

Isothermal microcalorimetry – A quantitative method to monitor *Trypanosoma congolense* growth and growth inhibition by trypanocidal drugs in real time

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ABSTRACT

Trypanosoma congolense is a protozoan parasite that is transmitted by tsetse flies, causing African Animal Trypanosomiasis, also known as Nagana, in sub-Saharan Africa. Nagana is a fatal disease of livestock that causes severe economic losses. Two drugs are available, diminazene and isometamidium, yet successful treatment is jeopardized by drug resistant *T. congolense*. Isothermal microcalorimetry is a highly sensitive tool that can be used to study growth of the extracellular *T. congolense* parasites or to study parasite growth inhibition after the addition of antitrypanosomal drugs. Time of drug action and time to kill can be quantified in a simple way by real time heat flow measurements. We established a robust protocol for the microcalorimetric studies of *T. congolense* and developed mathematical computations in R to calculate different parameters related to growth and the kinetics of drug action. We demonstrate the feasibility and benefit of the method exemplary with the two standard drugs, diminazene aceturate and isometamidium chloride. The method and the mathematical approach can be translated to study other pathogenic or non-pathogenic cells if they are metabolically active and grow under axenic conditions.

1. Introduction

Nagana, or African Animal Trypanosomiasis (AAT), is a parasitic livestock disease in sub-Saharan Africa caused by *Trypanosoma congolense*, *T. vivax* and, to a lesser extent, *T. brucei brucei*, and is transmitted by tsetse flies (*Glossina* spp.). If left untreated, infected animals will die (Auty et al., 2015). Nagana causes annual economic losses of US \$ 4.5 billion to the agricultural industry due to unproductive livestock farming (Shaw et al., 2014). *T. congolense* has the highest prevalence and is considered, together with *T. vivax*, the main livestock pathogen in cattle. *T. congolense* Savannah is the most virulent and the main cattle-infecting subgroup across sub-Saharan Africa (Auty et al., 2015).

In the absence of a vaccine, antitrypanosomal drugs are crucial in the control of AAT. Two trypanocides, which have been on the market for more than 50 years, are mainly used to treat Nagana. Diminazene aceturate is only administered as a therapeutic agent, whereas isometamidium chloride can also be employed for prophylaxis (Geerts et al., 2001). Due to extensive drug use, resistance has been spreading. Drug resistance was reported from 21 African countries and multi-drug

resistance from 10 African countries (Tsegaye et al., 2015). Therefore, the discovery and development of new drugs is needed desperately. This, in turn, requires robust *in vitro* cultivation and drug efficacy tests for *T. congolense*.

Different viability assays (Rüz et al., 1997; Vennerstrom et al., 2004; Cal et al., 2016) are routinely used to test compounds against various protozoan pathogens *in vitro*. A cheap, simple and reliable drug sensitivity test was also established for *T. congolense* using the viability marker Alamar blue (Rüz et al., 1997; Gillingwater et al., 2017). However, since this is an endpoint read-out, several Alamar blue assays with different exposure times have to be carried out to assess the time of drug action. This is highly labour-intensive and yet only provides a rough estimate of the kinetics of drug action.

Isothermal microcalorimetry is a simple tool that measures the heat flow of processes of biological, physical or chemical nature in real time. The heat produced by cell samples can be attributed to metabolic activity of the cells and to changing number of cells during growth or decay. For review see Braissant et al. (2010a). The continuous recording allows phenotypic analysis of cell growth, as the metabolic

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activity per cell is more or less constant, especially in the exponential growth phase (Kemp and Guan, 1999), and it can also be used to determine pharmacodynamic parameters such as onset of drug action and time to kill. The method has already been established for the human pathogenic protozoans *Trypanosoma brucei* and *Plasmodium falciparum* (Wenzler et al., 2012), for pathogenic helminths (Manneck et al., 2011; Keiser et al., 2013) and prokaryotes (Baldoni et al., 2010; Braissant et al., 2010a, 2010b).

Here, we validate the potential of isothermal microcalorimetry to measure growth and drug action in *T. congolense* cultures. We establish a protocol to monitor *T. congolense* growth in real time, apply this protocol to determine time of drug action, and validate it with the reference drugs diminazene aceturate and isometamidium chloride.

