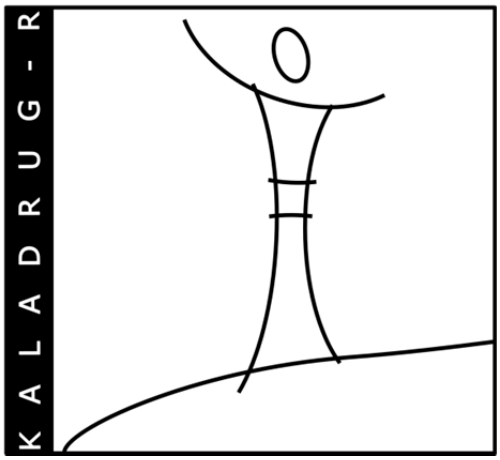
Outline

1. Kaladrug-R: concept and main findings

Visceral leishmaniasis (VL), one of the most-neglected infectious diseases, has an annual incidence of up to 400,000 cases (*Alvar J, Velez ID, Bern C, Herrero M, Desjeux P, et al. 2012 Leishmaniasis Worldwide and Global Estimates of Its Incidence. PLoS ONE 7(5): e35671*). Early treatment is a major pillar of the current programme for VL elimination in the Indian sub-continent. However, the arsenal of available drugs is very limited, and their indiscriminate use is jeopardized by drug resistance. Combination regimens for VL are under clinical development, but it will take several more years to change the drug policy. Meanwhile, the effectiveness of current drugs needs to be safeguarded in order to cure patients and ensure unremitting sustainment of VL control.



For this, the uninterrupted supply of quality drugs, the promotion of treatment compliance and the monitoring of treatment effectiveness and of drug resistance will be pivotal. The latter demands improved knowledge and know-how, hence clinical and laboratory research is urgently needed to support the drug policy of the VL elimination programme. Kaladrug-R addresses these needs: we aimed to develop, evaluate and disseminate new tools for the assessment of drug resistance in *L. donovani* as well as innovative methodologies for monitoring Kala-Azar treatment effectiveness under routine conditions.

1.1. Activities

This aim was further articulated around 8 specific objectives.

1. Development of an innovative approach for monitoring the effectiveness of Kala-Azar drug treatments in routine conditions. Simple clinical and epidemiological tools were developed and standardized based on the case definitions and retrospective cohort monitoring methodology[6,18,31].

2. Recruitment and follow-up of cohorts of patients treated with Miltefosine (MIL) in India and Nepal, provide complete clinical documentation and obtain pre-treatment samples as well as samples from treatment failure for validation of the above mentioned assays. In total, 567 and 253 VL patients were recruited for the project in India and Nepal, respectively. In India, at the end of treatment the initial cure rate was 98.06% (intention to treat) and at six month the final cure rate was 90.48%[21]. In Nepal,

in a cohort with 12 months of follow-up, the initial cure rate was 91.6%, at 6 months 77.6% and at 12 months post-treatment 69.2% (intention to treat)[30]. Altogether, these data indicate a substantial decline in the efficacy of oral miltefosine for the treatment of Indian VL.

We are currently exploring the possible causes for this phenomenon[30]:
- no significant clinical risk factors or predictors of relapse apart from age < 12 years were found.
- parasite fingerprints of pre-treatment and relapse bone marrow isolates were similar within 8 tested patients, suggesting that clinical relapses were not due to re-infection with a new strain, but to true recrudescences.
- MIL blood levels at the end of treatment were similar for cured and relapsed patients.
- the MIL-susceptibility of 131 VL isolates was analysed *in vitro* with a promastigote assay. The mean promastigote MIL-susceptibility (IC50) of isolates from definite cures was similar to that of relapses. A small pilot study showed that PKDL isolates were more tolerant towards MIL in comparison with VL isolates, and even more in case of PKDL treatment failure[24]. The results highlight the need for keeping a close parasitological monitoring of the MIL-susceptibility in the field.

Other factors should be explored, such as (i) host-related factors (immune status), or (ii) other parasitological factors (not assessed in the current MIL susceptibility assay), like the virulence of the parasites. We are also checking if any MIL-resistance phenotype might not be lost during isolation of parasites from patient isolates.

3. Development of new tools for the assessment of drug resistance in *L. donovani* parasites. A biological assay to test the *in vitro* susceptibility to MIL (with promastigote stages)[29] is currently running as a routine assay in 4 laboratories of the Indian sub-continent and could be further disseminated. We are currently checking the opportunity to keep the parasite under minimal MIL pressure at isolation time, to retain the susceptibility phenotype that was present in the patient. For other drugs (SSG, PMM, AmB), *in vitro* susceptibility assays can only run with intracellular amastigotes. Anyway, *in vitro* assays are complex and time-consuming; they should be replaced by molecular tools as soon as possible.

Molecular tools for the detection of drug resistance require the identification of molecular markers. This research is more advanced in the frame of SSG-resistance and several markers were identified: differences in gene expression, in fluidity of the parasite's membrane as well as in the presence of specific sugars on the cell membrane[13]. Using next generation sequencing, we deciphered the complete genome of 200 clinical isolates. SNP analysis revealed a relatively homogeneous group within our sample (in average 173 SNPs/strain in 94 % of

the strains) and different signatures of *in vitro* resistance, suggesting multiple and independent events of resistance emergence[14]. In contrast with the sequence homogeneity, we discovered an unprecedented level of aneuploidy and local gene copy number variations (CNVs, among others through circular episomes) among the clinical lines[14,15,26]: CNVs are known to play an important role in drug resistance in *Leishmania* and we indeed found correlations between some of these and *in vitro* drug resistance. Interestingly, SNPs or CNVs were found to correlate better with the *in vivo* phenotype than with the *in vitro* susceptibility assays. Altogether our results indicate a huge adaptive capacity of *L. donovani* (it probably uses different mechanisms to get resistant to SSG[19]); practically it means that molecular monitoring of resistance will require multi-locus approaches. As such, we showed that just 3 genetic markers proved sufficient to detect parasites that contribute to SSG-treatment failure and that these molecular markers significantly outperformed the current *in vitro* SSG-susceptibility test in terms of power, predictive value and practicability[20].
With respect to MIL-resistance, our work essentially focused on experimentally induced resistant strains as we did not yet find clinical drug-resistant strains: (i) we found point mutations and expression differences in two genes already highlighted in the literature in other species[24], suggesting a universal mechanism for acquiring resistance in experimental conditions, but (ii) also found additional and new molecular adaptations. Genome sequencing of natural parasites from the MIL-treated patients did not evidence any clear pattern so far: isolates from relapsing patients were scattered throughout the different genetic groups here encountered. None of them showed so far some of the signatures of resistance encountered in experimentally induced resistant strains.

