Report on the outcomes of a Short-Term Scientific Mission[1]

Action number: CA21111

Grantee name: Lorenzo Tagliazucchi

Details of the STSM

Title: **Revealing the Mechanism of action and the toxicity of the innovative antileishmanial agent H80, leveraging MS omics tools combined with ADME/Tox chemoinformatics.**

Start and end date: 02/04/2024 to 14/06/2024

Description of the work carried out during the STSM

Description of the activities carried out during the STSM. Any deviations from the initial working plan shall also be described in this section.

Purposes of the STSM (max 200 words):

The aim of this STSM is the characterization of L. infantum promastigotes metabolomic response to H80 and miltefosine administration, and its integration with MS Proteomics results to obtain a multi-omics biochemical asset. Along with computational bioinformatic approaches, MS metabolomics analysis is a powerful way to disclose the involvement of specific biological pathways to different cell stimuli, and, by extension, proteins that are involved in certain conditions to find the mechanisms of action of drugs, and their putative off target activity. When combined with MS Proteomics, this approach can also help finding molecules that can overcome drug resistance in parasitic diseases and improve existing hit compounds to refine their specificity and cell pharmacokinetics properties.

Based on these assumptions, our research group has identified H80 as the best hit compound from a phenotypic screening against the enzyme pteridine reductase 1 a target for anti-leishmania drug. The enzyme inhibition was not the main event related to the mechanism of action of the compound. The profile of the compound was very interesting because it showed

[1] This report is submitted by the grantee to the Action MC for approval and for claiming payment of the awarded grant. The Grant Awarding Coordinator coordinates the evaluation of this report on behalf of the Action MC and instructs the GH for payment of the Grant.

a low IC_{50} against Leishmania spp. and no drug resistance development. H80 was synthetically modified to improve selectivity between the parasite and human cells [1]. Potency and efficacy of H80 were studied with direct cytotoxicity assays and fluorescence microscopy. The co-localization of the molecule in cell cytosol suggests that its mechanism of action is developed in the cytosolic fraction. This evidence was corroborated by a cell fractionated MS proteomics experiment, from which we could clearly distinguish the differentially expressed proteins (DEPs) induced by H80 administration (mainly cytosolic) with respect to the DEPs modulated by miltefosine, that are mostly localized in the membranes.

Aim of the STSM

The aim of this STSM is the characterization of *L. infantum* promastigotes metabolomic response to **H80** in comparison with miltefosine, and its integration with MS Proteomics results to obtain a multi-omics biochemical asset leading to the evaluation of the metabolome modulation. This will help in the understanding of the mechanism of action of H80 and will help to design new derivatives with a higher potency but similar profile. Along with computational bioinformatic approaches, MS metabolomics analysis is a powerful way to disclose the involvement of specific biological pathways to different cell stimuli, and, by extension, proteins that are involved in certain conditions to find the mechanisms of action of drugs, and their putative off target activity. When combined with MS Proteomics, we may have a full profile to characterize the cellular mechanism of action.

The MS Metabolomics experiment will be performed on the same parasitic samples on which proteomics was conducted (i.e. EC_{10} and EC_{50} concentrations). Our aim is to complete the biochemical panel of subcellular modification induced by H80, in an optic to find the specific drug targets and the metabolic paths involved in drug response. Chemoinformatic tools and ecotoxicological platforms like SwissADME and ADMET2.0 will help understanding the chemical impact of the flavonoid scaffold of H80 compared to miltefosine, the currently available antileishmanial agents. The ADME-Tox tools will be used to compare the metabolomic results with the experimentally identified pathway to confirm the predictive metabolic pattern [2,3]. The drug target identified from the *Omic* studies will then be subjected to the SeqAPASS analysis to establish the predictive impact of the compound in the environment.

Description of the work carried out during the STSM (max 500 words):

Preliminary data included fluorescence analysis and MS proteomics analysis of EC10 concentration. During my stay at the National Hellenic Research Foundation (NHRF), as a **first**

experiment, I completed the MS proteomics and bioinformatic analysis of the parasites treated at the EC_{50} concentrations of miltefosine and H80. These data were compared with the same analysis on EC_{10} samples, and 8 proteins resulted as differentially expressed (DEPs) in all the four conditions (Figure 1A). Interestingly, both for EC_{10} (Figure 1B) and for EC_{50} treatments, all the mutual DEPs in miltefosine and H80 share the same trend of protein expression, i.e. up regulation for miltefosine corresponds to up-regulation in H80 sample, and vice versa. In H80 samples, both EC_{10} and EC_{50}, there's a strong and significant modulation of cytosolic proteins (data not shown), whereas miltefosine seems to affect mainly membrane proteins, corroborating its structure activity relationship. DEPs were furtherly investigated with bioinformatic tools, including STRING and Gene Onthology [4,5].

