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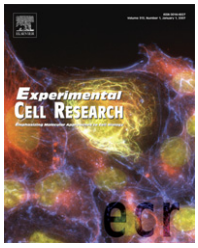

Research Article

Troglitazone induces differentiation in *Trypanosoma brucei*

Viola Denninger[a], *Katherine Figarella*[a], *Caroline Schönfeld*[a], *Stefanie Brems*[b], *Christian Busold*[b], *Florian Lang*[c], *Jörg Hoheisel*[b], *Michael Duszenko*[a,*]

[a]*Interfakultäres Institut für Biochemie, Universität Tübingen, Hoppe-Seyler-Str. 4, D-72076 Tübingen, Germany*
[b]*Functional Genome Analysis, DKFZ, D-69120 Heidelberg, Germany*
[c]*Institut für Physiologie, Gmelinstr. 5, D-72076 Tübingen, Germany*

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ABSTRACT

Trypanosoma brucei, a protozoan parasite causing sleeping sickness, is transmitted by the tsetse fly and undergoes a complex lifecycle including several defined stages within the insect vector and its mammalian host. In the latter, differentiation from the long slender to the short stumpy form is induced by a yet unknown factor of trypanosomal origin. Here we describe that some thiazolidinediones are also able to induce differentiation. In higher eukaryotes, thiazolidinediones are involved in metabolism and differentiation processes mainly by binding to the intracellular receptor peroxisome proliferator activated receptor γ. Our studies focus on the effects of troglitazone on bloodstream form trypanosomes. Differentiation was monitored using mitochondrial markers (membrane potential, succinate dehydrogenase activity, inhibition of oxygen uptake by KCN, amount of cytochrome transcripts), morphological changes (Transmission EM and light microscopy), and transformation experiments (loss of the Variant Surface Glycoprotein coat and increase of dihydroliponamide dehydrogenase activity). To further investigate the mechanisms responsible for these changes, microarray analyses were performed, showing an upregulation of expression site associated gene 8 (ESAG8), a potential differentiation regulator.

Introduction

The protozoan parasite *Trypanosoma brucei* undergoes a complex life cycle. Upon transmittance to the mammalian host, the metacyclic insect form transforms readily to the long slender bloodstream form, which multiplies logarithmically thus populating the bloodstream and lymphatic system. During the course of infection, a proportion of the long slender form differentiates into the non-dividing short stumpy form via an intermediate state. Differentiation from slender to stumpy forms is induced by a differentiation factor [1–3] and causes morphological changes and rearrangements of the parasite's metabolism [4–6]. While the slender form relies exclusively on glycolysis for energy generation, differentiation leads to an up-regulation of the activity of some mitochondrial enzymes like succinate dehydrogenase [7,8], and an increased appearance of transcription products like cytochromes [9,10]. Due to this preadaptation to conditions observed in the digestive tract of the tsetse fly, only metabolically changed parasites can transform to the procyclic midgut form while slender parasites die due to the lack of glucose. This transformation process can be mimicked *in vitro* by addition of *cis*-aconitate and citrate and a temperature shift from 37 °C to 27 °C [11]. Under these conditions the variant surface glycoprotein (VSG) coat is replaced by

* *Corresponding author*. Fax: +49 7071 29 5009.
E-mail address: michael.duszenko@uni-tuebingen.de (M. Duszenko).

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procyclin within 3 to 6 h in pleomorphic parasites [12,13]. Following this process, changes of enzyme activities, commenced in the stumpy form, are completed and the mitochondrion becomes fully active [14,15]. Finally, cells leave the G_0 phase and re-enter the cell division cycle as procyclic parasites.

To date, the trigger for the slender to stumpy differentiation has not been identified. Nevertheless the responsible substance is supposed to be a small soluble molecule of trypanosomal origin, called the "stumpy-induction-factor" (SIF), which acts via a cAMP-mediated pathway [3]. Pleomorphic as well as monomorphic strains, which are adapted to mammalian hosts by sustained syringe infections, are capable of producing this factor, although the latter seem to be less sensitive to it [1,14]. Therefore transformation of monomorphic bloodstream form cells to procyclic forms takes more time, but is nevertheless achieved. Beside the use of 50% (v/v) conditioned medium [1,14] or cell density triggers [2], which cause a synchronous and physiological differentiation to stumpy parasites, several substances have been shown to force cell cycle arrest, followed by formation of stumpy-like forms. Examples are DL-α-difluoromethylornithine, which causes a depletion of polyamines like putrescine and spermidine by inhibition of the ornithine decarboxylase [16], and 1,2-bis(methylsulfonyl)-1-methylhydrazine, a DNA-methylating agent [17]. The resulting cells showed a stumpy-like morphology, accompanied however by the occurrence of enlarged or more than one nuclei, indicating nonphysiological changes. In spite of a positive diaphorase staining, a full transformation to procyclics was not satisfactory or even impossible [16].

Since we investigated the effects of prostaglandin D_2 and its metabolites like Δ^{12} PGJ_2 and 15-deoxy PGJ_2 on bloodstream form trypanosomes [18,19], we questioned if these metabolites bind to a soluble receptor within the cell. There are several possible protein candidates and among those the peroxisome proliferating activated receptor γ (PPARγ) [20]. Troglitazone is a member of the family of thiazolidinediones, which are currently used as antidiabetic drugs due to their effects on fatty acid and glucose metabolism via PPARγ activation [21–23]. This receptor belongs to the nuclear receptor superfamily of ligand-activated transcription factors of higher eukaryotes and binds polyunsaturated fatty acids like linolenic acid or 15-deoxy PGJ_2 [24,25].

Additionally, troglitazone reduces cell proliferation and induces differentiation in different eukaryotic cells like adipocytes, keratinocytes or tumor cells [26,27]. These effects are supposed to be mediated by PPARγ activation. Nevertheless, experimental evidences have been published that both effects also could be caused by interaction of thiazolidinediones with other target proteins [28,29]. Upon treatment of bloodstream form trypanosomes with troglitazone we observed very similar effects to those described in higher eukaryotic cells, especially induction of differentiation.