As an alternative way to identify molecular markers of resistance, metabolomics was applied[2,9,35,36]. This new method allows unprecedented studies of the parasite biochemistry[4]: analysing metabolites (the ultimate expression of the genotype), they provide a perception which is closest to the phenotype. This revealed that drug resistance was associated with dramatic changes across entire biochemical pathways[10] and revealed molecular adaptations which were not detected so far at genomic level, hereby demonstrating the complementarity of different 'omic approaches[25]. Similar changes were found in SSG-R and MIL-R strains, such as in the composition of lipids and the membrane fluidity (very important for drug trafficking). In theory, some of the adaptations to a previous drug might thus favour adaptations to new drugs. We recommend any new drug to be tested against a panel of strains circulating in the region where it will be implemented; similarly, molecular adaptations should be monitored in clinical strains after introduction of new drugs.
In this context and albeit not initially foreseen in our workplan, we tested the efficacy of a cationic amphiphilic drug, imipramine, commonly used for the treatment of depression in humans. The drug was found to kill intracellular amastigotes very efficiently *in vitro* and *in vivo*, and this independently of the SSG-susceptibility background of the parasites[37]. The 4-week drug treatment in

normal hamsters did not change hepatic enzyme activities and serum creatinine level when compared to untreated group. The dose of imipramine expressed in terms of human equivalent was remarkably less compared to the dose in use for the treatment for depression in human. Further studies are required to confirm that the old drug imipramine might qualify for the treatment of VL.

4. Exploration, in experimental conditions, of the pathways leading to parasite resistance to Paromomycin. In the present project, there was no cohort of patients treated with PMM. However, in order to anticipate future implementation of the drug, we aimed to understand PMM-resistance in experimental conditions. A series of clinical isolates with different backgrounds of SSG-susceptibility were used to induce PMM-resistance. Selection of resistance at the intracellular amastigote level was very rapidly achieved (two selection cycles only)[23]. These data provide concerns on the propensity of rapid resistance development if PMM would be used in monotherapy and endorse the stringent need for close epidemiological monitoring. All PMM-resistant strains are being submitted to whole genome sequencing and metabolomic analysis.

5. Building models to understand the dynamics of the past spread of parasite SSG resistance as a model for resistance to MIL or future drugs. Before building a mathematical model on the emergence and spread of drug resistance, we first had to elaborate a basic transmission model for anthroponotic VL (not existing at the onset of our project)[16]. Our simulation results suggest that transmission of *L. donovani* is predominantly driven by asymptomatically infected hosts who are not eligible for treatment, hereby strengthening the importance of vector control in the frame of the Kala-Azar Elimination Programme. The extended VL model was used to explain the observed increase in the SSG treatment failure rate from about 5% in 1980 to about 64% in 1997. The model showed that such a quick rise in treatment failure cannot be reproduced even if first-line treatment fails in 100% of cases infected with the resistant strain[34]. Thus, additional assumptions are required e.g. that resistant parasites are transmitted more effectively than non-resistant parasites (see section herebelow on fitness).

6. Study of the impact of drug resistance on the parasite fitness. Different experimental lines indicated that SSG-R parasites had better survival skills and were more virulent than their sensitive counterparts[7,11-13]. This was further supported by the high prevalence (83%) of SSG-R isolates in recent patients[13] (thus in a context in which SSG is not used anymore). Accordingly, SSG-R *L. donovani* would constitute a unique example and model of drug-resistant pathogens with traits of increased fitness[27]. Our results corroborate the prediction made by the mathematical modelling and raise questions about the 'heritage' of the SSG era on the outcome of new drug therapies[27]; we recommend a particular attention to drugs interfering with the human immune system, such as SSG, as they might not be very effective against the current

background of SSG-R parasites. Similar studies are in progress in the frame of MIL-resistance.

7. Genetic structure of parasite populations. *L. donovani* is generally considered as a very homogeneous population within the Indian sub-continent (ISC); this depends of course on the sample considered and the discrimination power of the methods used for genotyping. Microsatellite typing showed to be poorly resolutive to study the micro-evolution within the ISC, but it could clearly resolve the phylogenetic relationships of the strains between continents, indicating that certain older Indian strains were closely related to African strains, highlighting mobility of the parasites between these continents[17]. Whole genome sequencing was much more discriminatory and quite powerful for understanding evolution of *L. donovani* within the ISC. In the context of the anti-malaria spraying campaigns in the 1960s, our results were consistent with a major bottleneck followed by clonal expansions[17]. Interestingly, we discovered a parasite population in Nepal, which was genetically very different from the main population of the ISC; these so called 'Yeti' strains seem to originate from Himalayan valleys possibly not covered by the DDT campaign and hence could represent pre-bottleneck strains. The clinical and epidemiological importance of these strains should be further studied. We developed a simple PCR assay allowing to track them easily.

8. Getting research results into policy at regional level and disseminating the generated knowledge and the validated tools in other regions in the world endemic for leishmaniasis.
We involved Indian and Nepalese representatives of the Kala Azar Elimination Programme in our plenary coordination meetings and informed relevant stakeholders at WHO/TDR, WHO/SEARO, Ministries of health in India and Nepal about the KALADRUG project. We also established a close interaction with the 2 other FP7 consortia active in the frame of chemotherapy and plan joining our efforts in future research activities[5]. Our website is operational (www.leishrisk.net/kaladrug) and regularly updated; so far, we published (or submitted) 37 papers and gave 46 presentations in congresses.

