Drug effect of clofazimine on persisters explain an unexpected increase in bacterial load from patients

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Running head: Increase in bacterial load during CLO monotherapy

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Abstract

Tuberculosis (TB) drug development is dependent on informative trials to secure development of new antibiotics and combination regimens. Clofazimine (CLO) and pyrazinamid (PZA) are important components of recommended standard multi-drug treatments of TB. Paradoxically, in a Phase IIa trial aiming to define the early bactericidal activity (EBA) of CLO and PZA monotherapy over the first 14 days of treatment, no significant drug effect was demonstrated for the two drugs using traditional statistical analysis. Using a model-based analysis we characterized statistically significant exposure-response relationships for both drugs that could explain the original findings of increase in colony forming units (CFU) with CLO treatment and no effect with PZA. Sensitive analyses are crucial for exploring drug effects in early clinical trials to make right decisions for advancement to further development. We propose that this quantitative semi-mechanistic approach provides a rational framework for analysing Phase IIa EBA studies, and can accelerate anti-TB drug development.
**Introduction**

Tuberculosis (TB) is the main cause of death from an infectious disease (1) and new drugs are urgently needed to not only shorten treatment but also to manage the rising numbers of cases with drug resistant TB (2). Accompanying the need for new drugs, older approved drugs are repurposed (3, 4) to be included in new regimens, based on preclinical and clinical information.

Clinical symptoms appear when active infection is established. Stationary phase infection in pulmonary tuberculosis is characterized by stable colony forming unit (CFU) counts over time grown from sputum collected from untreated patients (5, 6). Experiments using resuscitation-promoting factors have emphasized that the majority of bacteria in a clinical sputum sample are non-cultivable, non-multiplying bacteria (7), which are undetectable using CFU as a biomarker. However, CFU is often applied in TB drug development and Phase IIa studies particularly. A decline in CFU in the first days of treatment is generally considered a desirable treatment response and a lack thereof makes the treatment appear unlikely to be clinically useful (8).

The aim of Phase IIa TB trials is to assess the early antimycobacterial activity and safety in patients and guide informed decisions about which drug or regimen to move forward to more comprehensive and costly Phase IIb trial evaluations. Usually, Phase IIa is designed to quantify change in mycobacterial load during the first 7-14 days of treatment. It is performed by quantifying early bactericidal activity (EBA) defined as the daily fall in \(\log_{10}\)CFU/ml of sputum (9). Further, empirical model-based approaches is frequently used to measure change in CFU. These include mono-, bi- or multiexponential regression models (10, 11), and simultaneously involve all data from one patient in the estimation of change in CFU. It is
generally accepted that a bi-phasic decline is due to a drug effect on different subpopulations of bacteria. As the types of bacteria exhibit different susceptibility, an initial rapid decrease of CFU occurs due to the effect on the most susceptible subpopulations, followed by a slower decline representing the killing of the less susceptible subpopulation.

However, these models only account for the effects on viable bacteria that can grow CFU and not the drug effect on semi-dormant or non-multiplying bacteria (persisters) which are thought to be majority of the bacterial population and do not grow on culture media used for assessing CFU. The Multistate Tuberculosis Pharmacometric (MTP) (12) model is a semi-mechanistic model combining three different bacterial subpopulations and the transfer between them (Figure 1). Different bacterial subpopulations are defined by heterogeneity in metabolic activity, corresponding to fast (F)-, slow (S)- and non (N)-multiplying bacteria, and will be referred to as multiplying (F), semi-dormant (S) and persistent bacteria (N) for uniformity. It has previously been successfully applied to preclinical (12, 13) and clinical data (14) for determination of exposure-response relationships i.e. drug effect. Initially developed on in vitro data, the MTP model approach has confirmed its role in translational medicine of TB where exposure-response relationships based on in vitro information have successfully been used to predict clinical trial response using clinical trial simulations (15, 16). Combined with the General Pharmacodynamics Interaction (GPDI) model (17, 18), a method for assessment of pharmacodynamics (PD) interactions, the MTP model has also successfully been applied to assessment of PD interactions of TB drugs both in vitro (19) and in vivo (16).

The approach on which this work was built upon was selected by The Impact and Influence Initiative of the Quantitative Pharmacology (QP) Network of the American Society for Clinical Pharmacology and Therapeutics (ASCPT) and presented in a recent publication.
which highlighted the most impactful examples of QP application where QP played a transformational role resulting in increased confidence in biomarker-driven decisions (20).

