Metabolomics, lipidomics and proteomics profiling of myoblasts infected with *Trypanosoma cruzi* after treatment with different drugs against Chagas disease

K. Hennig[1], J. Abi-Ghanem[1], A. Bunescu[1], X. Meniche[1], E. Biliaut[1], A.D. Ouattara[1], M.D. Lewis[3], J.M. Kelly[3], S. Braillard[2], G. Courtemanche[1], E. Chatelain[2], F. Béquet[1 *]

[1] BIOASTER, 40 avenue Tony Garnier, 69007 Lyon, France

[2] Drugs for Neglected Diseases Initiative (DNDi), 15 Chemin Louis-Dunant, 1202 Geneva, Switzerland.

[3] Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK.

[*] Frederic.BEQUET@bioaster.org

Keywords: Chagas disease; Trypanosoma cruzi; fingerprinting; metabolomics; lipidomics; proteomics; LC-HRMS; NMR;

Abstract

Introduction

Chagas disease, the most important parasitic infection in Latin America, is caused by the intracellular protozoan *Trypanosoma cruzi*. To treat this disease, only two nitroheterocyclic compounds with toxic side effects exist and frequent treatment failures are reported. Hence there is an urgent need to develop new drugs. Recently, metabolomics has become an efficient and cost-effective strategy for dissecting drug mode of action, which has been applied to bacteria as well as parasites, such as different *Trypanosome* species and forms.

Objectives

We assessed if the metabolomics approach can be applied to study drug action of the intracellular amastigote form of *T. cruzi* in a parasite-host cell system.

Methods

We applied a metabolic fingerprinting approach (DI-MS & NMR) to evaluate metabolic changes induced by 6 different (candidate) drugs in a parasite-host cell system. In a second part of our study, we studied the impact of two drugs on polar metabolites, lipid and proteins to evaluate if affected pathways can be identified.

Results

The metabolic signatures obtained by the fingerprinting approach clustered according already described, similar mode of drug actions and that were different from three candidate drugs. Significant changes induced by drug action were observed in all the three metabolic fractions (polar metabolites, lipids and proteins). We identified a general impact on the TCA cycle, but no specific pathways could be attributed to drug action, which might be caused by a high percentage of common metabolome between a eukaryotic host cell and a eukaryotic parasite. Additionally, ion suppression effects due to differences in abundance between host cells and parasites may have occurred.

Conclusion

We validated the metabolic fingerprinting approach to a complex host-cell parasite system. This technique can potentially be applied in the early stage of drug discovery and could help to prioritize early leads or reconfirmed hits for further development.

1 Introduction

Chagas disease, the most important parasitic infection in Latin America, is caused by the intracellular protozoan *Trypanosoma cruzi*. The nitroheterocyclic compounds benznidazole and nifurtimox are the only drugs available to treat *T. cruzi* infections. They have been in use for decades, despite a requirement for long administration periods (60-90 days), frequent reports of treatment failure and toxic side-effects (Gaspar *et al.*, 2015; Molina *et al.*, 2014; Morillo *et al.*, 2015; Morillo *et al.*, 2017; Wilkinson and Kelly, 2009). Both compounds are pro-drugs and are activated within the parasite by the mitochondrial nitroreductase, TcNTR-1 (Mejia *et al.*, 2012; Wilkinson *et al.*, 2008), giving rise to reactive metabolites that have trypanocidal activity. In the case of benznidazole, these metabolites are highly mutagenic and can cause widespread damage to genomic DNA (Campos *et al.*, 2017).

The urgent need to develop new drugs against Chagas disease is being tackled at an international level by large multidisciplinary teams (Chatelain, 2017; Katsuno *et al.*, 2015) , with expertise from both the academic and commercial sectors. The main approach involves high-throughput phenotypic screening of large compound libraries, followed by downstream lead optimization studies including target deconvolution and identification of potential resistance mechanisms. Recently, metabolomics has become an efficient and cost-effective strategy for dissecting drug mode of action (MoA) (Zampieri *et al.*, 2018). Metabolic perturbations induced by drug activity can be detected as a change in the metabolome, since small molecules are downstream products of biological changes. Even if the drug target is not directly metabolic, specific changes in the metabolome can be observed (Zampieri *et al.*, 2018). NMR spectroscopy and mass spectrometry (MS) have been applied to predict drug mode of action (MoA) of antibiotics (Halouska *et al.*, 2012; Zampieri *et al.*, 2018). Based on the same technology we developed an automated screening method for bacteria, Met-SAMoA® (Metabolic screening of antimicrobial mode of actions). The approach is based on the comparison of the metabolic signatures induced by drugs with known MoA and to new drug candidates. Antibiotics are available covering different MoA, but also different drugs with the same MoA, which allows the construction of a robust database. Studying metabolic changes induced by drugs has also been applied to parasites and more specifically to trypanosomes. The effect of nifurtimox and pentamidine on T. *brucei* (Creek et al., 2013; Vincent et al., 2012) and of benznidazole on *T. cruzi* (Trochine et al., 2014*)* has been investigated. In comparison to bacteria, studying trypanosomes adds an extra difficulty because the parasite exists in different forms between insect vector and host. Metabolomic studies have been performed on the isolated bloodstream typomastigotes for *T. brucei* and on epimastigotes, the insect vector form of the parasites, for *T.cruzi*. The amastigote form of *T. cruzi*, however, has not been investigated with a metabolomics approach. Studies of antimalarial drugs against Plasmodium falciparum in red blood cells, however, have been performed (Allman *et al*., 2016; Cobbold *et al*.,2016).

