Clinical pharmacology in leishmaniasis: treatment optimization of a neglected disease
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Failure of miltefosine in visceral leishmaniasis is associated with low drug exposure

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Abstract

Recent reports indicated high miltefosine treatment failure rates for visceral leishmaniasis (VL) on the Indian subcontinent. To further explore the pharmacological factors associated with these treatment failures, a population pharmacokinetic-pharmacodynamic study was performed to examine the relationship between miltefosine drug exposure and treatment failure in a cohort of Nepalese VL patients treated with miltefosine monotherapy according to standard treatment protocols. Sparse blood samples were collected nominally at the end of treatment (EOT) and the miltefosine steady-state concentrations in these samples were analyzed using liquid chromatography coupled to tandem mass spectrometry. A population pharmacokinetic-pharmacodynamic analysis was performed in a sequential manner using non-linear fixed-effects modeling and a logistic regression model for the binary treatment outcome (failure vs. cure). Various individual estimates of miltefosine exposure were explored for their relationship with treatment failure. A population pharmacokinetic model for miltefosine was developed and used to derive several measures of individual drug exposure. The overall probability of treatment failure was 21%. The time the plasma concentration exceeded 10 times the EC$_{50}$ of miltefosine (median 30.2 days) was significantly associated with treatment success and failure: the odds ratio for treatment failure increased with 1.08 (95% CI 1.01-1.17) for every day that the EOT blood concentration did not exceed 10x EC$_{50}$. We here established that achieving sufficient miltefosine exposure is a significant and critical factor for VL treatment success, which urges the evaluation of the recently proposed optimal allometric miltefosine dosing regimen. This study constitutes a first step towards the definition of pharmacokinetic-pharmacodynamic targets to be attained for miltefosine in the treatment of VL.
Introduction

Miltefosine is currently still the only oral effective drug available to treat the neglected tropical parasitic disease visceral leishmaniasis (VL) [1]. Excellent efficacy of miltefosine in the treatment of VL was established in controlled clinical trials more than a decade ago in the Indian state of Bihar, one of the major areas of endemcity of VL [2]. Subsequently, miltefosine was introduced as first-line therapy for VL in most of the Indian subcontinent, including Nepal, where the drug was adopted in a multilateral program to eliminate the disease from the subcontinent [3,4]. Miltefosine replaced sodium stibogluconate [5,6], the conventional treatment for VL for the past 50 years, which suffered from increasing treatment inefficacy, both in Nepal and India [7,8].

Recently, the first reports appeared on the efficacy of miltefosine in the treatment of VL following its roll-out in primary health clinics. For both Nepal and India, after five and ten years of use respectively, disturbingly lower final cure rates were reported than the reported efficacies from previous clinical trials. This mainly concerned high relapse rates during follow-up; in India 7% of infections relapsed within 6 months [9]; in Nepal 11% relapsed within 6 months and 20% in total within 12 months (Rijal et al., submitted). To identify the cause of these high failure rates, several factors were further investigated in a prospective cohort study in Nepal, including parasite susceptibility, patient (or host) risk factors, and also quality of and exposure to the drug (Rijal et al., submitted). Patients were followed for a total of 12 months post-treatment, instead of the conventional 6 month follow-up period, to obtain data on long-term relapses. The analysis of risk factors and following implications for the VL elimination initiative are elaborately described elsewhere (Rijal et al., submitted).

In regard to drug exposure, end of treatment (EOT) miltefosine concentrations were studied as a general measure of exposure in a subset of this Nepalese cohort of patients receiving conventional miltefosine treatment (~2.5 mg/kg body weight for 28 days). Due to the extremely slow elimination of miltefosine, blood concentrations keep accumulating until EOT [10,11]. This would imply that drug exposure can adequately be assessed by studying the EOT plasma concentrations (equivalent to \( C_{ss} \) or \( C_{max} \)). Indeed, the mean miltefosine EOT concentration was lower for patients that failed versus those that cured, however, this effect did not reach statistical significance (Rijal et al., submitted).