Materials and methods

Trypanosomes and chemicals

Bloodstream forms of a monomorphic strain (EATRO 427, clone MITat 1.2) were used. Procyclic forms were obtained from the same strain, using a standard transformation protocol [30]. For additional morphological studies we used the pleomorphic strain AnTat1.1. Troglitazone was purchased from Biomol (Hamburg, Germany), rosiglitazone was obtained from IBL (Hamburg, Germany), ciglitazone from Calbiochem (Darmstadt, Germany); all substances were dissolved in DMSO. The final concentration of solvents in cultures was lower than 0.1% [v/v] to avoid toxic side effects. Nevertheless, the respective amount of solvent was always added to control cells as well.

Culturing conditions for growth

Monomorphic bloodstream forms were grown in axenic culture using modified MEM [14]. To start a cultivation experiment, stabilates of bloodstream form cells (stored in liquid nitrogen), were seeded at a density of 2.5×10^5 cells/ml in culture medium and propagated at 37 °C in a CO_2 atmosphere. After 20 h, cells were diluted to 2.5×10^5 cells/ml, transferred to fresh culture medium and treated with troglitazone (5 μM). Procyclic forms were cultivated in a modified MEM at 27 °C as described earlier [1]. After sterile isolation of parasites from infected mouse blood, pleomorphic cells of the strain AnTat1.1 were grown for 16 h in HMI-9 complete medium [31] containing methylcellulose (Engstler, M., personal communication).

FACS analysis

Investigation on cell cycle

The DNA-content of the nuclei was measured using propidium iodide. Cells were hypotonically lysed in 100 μl phosphate buffer (10 mM, pH 7.4, containing 100 ng/ml Rnase and 64 μM digitonine). After addition of the same volume of propidium iodide solved in PBS, samples were transferred into FACS tubes for measurements.

Determination of necrosis

Cellular permeability was assayed using propidium iodide, which stains the nuclei of necrotic cells due to plasma membrane disruption. Treated and control cells were incubated for 10 min at room temperature with propidium iodide (0.5 μg/ml) and transferred into FACS-tubes for measurements. Digitonin (6 μM) was used as a positive control to induce necrosis.

Monitoring the mitochondrial membrane potential

Following a 16 h treatment, control and treated cells were incubated at 37 °C for 30 min in the presence of tetramethylrhodamineethylester (TMRE, 25 nM). Thereafter, cells were transferred into FACS-tubes and kept on ice until measurement. Valinomycin (100 nM) was used as a positive control to abolish the mitochondrial membrane potential.

All FACS analyses were performed using a FACScalibur® cell sorter (BD Biosciences, Germany).

Electron microscopy

Treated or untreated cells (10^8 cells each) were harvested after 16 h in culture and prepared for TEM.

Fixation was performed in 2% (v/v) glutaraldehyde in 0.2 M sodium cacodylate buffer containing 0.12 M sucrose for 1 h at 4 °C. After washing four times (10 min each) and storage overnight in sodium cacodylate buffer, cells were post-fixed in osmium tetroxide (1.5%, wt/v) and stained in 0.5% uranyl acetate. Dehydration in ethanol, clearing in propylene oxide and embedding in Agar 100 was performed according to standard procedures [32]. Sections were stained in 5% (wt/v) uranyl acetate and 0.4% (wt/v) lead citrate.

Immunofluorescence

For immunofluorescence microscopy treated and control cells were harvested after the respective incubation times. 10^6 cells of each sample were spun down, washed with PBS, resuspended in 200 μl PBS and fixed by adding 300 μl of 4% paraformaldehyde/0.1% glutaraldehyde at 4 °C over night. After washing, cells were incubated for 15 min in 0.1 M Na_2HPO_4/0.1 M glycine (pH 7.2), permeabilized in 0.2% Triton-X100 in PBS and resuspended in PBS containing 1% BSA (bovine serum albumin). For staining, rabbit antiserum raised against the VSG antigen-variant 221 and the corresponding second antibody labeled with Alexa 594 (Invitrogen/Molecular Probes, Karlsruhe, Germany) were used; cells were incubated for 1 h at 4 °C each. For counterstaining DNA of all cells was stained with bisbenzimide. Images were taken by using a fluorescence microscope (Olympus BH2 RFCA/camera Olympus U-PMTVC) and processed using Adobe Photoshop.

Determination of SDH-activity

To determine succinate dehydrogenase activity, 3×10^7 cells were lysed in 10 mM phosphate buffer containing 0.01% Triton X-100. Lysates were spun down and the supernatant was replaced in phosphate buffer (17 mM; pH 7.0, containing 3 mM sodium azide and 50 μM 2,6-dichloroindophenole), before the reaction was started by addition of 100 μl succinate (final concentration: 17 mM). Formation of the reduced uncoloured form was monitored spectrophotometrically for 2 min at λ=600 nm (Ultrospec 3000; Pharmacia). The specific SDH activity was calculated by using the extinction coefficient $\varepsilon_{600\ nm}$ [DCPIP] = 21 mM^{-1} cm^{-1} [33,34] and the Bradford protein determination method [35].

Measurement of oxygen consumption

After 16 h in culture, aliquots of 3×10^7 cells each were spun down, washed twice with ice-cold phosphate buffer (20 mM, pH 7.4, containing 5 mM KCl, 80.1 mM NaCl, 2 mM $MgCl_2$, 0.15% BSA, 5 mM glucose), resuspended in 60 μl of the same buffer and stored on ice until for up to 1 h. A volume of 350 μl phosphate buffer was filled into an air-tight temperature controlled (25 °C) chamber fitted with a Clark-type oxygen electrode. After stabilization of the oxygen content, 20 μl aliquots of the cell suspensions were injected into the chamber. Inhibition of cell respiration was measured by consecutive addition of 50 μM KCN and 500 μM salicylhydroxamic acid [14,30].

Northern blotting

Northern blot analysis was performed as described earlier [36]. Briefly, total RNA from procyclic or bloodstream forms (about 2×10^8 cells each) were prepared as described in the manufacturers guidelines (QIAGEN). RNA samples were separated on a 1.2% agarose gel containing 1.2 M formaldehyde. Large rRNAs (2250, 1850, 1350 nt) were used as loading controls. RNA was transferred to a nitrocellulose membrane and hybridized with [^{32}P]-labeled DNA-probes from cytochrome C transcripts, 9S rRNA or the β-tubulin gene as control. Northern blots were visualized by autoradiography, using an X-ray developer (SRX-101A, Konica).