Since we were interested in drugs that are effective against the intracellular amastigote form of *T. cruzi*, we studied a complex system, host cells infected with parasites. We employed a metabolic fingerprinting approach (direct injection (DI)–HRMS and NMR) to analyse this system treated under different anti-parasitic drug treatment conditions. Since only two drugs, with overlapping MoAs, are in use for treatment against Chagas disease, the number of compounds with known MoA is limited and hence a robust database construction for a screening approach is not yet possible. The objective of this study was

to test if we can obtain metabolic signatures of drugs in a complex host cell – parasite-system, relative to their MoA. We tested the reference drugs and experimental drugs, which might have different MoA. In the second part of our study, we applied a more comprehensive metabolomics, lipidomics and proteomics approach to evaluate if metabolic changes induced by drug action can be attributed to a specific metabolic fraction or if they can be correlated to a particular pathway. We show here that the fingerprinting approach can be successfully applied to study different drugs in a complex host cell-parasite system. Furthermore, we demonstrate that comprehensive metabolomics and proteomics reveal metabolic differences relative to drug action, but interpretation towards affected pathways remains challenging due to the common metabolome of a eukaryotic parasite in a eukaryotic host cell.

2 Materials and methods

An overview of the applied experimental strategies is presented the supplementary materials **Error! Reference source not found.**, as well as a detailed description of the cell culture preparation, sample preparation, NMR and LC-HRMS experiments.

2.1 Cell culture and sample collection

2.2 The L6 rat myoblast cell line (L6.G8.C5 (ECACC 92121114)) and *T. cruzi* clone CL Brener (DTU VI) were used as the infection model. L6 cells were grown to 70 % of confluence and infected with trypomastigotes at a ratio of 10 parasites per myoblast cell for 16 hours. After removal of extracellular parasites, rat myoblasts were incubated for 48 hours to establish infection. Subsequently, cultures were treated with 6 different drugs (benznidazole, nifurtimox, posaconazole, S205, S448 and S1000) at their respective IC50 concentrations and infected and uninfected treated cultures were kept in parallel as controls. The DMSO concentration was adjusted to 0.125% in all the conditions. Since a direct cell count is not possible for each well, we estimate the typical infected untreated samples to have had ~4.8 x 10^5 L6 cells, ~10% of them infected and the average number of amastigotes per infected cell would have been approximately 8 and 16 for the 24 hr and 48 hr time points respectively.For the fingerprinting experiments, five replicates after 24 h and 48h of treatment were prepared. For the profiling experiments were performed with ten replicates after 24 h of treatment with two drugs. At the end of incubation time, medium and cells were separated, cells were washed, quenched and detached with cold methanol/ water (50/50, v/v) and snap frozen in liquid nitrogen and stored at -80°C until further processing. Methanol/ water (50/50) was described by Sapcariu et al. (2014) as suitable solvent to detach adherent cells. Furthermore, own tests showed that it was well suited and allowed to process a high quantity of samples in a short time. Sample preparation

For the metabolic fingerprinting experiments, culture supernatants were prepared for NMR analysis to obtain the extracellular metabolome and the cellular lysate in methanol/ water (50/50, v/v) were used for DI-MS analysis to obtain the intracellular metabolome.

For the metabolic profiling experiments the supernatant samples were prepared for NMR analysis. Myoblast cells were extracted with a modified Folch method (Folch *et al.*, 1957) to obtain three fractions of the intracellular metabolome: proteins, polar metabolites and lipids. Protein analysis was performed using a bottom-up approach, by proteolytic digestion of proteins with trypsin prior to LC-MS/MS analysis.

2.3 NMR and mass spectrometry data acquisition

The extracellular metabolite spectra for the fingerprinting, the extracellular and the intracellular polar fraction for the metabolomics approach were acquired by NMR. A one dimensional proton spectrum was acquired at 298K using a 600 MHz Avance III HD NMR from Bruker, equipped with quadruple cryogenic inverse probe for 1H/13C/15N/31P detection. For the fingerprinting approach, intracellular metabolite spectra were obtained by direct infusion high resolution mass spectrometry (DI-HRMS) on a Q-Exactive mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 liquid chromatography chain (Thermo Scientific). The mass spectrometer was operated in positive mode at 3.5kV, at a resolution of 140 000.

For the profiling approach the intracellular lipids and proteins were analyzed by LC-HRMS. Lipids were separated on an Xselect CSH C18 column (1 x 150 mm, 3.5µm i.D.) using water/acetonitrile (80/20, v/v) as solvent A, isopropanol/acetonitrile/water (88/10/2) as solvent B. Two separate injections were performed to acquire spectra in positive and negative ionization modes. Data were acquired in full scan alternating with data dependent acquisition (top 5) to obtain MS/MS spectra. For the proteomics analysis, capillary LC-MS/MS analysis was performed using an ultimate 3000 RS system (Themo Scientific) coupled to a Q-TOF Maxis HD mass spectrometer (Bruker Daltonics) operating in positive mode. Samples were pre-concentrated on a C18 µ-precolumn (300 µm i.d. x 5 mm Acclaim PepMap) and subsequently separated on a C18 capillary column (300 µm inner diameter x 15 cm, acclaim PepMap RSLC, Thermo Scientific) with H_2O/ACN 98/2 (v/v) as solvent A and ACN as solvent B, both containing 0.1% formic acid.The Maxis HD mass spectrometer was operated using the Instant Expertise data acquisition mode (self-optimizing MSMS acquisition) selecting up to sixteen of the most intense multiply charged ions (2+, 3+ and 4+) for MS/MS analysis. For both, lipid and proteins, quality control samples were used to monitor the analytical variability along the runs.

