mtb* spreads to other parts of the lungs and again causes caseous necrosis. However, now this develops into cavities that enable *Mtb* to also spread into the vascular system causing systemic miliary TB that has a large range of symptoms throughout the organs. Furthermore, in its active state TB is also contagious (Kaufmann and McMichael, 2005).



**Figure 1:** After infection by *Mycobacterium tuberculosis*, it may develop into three possible ways. Generally, *Mtb* will unfold as latent TB and is effectively encapsulated in granuloma. An infection can also result in acute TB for immunocompromised people, or spontaneous healing if *Mtb* cannot effectively reside within macrophages. Latent TB may develop into active TB when patients have a weak immune defence system due to for instance HIV or ageing (Kaufmann and McMichael, 2005).

## **Aim of Thesis**

This thesis attempts to describe strategies to find new drugs against tuberculosis. As target based drug design targeting cholesterol catabolism seems a viable strategy, the thesis will further zoom in on this pathway to discuss the feasibility hereof. All enzymes in this pathway are discussed in detail to be able to draw a conclusion on potential drug design. Therefore the research question of this thesis is: 'What therapeutic inhibitors for enzymes in cholesterol catabolism can be developed, and why is this a viable strategy for future drug design?'

## 2. Drug targets

Currently, a large set of drugs already exists that act effectively against TB. Most employed first-line drugs contain isoniazid, rifampicin, pyrazinamide and ethambutol, and are most effective against actively replicating *Mtb* (Wippenman et al., 2014). As these compounds act on different targets of *Mtb*, they can be readily used as combinatory drugs. Isoniazid inhibits fatty acid biosynthesis; rifampicin blocks the mycobacterial RNA polymerase; ethambutol inhibits bacterial cell wall synthesis. Unusually long period of antibiotic intake is necessary due to the slow replication of *Mtb*; during its latent stage replication cycles may last as long as several days (Wippenman et al., 2014).

Unfortunately there has been a rise of drug resistant strains of *Mtb* causing MDR-TB (multi drug resistant TB) or XDR-TB (extremely drug resistant TB) that are very difficult to treat (Udwadia et al., 2012). This makes the search for new antibiotic compounds very relevant as a TB infection becomes increasingly difficult to treat. A very useful and well maintained website updates frequently about novel TB drugs and their clinical phase ([www.newtbdrugs.org](http://www.newtbdrugs.org)). These may be promising drugs for the near future, but as drugs resistance is a constantly arising problem, there is always an urge for novel antibiotics. Several ways can be employed to achieve this goal, such as improving existing compounds, discovering new drugs by means of high throughput screening, or by target based drug design that inhibits systems *Mtb* needs for its virulence (Mdluli et al., 2015).

Existing drugs that are already successfully clinically utilised can be further improved to enhance their efficiency or avoid gained resistance in cases of MDR-TB or XDR-TB. An interesting example of such drug improvement is the large range of chemical modifications that has been performed on isoniazid (INH), a first-line drug (Velezheva et al., 2016). Many of these novel drugs showed decent inhibitory effects against *Mtb* H37Rv, the standard strain for TB research. But surprisingly, these compounds were also effective against INH insensitive strains, albeit with relatively high MIC (minimum inhibitory concentrations) values (Velezheva et al., 2016).

Whole-cell antitubercular drug screening has proven to be a successful method to identify effective antibiotics (Mdluli et al., 2015). The advantages of high throughput screening (HTS) on whole cells over target based drug design are numerous. Unpredicted targets can be elucidated from these screens, the *in vivo* efficacy of the compounds is directly shown, both non-replicating and replicating *Mtb* can be tested against, and it automatically accounts for unfavourable cell penetration and efflux issues (Mdluli et al., 2015). A large range of compounds has been discovered using HTS discovered (Rebollo-Lopez et al., 2015; Sorrentino et al., 2016), including natural products that cover a very large chemical space (Nguta et al., 2015).

Target based drug design revolves around specifically targeting systems *Mtb* requires for its virulence. Most relevant emerging targets include the iron acquisition and storage, the MmpL membrane protein family, cholesterol metabolism, *Mtb* proteasome, the central carbon metabolism, ATP synthesis, and ROS generation (Mdluli et al., 2015). An example of target based drug design that is currently researched is the selective inhibition of *Mtb* proteasome. A big advantage of proteasome inhibition is that it also kills non-replicating *Mtb* (Lin et al., 2009). Oxathiozol-2-one compounds act as suicide inhibitors on the proteasome, effectively killing non-replicating *Mtb* without affecting its human analogue (Lin et al., 2009). Further improvements in this field has been performed to further enhance its efficiency on non-replicating *Mtb* as well as preventing side effects in human cells (Yang et al., 2013; Russo et al., 2015).

## 2.1 Cholesterol metabolism as drug target

Cholesterol metabolism of *Mtb* is very interesting for target based drug design. Although cholesterol is not required for *Mtb* infection, it is necessary for persistence in lungs of infected animals, and in activated macrophages (Pandey and Sasseti, 2008). Activation of macrophages by IFN- $\gamma$  results in deprivation of commonly used nutrients such as glucose. It has been suggested that due to its unusual capability to metabolise sterols, *Mtb* is able to persist for so many years in alveolar macrophages (Pandey and Sasseti, 2008). Therefore drugs acting against cholesterol metabolism would mainly deprive non-replicating *Mtb* and eradicate latent TB.

Furthermore, cholesterol is a very versatile compound as its degradation products can be used in a large range of anabolic processes and for its energy metabolism. Metabolites that are produced during cholesterol breakdown are pyruvate, acetyl-CoA, and propionyl-CoA (Wippenman et al., 2014). Latter compound must be converted to methylmalonyl-CoA or succinate before it can enter the central carbon metabolism (Wippenman et al., 2014). Therefore, shutting down the cholesterol catabolism would require *Mtb* to find other ways to meet its metabolic necessities.

Lastly, interruption of the cholesterol degradation pathway can result in accumulation of toxic intermediates. For instance, high concentrations of catechol intermediates has a detrimental effect on *Mtb* growth even when grown on different carbon sources (Yam et al., 2009). Likewise, if the enzymes that convert propionyl-CoA in the methylcitrate cycle are inhibited, this results in intoxication by propionyl-CoA (Lovewell et al., 2016). This highlights the necessity of complete conversion of cholesterol.

Two viable therapeutic strategies arise using *Mtb* cholesterol catabolism to treat TB. As a first option, cholesterol analogues containing modified groups are converted to the toxic cholesterol intermediates that are then unable to be further degraded. For this, it is required that the uptake of these analogues is mediated by the same ABC-like cholesterol import system that *Mtb* utilises (Mohr et al., 2008). Another possibility is the design of inhibitors that specifically inhibit enzymes of the cholesterol degradation pathway resulting in either accumulation of toxic compounds or deprivation of the necessary metabolites as described above. It is important that these inhibitors do not interfere with human analogues as this would cause side effects.