This finding does not reject the hypothesis that low drug exposure leads to treatment failure, because several factors may be obscuring this comparison. For instance, there was high between-subject variability in the time of sampling at EOT. Moreover, the EOT-concentration is perhaps not the best proxy value of total drug exposure and is probably not the best measure that relates drug exposure to antiparasitic activity, certainly not when miltefosine’s action is time-dependent rather than concentration-
dependent. Overall, very little is known about the exposure-effect relationship of miltefosine in the treatment of VL [1]. To gain further insight into the possible correlation between various measures of exposure, pharmacokinetic targets and efficacy of miltefosine, and to overcome the limitations of untimely sampling at EOT, we analyzed the data from the Nepalese cohort using a combined sequential population pharmacokinetic-pharmacodynamic analysis, with the objective to identify an exposure-effect relationship as a possible explanation for the observed high relapse rate.

Methods

Patients

The population of patients in this pharmacokinetic-pharmacodynamic study is a subset of the Nepalese cohort of VL patients treated with miltefosine and studied in the framework of the Kaladrug-R project (Rijal et al., submitted). This study was conducted between March 2010 and August 2011 in a Nepalese referral hospital, BP Koirala Institute of Health Sciences (BPKIHS), which is a centre of excellence for VL treatment and research, located in the Eastern region of Nepal. VL patients who met previously described inclusion criteria (Rijal et al., submitted), for instance confirmed VL by a positive bone marrow aspirate, who had given consent for the Kaladrug-R study and from whom a blood sample was obtained around the EOT (~day 28) were eligible for this pharmacokinetic-pharmacodynamic study. Individual fat-free mass was estimated from each patient’s weight and height using Janmahasatian’s formula [12]; if height was unavailable (for 3 out of 81) fat-free mass was assumed to correspond to 90% of the total body weight of the individual, based on the mean observed fat-free mass from this cohort and previous cohorts of VL patients from the Indian subcontinent [11]. Patients were followed for a total of 12 months post-treatment, with follow-up visits at 3, 6, and 12 months after completion of therapy. They were examined for clinical signs of relapse and, if found bone marrow was re-examined for Leishmania parasites to confirm treatment failure. Patients who did not visit BPKIHS for their scheduled follow-up were actively traced in their homes. The research protocol of this prospective study was approved by the ethics committees of the Nepal Health Research Council and the University of Antwerp in Belgium.

Treatment

Patients were treated with miltefosine (Impavido®, Paladin Labs Inc, Montreal, Canada), for a total of 28 days, according to the national guidelines: adults (≥12 years of age) with a body weight of >25 kg received 50 mg twice daily (total dose: 100 mg/
day), adults with a body weight of $\leq 25$ kg received 50 mg once daily (total dose: 50 mg/day); whereas children (2-11 years of age) received 2.5 mg/kg body weight/day rounded to the nearest 10 mg.

**Samples & bioanalysis**

A single whole blood sample was obtained per patient on the occasion of their EOT-visit, approximately at day 28 after start of miltefosine treatment. Samples were kept frozen, minimally at -20°C, both during storage in Dharan, Nepal, and during transport from Dharan, Nepal, to Antwerp, Belgium, and subsequently from Antwerp, Belgium, to the bioanalytical laboratory of Slotervaart Hospital in Amsterdam, the Netherlands. Miltefosine concentrations were measured using a validated liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method, as described previously, with a limit of detection of 4 ng/mL, as described previously [13]. In short, samples were diluted in blank human plasma (50x) to avoid any unaccounted matrix effects, and were further prepared using solid phase extraction (phenyl-bonded solid phase) from which miltefosine was eluted using 0.1% triethylamine in methanol. An aliquot of 10 µL eluate was subsequently injected on the reversed-phased chromatography column (Gemini C18, 150 mm x 2.0 mm I.D., 5 µm) using an isocratic highly organic alkaline solvent (10mM NH₄OH in 95% methanol:water [v/v]) with a flow rate of 0.3 mL/min to achieve adequate separation. Detection was performed by positive ion electrospray ionization followed by triple-quadrupole mass spectrometry (API3000, Applied Biosystems), monitoring the specific mass transition of 408.6 m/z to 124.8 m/z for miltefosine. Concentrations were calculated based on the analyte/internal standard area ratio, using perifosine as internal standard.