2.4 Data processing

NMR and DI-MS data were preprocessed with an in-house workflow developed in Matlab to extract metabolic features. Quantification of relevant metabolites detected by NMR was performed using the Chenomx NMR suite 8.31 (Alberta, Canada). The Chenomx database was completed with new metabolites for those which were missing from original one using the compound builder module. Extraction and alignment of LC-MS spectra issued from lipid and protein analysis were performed using OpenMS (v. 2.1.0) software and in-house solutions developed with Matlab. QC samples were used to correct analytical drifts within and between batches. Features that were not present in 80% in the defined groups (QCs and the different tested conditions) were considered as unstable and were removed. Additionally, lipids features with a CV greater than 20% in QC samples were removed and correlated and co-eluting features from positive and negative mode were grouped to form putative

compounds. No normalization of the biomass was applied, because the biomass of the parasites was estimated to be 400-times lower compared to the myoblasts and hence a protein determination would not detect differences. The average volume of a *T.cruzi* amastigote was estimated to 14.5 fL (Rohloff et al., 2003) and we estimated the volume of myoblasts to 6.5 pL, based on the average size of 23 µm of spherical detached myboblasts. Since the infection rate of the myoblasts is about 10% with 8 and 16 parasites per myoblast after 24h and 48h respectively, we estimate the volume ratio parasites to myoblasts to 1: 450 after 24h and 1:420 after 48h and hence this difference in biomass negligible. The sum of all the signal intensities showed no difference in NMR and for the lipids 15% difference between infected and uninfected condition, but no differences between with and without treatment. After statistical analysis, relevant putative compounds were identified using the LipidMatch software (Koelmel *et al.*, 2017). Identification levels are reported according to the Metabolomics standard initative (Sumner *et al.*, 2007). Proteins were identified prior to statistical analysis via the Mascot server using the curated Uniprot databases *Rattus norvegicus* (8,036 sequences) and *Trypanosoma cruzi* (126 sequences). Identifications were validated when two peptides with a minimum length of 5 amino acids were detected and a false discovery rate of 5% was applied.

2.5 Statistical analysis

Discriminant analyses were performed using supervised multivariate analyses with the partial least square (PLS) algorithm (Barker and Rayens, 2003; Wold *et al.*, 2001). Subsequently, the coefficient of correspondence R2 and the cross-validation coefficient of correspondence Q2 were computed to evaluate the model performance. For the fingerprinting approach, the elastic net algorithm was used to select the most discriminant variables (Clemmensen *et al.*, 2011) to compare the signatures between different drugs via Venn Diagrams. In the profiling experiments, for the lipids and proteins, the 100 most important variables were selected and subsequently univariate differential analysis was performed to identify relevant metabolites ($p < 0.05$). For the polar metabolites, identification and quantification was performed prior to univariate differential analysis. Furthermore, z-scores were calculated to regulation direction. Positive and negative z-scores mean up- and down-regulations, respectively.

3 Results & discussion

3.1 Metabolic fingerprinting approach

The aim of the fingerprinting approach was to test if rat myoblasts infected with *T. cruzi,* and treated with different drugs, can be discriminated by their metabolic signatures. Infected rat myoblasts were incubated with six different drugs at their respective IC_{50} for either 24 or 48 hours. The selected agents included two reference drugs (benznidazole and nifurtimox), the candidate compound posaconazole, and three experimental drugs (S205, S448 and S1000). Extracellular metabolic signatures were obtained by NMR spectroscopy and intracellular signatures by DI-HRMS. The data were subjected to PLS analysis to test if metabolic signatures specific to each drug treatment could be identified (**Error! Reference source not found.**). For the intracellular metabolome, three different clusters were observed after 24 and 48 hours drug treatment, whereas the extracellular metabolome showed the same clusters only after 48 hours treatment. The signatures of benznidazole and nifurtimox form one group, the three

experimental drugs (S205, S448, S1000) form another, and Posaconazole is separated from the other two groups. Of the six drugs tested, the MoA for three of them has been described. Benznidazole and nifurtimox are pro-drugs that are activated by the parasite nitroreductase TcNTR-1, to generate nitro-species that react with the nucleic acids, causing significant DNA damage (Hall *et al.*, 2011; Hall and Wilkinson, 2012). Posaconazole is an ergosterol biosynthesis inhibitor that blocks growth since ergosterol is required for parasite membranes (Lepesheva *et al.*, 2010). The results of the PLS analysis reflected the differences and similarities between the three drugs, with separation of poscaconazole from the benznidazole-nifurtimox-cluster. Our results also suggest that the three experimental drugs have a MoAs distinct from the three reference drugs and that their MoAs may be similar, relatively to benznidazole, nifurtimox and posaconazole.

To test if specific signals for each drug treatment could be obtained, PLS analysis of the infected myoblasts and the infected, treated myoblasts were performed for each drug separately. The results are presented in figures **Error! Reference source not found.** and **Error! Reference source not found.**. Despite visual separation was obtained, the obtained Q2 –values, which represent the predictive power of a model obtained by cross-validation, are not satisfactory for the majority of the tested conditions (< 0.75). A variable selection using the elastic net algorithm was performed to remove features not relevant for the model. The selection was performed with 1, 5, 10, 20, 50, 100, 150, 300, 400 and 600 variables. Of all the models, the best Q2-values were obtained between 50 and 100 variables and model performance was decreasing with increased number of variables (Table **Error! Reference source not found.**). Both, intra- and extracellular metabolome allowed discrimination of each drug treatment from the control after both 24 and 48 hours treatment (Q2 >0.85). Since both treatment time points enabled metabolic signatures to be obtained, we choose 24h of treatment for the subsequent profiling experiments because after 48 h of treatment the myoblasts are close to cell death and we want to avoid unspecific death signatures. Furthermore, the drugs benznidazole and S205 are separated after 24 h in the extracelluar medium and this are the drugs which are further investigated.

3.2 Profiling approach

3.2.1 Global, supervised statistical analysis

The aim of this comprehensive profiling approach, covering polar metabolites, lipids and proteins, was to test if metabolic changes induced by the drug treatment can be attributed to a specific fraction of the metabolome and if pathways affected by the drugs can be identified. Next to the treatment of the infected myoblasts with the drugs, we also treated the uninfected myoblasts to test if we can detect an impact of the drug on the host cell. We focused on benznidazole as reference drug and the S205 as experimental drug. In total six conditions were prepared: 1) infected myoblasts (cInf), 2) infected myoblasts treated with benznidazole (cInfBz), 3) infected myoblasts treated with S205 (cInfS2), 4) uninfected myoblasts, (cUnInf) 5) uninfected myoblasts treated with benznidazole (cUnInfBz) and 6) uninfected myoblasts treated with S205 (cUnifS2). Twenty-four hours after the initiation of treatment, culture medium and cells were separated. From the cell culture medium (extracellular metabolome), only polar metabolites were studied. Cells were processed to obtain three different intracellular fractions: polar metabolites, lipids and proteins. Polar metabolites were analyzed by NMR, lipids and proteins by LC-HRMS. Supervised statistical analysis (PLS) was performed with the extracted features and