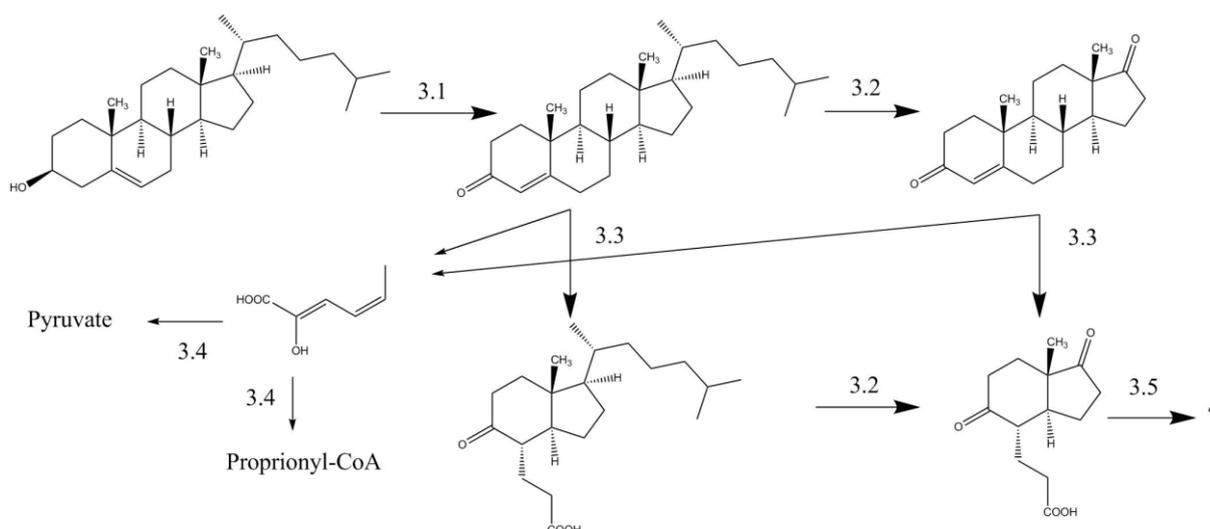
Cholesterol catabolism can be hijacked to let *Mtb* produce inhibitory compounds for its own pathway. An intermediate, cholest-4-ene-3-one, is known to inhibit *Mtb* growth on several carbon sources as glycerol, glucose, acetate and cholesterol (Frank et al., 2016). Cholesterol analogues with degradation resistant side chains can be converted to this intermediate, but not further metabolised by this pathway, resulting in accumulation of an inhibitory compound (Frank et al., 2016).

Although the use of inhibitory analogues seems promising, design of specific inhibitors is more versatile as it can both cause accumulation of toxic intermediates and deprivation of important metabolites. As it is important that the cholesterol degradation pathway is fully characterised, the next chapter attempts to describe each step of this pathway and the enzymes involved. We will zoom in and see whether the enzymes are still in the stage of discovery, if there is any evidence for their *in vivo* role and *in vitro* conversions, and what steps have been taken to find suitable inhibitors, or what are the possibilities of structure based drug design.

### 3. Cholesterol degradation in *Mtb*

As many genes are required for successful cholesterol degradation, this gene cluster is well conserved amongst several Actinobacteria (Wippenman et al., 2014). Cholesterol degradation can be subdivided into several stages, and for clarity this chapter is subdivided likewise. Although some controversy existed hereabout, the first two steps are catalysed by a single enzyme HsdD that oxidises the 3-hydroxyl and isomerises the double bond into a  $\alpha,\beta$ -enone. Afterwards, both side chain and steroid nucleus are catabolised simultaneously by a large series of enzymes. Steroid nucleus degrading enzymes can convert partially side chain degraded substrates, and vice versa. Thus, cholesterol degradation is not a linear process, but it can take many routes. However, the C & D rings can only be degraded when the cholesterol side chain is fully metabolised, so from this point on the degradation process is again linear. The final fate of C & D rings is only partially understood.

The enzymes in the degradation pathway will be discussed insofar as they are characterised. Some parts of cholesterol catabolism are very well understood, for instance the steroid nucleus cleavage. Side chain degrading enzymes are more difficult to assign to specific steps as there is a large redundancy in the *Mtb* genome regarding  $\beta$ -oxidation enzymes (Wippenman et al., 2014). In this chapter, mutant studies are described that either prove their requirement for growth on cholesterol or in macrophages, or accumulation of substrate compounds upon deletion of respective gene. Enzymatic assays serve as complementary evidence of the proposed *in vivo* role of the enzymes in the cholesterol catabolism. Insofar as inhibitors are known for this enzyme or class of enzymes, these inhibitors are discussed on their effectiveness and feasibility. The effectiveness is expressed in the inhibitory potential (*i.e.* inhibitory binding coefficient  $K_i$ ), and the feasibility is assessed by potential interference with orthologous human enzymes. Furthermore if protein structures of the enzymes are solved, these are also added as they may be leads for further drug design by molecular docking or cocrystallisation with inhibitory compounds.



**Figure 2:** Catabolism of Cholesterol subdivided in several stages: 3.1 is the initial dehydrogenation and isomerisation; 3.2 is the side chain degradation; 3.3 is the steroid nucleus cleavage into A & B rings, and C & D rings; 3.4 is the further degradation of A & B ring remnants into pyruvate and propionyl-CoA; 3.5 is the degradation of C & D rings, which is only partially known. The processes of 3.2 and 3.3 can occur simultaneously.