**Population pharmacokinetic analysis**

All calculations, simulations and estimations were performed on a dual-core desktop computer running NONMEM 7.2 (ICON Development Solutions, Hanover, MD, USA) [14], the R statistical software package (version 2.14.1; http://www.r-project.org/) [15], and Perl speaks NONMEM (PsN, version 3.5.3; http://psn.sourceforge.net) [16,17]. Pirana (version 2.5.0; an interface to NONMEM, PsN, and our cluster; http://www.pirana-software.com) was used to structure and document the model development work and interpret the output [18].

Non-linear mixed effects modeling was performed using a previously developed and extensively evaluated open two-compartment population pharmacokinetic model of miltefosine with first-order absorption and elimination from the central compartment as the structural base model [10,11], using the first-order conditional estimation procedure with interaction between between-subject variability and residual error components. Clearance ($CL/F$) from and volume of distribution ($V_z/F$)
of the central compartment was allometrically scaled with a power of 0.75 and 1, respectively, using individual fat-free mass as the optimal body size descriptor, as previously established for miltefosine over a wide range of body sizes [11]. Since the data from Nepal represent a sparse dataset with only a single observation per patient at EOT, the estimation of population pharmacokinetic parameters was performed in NONMEM combining the current sparse dataset with all other prior obtained miltefosine pharmacokinetic data (from an adult European study [10], an adult Indian study [19], and a paediatric Indian study [20]). To enable handling of whole blood concentrations (as collected in the Nepalese cohort) in combination with plasma concentrations (as collected in the other studies) a correction factor \(f_c\) was introduced to convert the predicted plasma concentrations \(C_{pl,pred}\) to whole blood concentrations \(C_{wb,pred}\). The value of \(f_c\) was fixed at 0.25 as derived from blood distribution studies [1]. Between-subject variability in \(CL/F\), \(V_{2}/F\) and the absorption rate \(k_a\) was modeled with an exponential error. Residual error (including within-subject variability) was modeled with a proportional error, using a separate estimate for the data from Nepal, since it was obtained from a distinctly different study with a different population. Bioavailability \((F)\) was unknown, parameters were therefore used and reported relative to bioavailability \((CL/F, V_{2}/F, \text{etc.})\). Model adjustments were evaluated for their goodness-of-fit. Model evaluation was guided by the objective function value (OFV; equal to \(-2\log\text{likelihood}\)) and by graphical goodness-of-fit assessment through a visual predictive check (using PsN and Xpose).

When data are sparse and less informative on individual parameters, it is expected that the empirical Bayes estimate will be shrunk towards the population mean. Shrinkage in empirical Bayes estimates of between-subject variability of parameter \(j\) \((\eta_j)\) was calculated for the Nepalese data using [21]:

\[
\text{Shrinkage}_{\eta_j} = 1 - \frac{\text{std}(\eta_{i,j})}{\omega_j}
\]

(Eq. 1)

Where \(\text{std}(\eta_{i,j})\) is the standard deviation of the distribution of individual estimates of between-subject variability for parameter \(j\) for \(i\) individuals, and \(\omega_j\) is the population model estimate of the standard deviation in \(\eta_j\). Uninformative data will lead to shrinkage of the distribution of \(\eta_{i,j}\) towards 0 and thus an individual empirical Bayes estimate closer to the population mean.

Individual estimates of drug exposure were calculated in NONMEM using a differential equation solver, dummy compartments and the individual population pharmacokinetic model parameter estimates. Amongst others were calculated the area under the concentration-time curve from day 0 till end of treatment (AUC\(_{0-EOT}\)), the AUC from 0 to infinity (AUC\(_{0-\infty}\)), and the period of time that the miltefosine blood concentration was either above the mean EC\(_{50}\) value (\(T>\text{EC}_{50}\)) or above 10x the mean
EC₅₀ value (T>10xEC₅₀), which were determined with intracellular drug sensitivity testing of available clinical *Leishmania* isolates from this particular Nepalese cohort (mean EC₅₀: 4.4 µM or ~17.9 µg/mL) [22].