Clofazimine (CLO) and pyrazinamide (PZA) are established anti-TB drugs with efficacy proven in clinical trials. CLO and PZA have also been studied in two monotherapy arms in a recent Phase IIa trial (NCT-01691534) (21). Although both drugs are recommended by the World Health Organization (WHO) as part of standard treatment regimens for drug-susceptible and drug-resistant TB (4), unexpectedly, no statistically significant drug effects were observed during 14 days of monotherapy. CLO, studied in an EBA study for the first time, even showed a numerical increase in CFU (21). This was surprising because CLO exhibits sterilizing activity in patients (22, 23) with multi-drug resistant TB (MDR-TB, defined as TB resistant to at least isoniazid (INH) and rifampicin (RIF)). The paradoxical lack of EBA is well-known for PZA (6) and is in contrast to its ability to shorten TB treatment to 6 months when added to INH and RIF in the first 2 months.

In this work, the MTP model was linked to pharmacokinetic (PK) models, and thereby used to investigate exposure-response relationships of CFU after CLO and PZA monotherapy during 14 days to explain the paradoxical increase of CFU during CLO treatment, and to assess PZA monotherapy efficacy. The analysis revealed significant activity of CLO and PZA on persistent and semi-dormant mycobacteria, respectively, that remained undetected with traditional quantification methods of anti-TB drug effects.
Results

Population pharmacokinetic modeling

The final CLO PK model consisted of a two-compartment disposition model with first-order absorption and elimination. Additionally, a parameter explaining lag-time in absorption was supported by the data. No statistically significant covariate relationship was found using body weight, sex or age, on apparent oral clearance (CL/F) or apparent volume of distribution (V/F). Inter-individual variability (IIV) expressed as percentage coefficient of variation (%) CV was supported for CL/F (75%), first-order absorption ka (35%) and V/F (23%), whereas inter-occasional variability (IOV) was statistically significant for bioavailability (F, 26%). The residual error model consisted of a proportional error model with the magnitude of 13.9 %.

A previously developed PK model for PZA (24) with a modest modification was found to describe the PZA PK data well. The original PK model included a bimodal distribution in ka values representing slow and fast absorbers which was not supported by the data in this work. The data supported a unimodal distribution of ka values, corresponding to the fast-absorbers proportion in the original publication. Hence, only the fast-absorbers ka value was applied in this analysis. With this modest discrepancy, the earlier developed PK model (24) was able to predict population and individual PK profiles of PZA without re-estimation as seen in Figure 2B and Supplementary Fig. S1B, respectively. All final PK parameter estimates are presented in Table 1.

Visual predictive checks (VPCs) were performed, illustrating the observed data and how the final models adequately predicted the PK data (Figure 2A and 2B). As the pharmacokinetic-
pharmacodynamic (PK-PD) effect evaluation was driven by input from the individual PK profiles for each patient, the observed and adequate model predicted PK profile for each patient was important. The individual PK profiles of PZA and CLO were well predicted by the final PK models (Supplementary Fig. S1).

Pharmacokinetic-pharmacodynamic modeling

The MTP model (12, 14) was used as the underlying disease model to describe PD (i.e. CFU) data from the two monotherapy treatment groups separately. All MTP model parameters except $B_{\text{max}}$, were fixed to estimates derived from in vitro natural growth data (12, 14). When modeling the CLO data, estimation of CFU baseline i.e. $B_{\text{max}}$, resulted in statistically significant drop in the objective function value ($\Delta \text{OFV} = -3212$), compared to using the in vitro estimate, adjusting for the magnitude of bacterial load in the clinical CFU data, at stationary phase for the typical patient on CLO treatment. Introduction of IIV in $B_{\text{max}}$ was statistically significant as the $\Delta \text{OFV}$ dropped by 106 points, enhancing model fit and functionality of the model as it allowed for adjustment for individual CFU baseline. Similarly, estimating $B_{\text{max}}$ and implementation of IIV in $B_{\text{max}}$ gave statistically significant OFV drops, for the PZA CFU analysis.

The Bayesian posthoc PK estimates for each individual, based on the final PK model for each drug were used as input to the PK-PD modeling. A statistically significant exposure-response relationship ($p<0.05$, OFV drop of 5.12) was found between adequately predicted individual CLO plasma concentrations, derived from the developed population PK model, and killing of persistent bacteria. In this analysis, the discovered significant drug effect, denoted as $\text{ND}_k$, was a linearly concentration dependent, second order killing rate. The data did not support CLO killing of the other states alone nor in combination. Further, the data did not support an
inhibition on the growth of the multiplying bacterial sub-state. The identified CLO drug effect after monotherapy in this analysis was in contrast to the analysis presented in the original publication (21), where no CLO drug effect in monotherapy was discovered using CFU as a biomarker. This means that the original analysis could have missed significant drug effects of CLO on persisters and if CLO were a drug in development it might been unjustly abandoned.

For the exposure-response analysis of PZA in monotherapy, a statistically significant linear relationship (SDL) between individual PZA plasma concentrations and killing of the semi-dormant bacterial sub-state was found (p<0.05, OFV drop of 5.81). This was in contrast to the analysis presented in the original publication (21) where no PZA drug effect in monotherapy was discovered using CFU as a biomarker. The final exposure-response relationship predicted a decrease in CFU over time (Figure 3). The data did not support PZA inhibiting the bacterial growth or killing of the other states alone nor in combination. This can explain the long debated paradox that PZA is exhibiting low EBA yet has proven to be able to shorten TB treatment to the current standard short-course of 6 months.