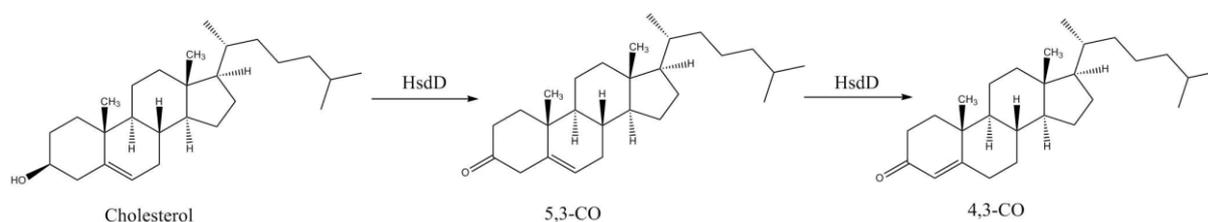
### 3.1 Initial dehydrogenation and isomerisation

#### *HsdD*

The first two conversions of cholesterol in its degradation pathway are catalysed by HsdD, a  $\beta$ -hydroxysteroid dehydrogenase (HSD). This is a multifunctional enzyme that acts both in the dehydrogenation of 3-hydroxyl and the isomerisation of the  $\beta,\gamma$ -unsaturated bond into the  $\alpha,\beta$ -unsaturated bond that is in conjugation with the 3-keto (Yang et al., 2007). *In vivo* studies in *Mtb* by growing a  $\Delta hsdD$ -mutant on cholesterol as carbon source shows no conversion for this compound. Enzymatic assays revealed that NAD<sup>+</sup> is the cofactor for this conversion, and both intermediate (cholest-5-en-3-one, 5,3-CO) and product (cholest-4-en-3-one, 4,3-CO) were observed. Trilostane acts as an uncompetitive inhibitor on this enzyme. This compound is already used for treatment of Cushing's syndrome (Komanicky et al., 1978), but shows severe side effects as it affects steroid metabolism systematically. Further research into other inhibitory compounds resulted in a series of inhibitory azasteroids (Thomas et al., 2011a). Usage of these compounds in human patients could interfere with mammalian HSD that are used by host steroid metabolism, but this has not yet been investigated. As no structure of this enzyme nor any closely related homologous proteins has yet been resolved, it may not be straightforward to design inhibitors that specifically target *Mtb* HsdD without inhibiting human HSD.

#### *ChoD*

Another protein was annotated to oxidise cholesterol by means of an extracellular enzyme using molecular oxygen as oxidant. However, conversion of cholesterol is not observed in enzymatic assays and ChoD is not essential in cholesterol metabolism as a  $\Delta ChoD$ -mutant is still able to convert cholesterol (Yang et al., 2011). Although in the related *M. neoaurum*, ChoD was able to oxidise cholesterol and similar compounds (Ivashina et al., 2012), this has never been proved for *Mtb*. Therefore, HsdD is the sole cholesterol oxidising enzyme. Interestingly, this enzyme is very important for *Mtb* virulence as it is essential for *Mtb* proliferation in macrophages (Brzostek et al., 2007). Later research has shown it interrupts the TLR2-mediated signal transduction of macrophages and is therefore important for the initial stages of *Mtb* infection rather than cholesterol degradation in the latent phase (Bednarska et al., 2014).



**Figure 3:** The first two steps in cholesterol degradation pathway. The hydroxyl moiety is reduced and the unsaturated bond is isomerised, both by the same enzyme, HsdD. Abbreviations of the compounds are written down above.

## 3.2 Side Chain degradation

### *Cyp125*

Degradation of cholesterol side chain is initiated through terminal oxidation by cytochrome P450 (Cyp125), that oxidises the terminal methyl moiety to a carboxylate. The product of this stereo selective conversion is (25S)-3-oxocholest-4-en-26-oate (25S-OCO). For *Mtb* CDC1551,  $\Delta$ *cyp125* were not able to grow on cholesterol and accumulated the toxic substrate 4,3-CO (Ouellet et al., 2010), and Cyp125 was shown to convert 4,3-CO (Ouellet et al., 2010). After the crystal structure of Cyp125 was solved at 1.4 Å resolution (McLean et al., 2009), structural studies with inhibitory azoles were also employed, but these compounds only showed moderate inhibitory effects (Ouellet et al., 2011).

### *Cyp142*

Cytochrome P450 (Cyp142) provides an alternative way for oxidation of cholesterol side chain. Interestingly, this Cyp142 is only present in the H37Rv strain, but not in CDC1551 (Ouellet et al., 2010). Like Cyp125, Cyp142 catalyses the stepwise terminal oxidation of the cholesterol side chain, but its product is the 25R-enantiomer rather than the 25S-enantiomer (Johnston et al., 2010) as was shown by enzymatic assays. Spectroscopic analyses also revealed that Cyp142 has a high binding affinity towards inhibitory azoles (Driscoll et al., 2010). Its protein structure was also solved at a resolution of 1.6 Å (Driscoll et al., 2010).

### *FadD19*

The ligase FadD19 catalyses the thioesterification of 3-oxocholest-4-en-26-oate (OCO) to 3-oxocholest-4-en-26-oyl-CoA (OCO-CoA). It accepts both enantiomers as substrate, and the product retains its stereochemistry (Yang et al., 2015). Although necessity of FadD19 has not been shown for mutants growing on cholesterol, this conversion was confirmed by enzymatic assays (Casabon et al., 2014). No protein structure is available for FadD19; this is necessary for drug design as general inhibitors against acyl-CoA ligases would interfere with similar human enzymes.

### *Mcr*

Degradation pathways of both enantiomers merge after 25R-OCO-CoA is converted to 25S-OCO-CoA by the racemase Mcr. Although Mcr has a large substrate susceptibility (Savolainen et al., 2005), it is also involved in the racemisation of OCO-CoA as demonstrated in enzymatic assays (Lu et al., 2015). A  $\Delta$ *cyp125* mutant is still able to metabolise cholesterol in presence of *cyp142* and Mcr, and thus it has been shown there are two initial degradation pathway for the cholesterol side chain (Lu et al., 2015). The first crystal structure of *Mtb* Mcr was solved in its substrate-free form at 1.8 Å resolution (Savolainen et al., 2005). Interestingly, in many forms of cancer methyl acyl-CoA racemases are overexpressed. Therefore a lot of inhibitors have been developed and tested (Carnell et al., 2007). However, inhibitors that specifically target *Mtb* Mcr are preferred over general inhibitors to prevent side effects. Recently, a compound has been discovered that only targets *Mtb* Mcr (Pal et al., 2016). Effectiveness of this inhibitor needs to be tested with clinical trials, and furthermore only works if *cyp125* pathway is also effectively hindered by inhibitors.

### *ChsE4; ChsE5*

A complex of ChsE4 and ChsE5 catalyses the dehydrogenation reaction of 25S-OCO-CoA to 3-oxocholest-4,24-dien-26-oyl-CoA (26-OCDO-CoA), the first step of first cycle of  $\beta$ -oxidation. However, it can also accept substrates of the second and third cycles of  $\beta$ -oxidation, albeit with a lower catalytic turnover (Yang et al., 2015). Although the *in vivo* role is not proven by mutants, enzymatic

assays show highest turnover for 25S-OCO-CoA (Yang et al., 2015). Furthermore, a crystal structure of ChsE4ChsE5 complex was solved at a resolution of 2.0 Å (Yang et al., 2015).