For confirmation of the microarray results the procedure was performed analogously, using total RNA from cells isolated directly from infected blood versus treated and non-treated cells after 14 h or 36 h incubation in bloodstream form medium (BFM) respectively, and [^{32}P]-labeled DNA-probes for the respective genes.

Transformation

After 16 h in culture, control and treated bloodstream form cells were transformed to the procyclic form using transformation medium (procyclic medium containing 3.2 mM *cis*-aconitate, 13.7 mM citrate and 33 mM glucose; pH 7.4) as described earlier [14,30]. Transformed cells were incubated for several days in procyclic medium at 27 °C monitoring cell density regularly using a haemocytometer.

Western blotting

After 16 h of incubation in BFM, aliquots were removed from control and treated cultures. Cells were hypotonically lysed and aliquots of 20 μg protein, as determined by the Bradford method [35], were separated on a 10% gel by SDS PAGE. Thereafter, gels were blotted for 1 h on a nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech) and blocked overnight with 10% commercially available milk powder in twofold concentrated PBS. Detection of dihydroliponamide dehydrogenase was performed using a polyclonal antibody and a corresponding second antibody coupled to alkaline phosphatase (Sigma).

Cytotoxicity assay

199 μl of a cell suspension (2.5×10^5 cells/ml) in each well of a 96-well plate was treated with 1 μl each of a serial dilution of the respective inhibitor. Cells were further incubated for 24 h in a CO_2 incubator at 37 °C. The EC_{50} was measured thereafter according to Bodley et al. [37], using an ELISA reader (MRX II, Dynex Technologies, Middlesex, England).

Microarray analysis and annotation

Construction of slides and hybridization conditions were performed as described previously [38]. Arrays were produced with 24,567 PCR-products created by using independent random genomic clones from *T. brucei* 927, spotted on glass slides [39].

For RNA-samples, cells of the monomorphic strain 221 (MITat 1.2) were used. According to the manufacturer's guidelines (QIAGEN), total RNA was isolated from cells incubated for 14 h in bloodstream form medium in the presence or absence of troglitazone. After reverse transcription into the respective cDNA and fluorescence-labeling of the latter, these samples were hybridized against labeled cDNA obtained from cells isolated directly out of infected blood. Dye-swapping did not lead to significant differences. Six independent experiments concerning each condition were performed.

The obtained intensity data were analyzed using the MCHIPS software package [39]. In order to filter out clearly regulated clones, only those with a fitted intensity of at least 60,000, a ratio of 4.0 and a min/max-separation of 0.1 were considered for final analysis. 57 clones with the most prominent and highly reproducible regulation were chosen to be sequenced. Identification of the respective clones was done by database searches in GeneDB using the blastn algorithm.

Results

Growth inhibition

To analyze the effects of thiazolidinediones on growth of bloodstream forms, cells were cultivated in the presence of 2 to 10 μM troglitazone, rosiglitazone or ciglitazone, respectively. The concentrations used were in the range of those effective in cell culture experiments with higher eukaryotes [40,41] and reflect the extensive binding to serum albumin (>99% for troglitazone) [42]. All thiazolidinediones showed a dose-dependent growth inhibition, with troglitazone as the most potent of all (Fig. 1A). Furthermore, addition of troglitazone after 24 h or 40 h led to a cell growth arrest, but not to a decrease in cell density and was thus not cytotoxic (Fig. 1B). Due to structural similarities (Fig. 2), α-tocopherol was also tested but had no visible effects on cell growth in concentrations up to 500 μM (data not shown).

Measurement of membrane integrity and cell cycle progress

To further investigate troglitazone induced growth inhibition, whole cells or their respective nuclei were stained with propidium iodide for FACS (fluorescence activated cell sorter) analysis in order to control for necrotic processes as well as DNA damage or cell cycle arrest. Since we found no increase of cell membrane permeability as compared with untreated cells, necrosis was excluded (Fig. 3A). Additionally no increase of DNA-degradation or polyploides could be observed, so other ways of cell death were also unlikely. As confirmed by enumeration of cells stained with bisbenzimide cell cycle arrest of treated cells as a reason for a decreased cell density could also be ruled out for the proportions being quite similar (Fig. 3B).

Measurement of the mitochondrial membrane potential

In addition we determined changes of the mitochondrial membrane potential (ψ_m), as an apoptosis marker [18,19]. However, FACS analysis of troglitazone treated parasites revealed an increased rather than a decreased mitochondrial membrane potential after TMRE (tetramethylrhodamine ethyl ester) staining [43] (Fig. 4). Similar data were observed after treatment with rosiglitazone and ciglitazone (data not shown), consistent with observations in T-cells, which also showed an enhanced mitochondrial membrane potential after thiazolidinedione induced activation of PPARγ [40].