**Pharmacokinetic-pharmacodynamic analysis**

The pharmacokinetic-pharmacodynamic relationship between miltefosine exposure and final treatment outcome was explored with various individual drug exposure estimates from the population pharmacokinetic analysis. Patients were excluded from the pharmacodynamic analysis if: (i) they died, probably unrelated to VL, before the end of follow-up (12 months), or (ii) they experienced a treatment switch due to severe adverse events during the miltefosine treatment. Failure was defined as (i) no initial cure at EOT or (ii) as relapse i.e. initial cure at EOT but with reappearance of clinical symptoms and/or signs along with confirmation of *Leishmania* infection by a positive bone marrow aspirate smear during the 12 months follow-up. Probability of failure for the *i*th individual (*p*ᵢ) was modeled with linear logistic regression performed on the dichotomous treatment outcome data (0 = cure versus 1 = failure) with NONMEM using the Laplacian estimation method and with the conditional and likelihood options. The logit of *p*ᵢ (*Logitᵢ*) was defined as follows:

\[
\text{Logit}_i = \theta_1 + \theta_2 \cdot (MIL_i - MIL_\mu)
\]  
(Eq. 2)

Where \(\theta_1\) (see Equation 3) and \(\theta_2\) are the fixed effect parameters defining intercept and slope, respectively, MILᵢ is a covariate corresponding with an individual estimate of miltefosine exposure (i.e. \(C_{\text{EOT}}\), \(AUC_{0-\text{EOT}}\), \(AUC_{0-\infty}\) \(T>\text{EC}_{50}\) or \(T>10\times\text{EC}_{50}\)) centered around its respective population mean value (MIL_μ). The intercept \(\theta_1\) was defined as follows to estimate the baseline probability BASE of an outcome with value 1:

\[
\theta_1 = \ln\left(\frac{\text{BASE}}{1 - \text{BASE}}\right)
\]  
(Eq. 3)

Finally, the individual estimate of probability *p*ᵢ was calculated as follows:

\[
p_i = \frac{e^{\text{Logit}_i}}{1 + e^{\text{Logit}_i}}
\]  
(Eq. 4)

For which an outcome of 1 corresponds with a prediction equal to *p*ᵢ and an outcome of 0 with a prediction of 1 – *p*ᵢ. The likelihood ratio test (the OFV is equal to -2 log likelihood) was used to assess the improvement of fit and influence of miltefosine
exposure covariates (MIL) on the probability of treatment failure when compared to the model with the miltefosine exposure covariates excluded. The difference between a pair of log likelihood values (OFV) was tested for statistical significance: with the OFV approximating the \( \chi^2 \) statistic with 1 degree of freedom (1 per introduced parameter/covariate), a p-value of 0.05 corresponds with a \( \Delta \text{OFV} \) decrease of 3.84 (\( \alpha = 0.05 \)). For graphical presentation of observed probability versus model-estimated probability, observations were binned in groups of equal size to obtain an observed probability per bin.