2. Materials and methods

2.1. *T. congolense* cultivation

T. congolense IL3000 bloodstream forms, subtype Savannah (Wellde et al., 1974) were cultivated *in vitro* in Iscove's Modified Dulbecco's Medium (IMDM) supplemented according to Hirumi (Hirumi and Hirumi, 1991) with 1 mM sodium pyruvate, 0.5 mM hypoxanthine, 0.05 mM bathocuproinedisulfonic acid, 1.5 mM L-cysteine, 0.16 mM thymidine, 2 mM L-glutamine, 0.2 mM 2-mercaptoethanol and 20% (v/v) heat inactivated bovine serum. All cultures were maintained in a humidified atmosphere containing 5% CO₂ at 34 °C. This culture medium was used for all microcalorimetry experiments.

2.2. Standard drugs

The standard drugs, diminazene aceturate (Sigma, MW: 515.52 g/mol) and isometamidium chloride (Trypamidium-Samorin[®]; Merial, France; MW: 496.01 g/mol) were selected to study the anti-trypanosomal effect on *T. congolense*. Both standard drugs were dissolved in 100% dimethyl sulfoxide (DMSO) to obtain stock solutions of 10 mg/ml of which serial dilutions (1:10) were made in DMSO and stored at –20 °C.

2.3. Preparation of calorimetry ampoules

Growth phenotype of initially separated surface attached and detached *T. congolense* phases of a culture in the exponential growth phase were studied. Detached trypanosomes were directly transferred into a Falcon tube. Fresh culture medium was added to the surface attached phase, and the culture plate was gently scratched to collect the attached trypanosomes. Both separated phases were centrifuged for 10 min at 1840 g and 34 °C to replace the medium. The cell pellets were then resuspended in fresh culture medium to the desired initial cell density. Ampoules were filled with 1 ml of trypanosome suspension at 10⁵ cells/ml. Measurements were performed in two independent experiments on separate days.

To determine the optimal initial cell density for growth studies, ampoules were filled in triplicate with 2 ml trypanosome suspension of the mixed *T. congolense* phase containing 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10² and 10 cells/ml. Measurements were performed in two independent experiments on separate days.

Different sample volumes were tested in order to evaluate their influence on the heat flow curves. Ampoules were filled in triplicate with 0.25, 0.5, 1, 2 and 4 ml of mixed trypanosome suspension of an initial density of 10⁵ cells/ml. Measurements were performed in two independent experiments on separate days.

Viable parasite cells of samples from the ampoules (2 ml with initially 10⁵ cells/ml) were counted with a Neubauer chamber at least every 24 h for 6 consecutive days in three independent experiments, to correlate the heat flow signals with the number of viable cells. Parasite cells were detached from the ampoule surface by resuspending the

culture in the ampoule. A linear model for the regression was calculated using the statistical software R (version 3.3.2) (Fig. S2). The detection limit was calculated from the linear model using the data points of the exponential growth phase.

Antitrypanosomal effects of diminazene and isometamidium on *T. congolense* were studied in duplicate with 2 ml of 10⁵ cells/ml mixed trypanosome suspensions. The ampoules were supplemented with diminazene or isometamidium at various concentrations with a final DMSO concentration ranging from 0.03 to 0.1% for diminazene and from 0.01 to 0.1% for isometamidium. DMSO at concentrations of ≤0.1% has no effect on *T. congolense* growth (Fig. S6). Trypanosome free samples in the absence of drug served as negative controls, in duplicate. Drug free trypanosome samples served as positive controls, in triplicate. Measurements were performed in two independent experiments on separate days.

2.4. Calorimetric equipment and measurements

The isothermal calorimeter (TAM III Thermostat 249, TA Instruments, New Castle, DE, USA) was equipped with 48 channels and aluminium thermal references with a heat capacity equivalent to 2 ml of water. The calorimeter was calibrated using the built in electric heater as recommended by the manufacturer. The calibrated instrument was utilised to continuously measure heat flow of *T. congolense* samples at 34 °C. The gas phase was ambient air. Air-tight 4 ml glass ampoules were introduced into the calorimeter and remained for 45 min in the thermal equilibration position at 34 °C, before being lowered into the measuring position. The temperature of the instrument was maintained within 0.0001 °C. The sensitivity of the instrument was ± 0.2 μW, according to the manufacturer's instructions.

