

1 **Metabolomics, lipidomics and proteomics profiling of myoblasts**
2 **infected with *Trypanosoma cruzi* after treatment with different drugs**
3 **against Chagas disease**
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13 HRMS; NMR;

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15

16 **Abstract**

17

18 Introduction

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

25 Objectives

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

28 Methods

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

33 Results

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

41 Conclusion

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

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48 **1 Introduction**

49 Chagas disease, the most important parasitic infection in Latin America, is caused by the intracellular
50 protozoan *Trypanosoma cruzi*. The nitroheterocyclic compounds benznidazole and nifurtimox are the
51 only drugs available to treat *T. cruzi* infections. They have been in use for decades, despite a requirement
52 for long administration periods (60-90 days), frequent reports of treatment failure and toxic side-effects
53 (Gaspar *et al.*, 2015; Molina *et al.*, 2014; Morillo *et al.*, 2015; Morillo *et al.*, 2017; Wilkinson and Kelly,
54 2009). Both compounds are pro-drugs and are activated within the parasite by the mitochondrial
55 nitroreductase, TcNTR-1 (Mejia *et al.*, 2012; Wilkinson *et al.*, 2008), giving rise to reactive metabolites
56 that have trypanocidal activity. In the case of benznidazole, these metabolites are highly mutagenic and
57 can cause widespread damage to genomic DNA (Campos *et al.*, 2017).

58 The urgent need to develop new drugs against Chagas disease is being tackled at an international level
59 by large multidisciplinary teams (Chatelain, 2017; Katsuno *et al.*, 2015), with expertise from both the
60 academic and commercial sectors. The main approach involves high-throughput phenotypic screening of
61 large compound libraries, followed by downstream lead optimization studies including target
62 deconvolution and identification of potential resistance mechanisms. Recently, metabolomics has
63 become an efficient and cost-effective strategy for dissecting drug mode of action (MoA) (Zampieri *et al.*,
64 2018). Metabolic perturbations induced by drug activity can be detected as a change in the metabolome,
65 since small molecules are downstream products of biological changes. Even if the drug target is not
66 directly metabolic, specific changes in the metabolome can be observed (Zampieri *et al.*, 2018). NMR
67 spectroscopy and mass spectrometry (MS) have been applied to predict drug mode of action (MoA) of
68 antibiotics (Halouska *et al.*, 2012; Zampieri *et al.*, 2018). Based on the same technology we developed an
69 automated screening method for bacteria, Met-SAMoA[®] (Metabolic screening of antimicrobial mode of
70 actions). The approach is based on the comparison of the metabolic signatures induced by drugs with
71 known MoA and to new drug candidates. Antibiotics are available covering different MoA, but also
72 different drugs with the same MoA, which allows the construction of a robust database. Studying
73 metabolic changes induced by drugs has also been applied to parasites and more specifically to
74 trypanosomes. The effect of nifurtimox and pentamidine on *T. brucei* (Creek *et al.*, 2013; Vincent *et al.*,
75 2012) and of benznidazole on *T. cruzi* (Trochine *et al.*, 2014) has been investigated. In comparison to
76 bacteria, studying trypanosomes adds an extra difficulty because the parasite exists in different forms
77 between insect vector and host. Metabolomic studies have been performed on the isolated bloodstream
78 trypomastigotes for *T. brucei* and on epimastigotes, the insect vector form of the parasites, for *T. cruzi*.
79 The amastigote form of *T. cruzi*, however, has not been investigated with a metabolomics approach.
80 Studies of antimalarial drugs against *Plasmodium falciparum* in red blood cells, however, have been
81 performed (Allman *et al.*, 2016; Cobbold *et al.*, 2016).

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

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

97 **2 Materials and methods**

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

101 **2.1 Cell culture and sample collection**

102 **2.2 The L6 rat myoblast cell line (L6.G8.C5 (ECACC 92121114)) and *T. cruzi* clone CL Brener**
103 **(DTU VI) were used as the infection model. L6 cells were grown to 70 % of confluence**
104 **and infected with trypomastigotes at a ratio of 10 parasites per myoblast cell for 16**
105 **hours. After removal of extracellular parasites, rat myoblasts were incubated for 48**
106 **hours to establish infection. Subsequently, cultures were treated with 6 different drugs**
107 **(benznidazole, nifurtimox, posaconazole, S205, S448 and S1000) at their respective**
108 **IC50 concentrations and infected and uninfected treated cultures were kept in parallel**
109 **as controls. The DMSO concentration was adjusted to 0.125% in all the conditions.**
110 **Since a direct cell count is not possible for each well, we estimate the typical infected**
111 **untreated samples to have had $\sim 4.8 \times 10^5$ L6 cells, $\sim 10\%$ of them infected and the**
112 **average number of amastigotes per infected cell would have been approximately 8 and**
113 **16 for the 24 hr and 48 hr time points respectively. For the fingerprinting experiments,**
114 **five replicates after 24 h and 48h of treatment were prepared. For the profiling**
115 **experiments were performed with ten replicates after 24 h of treatment with two**
116 **drugs. At the end of incubation time, medium and cells were separated, cells were**
117 **washed, quenched and detached with cold methanol/ water (50/50, v/v) and snap**
118 **frozen in liquid nitrogen and stored at -80°C until further processing. Methanol/ water**
119 **(50/50) was described by Sapcariu et al. (2014) as suitable solvent to detach adherent**
120 **cells. Furthermore, own tests showed that it was well suited and allowed to process a**
121 **high quantity of samples in a short time. Sample preparation**