Our research demonstrates the complexity of the problem of clinical drug resistance in VL and highlights the need of multidisciplinary approaches to tackle it. We provided new tools and new knowledge that could contribute to practical recommendations. We also raise new questions that motivate a continuous support to research both at basic and applied levels.

More info is available on the Kaladrug-R website: www.leishrisk.net/kaladrug

1.2. Kaladrug-R papers

1. Jean-Claude Dujardin. Structure, dynamics and function of *Leishmania* genome: resolving the puzzle of infection, genetics and evolution? (2009) IGE, 9(2):290-7

2. Richard A. Scheltema, Saskia Decuypere, Jean-Claude Dujardin, David G. Watson, Ritsert C. Jansen, and Rainer Breitling. (2009) A simple data reduction method for high resolution LC/MS data in metabolomics. Bioanalysis, 1: 1551-7

3. Johan van Griensven, Manica Balasegaram, Filip Meheus, Jorge Alvar, Lut Lynen, Marleen Boelaert. (2010) Combination therapy for visceral leishmaniasis. Lancet Infect Dis. 10(3):184-94.

4. Richard A. Scheltema, Saskia Decuypere, Ruben t'Kindt, Jean-Claude Dujardin, Graham H. Coombs and Rainer Breitling. (2010) The potential of metabolomics for *Leishmania* research in the post-genomics era. Parasitology, 137: 1291-302.

5. Jean-Claude Dujardin, Dolores González Pacanowska, Simon L. Croft, Ole F. Olesen, and Gerald F. Späth. (2010) Collaborative actions in anti-Trypanosomatid chemotherapy with partners from disease-endemic areas. Trends in Parasitology, 26: 395-403

6. Epco Hasker, Shri Prakash Singh, Paristosh Malaviya, Rudra Pratap Singh, R Shankar, Marleen Boelaert, Shyam Sundar. (2010) Management of visceral leishmaniasis in rural primary health services in Bihar, India. TMIH, Jul;15 Suppl 2:29-35.

7. Manu Vanaerschot, Ilse Maes, Meriem Ouakad, Vanessa Adaui, Louis Maes, Simonne De Doncker, Suman Rijal, François Chappuis, Jean-Claude Dujardin, Saskia Decuypere. (2010) Linking *in vitro* and *in vivo* survival of clinical *Leishmania donovani* strains. PLOS one, 5(8): e12211

8. Pankaj Srivastava, Vijay Kumar Prajapati, Manu Vanaerschot, Gert Van der Auwera, Jean-Claude Dujardin, Shyam Sundar. (2010) Detection of *Leptomonas*-like parasites in clinical isolates of Kala azar from India. IGE, 10(7):1145-50

9. Ruben t'Kindt, Andris Jankevics, Richard A. Scheltema, Liang Zheng, David Watson, Jean-Claude Dujardin, Rainer Breitling, Graham Coombs and Saskia Decuypere. (2010) Towards an unbiased metabolic profiling of protozoan parasites: optimisation of a HILIC-Orbitrap protocol for *Leishmania*. Analytical and Bioanalytical Chemistry, 398, 2059-2069. (Erratum published in Analytical and Bioanalytical Chemistry: Volume 400, Issue 2 (2011), Page 635.)

10. Ruben t'Kindt, Richard A. Scheltema, Andris Jankevics, Kirstyn Brunker, Suman Rijal, Jean-Claude Dujardin, Rainer Breitling, David G. Watson, Graham H. Coombs and Saskia Decuypere. (2010) Metabolomics to unveil and

understand phenotypic diversity among pathogen populations. PLOS-NTD. 4(11):e904.

11. Manu Vanaerschot, Simonne De Doncker, Suman Rijal, Louis Maes, Jean-Claude Dujardin, Saskia Decuypere. (2011) Antimonial resistance in *Leishmania donovani* is associated with increased *in vivo* parasite burden. PLoS ONE, 6(8):e23120

12. Meriem Ouakad, Manu Vanaerschot, Suman Rijal, Shyam Sundar, Niko Speybroeck, Luc Kestens, Luc Boel, Simonne De Doncker, Ilse Maes, Saskia Decuypere and Jean-Claude Dujardin. (2011) Increased metacyclogenesis rate among antimony-resistant *Leishmania donovani* clinical lines. Parasitology, 138(11):1392-9.

13. Rupkatha Mukhopadhyay, Sandip Mukherjee, Budhaditya Mukherjee, Kshudiram Naskar, Dinesh Mandal, Saskia Decuypere, Bart Ostyn, Vijay Kumar Prajapati, Shyam Sundar, Jean-Claude Dujardin, Syamal Roy. (2011) Characterisation of antimony resistant *Leishmania donovani* isolates: biochemical and biophysical studies and interaction with host cells. IJP 41(13-14):1311-21.

14. Tim Downing*, Hideo Imamura*, Saskia Decuypere, Taane G. Clark, Graham H. Coombs, James A. Cotton, James D. Hilley, Simonne de Doncker, Ilse Maes, Jeremy Mottram, Mike A. Quail, Suman Rijal, Mandy Sanders, Gabriele Schönian, Olivia Stark, Shyam Sundar, Manu Vanaerschot, Christiane Hertz-Fowler, Jean-Claude Dujardin and Matthew Berriman. (2011) Whole genome sequencing of multiple *Leishmania donovani* clinical isolates provides insights into the evolution and mechanisms of drug resistance. Genome Research, 21(12):2143-56. * Equal contribution.

15. Matthew B. Rogers, James D. Hilley, Nicholas J Dickens, Jon Wilkes, Paul Bates, Daniel P. Depledge, David Harris, Yerim Her, Pawel Herzyk, Hideo Imamura, Thomas Dan Otto, Walide Saad, Kathy Seeger, Jean-Claude Dujardin, Matthew Berriman, Deborah F. Smith, Christiane Hertz-Fowler, Jeremy C. Mottram. (2011) Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*. Genome Research, 21(12):2129-42.