2.5. Analysis of calorimetric data

Heat flow changes were recorded as an electrical signal converted into watt (W). After the measurements had been recorded, the number of data points were reduced to 1 data point per 90 s intervals and exported into Microsoft Excel spreadsheets. The time required for the preparation of the ampoules was added to the final dataset. Time of drug action and growth parameters, such as onset of drug action, time to peak, time to kill and maximum growth rate μ , were calculated using the statistical software R.

The maximum growth rate μ was estimated using the heat over time curve and fitted with a logistic model (Braissant et al., 2013) using the grofit package (Kahm et al., 2010) (Fig. S4). Curves were first smoothed using a bicubic spline (Legendre and Legendre, 2012) prior to the estimation of the onset of drug action, time to peak and time to kill, because of short term noise and metabolic oscillation sometimes perturbing the signal. The time to peak was calculated as the time until the maximum of the smoothed curve was reached. Similarly, the time to kill was calculated as the time until the slope of the smoothed curve could be considered as flat again (a slope below 0.005 μW/hour, Fig. S3b). We estimated that the chosen cut-off corresponded to a detection limit/sensitivity for time to kill of 1.3×10^3 cells (assuming 4 pW/cell and 0.005 μW detection limit). Finally, for the onset of drug action, the slope of averaged controls (untreated parasites; drug free) was compared to the slope of the drug containing sample. The time at which the slopes diverged by more than 0.04 μW/hour (i.e. activity of the control was still increasing while the treated samples showed levelling and then decrease) was considered as the onset of drug action (Fig. S3a). Slopes were used for the calculations instead of raw heat flow threshold to avoid errors due to baseline shift of the calorimetric measurements.

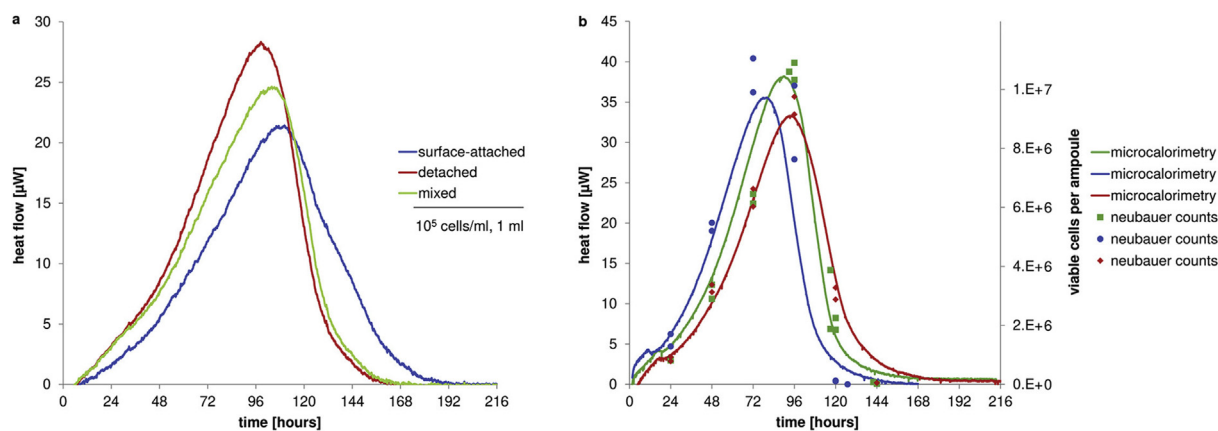


Fig. 1. Growth phenotype of initially separated and mixed *T. congolense* phases and correlation of the heat flow of *T. congolense* samples with the number of viable cells. (a) Heat flow signals over time from separated surface attached, detached and mixed *T. congolense* phases at a parasite density of 10^5 cells/ml in a 1 ml sample volume. Each heat flow curve is the mean of a single value from two independent experiments. (b) Heat flow measurements of samples with the mixed *T. congolense* phase and initial density of 10^5 cells/ml in a 2 ml sample volume and the number of viable cells per ampoules. Each heat flow curve is the mean of triplicates from one experiment.