**Results**

**Patients**

Eighty-one patients were enrolled in this pharmacokinetic study of which baseline characteristics, demographics and general outcome can be found in Table 1. The majority of patients was male (62%). The included patients were relatively young (mean age 22.9 years); 20% were children below 12 years of age. Five patients were excluded from the pharmacodynamic analysis: 2 patients were lost to follow-up due to untimely death unrelated to VL and 3 patients had a treatment switch due to severe adverse events during their miltefosine regimen. Among the remaining 76 patients the overall treatment failure rate was 21%.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients enrolled</td>
<td>81</td>
</tr>
<tr>
<td>No. of males/females</td>
<td>50/31</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>22.9 (12 – 32)</td>
</tr>
<tr>
<td>No. of patients aged &lt; 12 yrs</td>
<td>20</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>35.9 (24 – 46)</td>
</tr>
<tr>
<td>Height (cm)*</td>
<td>139 (127 – 155)</td>
</tr>
<tr>
<td>Body Mass Index (kg/m(^2))*</td>
<td>17.9 (16 – 20)</td>
</tr>
<tr>
<td>No. of days of miltefosine treatment</td>
<td>29.3 (28 – 31)</td>
</tr>
<tr>
<td>Daily miltefosine dosage (mg/kg/day) - overall</td>
<td>2.4 (1.67 – 4)(^b)</td>
</tr>
<tr>
<td>Children (&lt; 12 yrs)</td>
<td>2.7 (2 – 3)(^b)</td>
</tr>
<tr>
<td>Adults ((\geq 12) yrs)</td>
<td>2.4 (1.79 – 4)(^b)</td>
</tr>
<tr>
<td>No. of patients included in the pharmacodynamic analysis</td>
<td>76</td>
</tr>
<tr>
<td>No. of patients failed/cured</td>
<td>16/60</td>
</tr>
</tbody>
</table>

*Height, and thus body mass index, was unavailable for 3 patients

\(^b\) Range
Population pharmacokinetic analysis

The measured miltefosine EOT concentrations of the Nepalese VL patients are shown in Figure 1. Miltefosine EOT concentrations were significantly lower in children compared to the adult concentrations, depicted in Figure 2, although daily miltefosine mg/kg body weight dosages were comparable (Table 1). This indicates, as demonstrated previously, that children are less exposed to miltefosine when they receive a similar mg/kg body weight dose as adults [11]. The previously developed open 2-compartment population pharmacokinetic model for miltefosine fitted the collected sparse Nepalese miltefosine EOT concentrations adequately and pharmacokinetic parameters and the associated variabilities could be estimated with high precision for the sparse dataset when combined with the rich miltefosine pharmacokinetic datasets (from Europe and India) already available. The population pharmacokinetic parameter estimates are shown in Table 2. The separately estimated residual error for the Nepalese miltefosine data was a modest 24.5% (RSE 36.4%). The visual predictive check of the model is shown in Figure 1, with the observed

Figure 1. Visual predictive check. Observed concentrations (open circles) are plotted over the 95% prediction interval (gray area) from 200 simulated concentration-time curves for the 81 subjects in our dataset. The dark gray line indicates the mean predicted concentration. The broken lines represent the 1x (lower line) and 10x (upper line) mean in vitro EC$_{50}$ value of miltefosine for the clinical Leishmania isolates tested for drug susceptibility in the Nepalese cohort.
concentrations plotted over it. Shrinkage of the empirical Bayes estimates of between-subject variability was evaluated specifically for the Nepalese subset and amounted 14.5% and 34.5%, for $CL/F$ and $V_2/F$ respectively, which is modest given the extreme sparseness of the Nepalese miltefosine pharmacokinetic data.

Various measures of miltefosine exposure were estimated for all enrolled subjects in population pharmacokinetic model based on individual parameter

### Table 2. Population pharmacokinetic model estimates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimate</th>
<th>Precision (RSE)</th>
<th>Between-subject variability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorption rate (/day)</td>
<td>9.6</td>
<td>Fixed</td>
<td>19.4%</td>
</tr>
<tr>
<td>Central clearance (L/day)</td>
<td>3.69</td>
<td>3.4%</td>
<td>35.1%a</td>
</tr>
<tr>
<td>Intercompartmental clearance (L/day)</td>
<td>0.0316</td>
<td>16.6%</td>
<td>Not estimated</td>
</tr>
<tr>
<td>Central volume of distribution (L)</td>
<td>38.5</td>
<td>4.5%</td>
<td>31.6%a</td>
</tr>
<tr>
<td>Peripheral volume of distribution (L)</td>
<td>1.69</td>
<td>8.6%</td>
<td>Not estimated</td>
</tr>
<tr>
<td>Residual variability (%)b</td>
<td>24.5</td>
<td>36.4%</td>
<td></td>
</tr>
</tbody>
</table>

a Between-subject variabilities of clearance and volume of the central compartment were correlated by 87%.

b Residual variability specifically for the Nepalese subset of data; separate residual variabilities were estimated for the other subsets (unreported here).