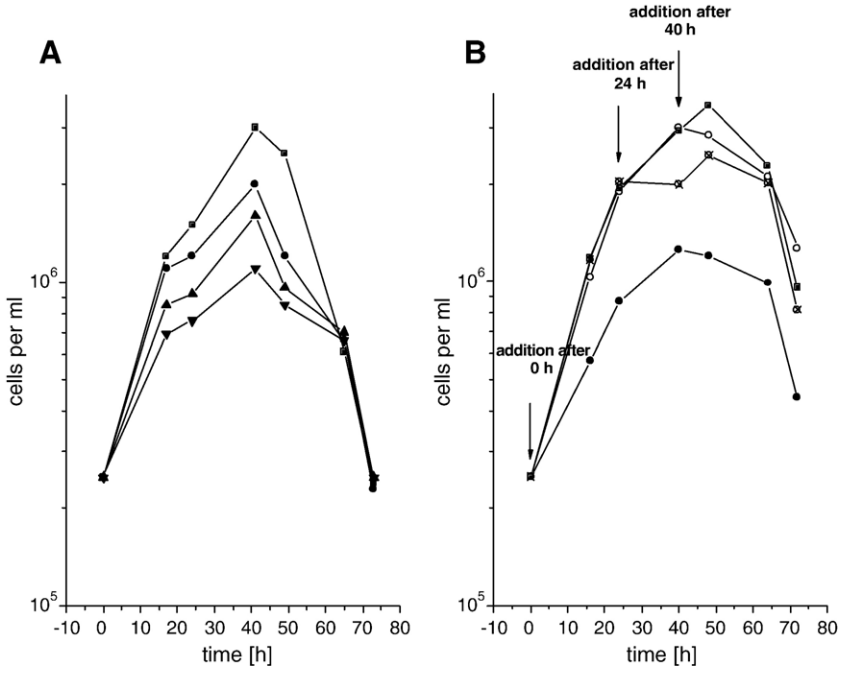


Fig. 1 – Troglitazone inhibits cell growth of monomorphic bloodstream form trypanosomes. (A) (■) control cells, (●) 2 μM troglitazone, (▲) 5 μM troglitazone, (▼) 10 μM troglitazone. (B) (■) control cells, addition of 5 μM troglitazone after (●) 0 h, (⊗) 24 h, (○) 40 h.

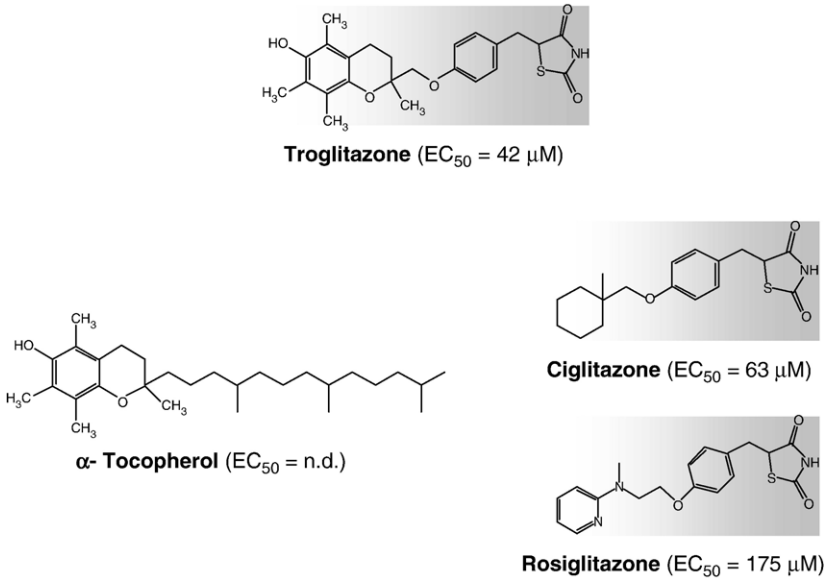


Fig. 2 – Structures and respective IC_{50} values for thiazolidinediones in comparison to α-tocopherol (n.d. = not detectable).

As confirmed by TEM, cells looked morphologically intact and showed no signs of cell damage. In addition, there was no chromatin-condensation or an elevated number of lysosomes visible. However, the mitochondrion of troglitazone-treated cells increased in size and showed an obvious augmentation of cristae structures, consistent with formation of stumpy-like forms, which usually appear only during the late stationary phase after more than 30 h in cell culture [14,15].

Activation of SDH

Since TEM revealed an elevated number of cristae structures, succinate dehydrogenase (SDH) activity was analysed. This enzyme is activated during the early stages of differentiation and serves as a mitochondrial marker for oxidative phosphorylation [4,8,10]. For this reason, bloodstream form trypanosomes were cultured for 16 h in the presence or absence of troglitazone, before SDH activity was determined. As shown in Fig. 5A, enzyme activity fairly increased in troglitazone treated parasites, as compared with control bloodstream form cells, but remained below the activity of procyclic insect forms. Since cultured control cells showed a detectable SDH activity even in the logarithmic growth phase, measurements were also performed with raw extracts of cells lysed directly after isolation from infected rat blood. These parasites expressed indeed no detectable SDH activity. It can thus be assumed that transfer to culture conditions triggers differentiation due to *quorum sensing* [1,44].