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

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

130 **2.3 NMR and mass spectrometry data acquisition**

131 The extracellular metabolite spectra for the fingerprinting, the extracellular and the intracellular polar
132 fraction for the metabolomics approach were acquired by NMR. A one dimensional proton spectrum
133 was acquired at 298K using a 600 MHz Avance III HD NMR from Bruker, equipped with quadruple
134 cryogenic inverse probe for $^1\text{H}/^{13}\text{C}/^{15}\text{N}/^{31}\text{P}$ detection. For the fingerprinting approach, intracellular
135 metabolite spectra were obtained by direct infusion high resolution mass spectrometry (DI-HRMS) on a
136 Q-Exactive mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 liquid
137 chromatography chain (Thermo Scientific). The mass spectrometer was operated in positive mode at
138 3.5kV, at a resolution of 140 000.

139 For the profiling approach the intracellular lipids and proteins were analyzed by LC-HRMS. Lipids were
140 separated on an Xselect CSH C18 column (1 x 150 mm, 3.5 μm i.D.) using water/acetonitrile (80/20, v/v)
141 as solvent A, isopropanol/acetonitrile/water (88/10/2) as solvent B. Two separate injections were
142 performed to acquire spectra in positive and negative ionization modes. Data were acquired in full scan
143 alternating with data dependent acquisition (top 5) to obtain MS/MS spectra. For the proteomics
144 analysis, capillary LC-MS/MS analysis was performed using an ultimate 3000 RS system (Thermo Scientific)
145 coupled to a Q-TOF Maxis HD mass spectrometer (Bruker Daltonics) operating in positive mode. Samples
146 were pre-concentrated on a C18 μ -precursor column (300 μm i.d. x 5 mm Acclaim PepMap) and subsequently
147 separated on a C18 capillary column (300 μm inner diameter x 15 cm, acclaim PepMap RSLC, Thermo
148 Scientific) with $\text{H}_2\text{O}/\text{ACN}$ 98/2 (v/v) as solvent A and ACN as solvent B, both containing 0.1% formic
149 acid. The Maxis HD mass spectrometer was operated using the Instant Expertise data acquisition mode
150 (self-optimizing MSMS acquisition) selecting up to sixteen of the most intense multiply charged ions (2+,
151 3+ and 4+) for MS/MS analysis. For both, lipid and proteins, quality control samples were used to
152 monitor the analytical variability along the runs.

153 **2.4 Data processing**

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

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

179 2.5 Statistical analysis

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

190 3 Results & discussion

191 3.1 Metabolic fingerprinting approach

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

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

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

227 **3.2 Profiling approach**

228 **3.2.1 Global, supervised statistical analysis**

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

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

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

266 3.2.2 Extracellular polar metabolite markers

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

282 pyruvate level in cUnInf and cUnInfS2 is the same, while in cUnInfBz it is lower. This suggests that, unlike
283 S205, benznidazole affects the host metabolism contrary, which is in agreement with the global PLS
284 analysis. As for S205, the pyruvate level in the clnfs2 condition is significantly higher compared to clnf,
285 similar to the uninfected conditions, and it looks like the metabolization of pyruvate by parasite is lower.
286 Similar finding could be observed for other TCA related metabolites lactate and citrate. Indeed, in clnfs2
287 condition these metabolites levels are normalized and closer to the uninfected conditions than clnf.
288 Similarly, S205 tends to decrease acetate level compared to infected conditions clnf or clnfs2, but the
289 effect is more limited and far from normalization.