the results are shown in **Error! Reference source not found.**. The strongest separation was observed between the infected and uninfected conditions for all metabolic fractions, which might be caused by the metabolome of the parasite and related changes of the myoblast metabolism. Unlike lipids and proteins, extracellular polar metabolites also clustered according to all the tested conditions (**Error! Reference source not found.**A). The cUnInfS2 condition clusters closely with cUnInf, indicating that the impact on the polar metabolome of S205 is limited, whereas cUnInfBz forms a separate group, indicating a higher impact on the host cell metabolome. For the intracellular, polar metabolome (**Error! Reference source not found.**B), the clusters are less pronounced, probably due to the lower signal intensity, close to the limit of detection compared to the extracellular metabolome (Figure **Error! Reference source not found.**). In PLS analysis of intracellular lipids (**Error! Reference source not found.**C) cInfS2 showed a separate group, whereas cInfBz shows some overlap with cInf. No separation subgroups could be observed for the uninfected conditions, indicating that the impact of the two drugs on the host lipidome is limited. Also the impact of the two drugs on the proteome of the host cell seems limited; no clusters were observed for the uninfected conditions (**Error! Reference source not found.**D). Interestingly, in the PLS analysis of the proteome (**Error! Reference source not found.**D), cInfS2 is clustered together with the non-infected conditions which shows that the proteome becomes similar to cUninf.

The impact of the drugs on the uninfected host cells was limited, except for the polar metabolome after benznidazole treatment, which is coherent with the unspecific radical mechanism described for this drug (Hall *et al.*, 2011; Hall and Wilkinson, 2012). According to internal data S205 had low cytotoxic effects on cell lines, which is in line with our results showing a low impact on the metabolome. In order to investigate more precisely the changes induced during the treatment of infected myoblasts with the drugs, we performed statistical analysis and identification of the underlying features for each of the three fractions separately. Due to low signal intensities of the intracellular polar metabolome by NMR analysis, the data were not considered for further analysis.

3.2.2 Extracellular polar metabolite markers

To determine pathways that are affected by the drug treatment, PLS models were calculated by comparing the infected condition with the infected treated condition for the two drugs separately. Metabolite concertations were quantified with the help of Chenomx NMR suite 8.31 software using internal standard reference DSS. Then, univariate analysis was performed for the different treatments to determine most relevant metabolites. Table **Error! Reference source not found.** shows the metabolites that are were significant in at least one of the conditions. For the treatment with S205, glucose and the metabolites of the TCA cycle pyruvate, citrate, succinate and acetate are clearly affected. For benznidazole only lactate levels are altered. The concentrations of glucose, TCA cycle metabolites and threonine are plotted in **Error! Reference source not found.** in order to understand if the drug acts on the host, amastigotes or both of them. Glucose consumption tends to be higher in the infected cInf, cInfBz and cInfS2 compared to the uninfected cUnInf, cUnInfBz and cUnInfS2 conditions, but high variations are observed. Only cInfS2 compared to cInf myoblasts has significantly lower glucose consumption and it seems to be similar to cUnInfS2 cells. The glycolysis metabolite pyruvate excreted in culture media in infected cInf and cInfBz conditions is lower compared to uninfected cUnInf and cUnInfBz counterparts, which could be explained by integration of this metabolite in parasite pathway. The

pyruvate level in cUnInf and cUnInfS2 is the same, while in cUnInfBz it is lower. This suggests that, unlike S205, benznidazole affects the host metabolism contrary, which is in agreement with the global PLS analysis. As for S205, the pyruvate level in the cInfS2 condition is significantly higher compared to cInf, similar to the uninfected conditions, and it looks like the metabolization of pyruvate by parasite is lower. Similar finding could be observed for other TCA related metabolites lactate and citrate. Indeed, in cInfS2 condition these metabolites levels are normalized and closer to the uninfected conditions than cInf. Similarly, S205 tends to decrease acetate level compared to infected conditions cInf or cInfS2, but the effect is more limited and far from normalization.

Surprisingly, we can observe a completely opposite effect of S205 in the TCA cycle on succinate. Its level in cInfS2 compared to cInf is approximately 2-fold higher, when in the uninfected condition this excreted succinate is absent. The production of succinate during infection could be a host response as well as a metabolic activity of the parasite. Indeed, it is known that heart cells under hypoxic stress can produce high amounts of succinate (Chouchani *et al.*, 2014) Trypanosomatids themselves may produce about 60% of all excreted succinate within glycosomes by NADH-dependent fumarate reductase (Besteiro *et al.*, 2002). Interestingly, accumulation of succinate was also observed *in vivo* for *T. cruzi* infected mice in heart tissue (Girones *et al.*, 2014). However, for the increased accumulation of succinate in the cInfS2 condition, compared to the infected untreated condition, we cannot distinguish if this is due to drug action on the parasite or if it is an indirect effect of the parasite or host cell. Only the use of labelled glucose could help understand the underlying mechanisms, as labeling of the resulting succinate would be differnt depending on its glycosomal or mitochondrial origin (Besteiro *et al.*, 2002; van Weelden *et al.*, 2003). Finally, looking at amino acids that have been observed as discriminant, the amount of threonine in all infected conditions was completely depleted (**Error! Reference source not found.**). Similar data were found for *Trypanosoma brucei* (Millerioux *et al.*, 2013) and actually, threonine is known to be the fastest amino acid to be metabolized by parasite for lipid biosynthesis. Glycine follows a different pattern, with a slight but significant increase during infection. However, even if global PLS models have identified these metabolites as significant in response to treatment, quantitative univariate results are less clear on the impact of the drugs. It enlightens the differences between global models and targeted approaches. Indeed, it shows that global models are able to identify subtle impact on some metabolic pathways that are not easily observed with targeted methods. While we observed variability due to biological batch effect between fingerprinting experiment and profiling one, for example threonine degrades slower than in the first compared to the second experiment. Generally acetate, glycine, pyruvate, lactate show the same trends in both experiments (data not shown) proving the repeatability and the consistency of experimental data.

