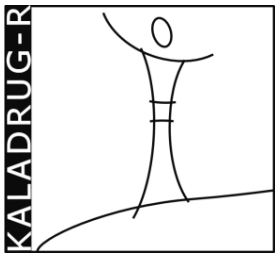
Final publishable summary report

a. Executive summary

Early treatment is a major pillar of the program for Visceral Leishmaniasis (VL) elimination in the Indian sub-continent (ISC). While waiting for new drugs or therapeutic schemes, the effectiveness of current drugs needs to be safeguarded. In the frame of the Kaladrug-R initiative we contributed to this effort and aimed to understand drug resistance and develop tools to monitor it. **1.** We developed simple clinical and epidemiological tools for monitoring the effectiveness of VL drug treatments in routine conditions. **2.** We report a substantial decline in the efficacy of MIL (90.48% cure rate at 6 months in India and 73.3% at 12 months in Nepal). Although MIL-tolerant strains occur, the observed decline of MIL effectiveness (i) is not related to MIL resistance of the parasite, as currently measured with a simplified in vitro assay, but (ii) possibly related with parasite virulence. MIL blood levels at the end of treatment were similar for cured and relapsed patients, but lower in children <12yrs. Incidence risk for relapse in the Nepal study was 3 times higher in children compared to adolescents and adults, possibly due to underdosing. **3.** The decreasing efficacy of MIL could be an heritage of the past pressure of antimonials (SSG). Mathematical modelling predicted that SSG-resistant (SSG-R) parasites were likely more fit than sensitive ones. Different experimental lines confirmed this prediction and showed that SSG-R parasites had better survival skills and might be more virulent than their sensitive counterparts. **4.** Therefore, we further focused research on SSG-resistant strains. Using next generation sequencing, we deciphered the complete genome of 200 clinical isolates and described different signatures of resistance (SNPs and structural variation), opening the way to simplified molecular assays for resistance screening. Metabolomic profiling evidenced molecular adaptations which could jeopardise the efficacy of the other drugs (a.o. membrane fluidity). We also showed that SSG-R parasites better manipulated the macrophage for their own survival. We found two drugs commonly used for other indications (Imipramine and Quercetine) which are able to revert this phenotype and could be used in combination therapy **5.** We experimentally induced resistance to MIL and Paromomycin on strains from the SSG era and described the respective molecular adaptations. **6.** We described the evolution of *L. donovani* through the SSG and MIL eras and situated it in a worldwide context: our results suggest that the main population of parasites originate from strains probably introduced from East Africa less than 200 years ago. Our research demonstrates the complexity of the problem of clinical drug resistance in VL and highlights the need of multidisciplinary approaches to tackle it.

b. Summary description of the project context and objectives

Visceral leishmaniasis (VL), one of the most-neglected infectious diseases, has an annual incidence of 500,000 cases. Early treatment is a major pillar of the current program for VL elimination on 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 are urgently needed to support the drug policy of the VL elimination program. Kaladrug-R addresses these needs: we aim 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.

c. Description of main S&T results

Our general 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.

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%. 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). Altogether, these data indicate a substantial decline in the efficacy of oral miltefosine for treatment of Indian VL.

We are currently exploring the possible causes for this phenomenon:

- 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. The results highlight the need for keeping a close parasitological monitoring of the MIL-susceptibility in the field.
- a preliminary study showed that parasites from MIL-failure were twice more virulent (as measured by resistance to complement lysis) than those from cure. This could be an heritage from the SSG era.

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

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) is currently running in 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 keep the susceptibility phenotype 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. 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, a.o. through circular episomes) among the clinical lines: 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 *in vivo* phenotype than the *in vitro* susceptibility assays did. Altogether our results indicate a huge adaptive capacity of *L. donovani* (it probably uses different mechanisms to get resistant to SSG); 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-treament failure and that these molecular markers significantly outperformed the current in vitro SSG-susceptibility test in terms of power, predictive value and practicability. With respect to MIL-resistance, our work essentially focused on experimentally induced resistant strains as we did not yet find clinically resistant strains: (i) we found point mutations and expression differences in two genes already highlighted in the literature in other species, 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. This new method allows unprecedented studies of the parasite biochemistry: 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 and revealed molecular adaptations which were not detected so far at genomic level, hereby demonstrating the complementarity of different 'omic approaches. 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 human. 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. 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. A similar approach was followed with a second drug, quercetin, with similarly promising results.