3. Results

3.1. Growth phenotype of different *T. congolense* phases

Unlike other trypanosomes, *T. congolense* grow axenically *in vitro* in two distinct phases (Hirumi and Hirumi, 1991). Some trypanosomes are attached to the base of the culture vessel (attached phase), whilst others swim freely near the surface of the culture medium (detached phase). We monitored the growth of the individual and mixed *T. congolense* phases with the microcalorimeter. Both of the initially separated phases, as well as the mixed phase, grew well when transferred into the microcalorimeter ampoules (Fig. 1a). Only minor growth differences were observed, whereby the initially detached trypanosomes showed a slightly higher heat flow peak (28 μW) than the initially attached samples (21 μW). The heat flow peak of the mixed culture was in between. Given the small difference between attached and detached cells, we decided to perform all subsequent studies with the mixed phase.

3.2. Optimisation of initial *T. congolense* density

In order to explore the optimal inoculum for calorimetric studies with *T. congolense*, we tested starting densities from 10 to 10^7 cells/ml in a volume of 2 ml (Fig. 2a). The maximal heat flow (40 μW) was obtained starting with 10^7 cells/ml, but the parasites started to die after only 24 h. The method worked even with the lowest starting density of

only 10 cells/ml, but with this density it took almost 3 days to reach the detection limit. An inoculum of 10^5 cells/ml gave strong and robust heat flow signals that were detectable right from the beginning of the experiment and peaked after 3 to 4 days of incubation (Fig. 2a). Moreover, a comparable inoculum is used for the Alamar blue viability assay, allowing a better comparison between the results. Thus, 10^5 cells/ml was chosen as the inoculum for all subsequent *T. congolense* drug action studies.

3.3. Optimisation of sample volume

Next, we tested different culture volumes from 0.25 to 4 ml. As expected, maximal heat flow and maximal area under curve both increased with sample volume from 0.5 to 2 ml (Fig. 2b). However, at 4 ml the heat flow peak decreased again and the heat flow curve was not smooth showing humps within the first day. Thus, a volume of 2 ml was chosen for all subsequent experiments. This was in agreement with the fixed inert reference ampoule that has a heat capacity adjusted for a 2 ml specimen in our calorimeter.

3.4. Correlation of heat flow with the number of viable cells

We compared the heat flow generated by *T. congolense* cultures with the number of viable cells as determined microscopically (Fig. 1b). The heat flow strongly correlated with parasite density ($R^2 = 0.963$, Fig.

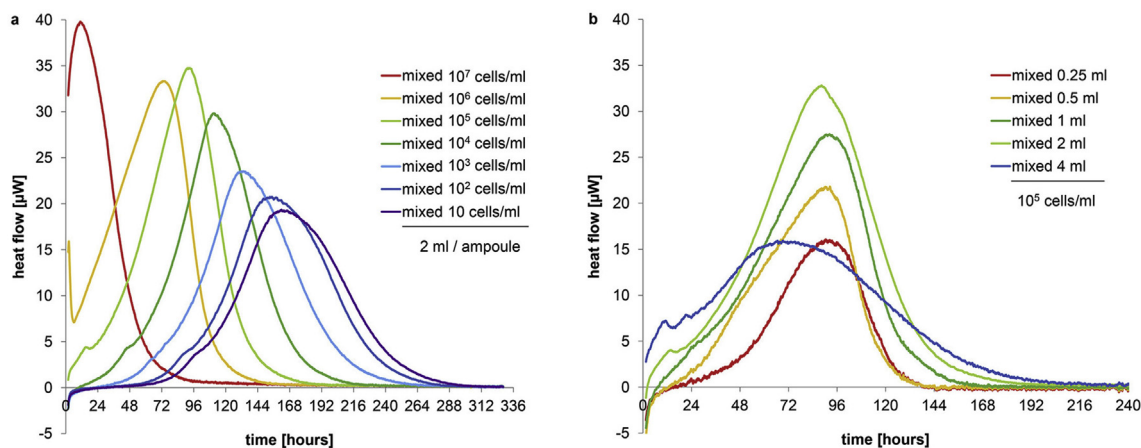


Fig. 2. Influence of *T. congolense* density and sample volume on heat flow slopes. (a) Heat flow curves of samples with different parasite densities, ranging from 10 to 10^7 cells/ml in a total volume of 2 ml. Each heat flow curve is the mean of two independent experiments, each performed in triplicate. (b) Heat flow curves of *T. congolense* parasites with an initial parasite density of 10^5 cells/ml in different sample volumes, ranging from 0.25 to 4 ml. Each heat flow curve is the mean of two independent experiments, each performed in triplicate.