The final differential equation system (Eq. 1-5) for the MTP model including CLO or PZA effect on the persistent or semi-dormant substate was:

\[
\frac{dF}{dt} = k_G \cdot \log \left( \frac{B_{\text{max}}}{F+S+N} \right) \cdot F + k_{SF} \cdot S - k_{FS} \cdot F - k_{FN} \cdot F \\
\frac{dS}{dt} = k_{FS} \cdot F + k_{NS} \cdot N - k_{SN} \cdot S - k_{SF} \cdot S - ESD \cdot S \\
\frac{dN}{dt} = k_{SN} \cdot S + k_{FN} \cdot F - k_{NS} \cdot N - END \cdot N
\]

where

\[ END = ND_k \cdot C_{CFZ} \]

And
\[ ESD = SD_k \cdot C_{PZA} \] (5)

END and ESD represent the linearly concentration dependent drug effects for CLO and PZA, respectively. The initial conditions of the differential equation system can be found in the NONMEM codes in supplementary materials (Text file 2 and 3). All final parameter estimates for discovered drug effects, using the MTP model as the underlying disease model can be found in Table 1.

The final MTP model provided adequate prediction of observed CFU data from patients in both treatment arms, as observed in Figure 3 and Figure 4, respectively. Typical bacterial simulations of each bacterial subtype and CFU counts can be found in the supplementary materials (Figure S2).

Sensitivity analysis

The performed sensitivity analysis covered the impact of relative amounts between the different sub-states of bacteria at baseline on the final model. For instance, changing the k_SN parameter was done so that the persistent bacterial subtype consisted of 99% to 90% of total bacteria. No significant drop in OFV (indicating that the alternative model did not improve the fit significantly) was observed following an empirical change of the original system parameters. This sensitivity analysis demonstrated that the CLO drug effect was statistically significant using the original system parameter estimates and that the model-fit did not improve by empirically changing the system parameter estimates.
Discussion

In this work, the MTP model was utilized as a framework, for studying anti-tubercular drug effects of CLO and PZA in monotherapy, using individual PK and PD (CFU) data from patients in a Phase IIa study. In contrast to the primary analysis of the data (21) where no statistically significant rate of decline in log_{10}CFU counts over the first 14 days of treatment were found, this analysis demonstrated statistically significant anti-mycobacterial activity for both CLO and PZA in monotherapy. The results indicate that the original analysis could have missed significant drug effects of CLO on persisters and if the substance was in development it might have been wrongly rejected. Further, the results might explain the low EBA of PZA, although proven to shorten TB treatment to the current standard short treatment of 6 months.

The results also indicate increased statistical power using the MTP approach, and emphasizes possible misinterpretations of potential drug effects, using traditional statistical analysis. As CFU is a summary biomarker of only multiplying bacilli, it is essential to emphasize that a drug with no decrease in CFU in EBA studies using a one-population model may be a drug with efficacy on bacterial populations other than those able to grow CFU, rather than having no effect as proposed in the original analysis. The findings are in accordance with previously published research (25), in which a simulated drug effect on the killing of the N sub-state (i.e. persisters), resulted in an indirect increase of CFU. The results are also in line with earlier work showing that the MTP model approach gives a higher power to find statistical significant drug effects compared to traditional statistical analysis (25). The semi-mechanistic MTP model has previously been utilized to describe drug effect on different TB bacterial sub-states in vitro (12), mouse (13), and clinical data (14). Additionally, the MTP model has been externally validated for clinical trial simulations, and proven to be able to predict decrease in CFU due to rifampicin treatment (14).
Clofazimine was suggested to have a significant, linearly concentration-dependent effect on the persistent sub-population. The data did not support any exposure-response relationship on the other sub-bacterial states, leaving the effect on the persistent state more interesting. Further, what constitutes this effect even more appealing, is that it explains the numerical increase in CFU, seen in the original publication (21). A model-based explanation of the increase in CFU, lies in a regrowth phenomenon caused by the Gompertz growth function in the MTP model. Growth of the different bacterial sub-populations is constrained by a growth capacity, which is defined by the $B_{\text{max}}$ term. When persistent bacteria are depleted, the density is decreased, paving way for regrowth proportional to the decrease of the persistent state. Mathematically, the Gompertz function which defines the growth of the multiplying state, according to equation (6):

$$k_G \cdot \log\left(\frac{B_{\text{max}}}{F+S+N}\right)$$

(6)

When persister bacteria (N) is killed, the growth of multiplying bacteria is enhanced due to a greater quota. This suggests an increase of multiplying bacteria. As CFU is a summary biomarker of multiplying (F) and semi-dormant (S) state bacteria, a total increase in CFU is seen. However, it is important to recognize that this is the explanations directly inferred from the presented model. There may be alternative explanations that appear more mechanistically relevant. To our knowledge, this is the first clinically defined exposure-response relationship of CLO using EBA data.