#### *FadA5*

Enzymes for the second and third step of the first cycle of  $\beta$ -oxidation are unknown, but 26-OCDO-CoA is converted via 24-hydroxy-3-oxocholest-4-en-26-oyl-CoA (26-HOCO) and 3,24-dioxocholest-4-en-26-oyl-CoA (26-DOCO) to 3-oxochole-4-en-24-oyl-CoA (24-OCO). The last step of  $\beta$ -oxidation is characterised and thiolase FadA5 is hypothesised to catalyse this last reaction, yielding propionyl-CoA as side product that can be further metabolised. FadA5 is also involved in the final step of the second cycle of  $\beta$ -oxidation in which 3,22-dioxochole-4-en-24-oyl-CoA (24-DOCO) is converted to 3-oxo-4-pregnene-20-carboxyl-CoA (OPC) and acetyl-CoA, again a functional metabolite for *Mtb* metabolism. Importance of FadA5 was shown in  $\Delta$ *fadA5* mutants that lost their virulence and capability to grow on cholesterol as carbon source (Nesbitt et al., 2010). Remarkably, only for substrate of the second cycle of  $\beta$ -oxidation enzymatic assays have been performed, and proven the catalytic role of FadA5 (Schaefer et al., 2015). For the first cycle of  $\beta$ -oxidation thiolase activity is therefore still obscure. Crystal structures were obtained for apo FadA5 and FadA5 C93S complexed to product OPC at a resolution of 2.7 Å and 1.7 Å respectively, revealing the steroid binding site (Schaefer et al., 2015). Drug design is not straightforward due to potential interference with human thiolases.

#### *ChsE3*

The second cycle of  $\beta$ -oxidation is initiated by the dehydrogenase ChsE3 that catalyses 24-OCO-CoA to 3-oxochole-4,22-dien-24-oyl-CoA (24-OCDO-CoA). In the second and third steps of this second cycle of  $\beta$ -oxidation, 24-OCDO-CoA is further catabolised to 22-hydroxy-3-oxochole-4-en-24-oyl-CoA (24-HOCO-CoA) and 24-DOCO-CoA respectively, but the enzymes responsible for these conversions still await characterisation. Unlike ChsE4ChsE5, ChsE3 specifically converts 24-OCO-CoA, and is not reactive towards 25S-OCO-CoA or OCP-CoA (Yang et al., 2015). This gene is required for *Mtb* growth on cholesterol (Griffin et al., 2011), and would thus be an interesting drug target. However, inhibitors have not been developed, and no ChsE3 structure is available.

#### *ChsE1;ChsE2*

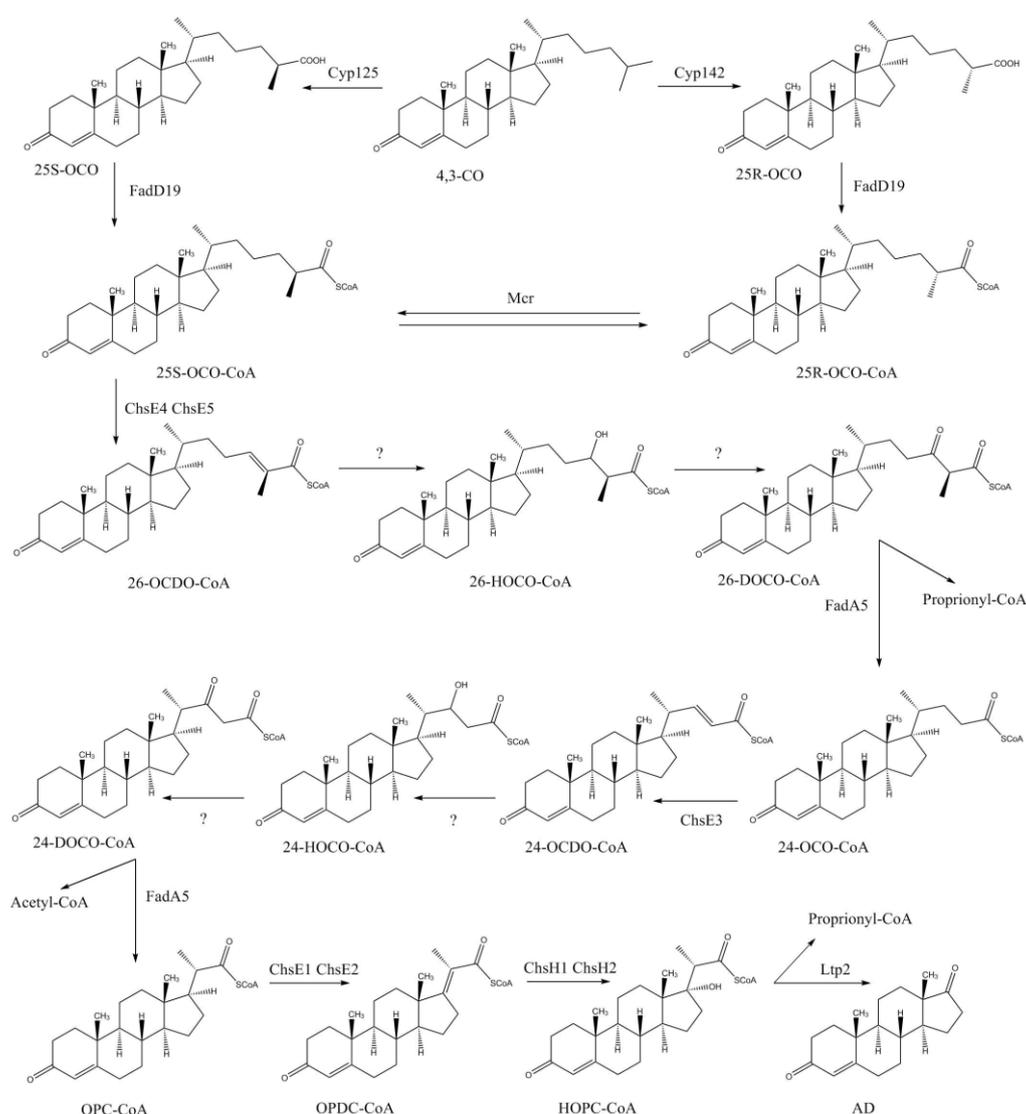
Finally, the third and last cycle of  $\beta$ -oxidation is initiated by the dehydrogenase complex ChsE1ChsE2 that catalyses the reaction of OPC-CoA to 3-oxo-4,17-pregnadiene-20-carboxyl-CoA (OPDC-CoA). This conversion was proven with enzymatic assays (Thomas et al., 2011). Although mutants lacking these genes show attenuated growth, the activity of this complex can be compensated by ChsE4ChsE5 (Yang et al., 2015). No structure is available for this complex, and no inhibitors have yet been designed.