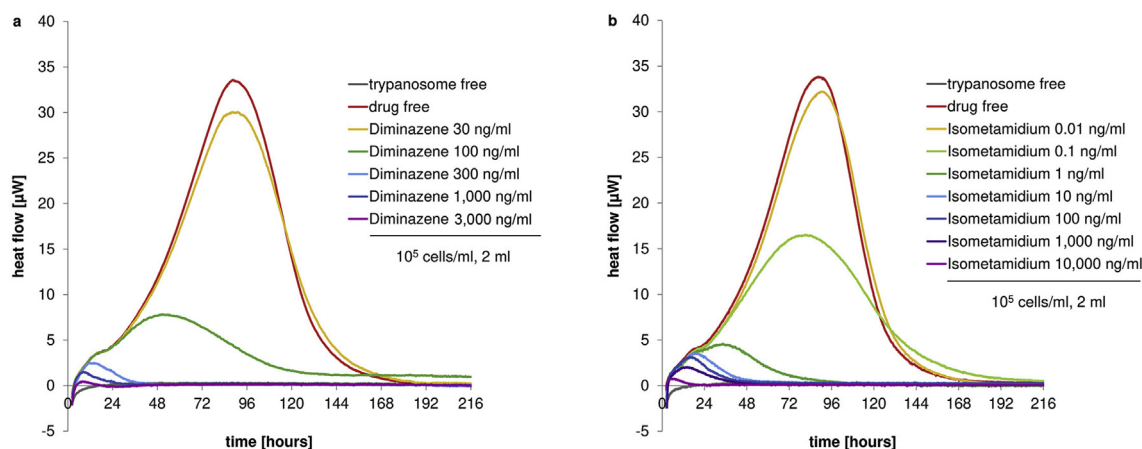


Fig. 3. Drug action by diminazene aceturate and isometamidium chloride. (a) Heat flow curves of *T. congolense* cultures with diminazene aceturate (30–3000 ng/ml) and (b) with isometamidium chloride (0.01–10,000 ng/ml). Each drug free heat flow curve is the mean of three independent experiments, each performed in triplicate and each drug containing heat flow curve is the mean of three independent experiments in duplicates.

S2). The average heat flow of a single *T. congolense* cell in the exponential growth phase was calculated to be 4 pW. The heat flow maxima were recorded after 3 to 4 days, whereas parasite numbers tended to peak a few hours later (Fig. 1b). After the stationary growth phase, the number of viable cells was slightly higher than estimated from the heat flow data, but then the cells were also slightly less motile than in the exponential growth phase (0–72 h), which probably correlates with a lower metabolic activity. The average population doubling time of the trypanosomes as calculated from the heat flow was 15 h during the exponential growth phase (0–72 h).

3.5. Time of drug action for standard drugs

Drug action was studied with the two reference drugs, diminazene aceturate and isometamidium chloride. Diminazene had an IC_{50} of 66 ± 11 ng/ml ($n = 7$) with a 3 day drug exposure time in the Alamar blue assay (Gillingwater et al., 2017). In the microcalorimeter, a dose-dependent growth inhibition was observed from 30 to 3000 ng/ml (Fig. 3a). At 100 ng/ml, diminazene substantially reduced the growth of *T. congolense*, but failed to kill all the cells as indicated by a stable heat flow signal at 2 µW (Fig. 3a). Microscopic examination of these cultures confirmed that they still contained viable trypanosomes, at a concentration of 7×10^4 cells/ml. At concentrations ≥ 300 ng/ml, diminazene quickly eliminated all parasites. The maximum growth rate μ of the integrated heat flow proved to be a sensitive parameter to monitor drug action, since for concentrations ≥ 100 ng/ml, substantially lower growth rates μ were obtained in a dose-dependent manner (Table 1). Growth inhibition was too strong at 1000 and 3000 ng/ml to be calculable in all experiments. Other *in vitro* pharmacodynamic parameters, such as onset of action and time to kill, were calculated as well and showed similar dose-dependent relationships (Table 1).