### Table 3. Miltefosine exposure estimates derived from the population pharmacokinetic model.

<table>
<thead>
<tr>
<th>Measure of exposure</th>
<th>Unit</th>
<th>Mean</th>
<th>(95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>End of treatment concentration ($C_{\text{EOT}}$)</td>
<td>µg/mL</td>
<td>38.0</td>
<td>(34.4 – 41.7)</td>
</tr>
<tr>
<td>Area under the curve from zero to end of treatment ($AUC_{0-\text{EOT}}$)</td>
<td>µg/mL·day</td>
<td>794</td>
<td>(725 – 864)</td>
</tr>
<tr>
<td>Area under the curve from zero to infinity ($AUC_{0-\infty}$)</td>
<td>µg/mL·day</td>
<td>1252</td>
<td>(1121 – 1385)</td>
</tr>
<tr>
<td>Time concentration above $EC_{50}$ ($T&gt;EC_{50}$)</td>
<td>day</td>
<td>58.2</td>
<td>(56.3 – 60.1)</td>
</tr>
<tr>
<td>Time concentration above 10x $EC_{50}$ ($T&gt;10\times EC_{50}$)</td>
<td>day</td>
<td>30.2</td>
<td>(28.3 – 32.0)</td>
</tr>
</tbody>
</table>
estimates and individual predicted plasma concentrations (Table 3).

**Pharmacokinetic-pharmacodynamic analysis**

The observed probability of miltefosine treatment failure in the Nepalese VL patients was 21%. Of these miltefosine treatment failures 37.5% had an age <12 years, compared to 25% in the full dataset. Correlations between the estimated miltefosine exposure values and observed probability of treatment failure were graphically explored by binning of the exposure values in three groups of equal size and plotting mean exposure values within each bin versus the observed probability of treatment failure within that bin. A more or less linear correlation between the various miltefosine exposure values (\(C_{\text{EOT}}\), \(\text{AUC}_{0-\text{EOT}}\), \(\text{AUC}_{0-\infty}\), \(T>\text{EC}_{50}\) and \(T>10\times\text{EC}_{50}\)) and probability of treatment failure could be observed.

A base pharmacodynamic logistic regression model was developed which accurately estimated the observed population probability of treatment failure (base probability of 0.211 [RSE 22.2%]). Miltefosine exposure values were introduced as covariates in the logistic regression model (Equation 1). All included measures of miltefosine exposure led to a decrease of OFV and thus an improved fit of the model.

**Figure 3.** Probability of treatment failure versus miltefosine exposure (time miltefosine concentration above 10\(\times\) EC_{50} \([T>10\times\text{EC}_{50}]\)). The solid line represents the logistic model predicted probability of treatment failure, the gray area its 90% confidence interval, the transparent bars indicate the interval of the observed \(T>10\times\text{EC}_{50}\) data covered by the bins, with approximately 25 observations in each bin, whereas the filled circles on top of the bins indicate the mean observed data-based probabilities of treatment failure per bin at the mean \(T>10\times\text{EC}_{50}\) of the bin.
but only inclusion of the exposure covariate \( T > 10 \times EC_{50} \) resulted in a significantly better fit of the model to the therapy outcome data (\( \Delta OFV: -5.19 \), corresponding with a \( p \)-value of 0.02 [\( \chi^2, 1 \text{ df} \)]) compared to the base model. The mean \( T > 10 \times EC_{50} \) in our population was 30.2 days. The final estimates (RSE) of intercept \( BASE \) and slope \( \theta_2 \), correlated to the centered effect of \( T > 10 \times EC_{50} \), were 0.195 (24%) and -0.08 (48%), respectively. This corresponds with a lower odds ratio for experiencing treatment failure of 0.92 (95% CI: 0.86-0.99) for each day increase of \( T > 10 \times EC_{50} \). Conversely, with 1 day less exposure above the concentration threshold of 10x \( EC_{50} \) the odds ratio for treatment failure would be increased to 1.08 (95% CI: 1.01-1.17). The mean model predicted probability of failure as a function of the achieved \( T > 10 \times EC_{50} \) is depicted in Figure 3 together with a 90% confidence interval and the observed probability of the binned \( T > 10 \times EC_{50} \) values as derived from our dataset.