In order to adjust for differences in baseline CFU, between the in vitro setting which the MTP model was developed on, and the clinical data in this work, the parameter $B_{\text{max}}$ was re-estimated. Re-estimation of $B_{\text{max}}$ had no effect on the relative amounts of bacterial sub-populations, but only the baseline CFU. Relative amounts of multiplying, semi-dormant, and persistent bacterial sub-states are determined by the transfer rates between the sub-
populations, which were fixed to in vitro estimates. The used set of parameter values resulted in prediction of 99% persistent bacteria at baseline, for a stationary phase infection. This assumes that the relative amounts of the different sub-populations are the same in vitro and in patients. As a sensitivity analysis, the different transfer rates was empirically manipulated resulting in different relative amounts, followed by re-estimation of the CLO drug effect. No significant improvement was observed which may provide further justification for the conclusion that CLO kills persistent bacteria (Supplementary Fig. S2).

Clofazimine is a highly lipophilic antibiotic with a log P value of >7 (26), exhibiting long half-life. Previous reports suggests half-life of 10 days after a single oral dose of 200 mg (27), while some reports suggests half-life of > 70 days in longer treatment (28). Due to the lipophilicity and high distribution into tissues, it is plausible and expectable that CLO exhibits a long half-life. Crystal-like inclusions composed of CLO have been reported in several tissues in vivo (29) and in patients (30), which demonstrates capability to accumulate. As absorption into plasma is vastly dependent on concentration of dissolved molecules in solution, the lipophilic nature of CLO causes large variability in absorption due to poor solubility in physiological fluids (31).

IIV was supported by the data for $\frac{CL}{F}$, $\frac{V}{F}$ and $k_e$, whereas IOV was included in F. Due to previously discussed physiochemical and PK properties of CLO, it is expected that bioavailability varies between individuals and occasions of intake. As oral clearance is dependent on F, and could be affected by $k_e$, high variability in absorption might have been the reason for the high uncertainty in the estimated typical $\frac{CL}{F}$ value. Reports have indicated significant food effects on the bioavailability of CLO, showing 45% increase when administered with high-fat meal compared to fasting (31). Rich PK sampling on day 14 revealed high variability in exposure data (Supplementary Fig. S3). The highly variable nature in PK properties of CLO introduced uncertainty in the estimated population PK parameters as
the total number of subjects was low. However, for the subsequent PD analysis using the MTP model, this was not a problem as the adequately model-predicted individual PK profiles were used for input to the evaluation of drug effects.

As a TB culture or infection enter stationary phase, the majority of the bacteria may not grow on solid media (32). Alternatively, they may grow better in liquid media. It has been demonstrated for a high-dose rifampicin trial that one subpopulation of TB were quantifiable using liquid but not on solid media (where rifampicin exhibited a dose-dependent effect on this sub-population) (33). The persistent (N) state of the MTP model, may partly or completely, correspond to the considered bacterial subpopulation that can grow in liquid culture. The effectiveness and low prevalence of resistant strains against CLO could be due to the fact that it rarely was used for TB in the past, and having several molecular mechanisms of action (34, 35) on persistent bacteria, and its propensity to accumulate in tissue. As CLO exhibits high lipophilicity, it is expected to penetrate lesions, and have been demonstrated to kill hypoxic non-replicating bacteria in vitro (36). Furthermore, it has been demonstrated to exhibit membrane destabilizing properties, an effect that was attenuated in presence of membrane stabilizing agents (37). In a clinical setting, the addition of CLO to multi-chemotherapy regimens resulted in significantly higher sputum culture conversion (22). Furthermore, treatment success-rate was higher in the CLO containing regimen with cavity closure occurring earlier compared to standard regimen. However, the exact mechanism of CLO mediated antimicrobial activity needs further investigation.

In contrast to the original analysis of the PZA CFU data, a statistical significant linearly concentration-dependent effect, was discovered for the killing of semi-dormant bacteria following PZA treatment. The effect was evaluated using adequately predicted individual...
exposures. These findings are in accordance with previous research stating that infected
macrophages contain phagolysosomes with a low pH, an environment that results in semi-
dormant bacteria as well as the activation of PZA (38). As an acidic environment shifts the
bacterial metabolism and creates adequate circumstances of PZA drug effect, the discovered
findings are plausible. These findings are also in accordance with the clinical situation where
PZA is effective during the first two months of the standard multi-drug TB treatment,
potentially eradicating the majority of slow multipliers. Furthermore, PZA is metabolized in
the tubercle bacterium into pyrazinoic acid, an active metabolite that has shown capability to
distribute into lesions to the same extent as PZA (38). As stated by the original authors, PZA
exhibits a slight EBA using other biomarkers than CFU, but this work demonstrates
statistically significant drug effect using CFU as well.