Inhibition of oxygen consumption by KCN

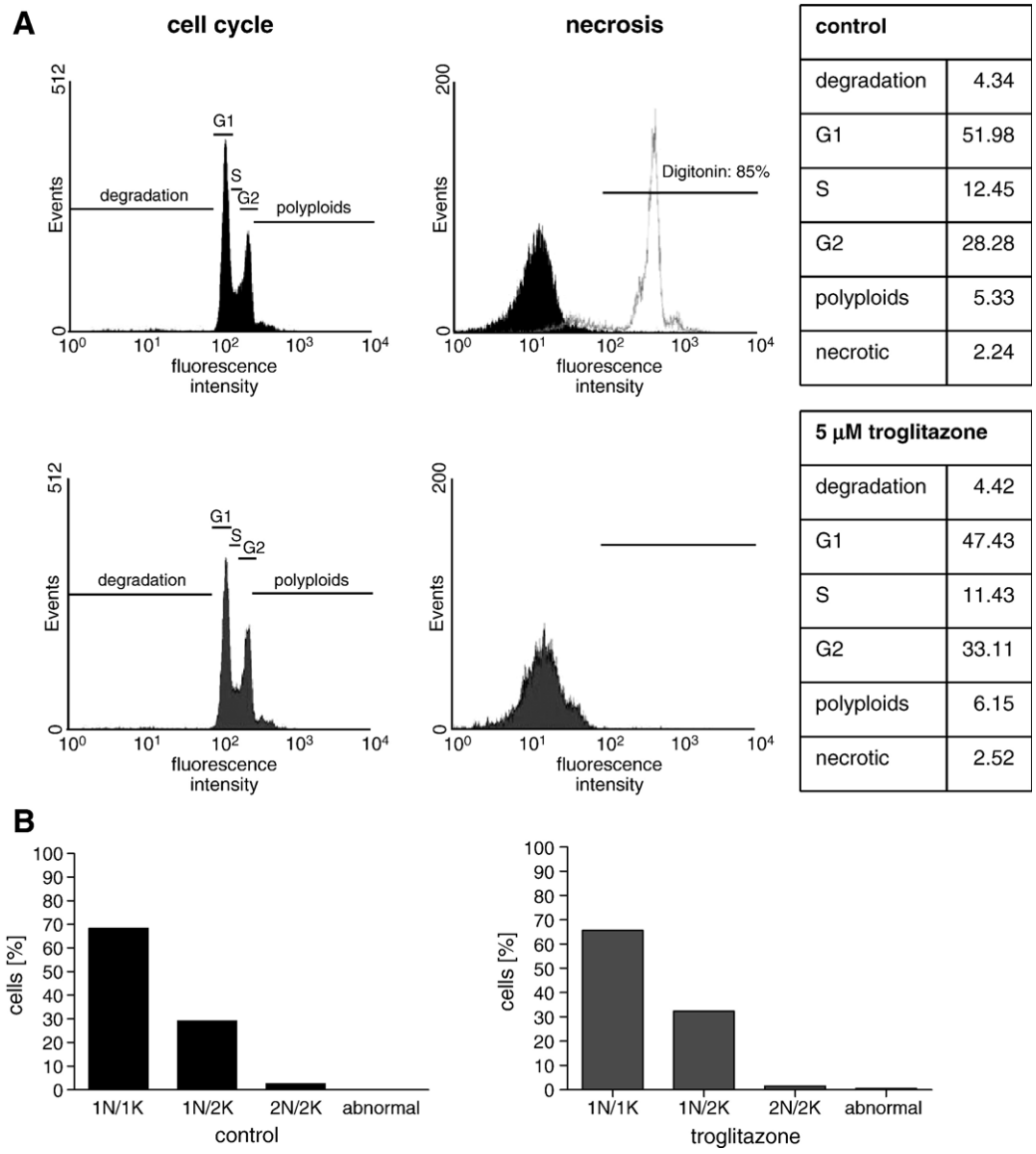
Respiratory chain reactivation is easily determined by measuring oxygen consumption. This process can be inhibited either by salicylhydroxamic acid (SHAM) or by KCN. Since SHAM blocks the alternative oxidase (TAO), an enzyme necessary to maintain the redox balance during glycolysis [45], its inhibition of oxygen uptake is indicative for the proportion of glycolysis on the total energy yield which is 100% in true slender forms. KCN, on the other hand, is an inhibitor of mitochondrial cytochromes and may thus serve as a marker for the activation of the respiratory chain. As shown in Fig. 5B, inhibition of oxygen uptake by KCN clearly shows a statistically significant increase in troglitazone treated cells, indicative for the presence of cytochromes. This confirms previous studies demonstrating a decreased inhibition by SHAM in bloodstream form cells of the stationary phase in contrast to slender forms [14]. Since TAO is active throughout the lifecycle, KCN inhibition never reaches 100%.

Cytochrome C transcripts and 9S rRNA

Due to post-transcriptional control, the amount of mitochondrial proteins is not only regulated by degradation of precursor proteins, but also by the half life time of the respective mRNAs [10]. Thus, expression of respiratory chain enzymes differs from bloodstream forms to procyclics and within bloodstream parasites from slender to stumpy. Examples for a regulation on the RNA level are cytochrome reductases, cytochrome oxidases and cytochrome *C*. Here we monitored the relative quantity of cytochrome *C* transcripts, which was reported to be 0.06 units in slender forms, 0.17 units in stumpy forms and 1.0 unit in procyclics [10].

Trypanosomes were treated for 16 h with 5 μM troglitazone, before total RNA was isolated and subject to Northern blotting; procyclic RNA was used as a positive control. Since bloodstream form cultures contain a certain proportion of the stumpy form after 16 h, a cytochrome *C* signal was also detectable in control cells. In troglitazone-treated cells, however, this band was more intensely stained. As expected, procyclics showed the highest intensity, thereby confirming published data (Fig. 5C).

In addition to the levels of mRNAs, 9S and 12S rRNA are also differentially regulated during the life cycle [9], depending on the stability of these transcripts [46]. Again, the well



control	
degradation	4.34
G1	51.98
S	12.45
G2	28.28
polyploids	5.33
necrotic	2.24

5 μM troglitazone	
degradation	4.42
G1	47.43
S	11.43
G2	33.11
polyploids	6.15
necrotic	2.52

Fig. 3 – Investigations on cell cycle and integrity after 16 h incubation with 5 μM troglitazone. (A) FACS analysis after staining of the nuclei with propidium iodide for determination of cell cycle stages, or after staining of the whole cells in order to test membrane integrity. Tests indicated no cell cycle arrest and no markers for cell death like DNA-degradation or loss of membrane integrity after treatment with troglitazone. (B) Measurement of dividing and non-dividing cell numbers by fluorescence microscopy after DNA staining with bisbenzimide, confirming the FACS data.

stained signal for the 9S rRNA in troglitazone treated cells as compared with control cells clearly indicates the higher proportion of stumpy forms under these conditions.

Transformation efficiency

All experiments described above are consistent with induction of differentiation by thiazolidinediones. Thus, transformation to the procyclic form should be readily achieved, because these parasites are preadapted for survival in the tsetse fly. As a proof of concept, trypanosomes were grown under regular bloodstream form culturing conditions for 16 h in the presence of troglitazone and subject to transformation thereafter, using a published transformation protocol [30]. To initiate transformation, cells were then incubated for 48 h in a so-called transformation medium, which is a procyclic medium containing *cis*-aconitate and citrate to induce transformation, and glucose to support ATP generation via glycolysis. Afterwards, cells were transferred into procyclic medium without glucose and with proline as primary carbon source. Stumpy parasites survive this procedure and turn readily into dividing procyclics. Indeed, the better and earlier adaptation of troglitazone treated cells was already observed during their incubation in transformation medium and was clearly obvious after transfer to procyclic medium. While the density of control cells decreased in transformation medium to a level of about 2×10^5 cells/ml and increased only very slowly in procyclic medium, the troglitazone-treated cells