16. Anette Stauch, Ram Rup Sarkar, Albert Picado, Bart Ostyn, Shyam Sundar, Suman Rijal, Marleen Boelaert, Jean-Claude Dujardin, Hans Peter Duerr. (2011) Visceral leishmaniasis in the Indian subcontinent: modelling epidemiology and control. PLOS-NTD, 5(11):e1405

17. Tim Downing, Olivia Stark, Saskia Decuypere, Simonne de Doncker, Jean-Claude Dujardin, Hideo Imamura, Ilse Maes, Suman Rijal, Mandy Sanders, Shyam Sundar, Manu Vanaerschot, Matthew Berriman, Gabriele Schönian. (2012) Genome-wide SNP and microsatellite variation unravel population-level *Leishmania donovani* species complex epidemiology. IGE, 12(1):149-59.

18. Paritosh Malaviya, Rudra Pratap Singh, Shri Prakash Singh, Epco Hasker, Bart Ostyn, R Shankar, Marleen Boelaert, Shyam Sundar. Monitoring drug effectiveness in kala-azar in Bihar, India: cost and feasibility of periodic random surveys vs. a health service-based reporting system. Trop Med Int Health. (2011) Sep;16(9):1159-66.

19. Saskia Decuypere, Manu Vanaerschot, Kirstyn Brunker, Hideo Imamura, Sylke Müller, Suman Rijal, Jean-Claude Dujardin, Graham H Coombs. (2012) Molecular adaptations of drug resistance in natural *Leishmania* populations vary with genetic background. PLOS-NTD, 6(2):e1514

20. Manu Vanaerschot, Saskia Decuypere, Tim Downing, Hideo Imamura, Olivia Stark, Simonne De Doncker, Syamal Roy, Louis Maes, Gabriele Schönian, Matthew Berriman, François Chappuis, Jean-Claude Dujardin, Shyam Sundar, Suman Rijal. (2012) Genetic markers for SSG-resistance in *Leishmania donovani* and SSG-treatment failure in visceral leishmaniasis patients in the Indian subcontinent. JID, 206(5):752-5.

21. Shyam Sundar, Anup Singh, Madhukar Rai, Vijay K Prajapati, Avinash K Singh, Bart Ostyn, Marleen Boelaert, Jean-Claude Dujardin, Jaya Chakravarty. (2012) Efficacy of Miltefosine in the treatment of Indian Visceral Leishmaniasis after a decade of use. CID, 55(4):543-50

22. Jean-Claude Dujardin & Saskia Decuypere. Epidemiology of leishmaniasis in the time of drug resistance. (2012) In: Drug Resistance in *Leishmania* Parasites. Consequences, Molecular Mechanism and Possible Treatments. Edited by Alicia Ponte-Sucre, Maritza Padron-Nieves and Emilia Diaz. Spinger-Verlag, in press.

23. Sarah Hendrickx, Raquel A. Inocêncio da Luz, Vasundhra Bhandari, Julien Lonchamp, Poonam Salotra, Katherine Carter, Shyam Sundar, Suman Rijal, Jean-Claude Dujardin, Paul Cos, Louis Maes. (2012) Experimental induction of paromomycin resistance in antimony-resistant strains of *L. donovani*: outcome dependent on selection protocol. PLOS-NTD, 6(5):e1664.

24. Vasundhra Bhandari, Arpita Kulshrestha, Deepak Kumar Deep, Olivia Stark, Vijay Kumar Prajapati, V. Ramesh, Shyam Sundar, Gabriele Schonian, Jean-Claude Dujardin and Poonam Salotra. (2012) Drug Susceptibility in *Leishmania* Isolates following Miltefosine treatment in cases of Visceral Leishmaniasis and Post Kala-Azar Dermal Leishmaniasis. PLOS-NTD , 6(5):e1657

25. Rainer Breitling, Barbara Bakker, Michael Barrett, Saskia Decuypere, Jean-Claude Dujardin. Metabolomic systems biology of protozoan parasites. (2012) In: Genetics meets Metabolomics: from Experiment to Systems Biology. Ed. Karsten Suhre. Springer, pp.73-84

26. An Mannaert, Tim Downing, Hideo Imamura & Jean-Claude Dujardin. (2012) Novel adaptive mechanisms in pathogens: universal aneuploidy in Leishmania. TiP, 28(9):370-6

27. Manu Vanaerschot, Saskia Decuypere, Maya Berg, Syamal Roy, Jean-Claude Dujardin. 2012. Drug-resistant microorganisms with a higher fitness – can medicines boost pathogens? (2012) Critical reviews in Microbiology, Epub ahead of print Sep 6.

28. Maartje Deschacht, Tim Van Assche, Sarah Hendrickx, Hidde Bult, Louis Maes, Paul Cos. (2012) Role of oxidative stress and apoptosis in the cellular response of murine macrophages upon *Leishmania* infection. Parasitology. 2012 Jul 10:1-9. [Epub ahead of print].

29. Arpita Kulshrestha, Vasundhra Bhandari, Rupkatha Mukhopadhyay, V. Ramesh, Shyam Sundar, Louis Maes, Jean Claude Dujardin, Syamal Roy, and Poonam Salotra. Concordance between inter-stage miltefosine susceptibility of Leishmania donovani isolates: utility of promastigote assay as a simplified biological tool. Submitted for publication.

30. Suman Rijal, Bart Ostyn, Surendra Uranw, Keshav Rai, Narayan Raj Bhattarai, Thomas P.C. Dorlo, Jos H. Beijnen, Manu Vanaerschot , Saskia Decuypere, Subodh S Dhakal, Murari Lal Das, Prahlad Karki, Rupa Singh, Marleen Boelaert, Jean-Claude Dujardin. Increasing failure of miltefosine in the treatment of kala-azar in Nepal and the potential role of parasite drug resistance, re-infection or non-compliance. Submitted for publication.