In the investigation of exposure-response relationships, it is favourable to have PK and PD
(CFU) data from the same individuals, which fortunately was the case for this clinical study.
Primarily, adequate individual model-predicted PK profiles relative to provided PK data was
ensured, and subsequently used to evaluate drug effects. Most Phase IIa trials are aiming to
investigate EBA in TB research involve 10-15 patients per arm (9). Despite this small number
of patients, the translational MTP model demonstrated higher power compared to the
traditional statistical analysis performed in the original publication. This demonstrates a clear
advantage by using a semi-mechanistic PK-PD model, such as the MTP model, compared to
traditional statistical methods. The reason of increased power is the use of a non-linear mixed
effects approach where all data is analysed simultaneously, and that the analysis includes a
semi-mechanistic structure of not only multiplying sub-states but also a non-replicating state
which consists of a majority of total bacterial burden. Due to the parameters of the semi-
mechanistic MTP model, it was possible to estimate a drug effect of CLO that suggests
mycobacterial killing, despite that CFU increased. Using traditional analysis, an increase
would not be interpreted as the drug being effective, whereas using the MTP approach
includes all data simultaneously, including individual PK exposures, and do not constrain the
analysis to decrease in CFU. To avoid unperceived exposure-response relationships in the
data, model based analysis utilize a PK-PD approach, and have previously demonstrated
higher statistical power compared to traditional statistical analysis (25, 39, 40). As the sizes of
Phase IIa trials in TB drug development constrains defining such relationships using
traditional analysis methods, it is important to emphasize more sensitive and modern
methodologies. By reducing number of patients required to detect significant drug effects, TB
drug development could be less costly and therefore enhanced. Although developed on in
vitro data, the model is translationally capable of describing clinical data as in the case of
CLO drug effect. However, in order to rationally implement these findings in the clinic,
further clinical trials investigating CLO drug effect is desired. To define CLO PK and its
variability components with higher precision, additional data is needed.

In contrast to the original analysis, statistically significant drug effects were discovered for
both PZA and CLO in monotherapy using CFU as a biomarker. Drug effect on persistent
tubercular bacilli explains the unexpected increase after CLO monotherapy, and sheds light on
possible misinterpretations of drug effects using CFU as a biomarker together with traditional
statistical analysis. Thus, the MTP model can be utilized for analysis and simulation of
clinical trials to accelerate TB drug development.
Materials and Methods

Patients and study design

Data was obtained from a 14 day phase IIa, two center, open-label, randomized clinical trial including a PZA (1500 mg o.d. n=15) and a CLO (300 mg o.d. on days 1-3, 100 mg o.d. on days 4-14, n=14) treatment arm (21). CFU counts of *Mycobacterium tuberculosis* were quantified daily from sputum collected 16 hours overnight, from two days before start of treatment to the last treatment day. PK plasma sampling was conducted hourly from pre-dose to 5 hours post-dose on days 1, 2, 3 and 8, with rich sampling on day 14 which also included time points at 10, 16 and 24 hours after dose. All patients were adults with confirmed treatment-naïve pulmonary TB. Ethical clearance was obtained from the local ethics committee and informed consent was obtained prior to the study, from all patients. The trial was conducted in accordance with Good Clinical Practice, and was approved by the Research Ethics Committee, University of Cape Town, South Africa and Pharma Ethics Pty Ltd, South Africa. More detailed information regarding the clinical trial design and baseline characteristics, can be found in the original publication (21).

Population pharmacokinetic modeling

One and two compartment disposition models were tested for CLO. Based on previously published work (31), a parameter accounting for an absorption lag-time was explored. To account for the difference in dosage between day 1-3 and day 4-14, a relative bioavailability (F) expressed as a quota was tested, and compared to a scenario of fixed typical bioavailability to 1. The stochastic model was developed by exploring IIV and IOV, alone and in combination, in all structural parameters. Non-significant parameters were not included. Proportional and/or additive residual error models were implemented, to elucidate the appropriate error model for the data. Allometric scaling was applied on oral clearance, inter-
compartmental clearance (Q/F), and both apparent volume of distribution in plasma (Vc/F) and periphery (Vp/F), according to Anderson and Holford (41).

\[ CL_i = CL_{typ} \cdot (WT_i/53)^{0.75} \]  \hspace{1cm} (7)

\[ V_i = V_{typ} \cdot (WT_i/53)^1 \]  \hspace{1cm} (8)

CLi and Vi are scaled typical value of CL and V for individual i, respectively. CLtyp and Vtyp corresponds to parameters for a typical individual of 53 kg (median weight of the study population). WTi is the bodyweight of individual i, in kg. Similar notation as observed in equation (7) and (8), was implemented for Q and Vp. Further, body weight, age and sex was tested as covariates on CL/F and V/F.