3.2.3 Lipid markers

The most pronounced separations in the global PLS model were investigated in more detail to determine pathways affected by infection and drug treatment. The selection of the 100 most important variables s was performed for the following models: A) cInf vs cUnif, B) cInf vs cInfBz, C) cInf vs cInS205 and D) cInfBz vs cInfS2. Subsequently, the variables which represent putative compounds were identified using the LipidMatch workflow (Koelmel *et al.*, 2017) and all non-identified compounds were removed. The main lipid classes that were identified are: phosphatidylethanolamines (PE), phosphatidylcholins (PC),

oxidized PC and PE, Lyso-PE (LPE) and lyso-PC (LPC), the plasmalogens with PE- and PC-head groups and the sphingolipids (SL) ceramides and sphingomyelin. Identified lipids showing significant differences ($p < 0.05$) between the peak areas in the different models are presented in Table **Error! Reference source not found.**. Furthermore, z-scores indicate the up- and down regulation in each of the conditions. The highest number of significantly different lipids was found for the model cInf vs cUnif. Twelve LPE and LPC differentiate infected from uninfected myoblasts as they are more abundant in the infected condition. This effect of the infection is in accordance with results of Gazos-Lopes *et al.* (2014), who identified LPC C18:1 as a platelet aggregation factor that is observed in myocarditis, whereas LPC with different chain length did not show this effect. Furthermore, 15 glycerophospholipids, mainly PCs, discriminated the infected from uninfected myoblasts which were all less abundant in the infected compared to the uninfected condition. Oxidated PCs and PEs also contribute to the separation of the conditions, but no clear up- or down regulation could be observed. Additionally, identifications are based on exact mass only and are hence not very precise due to high overlap of exact masses. Plasmalogens are positively and negatively correlated with infection and no coherence among head groups, fatty acid chains, alkyl- or alkenyl- linkage could be identified. Plasmalogens are abundant lipids in heart tissue (Braverman and Moser, 2012) and changes in this abundant lipid class might be related to remodeling of some lipid chains by *T.cruzi*. It was shown that *T.cruzi* incorporates host-glycerophospholipids by changing specific fatty acid chains (Gazos-Lopes *et al.*, 2017). The only two lipids that are only significant in the drug treatment condition (models B, C and D) and not in the control (model A) belong to the class of SL, more specifically a SM and a ceramide. Identification levels are not precise for this two lipids, hence no further conclusions can be drawn. Lipids are considered as targets for novel drug therapies against trypanosomatids since they exhibit critical functions, from building blocks of biological membranes to signal transduction, energy storage and virulence. In order to target the parasite the identification of unique lipid species or metabolic pathways is required (Biagiotti *et al.*, 2017). Guan and Maser (2017) characterized the sphingolipidome of different trypanosome species and identified aminoethylphosphonate ceramide and Inositolphosphoryl ceramide. The SL ceramides and SM we identified that discriminated between both treatments are, however, not specific to the parasite, but ceramides are precursors of parasite specific SL (Guan and Maser, 2017). SL have been described as being affected by drug treatment of trypanosomatids: Stoessel *et al.* (2016) identified an accumulation of ceramides in the bloodstream form of *T. brucei* after treatment with OXPA (3-(oxazolo[4,5-b]pyridine-2-yl)-anilide).

3.2.4 Protein markers

In order to investigate the proteins that explain the separations observed in the global PLS analysis (figure 2) more in detail, we performed univariate analysis on the following models: cInf vs cUnInf, cInf vs cInfBz, cInf vs cInfBz and cInfBz vs cInfS2. Only proteins that have a p-value less than 0.05 were kept for data mining and are listed in Table **Error! Reference source not found.Error! Reference source not found.**. The highest number of significantly different proteins was found for the model cInf vs cUnInf, in agreement with the lipidomics data. In cInf, host cell proteins (rattus norvegicus) that are involved in the glycolysis pathway (*e.g*. G6PI, KPYM) and de novo lipid synthesis (*e.g.* ACLY) are more abundant than in

the UnInf condition. This is in agreement with the polar metabolites results showing that infected cells consume more glucose than uninfected cells, and with data published by Shah-Simpson *et al.* (2017). The authors showed that *T. cruzi* amastigotes capitalize on the increase in glucose uptake by the infected cells to fuel their own metabolism and replication in the host cytosol. Interestingly, in S2-treated cells (model Inf-InfS205, table S8) the amount of G6PI enzyme go back down to the regular cell level as the Z score is similar to the uninfected cells (model Inf-Uninf, table S8 with Z scores at 2.8 and 2.7, respectively).From the protein list generated, and independently of the models, the first observations that we made was that only five proteins from *T. cruzi* were identified, two cytoskeleton proteins (TBB and TBA), the glycosomal D-glyceraldehyde-3-phosphate dehydrogenase (G3PG), the Ubiquitin-60S ribosomal protein L40 (RL40) and the mitochondrial Chaperonin HSP60 (CH60). There are two reasons to explain this observation; first we used the *T. cruzi* curated Swissprot database which contains 60 times less proteins than the *Rattus norvegicus* curated Swissprot database, and second, there is a high dynamic range between proteins of the host and those of the parasite. Myoblast proteins constitute the majority of abundant proteins that suppress identification of potential co-eluting peptides of *T. cruzi* during the LC-MS/MS runs. Amounts of these five proteins of *T. cruzi* in each condition tested, are shown in **Error! Reference source not found.**4. To confirm that peptides used for protein label-free quantitation are not shared between both eukaryotic organisms, myoblasts and parasites, we also performed the quantitation of the *T. cruzi* proteins for the uninfected conditions. As expected, no significant amount of *T. cruzi* proteins were observed for all the uninfected conditions tested. Therefore, we indeed used only unique peptides to specifically quantified proteins of *T. cruzi*. We observed significant differences for all of the five *T. cruzi* proteins only between infected cells (cInf) and infected cells treated with the S205 drug. Unlike the S205 drug, when infected cells are treated with Benznidazole, only two of the five *T. cruzi* proteins are significantly different (TBB and G3PG) compare to the infected cells. Altogether, these results might suggest that the S205 drug is more efficient than benznidazole to clear the parasites out of the infected cells, in agreement with the global PLS shown in **Error! Reference source not found.**. It has to be noted that for both TBB and CH60 proteins the difference between cInf and cS2inf is highly significant (p-value < 0.001). Unlike the CH60 protein, the TBB protein also show significant difference between cInf and cBzinf. We may propose two hypothesis, either these observations reflect parasite killing as we don't know the number of live parasites after the course of S205 treatment compare to untreated cells, or it might suggest that the S205 drug affects pathways that are involved in the stress response of the parasites. Like CH60, but to a lower extent, the ubiquitin-60S ribosomal protein L40 also shows significant difference only in infected cells treated with the S205 drug, but not with benznidazole. Again, that might suggest that treatment with S205 affects pathways involved in the regulation of protein translation or it might reflect parasites killing, as previously noted.