31. Bart Ostyn, Epco Hasker, Surendra Uranw, Shri Prakash Singh, Paritosh Malaviya, Suman Rijal, Shyam Sundar, Jean-Claude Dujardin, Marleen Boelaert. (2012) Retrospective Quarterly Cohort Monitoring for Visceral Leishmaniasis. Trop Med Int Health (in press)

32. Thomas P.C. Dorlo, Suman Rijal, Bart Ostyn, Peter J. de Vries, Jean-Claude Dujardin, Marleen Boelaert, Jos H. Beijnen, Alwin D.R. Huitema. Population pharmacokinetic-pharmacodynamic explorations of high miltefosine failure rates in visceral leishmaniasis in Nepal. Submitted for publication.

33. Surendra Uranw* & Bart Ostyn*, Suman Rijal, Epco Hasker, Thomas Dorlo, Bruno Dujardin, Jean-Claude Dujardin, Marleen Boelaert. Adherence to miltefosine treatment for visceral leishmaniasis under routine conditions in Nepal. Submitted for publication. * authors with equal contribution

34. Anette Stauch, Hans-Peter Duerr, Jean-Claude Dujardin, Shyam Sundar, Martin Eichner. Treatment of visceral leishmaniasis: model predictions into the spread of antimony-resistant *L. donovani* in Bihar, India. Submitted for publication.

35. Maya Berg, Manu Vanaerschot, Andris Jankevics, Bart Cuypers, Rainer Breitling, Jean-Claude Dujardin. LC-MS metabolomics from study design to data analysis – using a versatile organism as a model. (2012) Computational and Structural Biotechnology Journal. Accepted for publication in November issue.

36. Bedilu A. Ejigu, Dirk Valkenborg, Geert Baggerman, Manu Vanaerschot, Erwin Witters, Jean-Claude Dujardin, Tomasz Burzykowski, Maya Berg. Evaluation of normalization methods to pave the way towards large-scale LC-MS-based metabolomic profiling experiments. (2012) Submitted for publication.

37. Mukherjee S, Mukherjee B, Mukhopadhyay R, Naskar K, Sundar S, Dujardin JC, Das AK, Roy S. (2012) Imipramine is an Orally Active Drug Against both Antimony Sensitive and Resistant *Leishmania donovani* Clinical Isolates in Experimental Infection. PLoS NTD (Under revision)

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2. Abstracts

Retrospective Quarterly Cohort Monitoring for Visceral Leishmaniasis

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Introduction

Visceral Leishmaniasis (VL) is a neglected disease that affects yearly 500,000 people throughout the world, mainly in South-East Asia (Bangladesh, India and Nepal). It generally touches the poorest of the poor, and is fatal if left untreated. In the last decade, major advances were made with the development of a rapid diagnostic test (rK39) and new treatment options such as Miltefosine oral treatment, thus shifting diagnosis and treatment from specialized centers to the primary health care level, and reducing delay. However, little has been done to evaluate the effectiveness of this program, and prescribers show generally little concern on adherence and outcomes.

Method and tools

Within the Kaladrug-R research project, acronym for "New tools for monitoring drug resistance and treatment response in visceral leishmaniasis in the Indian subcontinent", we proposed a methodology for monitoring treatment outcomes, based on the internationally applied retrospective quarterly cohort monitoring methodology used in tuberculosis, developed the necessary tools to enable such monitoring, and piloted them in 5 health structures in VL endemic districts in India and Nepal.

Tools comprise a **register** and **reporting forms**. In the register (fig.1), all basic data for identification and for epidemiological purposes (name, ID, age, sex, address, contact (mobile phone)), are to be recorded, as well as data on diagnosis and treatment history, and current treatment choice (drug, dosage). (no major differences from the information that used to be collected before).

In the following columns, data are to be collected at two of three different moments in the follow up i.e. at the end of the treatment, at six (and eventually twelve) months. Purpose is to record treatment outcome, applying standardized case definitions (WHO-TDR) that feature on the lower part of the page.

Reporting forms are to be completed every three months, and compile the outcomes of every single VL case in the cohort of enrolled patients over a three months period:

- When a cohort closes, it takes one month before all enrolled patients all have finalized treatment, and End of Treatment outcome of that cohort can be calculated

- It takes 7 months before all enrolled patients all have reached 6 months post-treatment status

The retrospective quarterly cohort analysis (RQCA) method enables to calculate cure rates, defaulter rates and failure rates, provides immediate feedback to the health care center and the district health office e.g. in case of high defaulters or poor compliance, and enables to quantify effectiveness of VL treatment policy under field conditions on a regional level.
The method and tools were piloted in three PHCs in the district of Muzaffarpur, Bihar State India, and in two zonal hospitals in south-eastern Nepal.

Results

The methods and tools were piloted in three PHCs in the district of Muzaffarpur, Bihar State India, and in two district hospitals in south-eastern Nepal. The pilot period ran from January 2010 up to December 2011 and enabled us to improve the tools, identify the obstacles, and give feedback to the users. The major difficulty encountered was to collect late treatment outcomes as patients once cured are not motivated to present themselves at the clinics for a post-treatment check-up. This problem can be solved by dedicating the task of collecting treatment outcomes to the district health office and its network of village health volunteers such as ASHAs and ANMs[1], and the use of mobile phones to transfer the missing outcome.
The RQCA revealed the high proportion of defaulters (patients not returning for their second supply of miltefosine tablets), strongly affecting the cure rate. Non-response and treatment switch for SAE were relatively low (table). Due to the high number of "lost to follow-up" at the start of the pilot program (data collection through Health Workers not yet operational), final cure rates at 6 months were far below standards. Still, as we applied the same methodology in the two reference centers (KAMRC in Muzaffarpur, Bihar, and the Tropical ward at BPKIHS in Dharan, Nepal), we observed treatment failure rates, -in casu

relapses- in MIL-treated patients that were far higher than the ones reported during the phase IV trials hardly a decade earlier (in preparation).

Conclusion

Based on a standard applied methodology in TB programs, we developed a treatment outcome monitoring tool for VL that can be routinely used at primary health care level. It requires 1) a minimal training on treatment outcome definitions for PHC staff, and 2) a dedicated (VL) team at the district health office to organize the collection of late treatment outcomes through the ASHAs and/or ANMs. Involvement and training of these latter health volunteers will further benefit the VL elimination program as they could help in identifying suspected VL cases, and refer or eventually even use a rapid detection tests on the spot when adequately trained.
The tool can be used for any VL treatment program in the world, regardless of the treatment protocol(s) used. It should help in comparing the effectiveness in real life conditions of different treatment options, as well as monitoring the effectiveness of current treatment strategies over time in the light of possible emergence of resistance.