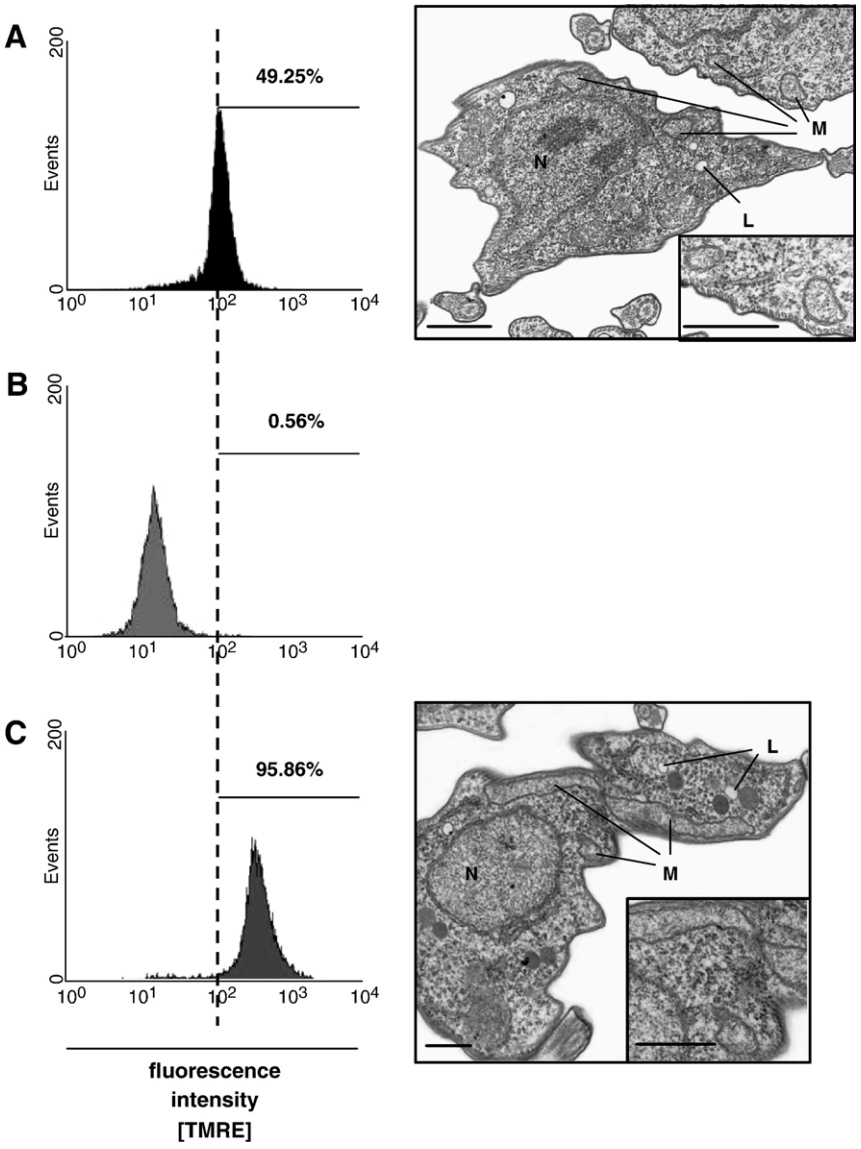
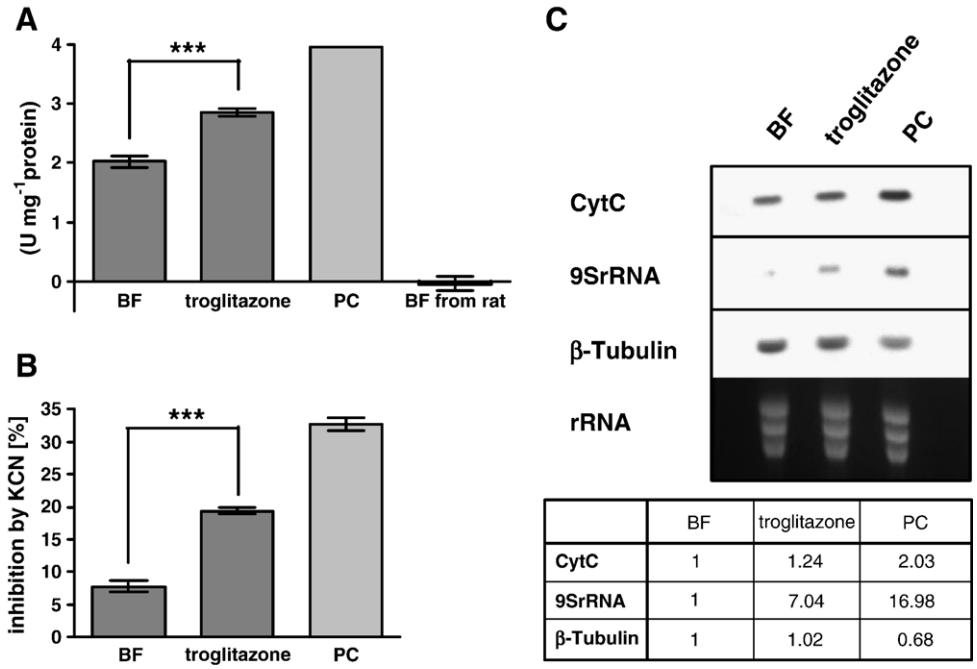


Fig. 4 – FACS analysis. Increase of the mitochondrial membrane potential by troglitazone. Cultures were seeded at a density of 2.5×10^5 cells/ml, substance solutions or solvents were added at $t=0$ min, cells were incubated for 16 h at 37 °C and stained with TMRE afterwards. (A) Control cells; (B) 100 nM valinomycin; (C) 5 μM troglitazone. Transmission EM micrographs were performed after 16 h incubation in bloodstream form medium with 5 μM troglitazone (bar = 0.5 μm). Control cells show a small and poorly developed mitochondrion, the mitochondrion of troglitazone-treated cells increases in size and shows more cristae. The nuclei of treated and untreated cells do not show any sign of DNA condensation and there is no increase in lysosomes visible. Nucleus (N); lysosomes (L); mitochondrion (M).

survived well and grew up immediately in procyclic medium (Fig. 6).