#### *ChsH1;ChsH2*

An  $\alpha_2\beta_2$ -heterotetramer complex of ChsH1 and ChsH2 form a MaoC-like enoyl-CoA hydratase (ECH), and catalyse the hydration reaction of OPDC-CoA to 17-hydroxy-3-oxo-4-pregnene-20-carboxyl-CoA (HOPC-CoA) (Yang et al., 2014). Mutants lacking either ChsH1 and ChsH2 lack growth in macrophages (Rengarajan et al., 2005). A complex of ChsH1 and C-terminally truncated ChsH2 was crystallised without substrate and with a substrate analogue at a resolution of 1.5 Å and 1.8 Å respectively (Yang et al., 2014). Conversion of OPDC-CoA was demonstrated, remarkably also for the C-terminally truncated ChsH2 (Yang et al., 2014).

## Ltp2

The last cycle of  $\beta$ -oxidation terminates atypically as the hydroxyl moiety is not reduced by a  $\beta$ -hydroxyacyl-CoA dehydrogenase. Instead, a retro aldol reaction is catalysed by Ltp2, and propionyl-CoA is removed from HOPC-CoA resulting in androst-4-ene-3,17 (AD) that is further metabolised in steroid nucleus cleavage. The *ltp2* gene is located in the *igr* operon, that also harbours *chsE1*, *chsE2*, *chsH1*, *chsH2* and *cyp125*. As with exception of *cyp125* all *igr* genes are involved in the third cycle of  $\beta$ -oxidation, *ltp2* is considered to be responsible for the last step in this cycle (Thomas et al., 2011). Only weak evidence exists as  $\Delta$ *igr* mutants accumulate  $1\beta$ -(2'-propanoate)- $3\alpha$ -H- $4\alpha$ -(3'-propanoic acid)- $7\alpha\beta$ -methylhexa-hydroindane-1,5-dione (1-PHIP) (Thomas et al., 2011), but this can also be explained due to the absence of the other genes involved in the third cycle of  $\beta$ -oxidation. Nevertheless, this result suggests that cholesterol side chain must be fully metabolised before further degradation of C & D rings. Heterologous expression of Ltp2 was not successful due to insolubility of the expressed protein (Thomas et al., 2011). Therefore, enzymatic assays and crystallisation trials could not be performed.



**Figure 4:** Cholesterol side chain degradation. After the initial bifurcation, both pathways converge into a single pathway that undergoes three cycles of  $\beta$ -oxidation (third, fourth and last rows). Abbreviations of the compounds are extended in the respective paragraph.

### 3.3 Steroid nucleus cleavage

#### *KstD*

A di- $\alpha,\beta$  enone cholesterol derivative is formed by KstD (3-ketosteroid- $\Delta$ 1-dehydrogenase) through a desaturation reaction. Initially, no enzymatic conversion was observed for androst-4-ene-3,17-dione (AD) as proposed substrate in the cholesterol degradation pathway (Knol et al., 2008). However, later studies showed that indeed AD is converted by KstD (Capyk et al., 2011), albeit in a relatively slower rate than cholesterol derivatives of which its side chain was partially degraded. This serves as additional evidence that steroid nucleus cleavage and side chain degradation are simultaneous processes. The role of this enzyme in this pathway is indirectly confirmed by a  $\Delta$ *kstD*-mutant in which accumulation of 9-hydroxy-4-androstene-3,17-dione (9-OHAD) is observed, the hydroxylated substrate for KstD (Brzostek et al., 2009). Hydroxylation at position 9 is catalysed by KshAB. Apparently, KstD and KshAB act together to cleave the steroid nucleus independent of the order of both conversions. Although no structure has yet been elucidated for *Mtb*, similar ketosteroid dehydrogenases of the evolutionary related *Rhodococcus erythropolis* SQ1 (Rohman et al., 2013) and *Rhodococcus jostii* RHA1 (van Oosterwijk et al., 2012) have been resolved at 2.0 and 1.6 Å respectively. Structure from *R. erythropolis* SQ1 is more relevant to *Mtb* since it catalyses desaturation on the same bond ( $\Delta$ 1-dehydrogenase), whereas structure of *R. jostii* RHA1 performs this reaction on a different bond ( $\Delta$ 4-dehydrogenase). As KstD has no human orthologues, this enzyme would be a potentially interesting target for drug design (Knol et al., 2008), but no inhibitors have been designed yet.

#### *KshAB*

Hydroxylation of ADD at C9 occurs by a two-component monooxygenase formed by KshA and KshB. Together, they catalyse the conversion of ADD to 9-hydroxy-androsta-1,4-diene-3,17-dione (9OHADD), which spontaneously rearranges to 3-hydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3-HSA). The driving force for this rearrangement is aromatisation of ring A, resulting in cleavage of ring B. KshB is a reductase that reduces a covalently bound FAD by NADH, and subsequently transfers the electrons via a plant type iron-sulphur cluster to a Rieske iron-sulphur cluster of KshA, the oxygenase component, and a non-heme iron. This iron ion coordinates and reduces molecular oxygen to a water molecule, while the other oxygen atom is incorporated at C9 (Petrusma et al., 2014). Both subunits are required for the conversion of ADD as has been proved by enzymatic assays on AD, ADD and intermediates of which the cholesterol side chain was only partially metabolised (Capyk et al., 2009). Furthermore, mutant studies with  $\Delta$ *kshA*,  $\Delta$ *kshB* and  $\Delta$ *kshA*/ $\Delta$ *kshB* double mutant confirm their essential role for growth on cholesterol or derivatives (Hu et al., 2010). Interestingly,  $\Delta$ *kshB* mutants also show altered penta-acylated trehalose (PAT) biosynthesis, suggesting multiple roles for KshB (Hu et al., 2010). A structure has only been resolved for KshA; at 2.3 Å resolution for apo KshA (Capyk et al., 2009), and 2.5 Å resolution KshA complexed with AD (Penfield et al., 2014). Subsequent drug design has not been performed for KshA. On the other hand, KshB is not structurally determined, but would be an interesting target for drug targeting as it is both involved in cholesterol catabolism as in PAT biosynthesis.