Examples of the Kala-Azar daily record register, the quarterly report form and SOPs on how to use them can be found under Kaladrug-R SOPs at www.leishrisk.net/kaladrug.

References

Hasker E, Singh SP, Malaviya P, Singh RP, Shankar R *et al.* Management of visceral leishmaniasis in rural primary health care services in Bihar, India. Trop Med Int Health. 2010 Jul;15 Suppl 2:55-62.

[1] ASHA: Accredited Social Health Activist ; ANM: auxiliary nurse midwife

Table 1: Early and late treatment outcomes (retrospective quarterly cohort monitoring) in 3 PHCs in India

Phc_Name (All)
Prev_treatment No previous treatment (naive VL cases only)
Current_Drug Miltefosine (MIL)

Early Treatment Outcome (at end of treatment)

Cohorts:	INITIAL CURE	DEFAULTER	DEATH	NON-RESPONSE	SAE_RELATED TREATMENT SWITCH	REFERRAL	*Grand Total*
2009-Q4	7	1					8
2010-Q1	33	5					38
Q2	51	5	1		1		58
Q3	34	11					45
Q4	33	5				1	39
2011-Q1	23	3	1			1	28
Q2	2					1	3
Total of cohorts	183	30	2	0	1	3	219
%	**83,6%**	**13,7%**	**0,9%**	**0,0%**	**0,5%**	**1,4%**	

Phc_Name (All)
Prev_treatment No previous treatment (naive VL cases only)
Current_Drug Miltefosine (MIL)

Late Treatment Outcome (6 months after end of treatment)

Cohorts:	FINAL CURE	DEATH	RELAPSE	LOST TO FOLLOW-UP	DEFAULTER	NON RESPONSE	SAE_RELATED TREATMENT SWITCH	REFERRAL OUT	*Grand Total*
2009-Q4	4			3	1				8
2010-Q1	30		3		5				38
Q2	48	1	3		5		1		58
Q3	29		1	4	11				45
Q4	30		3		5			1	39
2011-Q1	23	1			3			1	28
Q2	2							1	3
Total of cohorts	166	2	10	7	30	0	1	3	219
%	**75,8%**	**0,9%**	**4,6%**	**3,2%**	**13,7%**	**0,0%**	**0,5%**	**1,4%**	

Efficacy of Miltefosine in the Treatment of Visceral Leishmaniasis in India After a Decade of Use

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Background

Miltefosine is the only oral drug available for treatment of Indian visceral leishmaniasis (VL), which was shown to have an efficacy of 94% in a phase III trial in the Indian subcontinent. Its unrestricted use has raised concern about its continued effectiveness. This study evaluates the efficacy and safety of miltefosine for the treatment of VL after a decade of use in India.

Methods

An open-label, noncomparative study was performed in which 567 patients received oral miltefosine (50 mg for patients weighing <25 kg, 100 mg in divided doses for those weighing ≥25 kg, and 2.5 mg per kg for those aged <12 years, daily for 28 days) in a directly observed manner. Patients were followed up for 6 months to see the response to therapy.

Results

At the end of treatment the initial cure rate was 97.5% (intention to treat), and 6 months after the end of treatment the final cure rate was 90.3%. The overall death rate was 0.9% (5 of 567), and 2 deaths were related to drug toxicity. Gastrointestinal intolerance was frequent (64.5%). The drug was interrupted in 9 patients (1.5%) because of drug-associated adverse events.

Conclusions

As compared to the phase III trial that led to registration of the drug a decade ago, there is a substantial increase in the failure rate of oral miltefosine for treatment of VL in India.

The full article can be found in the journal *Clinical Infectious Diseases* (2012) 55(4):543-550.

Increasing Failure of Miltefosine in the Treatment of Kala-Azar in Nepal and the Potential Role of Parasite Drug-Resistance, Re-Infection or Non-Compliance

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Introduction

Miltefosine, the only oral drug for visceral leishmaniasis, is currently the 1st line therapy in the VL elimination programme of the Indian subcontinent. Given the paucity of anti-VL drugs and the looming threat of resistance, there is an obvious need for close monitoring of clinical efficacy of MIL and to develop adequate surveillance tools.

Methods

In a cohort study of 120 VL patients treated with MIL in Nepal, we monitored the clinical outcomes up to twelve months after completion of therapy and explored the potential role of drug compliance, parasite drug resistance and re-infection.

Results

The initial cure rate was 97.5% and relapse rate at six and twelve months was 10.9% and 20.2% respectively. No significant clinical risk factors or predictors of relapse apart from age < 12 years were found. Parasite fingerprints of pre-treatment and relapse bone marrow isolates were similar within 8 patients, suggesting that clinical relapses were not due to re-infection with a new strain. The mean promastigote MIL-susceptibility (IC_{50}) of isolates from definite cures was similar to that of relapses. Although more tolerant strains were observed, parasite resistance, as currently measured, is thus not likely involved in MIL treatment failure. Moreover, MIL blood levels at the end of treatment were similar for cured and relapsed patients.

Conclusion

Relapse in one fifth of the MIL treated patients observed in our study is an alarming signal for the VL elimination campaign, urging for further review and cohort monitoring of late treatment outcomes up to 12 months.

Pharmacokinetics of Miltefosine

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Within the KALADRUG-R project, increased treatment failure rates were demonstrated for miltefosine in the treatment of visceral leishmaniasis (VL) on the Indian subcontinent. To assess adherence to treatment, further explore the pharmacological factors associated with these treatment failures, and in general explore the pharmacokinetics (drug exposure) and pharmacodynamics (drug effects) of miltefosine in VL patients, miltefosine drug exposure was evaluated in a cohort of Nepalese VL patients treated with miltefosine monotherapy according to the current standard treatment protocols.