After a 16 h cultivation in bloodstream form medium, expression of an additional marker enzyme was monitored: dihydroliponamide dehydrogenase (DHLADH). This protein is encoded in the nucleus and is present in the plasma membrane and in the mitochondrion as part of the 2-oxo acid dehydrogenase complex [47,48]. Accordingly, bloodstream form trypanosomes possess a minor DHLADH activity, which is upregulated during differentiation [49] and reaches its maximum after transformation to procyclics. Using a DHLADH specific antibody [50], we investigated the level of enzyme expression by Western blotting. While the expression level of this enzyme was low in bloodstream form control cells and high in procyclics, troglitazone-treated cells showed an upregulated expression level, thus confirming an increased formation of stumpy forms (Fig. 7A).

In addition to enzyme expression, loss of VSG was monitored by SDS-PAGE and Coomassie staining of cell lysates and visualized by immunofluorescence of the cells. Aliquots were taken after 16 h in bloodstream form medium and then after 8, 24 and 48 h in transformation medium (Figs. 7B and C). As judged from immunofluorescence using VSG- or procyclin-specific antibodies respectively, shedding of VSG from troglitazone-treated cells was faster than from control parasites with the concomitant expression of procy-



	BF	troglitazone	PC
CytC	1	1.24	2.03
9SrRNA	1	7.04	16.98
β-Tubulin	1	1.02	0.68

Fig. 5 – Induction of enzyme expression by troglitazone. Bloodstream form trypanosomes were incubated with or without 5 μM troglitazone for 16 h and both cultures were measured using procyclics as a positive control. (A) Activity of succinate dehydrogenase increases by troglitazone. (B) Oxygen consumption of treated trypanosomes is more inhibited by KCN in comparison to untreated control cells. Statistical significance was evaluated by the Student's *t*-test (*$p<0.001$; **$p<0.01$; *$p<0.05$). (C) The amount of cytochrome *C* transcripts and 9S rRNA is up regulated by troglitazone as measured by Northern blot analysis. The UV-illuminated gel with the three large rRNA bands is shown below each blot. β-tubulin probes were used as a control. The differential integrated density (DID) of each spot was quantified by GelScan V5.1. Afterwards the DID's of the BF control were set as 1.0 and the correspondent DID's of the other samples were correlated.**

clin on the cell surface (data not shown). The differences concerning cell density were even more obvious and showed that 30% of control cells died within the first 24 h in transformation medium, while in the treated culture the cell number increased twofold.

Microarray experiments

In order to get further insight into the molecular changes, responsible for the induction of differentiation by troglitazone, we performed microarray experiments. Since populations of monomorphic parasites directly isolated from blood of an infected mouse consist exclusively of the slender form, we used RNA of these cells as control. In this way, regulation due to normal cultivating conditions after 14 h incubation (log-phase) and troglitazone induced changes could be measured and compared. In addition, Northern blots were performed to check consistency of the results.

Measurements of SDH activity and KCN inhibition (Figs. 5A and B) suggest that even untreated cells began to differentiate after transfer to culture conditions due to SIF accumulation. The microarray results of Fig. 8 confirmed this, because most of the differentially expressed genes of the log-phase control showed the same tendency of regulation as with troglitazone. Nevertheless we observed some differences and most of the regulations were more pronounced after troglitazone treatment.

Among the regulated clusters, upregulation of some of the expression site associated genes (ESAGs) was most striking. So far there are 12 ESAGs known which are located within the expression site cassette at telomeres and cotranscribed with VSG.

ESAG4 was upregulated *in vitro* with and without troglitazone. However, as shown by Northern blot analysis, the increase was most pronounced in the stationary phase after troglitazone treatment. This protein belongs to the group of adenylyl cyclases [51], which are also encoded in other areas of the genome, then called GRESAGs (genes related to ESAGs). They all have a conserved C-terminus and a single transmembrane domain, but differ in their ligand binding domain at the N-terminus outside of the membrane. ESAG4 was proven to be located on the surface of the flagellum [52] and responds to differentiation [3] and stress signals [53].

In contrast, ESAG8 is the only non membrane protein member of the expression site and is exclusively encoded there. Its structure, containing a ring finger motif and 18 leucine-rich repeats, seems to be involved in protein–protein interaction [54]. All functions which have been reported so far are indicative of a role in cell cycle regulation, either by binding to PIE8 and activation of the mitochondrion [55] or through interactions with a protein of the puf-family [56]. As supported by Northern blot analysis, the amount of ESAG8 increases after entering the stationary phase or after troglitazone treatment. Concerning the microarray experiments, this

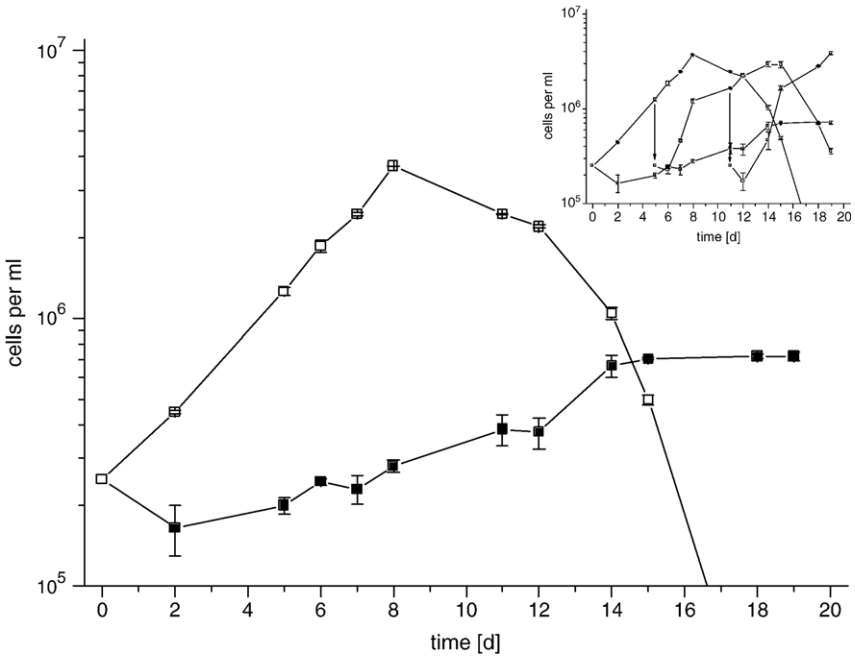


Fig. 6 – Improvement of transformation from bloodstream form trypanosomes to procyclic forms by troglitazone. Shown are the growth curves of procyclic cells, transformed out of treated and non-treated bloodstream form cells: (■) control cells and (□) 5 μM troglitazone. Parasites were incubated for 16 h in bloodstream form medium (BF medium), adjusted to a density of 1×10^6 cells/ml and transferred into transformation medium (TF medium) where they were incubated at 27 °C for 48 h. Finally, they were transferred into procyclic medium (PC medium) and cell density was adjusted to 2.5×10^5 cells/ml. In PC medium, only troglitazone treated cultures were diluted twice and grown up again (inserted image), because the cell density of the control culture was too low.