4 Overall discussion and conclusions

The objective of this study was to test if we can obtain metabolic signatures of drugs in a complex host cell – parasite-system. We showed in our first approach, the fingerprinting, that screening for metabolic differences after drug treatment is possible in a complex system of two eukaryotes. The two reference drugs with known MoA showed signatures that were different from a candidate drug and three experimental compounds in development. Both intra- and extracellular metabolome are suitable to

obtain these signatures. Hence, we validated the approach already applied to bacterial cultures and parasite cultures in isolation to a complex system of two eukaryotes. Today, the number of drugs available to treat Chagas disease is limited, hence a construction of a robust database and a prediction of MoA, is not yet possible. However, the construction of such a database during the development of new drugs would be relevant. It could accelerate drug research in Chagas disease, since compounds with potentially new MoA could be identified in an early stage based on differences of metabolic signatures. This technique can potentially be applied in the early stage of drug discover and could help to prioritize early leads or reconfirmed hits for further development.

In the second part of our study, we applied a more comprehensive metabolomics, lipidomics and proteomics approach to evaluate if metabolic changes induced by drugs can be attributed to a specific metabolic fraction or to common pathways. As for the fingerprinting, multivariate statistical analysis allowed the separation of the different conditions in all three fractions. Subsequent identification of the metabolites, lipids and proteins that are underlying these separations showed a strong impact of the infection. The majority of the metabolites and lipids that explain differences between infection and treatment are also discriminant for the infection only. The few metabolites and lipids that were only discriminant in the treated condition are metabolites that are in common between the host cell and the parasite, hence it is difficult to form hypothesis about pathways affected since we can't distinguish between host cell and parasite. The fact that we studied a eukaryotic parasite in a eukaryotic host cell is likely to be responsible for this high overlap. However, we are able to identify that S205 has an impact on, or close to, the TCA cycle from the parasite and/or the host and that it has a more focused action on the metabolism compared to benznidazole. Lipids that were described as specific for *T. cruzi* in the isolated and extracellular form of the parasite were not detected in our study. In the proteomics approach, the number of proteins attributed to the rat myoblasts was 60-times higher than the number of proteins attributed to *T. cruzi*. The databases used for identification also contain 60 times more rat than parasite proteins, which explains these differences. Furthermore, rat myoblasts were more abundant in the extracted samples compared to *T. cruzi,* hence parasite specific lipids and proteins might be masked by ion suppression in mass spectrometry. The isolation of the intracellular form of the parasite from the host cell after cultivation, as it was performed by Gazos-Lopes *et al.* (2017), would be a possibility to overcome this ion suppression problem and allows to lower the limits of detection, but it is much more laborious than our approach. Separation of the two species, would also allow to attribute changes in metabolites that are common between the host cell and the parasite to one of the two and allow more mechanistic insights of the drug action. As a screening approach, however it is not suitable. The magnetic purification of *Plasmodium falciparum* parasites from red blood cells was chosen by Allman et al. (2016) to study the mode of action of antimalarial drugs using a targeted metabolomics approach. The authors detected much higher fold-changes after purification of parasites compared to bulk extraction of infected and uninfected red blood cells. This approach allows a higher throughput than a manual isolation. Our results and the two studies show that separation of the two species seems necessary to obtain pathway information.

Author contributions

KH, JAB, AB, XM, MDL, ADO, JMK, SB, GC,EC, and FB designed, planned and interpreted the study. MDL prepared the cell cultures, collected the samples and supported the experimental setup. . EB extracted the samples and prepared them for LC-HRMS and NMR analysis. AB carried out the NMR, XM the proteomics and KH the fingerprinting and lipidomic analysis. JAB processed the data and performed with ADO the statistical analysis of the data. KH lead and all authors contributed to the writing of the manuscript.

Funding

We kindly acknowledge funding from the French Government through the Investissement d'Avenir program (Grant NO. ANR-10-AIRT-03) and from the Drugs for Neglected Disease *initiative* (DND*i*) for this project. DND*i* received financial support from the following donors: UK Aid, UK, and Reconstruction Credit Institution-Federal Ministry of Education and Research (KfW-BMBF), Germany. The donors had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards

This article does not contain any studies with human and/or animal participants performed by any of the authors.

Conflict of interest: All authors who have contributed to this research have declared no conflict of interests with respect to this article.

5 References

Barker, M. and Rayens, W. (2003) Partial least squares for discrimination. *Journal of Chemometrics* **17,** 166-173.

Besteiro, S., Biran, M., Biteau, N., Coustou, V., Baltz, T., Canioni, P. and Bringaud, F. (2002) Succinate secreted by Trypanosoma brucei is produced by a novel and unique glycosomal enzyme, NADH-dependent fumarate reductase. *J Biol Chem* **277,** 38001-12.