**Discussion**

In this study we established that the observed miltefosine treatment failure in Nepalese VL patients is significantly correlated to the obtained \( T > 10 \times EC_{50} \) values in VL patients. Miltefosine is an essential oral drug in the treatment of the neglected tropical disease VL, but the recently reported decaying efficacy rates under the current conventional miltefosine dose regimen may seriously threaten its future use and application in the field. To our knowledge the here reported study is the first study to date that investigates the drug exposure-effect relationship of any antileishmanial treatment.

Previously we described the body size-related differences in pharmacokinetics of miltefosine between adults and children [11]. Clearance of miltefosine is not linearly related to body size. Allometric scaling of clearance using an allometric exponent of 0.75 in combination with fat-free mass as body size descriptor adequately described the effect of body size on drug exposure. Consequently, we proposed a revised miltefosine dosage regimen based on the allometric relationship established to achieve equivalent exposure in children as compared to adults. However, this recently proposed dosing algorithm has not been clinically evaluated and has not been implemented yet in the miltefosine treatment guidelines. In the here described cohort, miltefosine was used according to the conventional miltefosine treatment guidelines, resulting in daily administration of 2.5 mg/kg body weight miltefosine, both to adults and children (Table 1). Similar to our previous findings, also in this cohort of Nepalese VL patients administration of this comparable mg/kg dose of miltefosine led to lower drug exposure in children compared to adults, with significantly lower EOT concentrations. Moreover, these observations were corroborated by the findings from the general cohort that age <12 years was the only
risk factor found to be correlated with treatment failure (Rijal et al., submitted). This underscores the need to evaluate safety and efficacy of the previously proposed allometric miltefosine regimen [11]. Nevertheless, EOT miltefosine concentrations were not found to be significantly different between treatment cures and failures, but this finding could be obscured by the fact that not all patients were sampled exactly at EOT. Therefore we decided to estimate more accurately individual miltefosine exposure by applying population pharmacokinetic modeling. The previously developed population pharmacokinetic model fitted the sparse miltefosine EOT concentrations well. Only modest $\eta$-shrinkage for $CL/F$ and $V_d/F$ was observed, probably indicating that the inclusion of allometric scaling based on fat-free mass as a covariate in the parameters corrected well for the present between-subject variability. Using this population pharmacokinetic model, various individual estimates of miltefosine exposure were derived for evaluation in the pharmacokinetic-pharmacodynamic analysis.

The proportion of miltefosine treatment failures in this Nepalese cohort of VL patients was significant. The urgent implications of this observation for the ongoing elimination program in Nepal are discussed in more detail elsewhere (Rijal et al., submitted). In the subset of patients who were enrolled in this pharmacokinetic study, the overall failure rate was 21% (the failure rate among children was 33%), which is much higher than the rates previously reported in the region which do normally not exceed 5-10% [9]. This observed higher failure rate might be caused by the study design, since patients were followed up for a total period of 12 months instead of the conventional 6 month follow up period. This is corroborated by the fact that most failures were actually detected between 6 and 12 months of follow up in our cohort, which might be failures otherwise missed. On the other hand, the emergence of miltefosine resistant *Leishmania* clones is anticipated in this region where anthroponotic transmission is the main route of transmission. Nevertheless, evidence for the emergence of resistant clones could not be demonstrated in this cohort (Rijal et al., submitted). Also it may be argued that these late treatment failures might be in fact reinfections. Nevertheless, the available results on the comparison of genetic profiles and fingerprinting studies of strains at pre-treatment and at relapse within failed patients did not indicate any genetic difference between these strains (Rijal et al., submitted). Additionally, no difference between the clinical isolates in *in vitro* drug susceptibility to miltefosine was found [22]. Moreover, reinfection within a period of 12 months is highly unlikely based on mathematical modeling of infection dynamics [23].