#### *HsaAB*

The next step in steroid nucleus cleavage is another hydroxylation catalysed by a two-component monooxygenase, HsaAB. These proteins cooperate to convert 3-HSA to 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (3,4-DSHA). Analogously to KshAB, subunit A acts as an oxygenase, whereas subunit B is a flavin dependent reductase (Dresen et al., 2010). An interesting

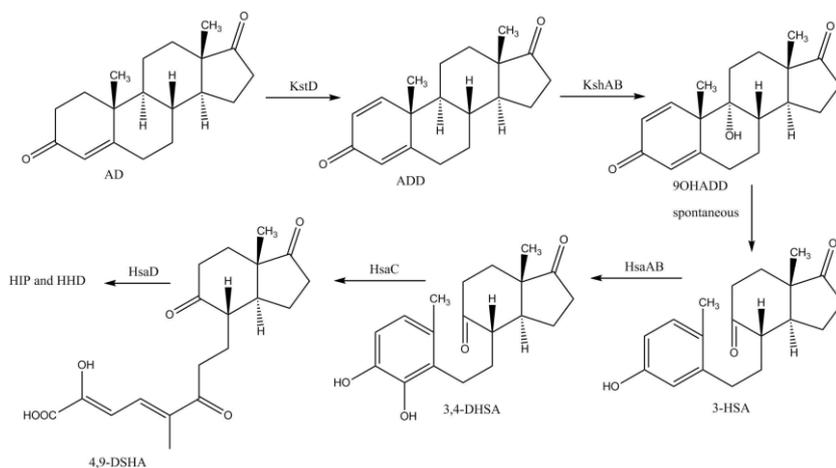
difference between KshAB and HsaAB is the absence of requirement for subunit B in the case of HsaAB. Mutant studies show only an absolute HsaA necessity for *Mtb* survival in macrophages (Rengajaran et al., 2005). This has not been shown for HsaB, suggesting no requirement for a specific reductase, but the possibility of other reductases to compensate for its activity. Enzymatic assays of purified proteins have shown that HsaAB is able to convert 3-HSA (Dresen et al., 2010). A crystal structure of HsaA resolved at 2.0 Å suggests that not only 3-HSA can bind into the active site, but also derivatives with partially degraded side chains (Dresen et al., 2010). Recently, two inhibitors were identified that specifically target the HsaAB complex resulting in the accumulation of 3-HSA (van der Ven et al., 2015). These compounds are interesting leads for novel drug development.

### HsaC

This dioxygenase catalyses the cleavage of the A ring, and converts 3,4-DHSA to 4,5-9,10-diseco-3-hydroxy-5,9,17-trioxoandrosta-1(10),2-diene-4-oic acid (4,9-DSHA). Molecular oxygen oxidises the C4-C5 aromatic bond to carbonyl moieties on both C4 and C5 resulting in aromatic ring cleavage of 3,4-DHSA. A  $\Delta hscC$  deletion mutant failed to grow on cholesterol and showed decreased pathogenicity (Yam et al., 2009). Conversion of 3,4-DHSA to 4,9-DSHA by HsaC was confirmed in presence of oxygen (Yam et al., 2009). Structures of both apo HsaC and HsaC complexed to 3,4-DHSA were solved at a resolution of 2.0 Å and 2.2 Å respectively (Yam et al., 2009). Based on homology to BphC (EC 1.13.11.39), two compounds were tested for inhibition, but were not very effective inhibitors to HsaC (Yam et al., 2009). Improved design of these inhibitors with regard to crystal structure of HsaC may result in more efficient inhibitors.

### HsaD

The final step of steroid nucleus cleavage is removing remnants of A & B rings from the intact C & D rings by the hydrolase HsaD. This enzyme first tautomerises 4,9-DSHA into a diketo intermediate. Subsequent hydrolysis results in formation of two products: 3 $\alpha$ -H-4 $\alpha$ (3'-propanoic acid)-7 $\beta$ -methylhexa-hydroindane-1,5-dione (HIP) and 2-hydroxy-hexa-2,4-dienoic acid (HHD). This gene is essential for *Mtb* survival in macrophages (Rengarajan et al., 2005). Enzymatic conversion of 4,9-DSHA by HsaD was confirmed by assays, and a S114A mutant was unable to perform this catalysis proving the catalytic function of this residue (Lack et al., 2010). Crystal structures of this S114A mutant in presence of 4,9-DSHA were obtained at a resolution of 1.8 Å (Lack et al., 2010). A large range of serine protease and acetylcholinesterase inhibitors was tested for their inhibitory effect on HsaD. Only two broad-spectrum inhibitors were found to have moderately high inhibition rates, but these may have side effects due to interference with similar human proteins (Ryan et al., 2013). Thus, further optimisation is necessary for these inhibitors to be utilised as drugs.



**Figure 5:** Cleavage of the steroid nucleus. KstD and KshAB activity can be inverted. After the spontaneous thermodynamically driven aromatisation, the A & B rings are further modified by HsaABC until these are cleaved off by HsaD. Resulting HHD and HIP are further catabolised by two different pathways. Abbreviations of the compounds are given in the respective paragraphs.

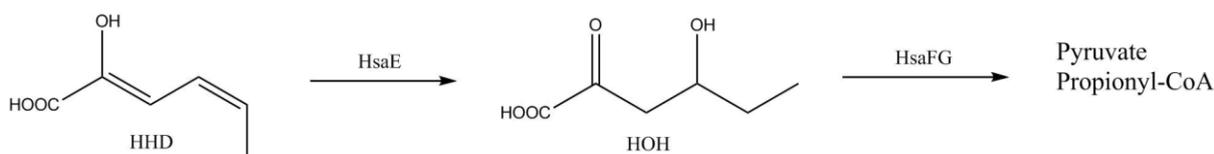
### 3.4 Remnant of A & B rings

#### *HsaE*

Based on homology of this protein to TesE of *Comamonas testosteroni*, this protein is annotated as a hydratase that catalyses the reaction of HHD to (S)-4-hydroxy-2-oxohexanoate (HOH). This conversion has been shown for TesE, but still has to be proven for *Mtb* (Horinouchi et al, 2005). A deletion mutant of HsaE only results in slower growth on cholesterol (Griffin et al., 2011). An explanation is that these mutants are still able to metabolise the C & D rings as carbon and energy source. Both TesE or HsaE lack crystal structures.

#### *HsaFG*

This complex of HsaF and HsaG is thought to catalyse two reactions in the A & B ring degradation, again based on homology to proteins of *C. testosteroni* (van der Geize et al., 2007). First, the aldolase HsaF performs a retroaldol reaction on HOH, producing pyruvate and propanal. Pyruvate can be used in the central carbon metabolism, but the volatile propanal has to be directly converted to propionyl-CoA by the dehydrogenase HsaG, and is subsequently thioesterified with coenzyme A. As for HsaE, mutant studies revealed these enzymes are not absolutely required, but result in attenuated growth in *Mtb* (Griffin et al., 2011). Although HsaG is able to act as a dehydrogenase on propanal, only a HsaFG complex is able to perform the initial aldolase reaction (Carere et al., 2013). This complex has been successfully crystallised in its apo structure at a resolution of 1.9 Å (Carere et al., 2013).