Biagiotti, M., Dominguez, S., Yamout, N. and Zufferey, R. (2017) Lipidomics and anti-trypanosomatid chemotherapy. *Clin Transl Med* **6,** 27.

Braverman, N.E. and Moser, A.B. (2012) Functions of plasmalogen lipids in health and disease. *Biochim Biophys Acta* **1822,** 1442-52.

Campos, M.C., Phelan, J., Francisco, A.F., Taylor, M.C., Lewis, M.D., Pain, A., Clark, T.G. and Kelly, J.M. (2017) Genome-wide mutagenesis and multi-drug resistance in American trypanosomes induced by the front-line drug benznidazole. *Sci Rep* **7,** 14407.

Chatelain, E. (2017) Chagas disease research and development: Is there light at the end of the tunnel? *Comput Struct Biotechnol J* **15,** 98-103.

Chouchani, E.T., Pell, V.R., Gaude, E., Aksentijevic, D., Sundier, S.Y., Robb, E.L., Logan, A., Nadtochiy, S.M., Ord, E.N.J., Smith, A.C., Eyassu, F., Shirley, R., Hu, C.H., Dare, A.J., James, A.M., Rogatti, S., Hartley, R.C., Eaton, S., Costa, A.S.H., Brookes, P.S., Davidson, S.M., Duchen, M.R., Saeb-Parsy, K., Shattock, M.J., Robinson, A.J., Work, L.M., Frezza, C., Krieg, T. and Murphy, M.P. (2014) Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature* **515,** 431-435.

Clemmensen, L., Hastie, T., Witten, D. and Ersbøll, B. (2011) Sparse Discriminant Analysis. *Technometrics* **53,** 406-413.

Creek, D.J., Nijagal, B., Kim, D.H., Rojas, F., Matthews, K.R. and Barrett, M.P. (2013) Metabolomics guides rational development of a simplified cell culture medium for drug screening against Trypanosoma brucei. *Antimicrob Agents Chemother* **57,** 2768-79.

Folch, J., Lees, M. and Stanley, G.H.S. (1957) A SIMPLE METHOD FOR THE ISOLATION AND PURIFICATION OF TOTAL LIPIDES FROM ANIMAL TISSUES. *Journal of Biological Chemistry* **226,** 497-509.

Gaspar, L., Moraes, C.B., Freitas-Junior, L.H., Ferrari, S., Costantino, L., Costi, M.P., Coron, R.P., Smith, T.K., Siqueira-Neto, J.L., McKerrow, J.H. and Cordeiro-da-Silva, A. (2015) Current and Future Chemotherapy for Chagas Disease. *Curr Med Chem* **22,** 4293-312.

Gazos-Lopes, F., Martin, J.L., Dumoulin, P.C. and Burleigh, B.A. (2017) Host triacylglycerols shape the lipidome of intracellular trypanosomes and modulate their growth. *PLoS Pathog* **13,** e1006800.

Gazos-Lopes, F., Oliveira, M.M., Hoelz, L.V., Vieira, D.P., Marques, A.F., Nakayasu, E.S., Gomes, M.T., Salloum, N.G., Pascutti, P.G., Souto-Padron, T., Monteiro, R.Q., Lopes, A.H. and Almeida, I.C. (2014) Structural and functional analysis of a platelet-activating lysophosphatidylcholine of Trypanosoma cruzi. *PLoS Negl Trop Dis* **8,** e3077.

Girones, N., Carbajosa, S., Guerrero, N.A., Poveda, C., Chillon-Marinas, C. and Fresno, M. (2014) Global metabolomic profiling of acute myocarditis caused by Trypanosoma cruzi infection. *PLoS Negl Trop Dis* **8,** e3337.

513 Guan, X.L. and Maser, P. (2017) Comparative sphingolipidomics of disease-causing trypanosomatids
514 reveal unique lifecycle- and taxonomy-specific lipid chemistries. *Sci Rep* **7,** 13617.

515
516 Hall, B.S., Bot, C. and Wilkinson, S.R. (2011) Nifurtimox activation by trypanosomal type I nitroreductases
517 generates cytotoxic nitrile metabolites. *J Biol Chem* **286,** 13088-95.

518
519 Hall, B.S. and Wilkinson, S.R. (2012) Activation of benznidazole by trypanosomal type I nitroreductases
520 results in glyoxal formation. *Antimicrob Agents Chemother* **56,** 115-23.

521
522 Halouska, S., Fenton, R.J., Barletta, R.G. and Powers, R. (2012) Predicting the in Vivo Mechanism of
523 Action for Drug Leads Using NMR Metabolomics. *ACS Chemical Biology* **7,** 166-171.

524
525 Katsuno, K., Burrows, J.N., Duncan, K., Hooft van Huijsduijnen, R., Kaneko, T., Kita, K., Mowbray, C.E.,
526 Schmatz, D., Warner, P. and Slingsby, B.T. (2015) Hit and lead criteria in drug discovery for infectious
527 diseases of the developing world. *Nat Rev Drug Discov* **14,** 751-8.

528
529 Koelmel, J.P., Kroeger, N.M., Ulmer, C.Z., Bowden, J.A., Patterson, R.E., Cochran, J.A., Beecher, C.W.W.,
530 Garrett, T.J. and Yost, R.A. (2017) LipidMatch: an automated workflow for rule-based lipid identification
531 using untargeted high-resolution tandem mass spectrometry data. *BMC Bioinformatics* **18,** 331.

532
533 Lepesheva, G.I., Hargrove, T.Y., Anderson, S., Kleshchenko, Y., Furtak, V., Wawrzak, Z., Villalta, F. and
534 Waterman, M.R. (2010) Structural insights into inhibition of sterol 14alpha-demethylase in the human
535 pathogen Trypanosoma cruzi. *J Biol Chem* **285,** 25582-90.