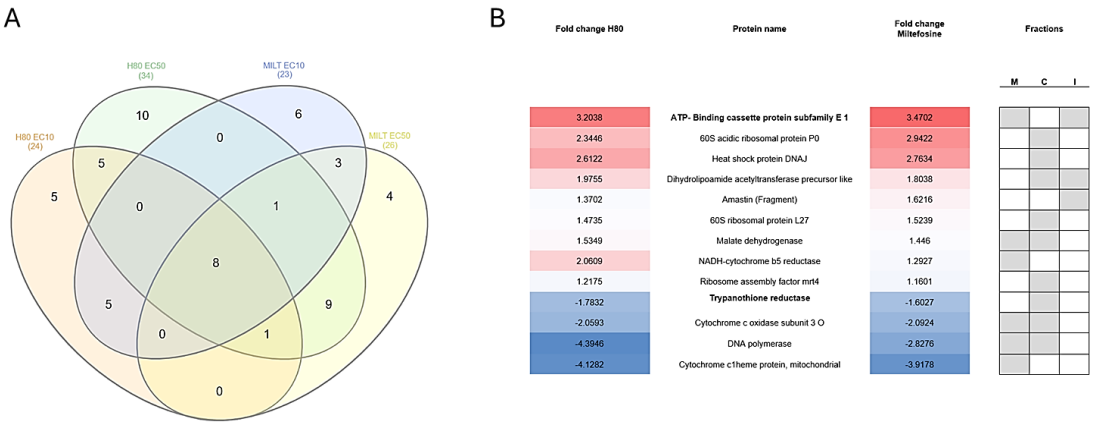

Figure 1. (A) Venn's Diagram representing the differentially expressed proteins per each treatment (Miltefosine and H80, EC10 and EC50 concentration, cross panel). 8 Proteins resulted differentially expressed in all four conditions. (B) HeatMap representing the DEPs mutual between Miltefosine (EC10) and H80 (EC10) treatments, with their subcellular localization as fractions. Interestingly, all the mutual DEPs have the same trend in each sample (i.e. up regulation for Miltefosine corresponds to up-regulation in H80 sample, and vice versa).

An example of bioinformatic analysis is reported in Figure 2. The biochemical networks were obtained with STRING and represent the pathway enrichment of DEPs from H80-treated sample at EC50 concentrations.

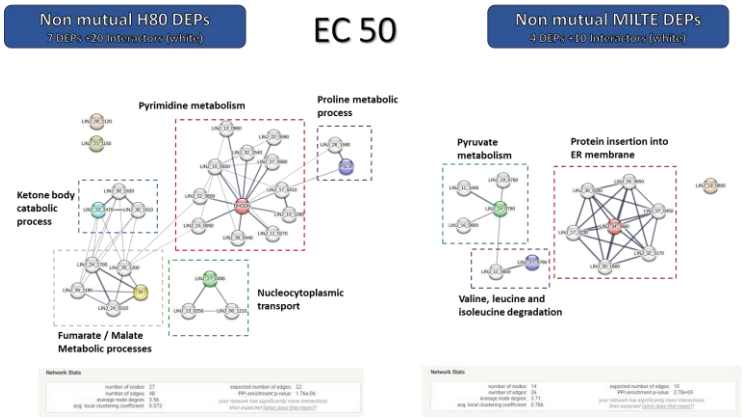

Figure 2. Example of bioinformatic analysis performed on H80 treated samples at EC50 concentration. Mutual (right) and non-mutual (left) DEPs were considered separately. Coloured spheres represent the loaded DEPs, grey spheres represent proteins obtained throughout the enrichment process. Clusters were obtained with MCL clustering algorithm, inflation parameters set at 3.0 *p*-val =1.76e-6 (left) and 2.78e-5 (right), significance 0.700 (medium level).