For the PZA PK data, a previously developed population PK model was utilized without re-estimation of population parameters (24). In brief, the PZA model consisted of a one-compartment distribution model with first-order absorption and elimination to and from the central compartment, respectively. The model included a component to account for bimodal distribution of ka values, and was therefore evaluated. Further, the model included a zero-order rate constant accounting for release of drug from the formulation expressed as input to the absorption compartment. IIV was included in CL/F, V/F and the duration of the zero-order release from the formulation. IOV was included in CL/F and ka. Residual variability was described using a combined error model consisting of a proportional error at high concentration and an additive error component at lower concentrations. Body weight was included as a covariate on CL/F and V/F, while sex was included as a covariate on V/F.
Pharmacokinetic-pharmacodynamic modeling

The MTP model (12), originally developed on \textit{in vitro} data, was applied to the clinical CFU counts from the different treatment arms, as a disease model to explore significant drug effects on the different bacterial subpopulations. CFU was predicted based on the sum of the multiplying (F) and semi-dormant (S) bacterial sub-states, whereas the persistent (N) bacterial sub-state was considered non-multiplying on solid media and as such a “hidden” state (42). In brief, the MTP model consists of a differential equation system accounting for the transfer rates and conversion from one sub-state to another, and reflected a change in metabolic activity. The assumed scientifically plausible direction of flows can be observed in Figure 1, as well as in the following differential equations (9), (10), and (11) which defined the MTP system:

\begin{align}
\frac{dF}{dt} &= k_G \cdot \log\left(\frac{P_{\text{max}}}{F+S+N}\right) \cdot F + k_{FS} \cdot S - k_{FN} \cdot F - k_{FS} \cdot F \\
\frac{dS}{dt} &= k_{FS} \cdot F + k_{NS} \cdot N - k_{SN} \cdot S - k_{SF} \cdot S \\
\frac{dN}{dt} &= k_{SN} \cdot S + k_{FN} \cdot F - k_{NS} \cdot N
\end{align}

in which the rate constant \( k \) labelled with two-letters represents origin as first letter and flow direction as second letter. Transition rate from multiplying (F) to semi-dormant (S) bacterial sub-state, denoted \( k_{FS} = k_{FSlin} \cdot t \) was unique, in terms of the time (t) after infection (days) dependency. Growth of the multiplying (F) state \( k_G \) was accounted for using a Gompertz function. F, S and N were the model predicted bacterial number (ml\(^{-1}\)) in multiplying-, semi-dormant-, and persistent states, respectively. All transitions were allowed to occur, except for the flow of persistent state to the multiplying state, although indirectly possible through the transition to semi-dormant. As represented in Figure 1 and in previous equations (9), (10) and (11).
direct growth of the multiplying (F) state was possible, whereas increase in the number of semi-dormant (S) and persistent (N) states occurred only indirectly as a result of bacterial transfer. The transfer rates of the MTP parameters were fixed except $B_{\text{max}}$, which was estimated, and represents the bacterial growth capacity of the system. Patients were assumed to have stationary phase infections at start of treatment which correspond to 150 days after infection in a model-based setting. An IIV component in $B_{\text{max}}$ was added to account for different baseline in bacterial load between patients. Further, residual variability was accounted for by applying two additive components on log-scale. One of the residual error components was implemented on all replicates ($\varepsilon$) whereas the other residual error component accounted for replicates from the same sputum sample ($\varepsilon_{\text{repl}}$).

Model-predicted individual PK profiles were utilized, to let adequate individual drug exposures drive an effect on different effect sites in the MTP model. Potential effect sites were defined as inhibition of the growth of multiplying bacteria, or stimulation of death of multiplying, semi-dormant, or persistent bacteria. Included drug effect models were on/off (constant fixed drug effect with exposures above 0 mg/L), linear, $E_{\text{max}}$ and sigmoidal $E_{\text{max}}$.

Exposure-response relationship in the clinical data was investigated in four sequential steps, according to Svensson and Simonsson (14). The first step incorporated univariate drug effects on the different effect sites, utilizing previously mentioned effect models. To not exclude effect sites only apparent in combination, the second step included combination of all effect sites, with at least a linear effect model. The third step re-evaluated the most significant exposure-response relationship at each of the effect sites, retesting all effect parameters. As the final step, a backward elimination of all exposure-response parameters was performed, to
exclude non-significant effect sites at 1% significance level (ΔOFV>6.63 for removal of one 438 parameter).

To evaluate the impact of the fixed system-related parameters on the final CLO drug effect 441 model, a sensitivity analysis was conducted. Each parameter was empirically subject to a 442 change of 15, 80, 120 and 185% of the original in vitro estimate followed by re-estimation of 443 the drug effect and comparison of OFV (Supplementary Table S1).