**Figure 6:** The remnant of A & B rings is converted into two metabolites that can be used in the central carbon metabolism (pyruvate and propionyl-CoA) in two steps by HsaE and HsaFG. Abbreviations of the compounds are given above.

### 3.5 Partial degradation of C & D rings

#### *FadD3*

The first step in degradation of C & D rings is the thioesterification of HIP to 3 $\alpha$ -H-4 $\alpha$ -(3'-propanoyl-CoA)-7 $\beta$ -methylhexa-hydroindane-1,5-dione (HIP-CoA) by the ligase, FadD3. For steroid nucleus cleavage, enzymes allowed a not fully metabolised cholesterol side chain, but for further degradation of C & D rings, this side chain needs to be fully removed (Casabon et al., 2013). A  $\Delta$ *fadD3* mutant of *Rhodococcus jostii* RHA1 accumulated and excreted HIP, but upon introduction of *Mtb fadD3* HIP was no longer excreted. Therefore, the *in vivo* function of *fadD3* was confirmed by functional complementation (Casabon et al., 2013). Furthermore, this conversion was also proved *in vitro* by enzymatic assays on HIP; the activity of FadD3 on HIP was 165 times higher than on 5OH-HIP affirming the suggested degradation pathway for C & D rings (Casabon et al., 2013). No structure of FadD3 has yet been elucidated, nor do any inhibitors show specific inhibition on FadD3.

#### *FadE30*

After reduction of the 5-keto to a 5-hydroxyl moiety by an unknown enzyme, FadE30 reduces the double bond of the HIP side chain to 5-hydroxyl-3 $\alpha$ -H-4 $\alpha$ -(3'-prop-2'-ene-oyl-CoA)-7 $\beta$ -methylhexa-hydroindane-1-one (HHIP-ene-CoA). This likely initiates another cycle of  $\beta$ -oxidation as was also observed for cholesterol side-chain degradation. In *Rhodococcus equi*,  $\Delta$ *fadE30* mutants accumulated

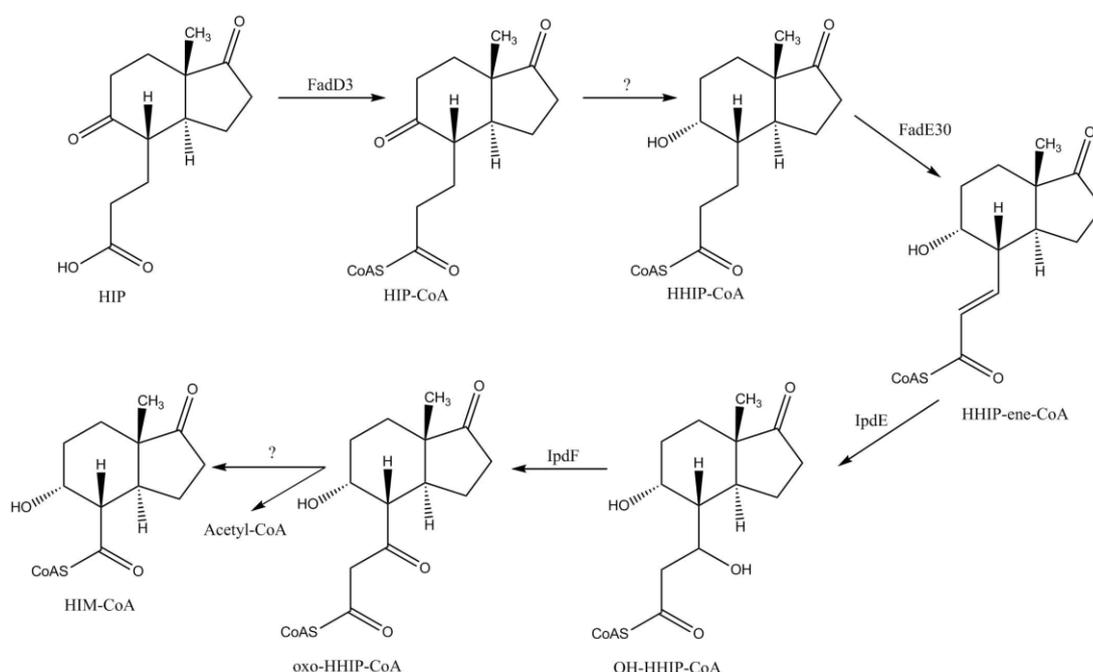
HIP and 5OH-HIP, suggesting this enzyme is involved in dehydrogenation, the first step of  $\beta$ -oxidation (van der Geize et al., 2011). However, this conversion is not confirmed *in vitro*.

#### *lpdE*

A putative ECH, *lpdE*, is thought to hydrate the double bond of HHIP-ene-CoA to 5-hydroxyl-3 $\alpha$ -H-4 $\alpha$ (1'-hydroxy-3'-propanoyl-CoA)-7 $\beta$ -methylhexa-hydroindane-1-one (OH-HHIP-CoA). For *Rhodococcus erythropolis* SQ1,  $\Delta$ *lpdE* mutants accumulate HHIP-ene-CoA (Petrusma, unpublished). However, this conversion has not yet been proven by enzymatic assays. Recently, a crystal structure of *lpdE* was solved at 1.9 Å resolution (dal Lago, unpublished), but cocrystallisation with its substrate has not been performed yet. In *Mtb*, the genes *Rv3541c* and *Rv3542c* are *lpdE* homologs and form a two-component system like *ChsE1/ChsE2*. These genes are essential for *Mtb* survival in macrophages (Rengajaran et al., 2005). However, enzymatic assays and protein structures for these *Mtb* genes are not available. Successful ECH inhibitors have been developed (Agnihotri and Liu, 2013), but these compounds must not interfere with human ECH to be utilised in drug design.

#### *lpdF*

The hydrated product of *lpdE*, 3-hydroxy-HHIP-CoA, is subsequently reduced to its respective ketone 5-hydroxyl-3 $\alpha$ -H-4 $\alpha$ (1'-oxo-3'-propanoyl-CoA)-7 $\beta$ -methylhexa-hydroindane-1-one (oxo-HHIP-CoA) by a putative dehydrogenase, *lpdF*. Afterwards, the  $\beta$ -oxidation is thought to be completed by an unknown thiolase yielding 5-hydroxyl-3 $\alpha$ -H-4 $\alpha$ (methanoyl-CoA)-7 $\beta$ -methylhexa-hydroindane-1-one (HIM-CoA). The fate of latter compound is still unknown. Again, *Rhodococcus erythropolis* SQ1  $\Delta$ *lpdF* mutants accumulate HHIP-ene-CoA (Petrusma, unpublished). Moreover, *Nocardia corallina* IFO 3338 mutants also show accumulation of this compound (Nakamatsu et al., 1980). The *Mtb* gene *Rv3548* encodes a protein homologous to *lpdF* and has a 70% amino acid similarity with a short chain dehydrogenase of *M. avium*. A crystal structure of this dehydrogenase was solved at a resolution of 1.8 Å (Baugh et al., 2015), and may serve as a good model for *lpdF*. However, the necessity of *Rv3548* for *Mtb* survival, its enzymatic conversion and its protein structure still need to be investigated.