The second experiment was the preparation of the samples for the metabolomic experiment. The same parasitic samples were processed for MS metabolomics experiments. Cells are lysed according to Chalikiopoulou et al and Bafiti et al [6,7]. Briefly, cell pellets were resuspended with 1mL ice cold MeOH/Water (80:20) and treated with two cycles of freeze-thaw. The supernatant was collected, and a second extraction was performed. Cell pellets were treated again twice with 0.5mL of ice-cold methanol, and the supernatants were pooled together. Samples were dried down in a SpeedVac (Savant SC210A, Thermo Fisher, Waltham, Massachusetts, U.S.) concentrator overnight (i.e. 18h), RT, and stored at -20°C until sample reconstitution. Samples were reconstituted with 0.2mL of initial mobile phase and analysed in a LTQ Ion Trap Mass Spectrometer (Thermo Fisher) according to Bafiti et al [6]. Quality control samples, used for normalization of the total metabolite content, were prepared by pooling together 1µL from every sample. Samples were separated with a C18 column (methanol and water were used as mobile phase) and analysed in an LTQ Orbitrap XL Hybrid FT Mass Spectrometer. Raw data are currently being processed and quantified with the online platform MetaboAnalyst v6.0 [8].

MS metabolomics data analysis

The software MetaboAnalyst allows for a qualitative and quantitative analysis of the LC-MS data. Initially, through the *Spectra Processing section*, raw mz.rt data will be isolated, re-aligned (i.e. normalized to adjust differences in signal intensity related to technical and instrumental inter-sample differences), and annotated. HMDB database will be used to identify the peaks [9]. The *statistical analysis* section will provide single and multivariate analysis and identify the most important features (metabolites), and the biochemicals that are significantly more or less concentrated with respect to control per both treatments at both concentrations. The quality of

each replicate and the differences of each treatment can be visualized by PCA and PLS-DA. In *Functional analysis* section, the entire metabolome for each treatment *vs* control sample will be characterized to evidence significantly overrepresented biochemical pathways/metabolic cycles through different statistical tools, like the Mummichog Activity Profile scatterplot [8]. Finally, with the *Enrichment section*, through Fisher's exact test or Hypergeometric Test, the most significant metabolites found in the previous analysis will be submitted to the Metabolite Set Enrichment analysis (MSEA) and compared to 15 libraries with 13.000 biologically meaningful metabolites.

The third analysis was performed in parallel with the metabolomic analysis, a chemoinformatic analysis was carried out on H80, miltefosine and Sodium stibogluconate (Sb(V)) on ADMETLab 2.0 and SwissADME online tools. The software's allowed the prediction of pharmacokinetics and ecotoxicological behaviours of the three chemical species. As shown in Figure 3, H80 is the most drug like compound according to the Physico-chemical descriptors and performs well in absorption and metabolism assays. These data were confirmed by enzymatic assays on CYP450 isoforms. Also, it seems to display a low toxicity profile when compared to miltefosine and Sb(V), even though it display a non-negligible interference activity on the androgen receptor. However, a small hormonal response it typical of most flavonoid-like products [10].