Statistical analysis
Models were primarily selected on the basis of VPCs, difference in OFV, parameter precision, 447 diagnostic plots and scientific plausibility. Minimizing the OFV value has been interpreted as 448 maximizing the likelihood of estimated model parameters, given the clinical data using the 449 first order conditional estimation method with interaction. Model parameters and OFV was 450 estimated using the software NONMEM (version 7.3; Icon Development Solutions, Hanover, 451 MD) (43). A nested hierarchical model with addition or exclusion of one parameter was 452 considered to be statistically significant, at 5% significance level, if OFV decreased by at least 453 3.84 for 1 degree of freedom (χ² distribution). All diagnostic plots and data visualization were 454 performed using R package Xpose version 4.5.2 (Department of Pharmaceutical Biosciences, 455 Uppsala University, Uppsala, Sweden) (44, 45). VPCs was generated using Pearl-Speaks-
NONMEM (PsN) version 4.3.2 (Department of Pharmaceutical Biosciences, Uppsala 457 University), using 1000 simulations (44, 46). VPCs were assessed, in order to evaluate the 458 95% confidence intervals for the median, 90th and 10th percentiles of the by model simulated 459 data. As the drug effect evaluation was driven by individual PK profiles, each patients 460 observed and model-predicted PK profile was assessed using the same R package as for 461 diagnostic plots and data visualization. In addition, a 1000 sample bootstrap using PsN was
utilized to generate non-parametric 90% confidence intervals for all parameters in the final models (44, 46). Adequate track of record and comparison of models was maintained using Pirana (version 2.9.7; Pirana Software&Consulting) (47, 48).

Data availability

NONMEM code for PK models and the PK-PD models can be found in supplementary materials. All relevant data are available from the authors upon reasonable request.
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Contributions

All authors contributed to the data analysis and writing of the manuscript. A.H.D was the principal investigator of the clinical study.

Competing interests

None to declare.

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Fig 1. Schematic illustration of clofazimine and pyrazinamide pharmacokinetics models together with the Multistate Tuberculosis Pharmacometric (MTP) model. Abs, absorption compartment; $k_a$, absorption rate constant; CL/F, apparent oral clearance; V/F, apparent volume of distribution; $C_{comp}$, central compartment; $P_{comp}$, peripheral compartment; $B_{max}$, system carrying capacity; $k_{FS}$, time-dependent linear rate parameter describing transfer from multiplying (F)- to semi-dormant (S) state; $k_{SF}$, transfer rate between S- to F state; $k_{FN}$, transfer rate between F- and persisters (N) state; $k_{SN}$, transfer rate between S- and N state; $k_{NS}$, transfer rate between N- and S state; $F_{growth}$, parameter consisting of $k_G$ (growth rate constant) and the Gompertz function, describing growth of F state bacteria; $F_{kill}$, killing of the F state; $S_{kill}$, killing of the S state; $N_{kill}$, killing of the N state. Dashed lines indicates identified exposure-response relationship.
Fig 2. Visual predictive check for the observed (a) CLO and (b) PZA concentrations following rich sampling from day 14. Open circles are the observations. Upper and lower dashed lines illustrate 90th and 10th percentiles of observed data, respectively. The solid line is the median of observed data. From top to bottom, shaded areas represent 95% confidence intervals of the 90th (light grey), median (dark grey) and 10th (light grey) percentiles of simulated data based on 1000 simulations.
Fig 3. Visual predictive check of the final exposure-response model for patients receiving PZA. Dashed lines represent 90th and 10th percentiles of observed CFU data, whereas the solid line is the median of observed CFU data. From top to bottom, the shaded areas represent the 95% confidence intervals of the 90th (light grey), median (dark grey) and 10th percentiles of simulated data based on 1,000 simulations. All open circles illustrate observation points.
Fig 4. Visual predictive check of the final exposure-response model for patients receiving CLO. Dashed lines represent 90th and 10th percentiles of observed CFU data, whereas the solid line is the median of observed CFU data. From top to bottom, the shaded areas represent the 95% confidence intervals of the 90th (light grey), median (dark grey) and 10th percentiles of simulated data based on 1,000 simulations. All open circles illustrate observation points.
Table 1. Parameter estimates based on the final models

| Parameter Description | Estimate (% RSE) | IIV % (% RSE) | IOV % (% RSE) |
|-----------------------|------------------|---------------|---------------|
| **Population pharmacokinetic parameters of clofazimine** |
| CL/F (L·h⁻¹) Oral clearance | 12.5 (145) | 74.8 (160) | - |
| Vc/F (L) Apparent volume of distribution | 1138 (18.4) | 23.0 (85.9) | - |
| kₚ (h⁻¹) Absorption rate constant | 0.67 (50) | 35.3 (95.3) | - |
| Q/F (L·h⁻¹) Inter-compartmental clearance | 63.3 (12.7) | - | - |
| Vp/F (L) Peripheral apparent volume of distribution | 8062 (82.7) | - | - |
| tLAG (h) Absorption lag-time | 0.62 (0.75) | - | - |
| F Bioavailability | 1 FIX | 43.8 (26.1) | - |
| **Residual error parameters** |
| εprop (CV%) Proportional error model parameter | 13.9 (0.08) | - | - |
| **Multistate Tuberculosis Pharmacometric model parameters** |
| | | | |