As with diminazene, a dose-dependent relationship of the *in vitro* pharmacodynamic parameters was also observed for isometamidium, albeit not as pronounced (Fig. 3b). A comparable inhibition spectrum was covered by a wider concentration range from 0.01 to 10,000 ng/ml for isometamidium than with only a 100-fold range for diminazene (Table 1). The stronger activity of isometamidium with a significant inhibition at ≥ 0.1 ng/ml, is in agreement with the higher activity of isometamidium in a 3 day Alamar blue assay showing an IC_{50} of 0.3 ± 0.1 ng/ml (Gillingwater et al., 2017).

4. Discussion

The study shows that isothermal microcalorimetry can be used as a simple, robust and sensitive technique to study small growth differences

between various *Trypanosoma congolense* samples. An expedient application is the determination of growth inhibition after the addition of antitrypanosomal drugs. The continuous measurements can provide valuable drug characterisation information, in particular regarding time of drug action. Especially, the time to kill is an essential parameter of potential new drug candidates, which can be visualised and quantified using the microcalorimetric data in a simple way without much data handling in Excel or R.

Unlike other human and animal infecting trypanosome species, *T. congolense* form *in vitro* two distinct layers. The first layer, which is placed at the bottom of the culture well, is formed by surface attached trypanosomes. This phase is always populated more densely than the second layer, herein called the detached trypanosome phase, and presents as a tight cluster of free-swimming trypanosomes. The phenomenon has already been described by Hirumi (Hirumi and Hirumi, 1991). However, no further investigations have been performed. The growth of both phases was monitored and it was observed that both showed a similar growth phenotype. Furthermore, the initially separated phases formed an equilibrium, forming new detached and attached trypanosome phases. Thus, we considered a pool of the two phases adequate for time of drug action studies.

Heat flow signals depend on the number of viable cells and their metabolic activity (Alklint et al., 2005; Braissant et al., 2013). Based on the assumption that each *T. congolense* cell has a constant metabolic activity, at least during the exponential growth phase, we plotted the heat flow versus the cell number and obtained a high correlation with an adjusted $R^2 = 0.963$ and a p-value of < 0.05 confirming our hypothesis (Fig. S2). The heat flow of a single cell in the exponential growth phase was calculated to be 4 pW. Heat flow rate and most likely metabolic activity per cell, declined slightly after the stationary phase as described previously also for mammalian cells by Kemp and Guan (1999). This demonstrates the high sensitivity of microcalorimetry measuring growth inhibition already before cell death appears. We used heat flow data (W) and not heat data (J) to correlate with viable, metabolically active cells because cells lyse and disappear after overgrowth or cell death. All the cells counted in the Neubauer chamber were viable, which was indicated by their high motility. This is different to bacteria, which accumulate in the sample and do not lyse as rapidly as trypanosomes after cell death. For bacteria, the total biomass produced correlates better with heat data (Braissant et al., 2013).

One essential parameter for the kinetics of drug action is the time to kill, which was determined by measuring the time the heat flow reached the baseline. The detection limit was around 1.3×10^3 trypanosomes (based on 4 pW/cell and 0.005 µW limit as defined for the baseline). Compared to a dense 2 ml culture with 2×10^7 cells per

Table 1
In vitro pharmacodynamic parameters of the standard drugs.

Drug	Drug dose [ng/ml]	Onset of drug action [hours] ± SD	Time to peak [hours] ± SD	Time to death/kill [hours] ± SD	Maximum growth rate μ [h ⁻¹] ± SD
Drug free	–	–	90 ± 5	187 ± 3	0.122 ± 0.003
Diminazene	30	55 ± 17	90 ± 6	191 ± 4	0.113 ± 0.005
	100	28 ± 2	52 ± 7	n.d. ^a	0.027 ± 0.005
	300	8 ± 2	13 ± 2	52 ± 7	0.009 ± 0.001
	1000	5 ± 1	9 ± 1	36 ± 8	< 0.004 ^b
	3000	< 4 ^b	< 7 ^b	< 27 ^b	< 0.004 ^b
Drug free	–	–	89 ± 5	181 ± 12	0.131 ± 0.016
Isometamidium	0.01	46 ± 21	90 ± 6	187 ± 10	0.127 ± 0.016
	0.1	34 ± 5	77 ± 8	230 ± 53	0.069 ± 0.037
	1	23 ± 2	33 ± 5	112 ± 20	0.017 ± 0.005
	10	13 ± 5	18 ± 2	60 ± 3	0.011 ± 0.004
	100	9 ± 3	16 ± 1	54 ± 6	0.009 ± 0.002
	1000	8 ± 3	14 ± 2	52 ± 8	0.006 ± 0.003
	10,000	< 3 ^b	6 ± 1	25 ± 6	0.001 ± 0.001