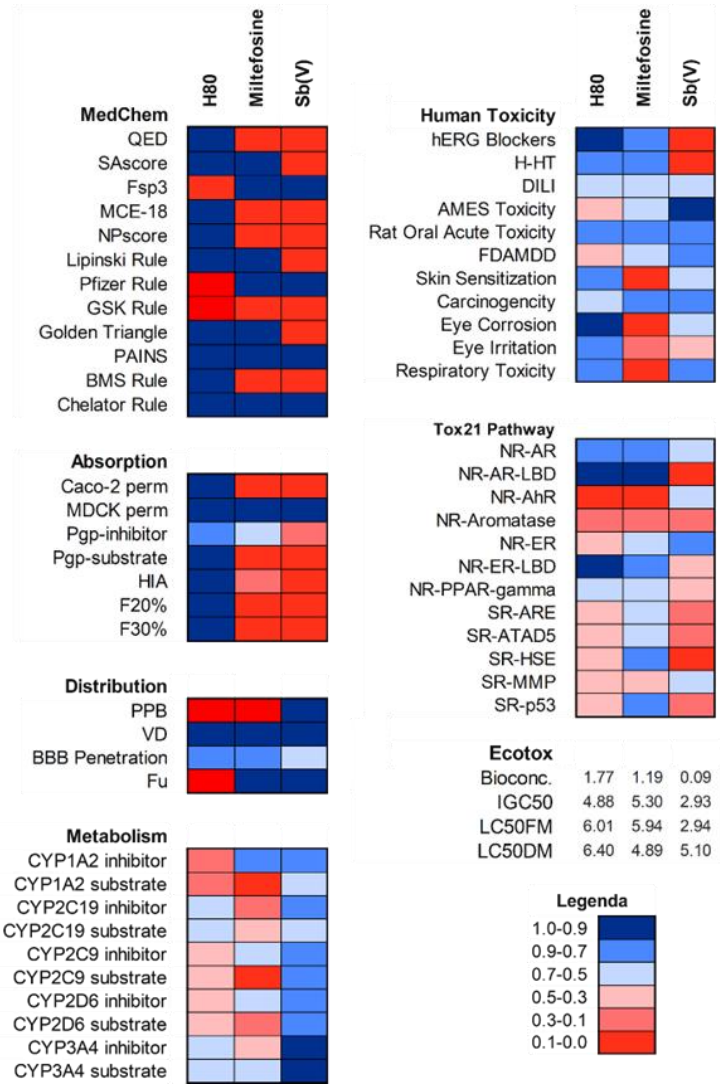

Figure 3. HeatMap representation of the predicted drug likeness and ADME-Tox parameters for H80, Miltefosine and Sb(V) through the software ADMETLab 2.0. The outcomes of the analysis are expressed as 'accepted/rejected' for the medicinal chemistry parameters, coloured respectively in blue and red, and in a colour-scale in six levels of percentage probability from green- highest predicted probability- to red- lowest predicted probability.

Ethics and transparency of research

Obtained data (Raw data from Mass Spectrometer and MS Metabolomics analysis outcome as *mzrt* file, along with the values of the differential analysis) will be deposited on FairDom database, and all the files will be commuted to open access after paper publication. All the data and outcomes from the experiments regarding this STSM will be treated according to the FAIR science guidelines. All the procedures are standardized through SOPs.

Description of the STSM main achievements and planned follow-up activities

Description and assessment of whether the STSM achieved its planned goals and expected outcomes, including specific contribution to Action objective and deliverables, or publications

resulting from the STSM. Agreed plans for future follow-up collaborations shall also be described in this section.

Next analysis steps will include the analysis of MS metabolomics samples with MetaboAnalyst platform v6.0. The analysis workflow will include peak identification against the HMDB, run normalization, statistical analysis and functional analysis. The outcome will provide biochemical information about the most involved networks in drug response, including singular specific metabolites associated to the drug's mechanism of action. Also, these data will be integrated with MS Proteomics outcomes to obtain a dual-omics panel that characterize the cellular response to H80 and Miltefosine.

Unfortunately, due to technical issues of the MS equipment of the hosting facility, samples were pre-processed and randomized, but not loaded into the LC-MS. For this reason, during the last period of staying at the NHRF, I wrote two SOPs (Standardized Operative Procedures) for i. Parasitic Sample Preparation and H80/Miltefosine administration, and ii. Sample processing for MS Metabolomics on parasitic cells, I was engaged in lab experiments of the hosting lab, I attended two seminars/workshops. Also, I spent most of my dry lab time in MetaboAnalyst *v*6.0 courses and internal lab tutorials to gain expertise in MS data analysis.

Attended workshops and other laboratory activities:

i. Mass Spectrometry Day 2024, Pharma, Biopharma & other Applications, Athens – Eleon Loft, 22nd May 2024, a 4h conference organized by Sciex + Antisel to present some biomedical and pharmaceutical applications of Sciex Mass Spectrometers.
ii. Open Day of the Laboratory of Biomarker Discovery & Translational Research, 7th June. Open day for MSc and BSc students.
iii. Internal short presentation about Cryo-EM and sample preparation
iv. Lab Meeting update (15th May 2024) with all lab members
v. Webinair Clinical metabolomics use cases to improve diagnosis and monitoring of endocrine disorders (Antonin Lamazière, Sorbonne Université), online 11th June (organized by MSACL Connect).

Mutual benefits for the Home and Host institutions:

Collaboration with Dr. Theodora Katsila, PhD, and her colleagues has been possible because of the STSM project. We plan to publish the current study of MS proteomics and metabolomics data integration in a prestigious journal with a significant influence. Furthermore, the relationship established by this initiative will facilitate future partnerships. This collaboration has enabled us to broaden our network and knowledge in the subject, perhaps resulting in significant advancements in our research projects.

Foreseen publications or conference presentations expected to result from the STSM

The current study and its outcomes will be included in my PhD thesis (2024-2025). The results will be presented in COST Action CA21111 internal meetings. In addition, the partnership between Prof. Costi's team and Dr Katsila's group will result in a joint publication in a high-impact specialist journal based on the project's results. A Poster presentation will be given at the XXVIII EFMC International Symposium on Medicinal Chemistry in Rome in September 2024.

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