In this study we investigated in more depth the relationship between measures of miltefosine exposure and the probability of treatment failure. The only identified risk factor for miltefosine exposure was the estimate of time that plasma concentrations were above 10x the mean *in vitro* $EC_{50}$ value established in this cohort of Nepalese patients. Other measures of exposure like the observed EOT concentration or various
AUCs were not found to be significantly correlated to treatment failure. This may indicate that the mechanism of action of miltefosine is defined by a time-dependent killing effect rather than a concentration-dependent effect. In turn this may explain why the duration of treatment of miltefosine was found to be important during the limited dose-finding studies of miltefosine in VL. In this perspective, it is of importance to find the optimal threshold concentration that needs to be attained for a period of time for miltefosine to exert its antileishmanial effect. For instance, we only found a significant correlation of treatment failure with time above 10x EC\textsubscript{50} but not with time above 1x EC\textsubscript{50}. Apparently, the concentration to be attained for a certain time period is also important to prevent treatment failure and should be substantially higher than the average EC\textsubscript{50} value. The value of 10x EC50 (17.9 µg/mL) corresponds with the highest level of miltefosine-resistance of Leishmania in vitro (40 µM or 16.3 µg/mL). This relatively high systemic concentration to be attained may indicate that miltefosine concentrations at the site of infection (spleen, bone marrow, liver) are lower, although this does not follow from animal distribution studies, or that there remain sanctuary or reservoir sites where the Leishmania parasites reside in which miltefosine penetration is far from optimal. Both these possible issues deserve further consideration and evaluation, for instance by measuring target site-specific pharmacokinetics. Preferably we would have used individual EC\textsubscript{50} values for these estimations, but individual values were unfortunately not available for every individual in our pharmacokinetic subset of the cohort. The mean estimated $T>10\times EC_{50}$ was 30.2 days. This value was associated with a failure rate of 19.5%. A decrease of the $T>10\times EC_{50}$ with 1 day was associated with a 1.08 times increased odds of treatment failure. In our cohort there were two patients for whom a $T>10\times EC_{50}$ was estimated of 0 days which therefore both had a probability of treatment failure of 73% and indeed both patients had failed treatment, one with a relapse within 6 months follow up and the other within 12 months. The use of dichotomous outcome data (cure versus failure) may not be optimal to fully characterize an exposure-response relationship. In future clinical trials or cohorts, the time until relapse occurs should be monitored more closely to get an impression whether drug exposure can be correlated to time until relapse, as is the case for other parasitic diseases such as malaria. Nevertheless, slow and variable onset of disease, between-patient variability in disease severity, and the general lack of markers to accurately monitor the pharmacodynamic response to treatment are all factors which may complicate the interpretation of the time until relapse. In future clinical trials more emphasis should be put on evaluation of pharmacodynamic markers, such as quantitative measurements of parasite load by PCR, to enable a better characterization of the exposure-response relationship of antileishmanial drugs which in turn would allow for a better prediction of possible relapse cases and ultimately optimal treatment protocols.

In conclusion, this study is the first to explore the pharmacokinetic-pharmacodynamic relationship between miltefosine exposure and VL treatment failure. Although
the reasons behind treatment failure in VL are probably far from singular, drug exposure may be one of them. This kind of pharmacological studies is therefore particularly needed now that increasing failure rates of VL under the conventional miltefosine treatment regimen are being reported in the Indian subcontinent. Again, we established that children are less exposed to miltefosine than adults under the current conventional 2.5 mg/kg body weight dosing regimen. Combined with the finding that being a child (<12 years of age) has been shown to be a significant clinical risk factor for treatment failure, the introduction and clinical evaluation of the previously proposed allometric miltefosine dosing regimen is urgently indicated. The only measure of miltefosine exposure in this study significantly associated with treatment failure was the time that miltefosine concentrations were above 10x the mean EC$_{50}$ value. This is the first step towards the definition of pharmacokinetic-pharmacodynamic targets to be attained for miltefosine in the treatment of VL.
References


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