290 Surprisingly, we can observe a completely opposite effect of S205 in the TCA cycle on succinate. Its level
291 in clnfs2 compared to clnf is approximately 2-fold higher, when in the uninfected condition this excreted
292 succinate is absent. The production of succinate during infection could be a host response as well as a
293 metabolic activity of the parasite. Indeed, it is known that heart cells under hypoxic stress can produce
294 high amounts of succinate (Chouchani *et al.*, 2014) Trypanosomatids themselves may produce about
295 60% of all excreted succinate within glycosomes by NADH-dependent fumarate reductase (Besteiro *et*
296 *al.*, 2002). Interestingly, accumulation of succinate was also observed *in vivo* for *T. cruzi* infected mice in
297 heart tissue (Girones *et al.*, 2014). However, for the increased accumulation of succinate in the clnfs2
298 condition, compared to the infected untreated condition, we cannot distinguish if this is due to drug
299 action on the parasite or if it is an indirect effect of the parasite or host cell. Only the use of labelled
300 glucose could help understand the underlying mechanisms, as labeling of the resulting succinate would
301 be different depending on its glycosomal or mitochondrial origin (Besteiro *et al.*, 2002; van Weelden *et al.*,
302 2003). Finally, looking at amino acids that have been observed as discriminant, the amount of threonine
303 in all infected conditions was completely depleted (**Error! Reference source not found.**). Similar data
304 were found for *Trypanosoma brucei* (Millerioux *et al.*, 2013) and actually, threonine is known to be the
305 fastest amino acid to be metabolized by parasite for lipid biosynthesis. Glycine follows a different
306 pattern, with a slight but significant increase during infection. However, even if global PLS models have
307 identified these metabolites as significant in response to treatment, quantitative univariate results are
308 less clear on the impact of the drugs. It enlightens the differences between global models and targeted
309 approaches. Indeed, it shows that global models are able to identify subtle impact on some metabolic
310 pathways that are not easily observed with targeted methods. While we observed variability due to
311 biological batch effect between fingerprinting experiment and profiling one, for example threonine
312 degrades slower than in the first compared to the second experiment. Generally acetate, glycine,
313 pyruvate, lactate show the same trends in both experiments (data not shown) proving the
314 repeatability and the consistency of experimental data.

315 3.2.3 Lipid markers

316 The most pronounced separations in the global PLS model were investigated in more detail to determine
317 pathways affected by infection and drug treatment. The selection of the 100 most important variables *s*
318 was performed for the following models: A) clnf vs cUnif, B) clnf vs clnfBz, C) clnf vs clnS205 and D)
319 clnfBz vs clnfs2. Subsequently, the variables which represent putative compounds were identified using
320 the LipidMatch workflow (Koelmel *et al.*, 2017) and all non-identified compounds were removed. The
321 main lipid classes that were identified are: phosphatidylethanolamines (PE), phosphatidylcholins (PC),

322 oxidized PC and PE, Lyso-PE (LPE) and lyso-PC (LPC), the plasmalogens with PE- and PC-head groups and
323 the sphingolipids (SL) ceramides and sphingomyelin. Identified lipids showing significant differences (p
324 <0.05) between the peak areas in the different models are presented in Table **Error! Reference source**
325 **not found..** Furthermore, z-scores indicate the up- and down regulation in each of the conditions. The
326 highest number of significantly different lipids was found for the model cInf vs cUnif. Twelve LPE and LPC
327 differentiate infected from uninfected myoblasts as they are more abundant in the infected condition.
328 This effect of the infection is in accordance with results of Gazos-Lopes *et al.* (2014), who identified LPC
329 C18:1 as a platelet aggregation factor that is observed in myocarditis, whereas LPC with different chain
330 length did not show this effect. Furthermore, 15 glycerophospholipids, mainly PCs, discriminated the
331 infected from uninfected myoblasts which were all less abundant in the infected compared to the
332 uninfected condition. Oxidated PCs and PEs also contribute to the separation of the conditions, but no
333 clear up- or down regulation could be observed. Additionally, identifications are based on exact mass
334 only and are hence not very precise due to high overlap of exact masses. Plasmalogens are positively and
335 negatively correlated with infection and no coherence among head groups, fatty acid chains, alkyl- or
336 alkenyl- linkage could be identified. Plasmalogens are abundant lipids in heart tissue (Braverman and
337 Moser, 2012) and changes in this abundant lipid class might be related to remodeling of some lipid
338 chains by *T. cruzi*. It was shown that *T. cruzi* incorporates host-glycerophospholipids by changing specific
339 fatty acid chains (Gazos-Lopes *et al.*, 2017). The only two lipids that are only significant in the drug
340 treatment condition (models B, C and D) and not in the control (model A) belong to the class of SL, more
341 specifically a SM and a ceramide. Identification levels are not precise for this two lipids, hence no further
342 conclusions can be drawn. Lipids are considered as targets for novel drug therapies against
343 trypanosomatids since they exhibit critical functions, from building blocks of biological membranes to
344 signal transduction, energy storage and virulence. In order to target the parasite the identification of
345 unique lipid species or metabolic pathways is required (Biagiotti *et al.*, 2017). Guan and Maser (2017)
346 characterized the sphingolipidome of different trypanosome species and identified
347 aminoethylphosphonate ceramide and Inositolphosphoryl ceramide. The SL ceramides and SM we
348 identified that discriminated between both treatments are, however, not specific to the parasite, but
349 ceramides are precursors of parasite specific SL (Guan and Maser, 2017). SL have been described as
350 being affected by drug treatment of trypanosomatids: Stoessel *et al.* (2016) identified an accumulation
351 of ceramides in the bloodstream form of *T. brucei* after treatment with OXPA (3-(oxazolo[4,5-b]pyridine-
352 2-yl)-anilide).

353

354 3.2.4 Protein markers

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

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

396 **4 Overall discussion and conclusions**

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

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

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

440 **Author contributions**

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

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454 **Compliance with ethical standards**

455 This article does not contain any studies with human and/or animal participants performed by any of the
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457 **Conflict of interest:** All authors who have contributed to this research have declared no conflict of
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459

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