Sparse blood samples were collected around the end of treatment (EOT) and the miltefosine steady-state concentrations in these samples were analyzed using liquid chromatography coupled to tandem mass spectrometry. Subjective personal accounts of treatment adherence could not be validated with the objective miltefosine exposure measurements, probably due to the sparseness of sampling. More importantly, a significant lower drug exposure was observed in children compared to adults, although receiving approx. the same mg/kg/day dosage. This confirms previous observations and might be an important impetus to adjust and optimize the current dosing guidelines for miltefosine. When comparing the EOT concentrations between cured and relapsed patients no significant difference could be observed, although there was a trend towards lower observed concentrations in relapsed patients. However, when applying more sophisticated mathematical modelling techniques, a significant factor of drug exposure could be identified: the individual time that miltefosine concentrations were above 10x the EC_{50} (indicating parasite drug susceptibility) which was significantly associated with treatment success and failure. Both these observations (lower exposure in children and the importance of achieving sufficient miltefosine exposure to prevent relapse of disease) urge the evaluation of a recently proposed optimal allometric miltefosine dosing regimen. All together this substudy constitutes a first step towards the definition of pharmacokinetic-pharmacodynamic targets to be attained for miltefosine in the treatment of VL.

Miltefosine in the Field, Susceptibility Testing, Tolerance and Genome Data

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Leishmania donovani is responsible for causing a potentially fatal disease Visceral Leishmaniasis (VL). Post Kala-azar dermal leishmaniasis (PKDL), a sequel of VL, constitutes an important parasite reservoir. Widespread resistance towards Sodium antimony gluconate (SAG) resulted in introduction of Miltefosine (MIL) as the first line drug. Anthroponotic VL transmission and long half-life of MIL poses threat of development of resistance. In a set of VL/PKDL cases recruited for MIL treatment, we determined the *in vitro* susceptibility of *L. donovani* parasites obtained pre and post-treatment. Susceptibility of post-treatment isolates from cured VL cases (mean IC_{50}±SD=2.43±1.44μM), was comparable ($p>0.05$) whereas that from relapses (mean IC_{50}=4.72±1.99μM) was significantly lower ($p=0.04$) than that of the pre-treatment group (mean IC_{50} =1.86±0.75μM). Similarly, PKDL isolates from relapse cases exhibited significantly higher MIL tolerance compared to pre-treatment isolates (IC_{50}=16.13±2.64μM and 8.63±0.94μM, respectively).

To understand the mechanism of resistance towards MIL, two Indian cloned clinical isolates (BHU573cl-3 and BHU568cl-1) were experimentally induced at the promastigote stage to become resistant to MIL (up to 74μM). The MIL-R isolates showed no change in susceptibility towards other antileishmanial agents. Further, we analyzed the expression of experimental MIL resistance markers, LdMT and LdRoS3 in MIL-R and MIL clinical isolates (pre-treatment, post treatment and relapse isolates). Down-regulated expression of these transporters was observed in MIL-R parasite but not in parasites from relapse cases. LdMT/LdRos3 genes therefore, do not appear to be suitable markers so far for monitoring MIL susceptibility. Transcriptomic profiling of MIL-R parasite led to identification of 395 differentially expressed genes which represented various functional categories including metabolic pathways, transporters and cellular components. Current results suggest several probable mechanisms employed by parasite to adapt to MIL exposure including reduced protein synthesis and degradation, altered energy utilization via increased lipid degradation and increased antioxidant defence mechanism via elevated trypanothione metabolism. Other genetic markers for parasites that contribute to ML-treatment failure might be identified by whole genome sequencing of natural strains from MIL-relapses, which is currently ongoing in the Kaladrug-R project.

Miltefosine in the Laboratory: Experimental Induction, Mechanisms and Fitness

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Leishmania donovani isolates from Nepal and India with different levels of resistance to antimonial drugs were grown at the promastigote stage in the presence of increasing amounts of miltefosine to produce parasites that could grow in higher levels of the drug. These *in vitro* induced drug-resistant parasites thus show the features of miltefosine resistant parasites that may occur in clinical isolates, and may indicate whether the ability to tolerate antimonial drugs had an influence on the characteristics of the resulting miltefosine-resistant strains. We used various methods (e.g. infectivity to cells, level of miltefosine resistance, ability of components of the immune system to kill parasites, compounds present in cells, expression of particular genes) to compare the *in vitro* generated miltefosine-resistant parasites with the original wild-type parasites. We found that the miltefosine-resistant parasites were easy to produce: it took only 31 weeks to generate parasites that were resistant to concentrations of miltefosine that are toxic to host cells. Resistant parasites were about 12x more resistant to the drug than the original strain in the intracellular amastigote stage and about 20x more resistant to the drug at the free-living promastigote stage. Resistance can easily be measured using a simple assay. Besides the changes related to experimentally induced miltefosine-resistance described in the previous abstract, we showed that there were also changes in the lipid composition of the miltefosine resistant parasites compared to the parent strain, which could be associated with lower uptake of lipids such as miltefosine. If similar changes would occur in parasites from miltefosine-relapsed patients as well, assays to detect these changes could be used to develop novel assays to monitor miltefosine resistance.

Experimental Induction of Miltefosine and Paromomycin Resistance on Intracellular *Leishmania* amastigotes

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Introduction/background

Following the increasing rate of treatment failures related to antimony resistance, miltefosine (MIL) and paromomycin (PMM) are being implemented more widely as first-line drug. However, once MIL and PMM will be routinely used in the field, they may also become at risk for development of resistance. Sporadic cases of MIL treatment failure and MIL relapses have indeed already been reported. To adequately cope with drug-resistance development in the field, close epidemiological monitoring and implementation of rigid treatment policies will have to ensure long term clinical efficacy of both drugs. To adequately achieve this, research on the factors that impact on resistance development is required.

Experimental approach

PMM and MIL resistance were experimentally induced *in vitro* in different *Leishmania* species/isolates by applying drug selection pressure specifically on the intracellular (mammalian) amastigote stage to mimic the situation as it occurs in the treated patient. Successive selection cycles were initiated with amastigotes surviving the highest drug pressure (after back-transformation to promastigotes and metacyclogenesis).