**Figure 7:** Metabolism of C & D rings is only partially understood. After reduction of a keto moiety, the side chain of HIP is removed by a cycle of  $\beta$ -oxidation. Abbreviations of the compounds are given in the respective paragraphs.

Metabolite	Gene name	Mutants studies	Enzymatic assays	Structure (res.)	Drug development
Cholesterol	hsdD	Confirmed	Confirmed	-	Interference
4,3-CO	cyp125;142	Confirmed	Confirmed	Solved (1.4-1.6 Å)	Inhibitors
25S-OCO	fadD19	-	Confirmed	-	-
25R-OCO	fadD19	-	Confirmed	-	-
25S-OCO-CoA	chsE4;E5	-	Confirmed	Solved (2.0 Å)	-
25R-OCO-CoA	mcr	-	Confirmed	Solved (1.8 Å)	Inhibitors
26-OCDO-CoA	-	-	-	-	-
26-HOCO-CoA	-	-	-	-	-
26-DOCOCO-CoA	fadA5	Confirmed	Confirmed	Solved (2.7 Å)	-
24-OCO-CoA	chsE3	Confirmed	Confirmed	-	-
24-OCDO-CoA	-	-	-	-	-
24-HOCO-CoA	-	-	-	-	-
24-DOCOCO-CoA	fadA5	Confirmed	-	Solved (2.7 Å)	-
OPC-CoA	chsE1;E2	-	Confirmed	-	-
OPDC-CoA	chsH1;H2	Confirmed	Confirmed	Solved (1.5 Å)	-
HOPC-CoA	ltp2	Indirect evidence	-	-	-
AD	kstD	Indirect evidence	Confirmed	Homologs (1.6 Å)	-
ADD	kshAB	Confirmed	Confirmed	Only KshA (2.3 Å)	-
3-HSA	hsaAB	Only HsaA	Confirmed	Only HsaA (2.0 Å)	Inhibitors
3,4-DSHA	hsaC	Confirmed	Confirmed	Solved (2.0 Å)	Poor inhibitors
4,9-DSHA	hsaD	Confirmed	Confirmed	Solved (1.8 Å)	Inhibitors
HHD	hsaE	Confirmed	-	-	-
HOH	hsaFG	Confirmed	Confirmed	Solved (1.9 Å)	-
HIP	fadD3	Confirmed	Confirmed	-	-
HIP-CoA	-	-	-	-	-
HHIP-CoA	fadE30	Homologs	-	-	-
HHIP-ene-CoA	ipdE	Confirmed	-	Homolog (1.9 Å)	Interference
OH-HHIP-CoA	ipdF	Homologs	-	Homolog (1.8 Å)	-
oxo-HHIP-CoA	-	-	-	-	-

**Table 1:** All known metabolites for the cholesterol degradation pathway. This table illustrates whether the respective genes for conversion of these metabolites are known, whether their *in vivo* role has been confirmed by mutational studies, whether the enzymatic conversion was demonstrated *in vitro*, whether protein structures were solved at to what resolution, and if compounds have been designed that can inhibit this enzyme. The respective paragraphs contain further information on these categories, and contain the full compound name of the abbreviations used in the table.

## 4. Discussion

A deeper knowledge of the cholesterol degradation pathway has demonstrated that this pathway harbours a large potential for future drug development against TB. Because of emerging drug resistance amongst *Mtb* strains, new fields for drug development have to be explored.

Several general techniques have been discussed that aim for novel drugs to treat TB. Enhancing or modifying existing drugs like INH to combat resistance may at first sight seem a good solution. However, as these compounds are only altered version of already existing drugs, *Mtb* may respond very quickly by gaining resistance through modifying its own set of enzymes. High throughput screening methods result in useful hits as therapeutic drugs, and can also reveal novel targets for drugs, and furthermore these hit compounds can be further optimised. Deeper understanding of processes in *Mtb* like cholesterol degradation pathway reveal interesting drug targets for the long term, as all individual steps of certain processes can be acted against by inhibitory compounds.

Several targets in *Mtb* are suggested for drug development, but cholesterol degradation seems a particularly interesting target. Interruption of this pathway by deletion mutants results in accumulation of toxic intermediates and prevents *Mtb* from using cholesterol as its carbon and energy source. This suggests that if interruption can also be achieved by inhibitors, these are potent antitubercular agents.

This pathway relies on many enzymes for full metabolism of steroids, so there are many potential inhibitors for this pathway. Unfortunately, many of these enzymes catalyse one of the four steps of  $\beta$ -oxidation. These follow a general mechanism that is also utilised by eukaryotes making it difficult to design very specific inhibitors that do not interfere with its human analogues. Furthermore, many putative  $\beta$ -oxidation enzymes are not yet even assigned to a specific step within the pathway. On the other hand, some parts of this pathway are very well studied, especially the steroid nucleus cleavage. Not surprisingly, the more characterised parts also yield more potential inhibitors. Therefore this suggests that by further elucidation even more inhibitors can be found.

Inhibitory compounds have to fulfil two important requirements. They must be able to effectively inhibit the respective enzymes, but must not interfere with analogous human enzymes. Some compounds have been discovered that potently inhibit cholesterol degradation enzymes, like HsdD and HsaD, but have interference issues. However, these may be modified in such a way they no longer interfere. On the other hand, some enzymes do not have human analogues, but no efficient inhibition has yet been accomplished, for instance KstD and HsaC.

More promising steps of the cholesterol degradation pathway for future drug design are for instance Mcr and HsaAB. Inhibitors of Mcr have been developed that are both specific and efficient. However, this enzyme is only active in one of the two side chain degradation pathways, so without inhibiting Cyp125 the effects would only be moderate. Another interesting enzyme complex is HsaAB, the inhibitors specifically and efficiently target *Mtb* HsaAB, resulting in accumulation of toxic intermediates.

In conclusion, studies on the cholesterol degradation pathway have revealed some interesting leads for future drug design, but some inhibitors still require further optimisation before clinical trials. Moreover, this pathway is not fully characterised and research into the undiscovered parts may reveal more potential drug targets in cholesterol catabolism.

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