536
537 Mejia, A.M., Hall, B.S., Taylor, M.C., Gomez-Palacio, A., Wilkinson, S.R., Triana-Chavez, O. and Kelly, J.M.
538 (2012) Benznidazole-resistance in Trypanosoma cruzi is a readily acquired trait that can arise
539 independently in a single population. *J Infect Dis* **206,** 220-8.

540
541 Millerioux, Y., Ebikeme, C., Biran, M., Morand, P., Bouyssou, G., Vincent, I.M., Mazet, M., Riviere, L.,
542 Franconi, J.M., Burchmore, R.J., Moreau, P., Barrett, M.P. and Bringaud, F. (2013) The threonine
543 degradation pathway of the Trypanosoma brucei procyclic form: the main carbon source for lipid
544 biosynthesis is under metabolic control. *Mol Microbiol* **90,** 114-29.

545
546 Molina, I., Gomez i Prat, J., Salvador, F., Trevino, B., Sulleiro, E., Serre, N., Pou, D., Roure, S., Cabezos, J.,
547 Valerio, L., Blanco-Grau, A., Sanchez-Montalva, A., Vidal, X. and Pahissa, A. (2014) Randomized trial of
548 posaconazole and benznidazole for chronic Chagas' disease. *N Engl J Med* **370,** 1899-908.

549
550 Morillo, C.A., Marin-Neto, J.A., Avezum, A., Sosa-Estani, S., Rassi, A., Jr., Rosas, F., Villena, E., Quiroz, R.,
551 Bonilla, R., Britto, C., Guhl, F., Velazquez, E., Bonilla, L., Meeks, B., Rao-Melacini, P., Pogue, J., Mattos, A.,

552 Lazdins, J., Rassi, A., Connolly, S.J. and Yusuf, S. (2015) Randomized Trial of Benznidazole for Chronic
553 Chagas' Cardiomyopathy. *N Engl J Med* **373,** 1295-306.

554
555 Morillo, C.A., Waskin, H., Sosa-Estani, S., Del Carmen Bangher, M., Cuneo, C., Milesi, R., Mallagray, M.,
556 Apt, W., Beloscar, J., Gascon, J., Molina, I., Echeverria, L.E., Colombo, H., Perez-Molina, J.A., Wyss, F.,
557 Meeks, B., Bonilla, L.R., Gao, P., Wei, B., McCarthy, M. and Yusuf, S. (2017) Benznidazole and
558 Posaconazole in Eliminating Parasites in Asymptomatic T. Cruzi Carriers: The STOP-CHAGAS Trial. *J Am*
559 *Coll Cardiol* **69,** 939-947.

560 Sapcariu, S.C., Kanashova, T., Weindl, D., Ghelfi J., Dittmar G., Hiller K. (2014) Simultaneous extraction of
561 proteins and metabolites from cells in culture : *Methods X* **1**, 74-80
562
563 Shah-Simpson, S., Lentini, G., Dumoulin, P.C. and Burleigh, B.A. (2017) Modulation of host central carbon
564 metabolism and in situ glucose uptake by intracellular Trypanosoma cruzi amastigotes. *PLoS Pathog* **13,**
565 e1006747.

566
567 Stoessel, D., Nowell, C.J., Jones, A.J., Ferrins, L., Ellis, K.M., Riley, J.S., Rahmani, R., Read, K.D., McConville,
568 M.J., Avery, V.M., Baell, J.B. and Creek, D.J. (2016) Metabolomics and lipidomics reveal perturbation of
569 sphingolipid metabolism by a novel anti-trypanosomal 3-(oxazolo[4,5-b]pyridine-2-yl)anilide.
570 *Metabolomics* **12,** 1-14.

571
572 Sumner, L.W., Amberg, A., Barrett, D., Beale, M.H., Beger, R., Daykin, C.A., Fan, T.W., Fiehn, O.,
573 Goodacre, R., Griffin, J.L., Hankemeier, T., Hardy, N., Harnly, J., Higashi, R., Kopka, J., Lane, A.N., Lindon,
574 J.C., Marriott, P., Nicholls, A.W., Reily, M.D., Thaden, J.J. and Viant, M.R. (2007) Proposed minimum
575 reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics
576 Standards Initiative (MSI). *Metabolomics* **3,** 211-221.

577
578 Trochine, A., Creek, D.J., Faral-Tello, P., Barrett, M.P. and Robello, C. (2014) Benznidazole
579 biotransformation and multiple targets in Trypanosoma cruzi revealed by metabolomics. *PLoS Negl Trop*
580 *Dis* **8,** e2844.

581
582 van Weelden, S.W., Fast, B., Vogt, A., van der Meer, P., Saas, J., van Hellemond, J.J., Tielens, A.G. and
583 Boshart, M. (2003) Procyclic Trypanosoma brucei do not use Krebs cycle activity for energy generation. *J*
584 *Biol Chem* **278,** 12854-63.

585
586 Vincent, I.M., Creek, D.J., Burgess, K., Woods, D.J., Burchmore, R.J. and Barrett, M.P. (2012) Untargeted
587 metabolomics reveals a lack of synergy between nifurtimox and eflornithine against Trypanosoma
588 brucei. *PLoS Negl Trop Dis* **6,** e1618.

589
590 Wilkinson, S.R. and Kelly, J.M. (2009) Trypanocidal drugs: mechanisms, resistance and new targets.
591 *Expert Rev Mol Med* **11,** e31.

592

Wilkinson, S.R., Taylor, M.C., Horn, D., Kelly, J.M. and Cheeseman, I. (2008) A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. *Proc Natl Acad Sci U S A* **105,** 5022-7.

Wold, S., Sjöström, M. and Eriksson, L. (2001) PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **58,** 109-130.

Zampieri, M., Szappanos, B., Buchieri, M.V., Trauner, A., Piazza, I., Picotti, P., Gagneux, S., Borrell, S., Gicquel, B., Lelievre, J., Papp, B. and Sauer, U. (2018) High-throughput metabolomic analysis predicts mode of action of uncharacterized antimicrobial compounds. *Sci Transl Med* **10**.

605