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\( k_0 \) (days\(^{-1}\))  Fast-multiplying bacterial growth rate 0.206 FIX  -  -  
\( k_{FSN} \) (days\(^{-1}\))  Transfer rate from fast- to non-multiplying state 8.98 \times 10^{-7} FIX  -  -  
\( k_{SN} \) (days\(^{-1}\))  Transfer rate from slow- to non-multiplying state 0.186 FIX  -  -  
\( k_{SSF} \) (days\(^{-1}\))  Transfer rate from slow- to fast-multiplying state 0.0145 FIX  -  -  
\( k_{NS} \) (days\(^{-1}\))  Transfer rate from non- to fast-multiplying state 0.00123 FIX  -  -  
\( k_{FSLin} \) (days\(^{-2}\))  Time-dependent transfer rate from fast- to slow-multiplying state 0.00166 FIX  -  -  
\( F_0 \) (ml\(^{-1}\))  Initial bacterial number of fast-multiplying state 4.11 FIX  -  -  
\( S_0 \) (ml\(^{-1}\))  Initial number of slow-multiplying state 9770 FIX  -  -  

Exposure-response parameters of clofazimine

ND\(_{50}\) (L·mg\(^{-1}\)·day\(^{-1}\))  Second-order non-multiplying state death rate 1.63 (11.5, 1.36-2.05\(^{\text{st}}\))  -  -  
\( B_{\text{max}} \) (ml\(^{-1}\))  System carrying capacity per ml sputum (CZN arm) 0.06-10\(^{\text{th}}\) (35.1, 0.03-0.10\(^{\text{th}}\)) 93.1-157\(^{\text{th}}\)  -  -  

Residual error parameters

\( \varepsilon_{\text{add}} \) (CV%)  Additive residual error on log scale for all replicates 128 (7.85, 110-144\(^{\text{th}}\))  -  -  

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| Parameter | Description | Value | 90% Confidence Interval |
|-----------|-------------|-------|------------------------|
| \( \varepsilon_{\text{total}} \) (CV\%) | Additive residual error on log scale for all replicates | 98.5 (6.12, 87.7-108\(^e\)) | - |
| \( \varepsilon_{\text{rep}} \) (CV\%) | Additive residual error on log scale between replicates | 39 (11.6, 31.4-46.4\(^e\)) | - |
| SD_{\text{logA}} (L·mg \(^{-1}\)·days\(^{-1}\)) | Second-order slow-multiplying state death rate | 0.02 (30.6, 0.01-0.04\(^e\)) | - |
| B_{\text{max}} (ml\(^{-1}\)) | System carrying capacity per ml sputum, (PZA arm) | 0.08·10\(^9\) (54.7, 0.04-217 (16.3, 149-268\(^e\)) | - |

**Exposure-response parameters of pyrazinamide**

- \( \varepsilon_{\text{rep}} \) (CV\%): Additive residual error on log scale between replicates: 49 (17.4, 36.9-63.6\(^e\))
- \( \varepsilon_{\text{total}} \) (CV\%): Additive residual error on log scale for all replicates: 98.5 (6.12, 87.7-108\(^e\))
- SD_{\text{logA}} (L·mg \(^{-1}\)·days\(^{-1}\)): Second-order slow-multiplying state death rate: 0.02 (30.6, 0.01-0.04\(^e\))
- B_{\text{max}} (ml\(^{-1}\)): System carrying capacity per ml sputum, (PZA arm): 0.08·10\(^9\) (54.7, 0.04-217 (16.3, 149-268\(^e\))

- a: shrinkage in IIV of apparent oral clearance, expressed in percentage (10.5%).
- b: shrinkage in IIV of the apparent volume of distribution, expressed in percentage (16.5%).
- c: shrinkage in IIV absorption parameter, expressed in percentage (38.3%).
- d: shrinkage in the proportional residual error model parameter, expressed in percentage (15.6%).
- e: 90% confidence interval computed from the non-parametric bootstrap (n=1000).
FIX, parameter was fixed during estimation. RSE=relative standard error. IIV=inter-individual variability expressed as coefficient of variation and in % of the parameter estimate. IOV=inter-occasional variability expressed as coefficient of variation and in % of the parameter estimate. All MTP model parameters were fixed to estimates reported by Clewe et al. (12), except B_{max}.