Results show the mean ± standard deviation of n = 9 for drug free samples and n = 6 for drug containing samples.

^a Heat flow is 2 μ W above the baseline.

^b Standard deviation could not be calculated for all samples since the drug action was too fast to calculate the parameters from all experiments.

ampoule, the detection limit corresponds to < 0.01%.

For the calculation of the maximum growth rate μ , we used heat data (J), since the slopes from the heat flow curves were similar in the exponential growth phase and changed only after the drug action had occurred (Fig. 3). Models based on heat data rely on the assumption that all cells produced, have a similar energetic turnover and remain measurable in the sample if one wants to assess correlation with cell count data (Braissant et al., 2013). As stated previously, *T. congolense* lyse rapidly in the declining phase, which would impair the use of the heat data approach. However, the heat data was used in this context as it describes how fast and how much cells grew in the presence or absence of drugs (i.e. the growth rate and the maximum growth or maximum number of cells produced). This indeed better reflects the influence of drug action on parasite growth.

Optimisation of the microcalorimetric protocol led to 10⁵ cells/ml in a 2 ml sample volume for *T. congolense* studies. Under these conditions, robust and stable signals were observed with good intra- and inter-experimental reproducibility (Fig. S1). This protocol is ideal for most research studies. However, since this technique is very robust, parameters can also be varied to address various research questions. For fast-acting drugs, it would be more favourable to use a higher initial cell density (e.g. 10⁶ cells/ml) in order to get a strong heat flow signal earlier in the experiment, enabling a read-out of an early onset of drug action. Slow-acting compounds, on the other hand, would best be studied with a lower inoculum (e.g. 10⁴ cells/ml) to avoid an overlap of drug action with cell overgrowth. The sample volume is dependent on the reference ampoule in the machine, which was fixed in our apparatus to 2 ml. Nevertheless, if the signal is strong enough, variations also of the sample volume can be applied.

In addition to the time to peak, growth rate and time to kill, also onset of drug action was determined. As seen in Table 1, onset of drug action was difficult to determine within the first 3 h. This was the time needed to prepare the samples, setting the ampoules into the machine and for the equilibration to 34 °C in the equilibration position and measuring position. In order to shorten the equilibration time, a simple injection system can be used, which offers fewer thermal disturbances and less data loss (Manneck et al., 2011).

Further expedient applications of the microcalorimetric method are studies of inoculum effect, reversibility of drug action by monitoring proliferation after a drug wash-out and drug resistance analysis; or without drugs, growth characterisation of different parasite isolates (e.g. Fig. S5) or genetic mutants.

In the present study, we demonstrated that *T. congolense* growth phenotype and drug inhibition can be monitored well by heat flow real

time measurements. In principle, this method can also be applied to other extracellular parasites. One requirement is a stable and continuous *in vitro* cultivation of axenic cultures. Next to *T. congolense* and *T. brucei*, cultivation of *T. evansi* and *T. equiperdum* has been established (Baltz et al., 1985). Hence, both of these trypanosome species have the potential to be studied in the microcalorimeter, as it was possible for *T. congolense* and *T. brucei* (Wenzler et al., 2012).

5. Conclusion

Microcalorimetry is a versatile and simple tool representing a valuable supplementation to the tool box in the drug discovery process. The microcalorimetric method can support phenotypic *T. congolense* growth studies and drug discovery programs by providing meaningful data especially for the time of drug action of new experimental compounds, as presented exemplarily with the two trypanocidal drugs, diminazene and isometamidium, used against Nagana disease.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ijpdr.2018.03.003>.

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