Results

PMM-resistant parasites could be selected within 2-3 induction cycles. The induced resistance was very stable (both after *in vitro* and *in vivo* passage) with some clones tolerating up to 10x higher levels of PMM compared to the non-selected parent clone. Selection for **MIL**-resistance did not lead to resistant parasites although recovery of promastigotes from exposed amastigotes was possible at increasing drug concentrations. Even after 8 successive selection cycles, no decrease in susceptibility could be observed in amastigotes and promastigotes.

Conclusions

Stable **PMM** resistance can rapidly be induced in *L. donovani* isolates. The resulting parasites show a susceptible phenotype at the promastigote stage, but a resistant phenotype at the amastigote stage, implying that only amastigotes should be used for monitoring of PMM resistance. **MIL** resistance is difficult to induce in *L. donovani* amastigotes for reasons still unknown. Further research is necessary to understand the underlying mechanisms of treatment failure and relapse in the field.

History of Kala-Azar Research in the Indian Subcontinent, Discovery of Organic Antimonials, Development of Antimony Resistance, Host Response and Pathogenesis

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The diseases Kala-azar or visceral leishmaniasis is widening its base in the different parts of the subcontinent and the development of resistance to common antileishmanial drugs is making the situation complex. The organic pentavalent antimonials were introduced for the treatment of Kala-azar with dramatic success. Unfortunately with time the drug lost its efficacy. The drug is of limited use in the subcontinent at present. However, resistance to antimonials is still prevailing in the field isolates. There are reports of cross resistance across antileishmanial drugs. This raises the major concern how the newer version of antileishmanial drugs would behave in the context of existing antimonial resistance in the field. It may be noted that Miltefosine relapse cases are on the rise. Our study showed that antimony resistant parasite behaves differently with the host cells as compared to sensitive ones. The resistant parasites induce huge surge of IL-10 from the host which may favour parasite growth in vivo. Oddly enough, endogenous IL-10 up-regulates multi-drug resistant protein-1 on the host cells which may in turn favour efflux of drugs. Thus infection of mammalian hosts either with antimony resistant versus antimony sensitive parasites may give rise to different outcome of pathogenesis. As such, we found that antimony-resistant parasites cause greater in vivo infections compared to their sensitive counterparts. These results suggest that antimony-resistant parasites have a greater fitness compared to their antimony-sensitive counterparts, which might make them even more difficult to eliminate.

Visceral Leishmaniasis Epidemics in the Indian Subcontinent – Origin and Relationships With Other VL Epidemics

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To understand the epidemics of VL in the Indian subcontinent, the diversity in *L. donovani* strains recently isolated in India, Nepal and Bangladesh had to be determined and compared to that found in European, African and other Asian strains of the *L. donovani* complex. This was done by investigating the variation in DNA microsatellite loci and genome-wide single nucleotide polymorphisms (SNPs). Microsatellite analyses revealed a remarkably homogenous 'Indian' *L. donovani* population compared to *L. donovani* from East Africa *and L. infantum* from the Mediterranean and Asia. Specific genetic groups or genotypes correlating either to clinical manifestations, resistance phenotypes or the geographical origin of the strains could not be identified. Identical microsatellite profiles were found for 66% of the strains from the Indian subcontinent, most other strains differed in only one marker. Considerably different genotypes were identified for 3 Indian strains most closely related to *L. donovani* from Kenya, and for 4 strains from Indian and Sri Lankan CL cases. SNP analyses largely confirmed the results of microsatellite typing but provided a higher resolution of strain variability. They identified four different lineages of Indian subcontinent strains, one of which was more closely related to African and European strains. Both microsatellite and SNP analyses appear to trace the origin of Indian strains back to common ancestors within the African cluster.

Certain Indian strains that were related to African strains had been isolated before the anti-malaria spraying campaigns in the 1960s. Our data favor the hypothesis that this campaign resulted in a bottleneck event exterminating the original *L. donovani* population(s) and leaving only few survivors. The recent circulation of a relatively homogeneous population of *L. donovani* in the Indian subcontinent is, most probably, related to the epidemic spread of parasites that evolved after the end of the spraying campaign.

Mathematical Modelling of Visceral Leishmaniasis and Drug Resistance

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We have established a data-based simulation model which describes the transmission of *Leishmania donovani* and the establishment of antimony-resistant parasites. It allows for various interventions, ranging from the treatment of cases with Kala-azar (VL) or PKDL to the control of breeding sites or indoor residual spraying. Although individual Kala-azar cases and (even more so) PKDL cases hugely contribute to the transmission of the infection, their role is overbalanced by asymptomatic infections which (although individually contributing little) basically drive the transmission of VL due to their abundance.

Due to this dominance of asymptomatic infection, the quick rise of treatment failure rates in Bihar, India could only be reproduced by assuming that antimony-resistant parasites are either more transmissible (at least 5% more transmissibility suffice) or that they cause much more KA cases (increasing the relative risk of infected individuals to at least 6.5; such drastic increase ought have been noticed in the field, though). Without such "additional fitness", the rise in treatment failure rates should have been much slower. Resistant parasites will eventually replace the sensitive ones, even in the absence of pressure due to treatment. Despite of this fact, about 90% of resistant infections which are newly introduced into a population fail to establish, but disappear within months or a few years. If they persist, it takes a few decades to completely replace the resistant parasites (depending on the population size).

Due to the dominance of asymptomatic infection, any improvement of treatment is of small or even negligible effect on population-level transmission, although it may be highly beneficial for individual cases. Breeding site control reduces transmission linearly and introduction of zooprophylactic animals even reduces it as a square function, but the potentially most powerful effect can be obtained by indoor residual spraying.

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- the District Health Office of Muzaffarpur, Bihar, India and the District Health Offices of Siraha and Mahottari in Nepal.

- all VL patients participating in this study. May all our efforts finally serve them and alleviate their suffering. And may this study contribute to the elimination of VL from this region.

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4. Notes

www.leishrisk.net/kaladrug

Thank you for your participation!