4. Exploration, in experimental conditions, of the pathways leading to parasite resistance to Paromomycin. In present project, there was no cohort of patients treated with PMM. Anyway, 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). 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). 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. 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. This was further supported by the high prevalence (83%) of SSG-R isolates in recent patients (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. 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; we recommend a particular attention with drugs interfering with the human immune system, like 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 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. Whole genome sequencing was much more discriminatory and quite powerful for understanding evolution of the parasite 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; our results suggest that ISC main parasite population originate from strains that were probably introduced from East Africa less than 200 years ago. 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. Our website is operational (www.leishrisk.net/kaladrug) and regularly updated; so far, we published (or submitted) 45 papers (10 more in preparation) and gave 84 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.

<u>d. Potential impact and main dissemination activities and exploitation of results</u>

Dissemination

Besides the classical dissemination channels (publications, scientific congresses, webpage…), we paid a particular attention to disseminate our findings among international policy makers as well as local and regional stakeholders. This allowed among others to communicate the tools (clinical and laboratory) that were generated by the consortium, but also to provide some recommendations based on the acquired knowledge. Our dissemination campaign culminated with the organisation of a dissemination workshop in Kathmandu in September 2012.

Impact

Our main messages/recommendations at the end of the Kaladrug-R project are the following:

- Late treatment outcome monitoring should be extended to 12M; encourage PKDL patients for treatment ; optimal dosage for children
- Monitoring is best done by an outcome-based recording and reporting system as done in TB programs (this system was adopted, with adaptations, by DNDi in the current field trials with new treatment regimens (i.e. combination treatment) in the region.
- To reduce defaulters and treatment failures through insufficient adherence, counseling of VL patients on treatment adherence and management of side effects is crucial
- Existing network of ANMs/ASHAs are well placed to help in referral of suspected VL cases, supervision of treatment, and treatment outcome follow up
- To coordinate: reinforce the role of the Kala azar Treatment Supervisor (KTS) at block level
- Need to (keep on) monitoring drug susceptibility (all drugs, also new ones, also combinations); we developed and validated the tools needed for it
- We document a significant decrease in the efficacy of MIL in India and even more in Nepal
- MIL-resistance is not yet detected, but virulence of the parasites could play a role and should be further monitored
- SSG-resistance is still abundant among circulating parasites; this may have clinical and epidemiological implications and might constitute an argument against the re-introduction of SSG; however, we found two drugs used in other applications (imipramine and quercetine), which are able to revert the SSG-resistance phenotype and could be used in combination therapy
- PMM-resistance was easily induced in vitro: vigilance required if implemented in clinical practice
- Kaladrug-R was a milestone in the history of parasitology: for the first time, full genome sequencing of 200 clinical isolates was successfully performed. This technology becomes more accessible and should be soon applicable directly in clinical samples; it could become an element of surveillance (resistance or tracking the origin of new outbreaks); further investments in infrastructure and people (bioinformatics) are needed.
- *L.donovani* from ISC is quite different from East Africa: results cannot a priori be extrapolated!!!
- Asymptomatically infected individuals might play an important role in transmission
- Treatment of cases must be supplemented by vector control as a major factor towards disease elimination
- Quality assurance of vector control measures is important
- Role of arsenic contamination in antimonial resistance?

Exploitation

- Continue routine monitoring (up to 12 m); optimal MIL dosing algorithm
- Feasibility of other treatment schemes
- What explains MIL failure (why different results in different settings?): continue research on parasite, host, drug (Pharmaco-Kinetics/Pharmaco-dynamics), vector
- Legacy of SSG on other drugs: study long-term influence on efficacy of other drugs
- Drug discovery: (i) include SSG-R (and others) from the region, in panel for compound screening; (ii) induce resistance against new lead compounds (part of screening pipeline?)
- Imipramine/Quercetine: more research before possible VL clinical trial?
- PMM-R: identify mechanisms and markers; be proactive, considering the high risk of drug-resistance emergence
- Develop Whole Genome Sequence platforms for direct application in clinical samples, simplify data analysis, integrate with clinical databases, train more people
- Revisit natural history of L.donovani (new variants, sandfly behaviour, asymptomatics, animals)
- Translate research (questions/tools) in other regions worldwide, where VL is endemic
- Continue the financial support of multi-disciplinary consortia like Kaladrug-R

e. Address of project public website and relevant contact details

Project website address: www.leishrisk.net/kaladrug

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