was the most striking upregulation found due to troglitazone treatment, being two-fold higher as during log-phase control conditions and over 11 times higher than under control conditions using fresh isolates.

Another regulation cluster concerns enzymes involved in proteolysis. We detected an increased amount of transcripts of the ubiquitin activating enzyme, involved in proteasome-dependent proteolysis, and of a calpain-like cystein peptidase. Normally, calpains are Ca^{2+}-dependent cytosolic enzymes involved in signal transduction and cell differentiation [57]. Several calpain-like peptidases exist with enough similarity to bind to the respective clone-sequence spotted onto slides. Nevertheless their function remains unclear, because they do not encode typical calpains as known from higher eukaryotes [58]. Irrespective of the fact that all kinetoplastid calpains lack a domain IV containing the EF-hand motive for Ca^{2+} binding, the transcript we detected by microarray analysis contains only the domain I^{κ}, a kinetoplastid specific sequence with a yet unknown function, which belongs to the "short calpain-like proteins lacking domain II" [58]. Using the transcript-sequence of the smallest candidate as a probe for Northern blotting, we got two different bands. The shorter transcript was quite strongly expressed at all times, whereas the higher molecular weight transcript was only slightly expressed during the log-phase of treated and untreated cells. Nevertheless the amount of both transcripts increased within the stationary phase and by troglitazone-treatment, in accordance with the microarray data.

The two upregulated heat shock proteins (HSP) are located in different compartments of the cell. Both HSP's are upregulated under culture conditions compared to RNA samples from fresh isolates, probably due to the change of the environment. HSP83 which increases slightly more during troglitazone treatment is a cytosolic protein with various functions in higher eukaryotic cells. Beside its function as a chaperone, it stabilizes transcription factors and interacts with cell cycle kinases [59]. In addition, the kinetoplastid homologue has a far stronger ATPase activity [60]. HSP60 prevents protein aggregation and is responsible for protein refolding after the precursers have passed the mitochondrial membrane [61].

The functions of other troglitazone-regulated proteins are so far unclear. Especially interesting is Tb927.3.3430, which is strikingly upregulated and thus likely to be involved in differentiation, but has not been correlated with cellular function yet.

Morphology study with the pleomorphic strain AnTat1.1

Differentiation from long slender to short stumpy forms not only concerns activation of the respiratory chain and easy transformation into procyclic forms, but also changes in morphology [7]. Monomorphic parasites are adapted to the mammalian host by sustained syringe infections and are thus selected for fast growth. Since we used the monomorphic strain MITat1.2 for our investigations, morphological differences are not so obvious to quantify by microscopy. We

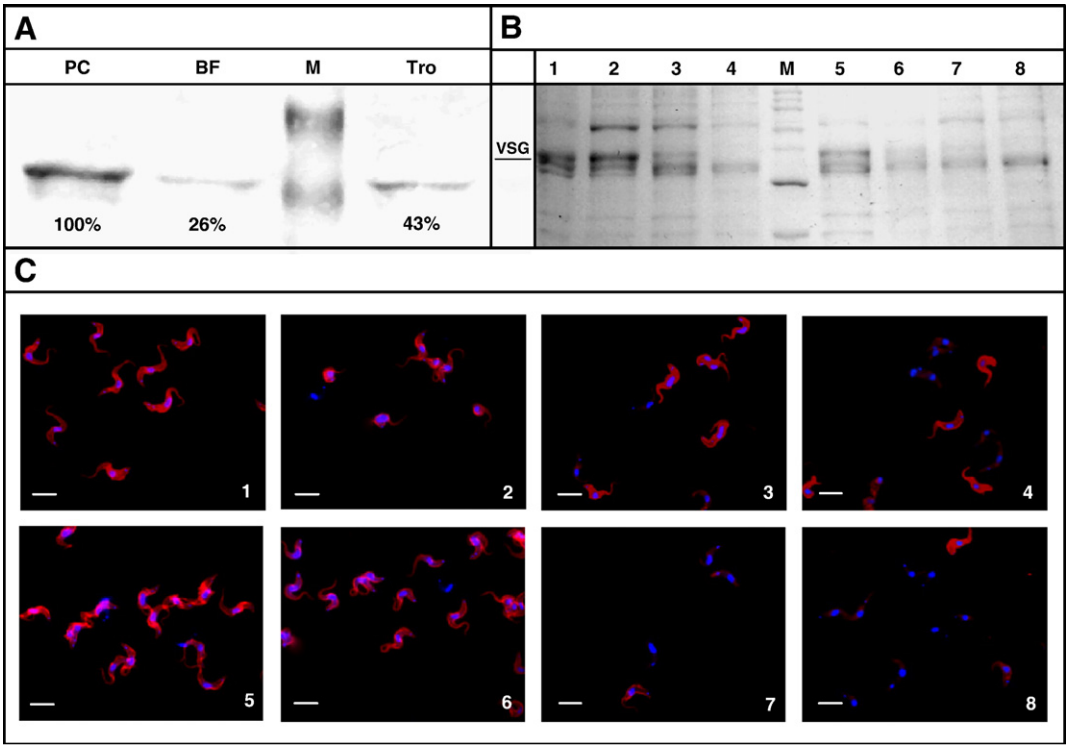


Fig. 7 – Observation of the transformation process shown in Fig. 6 by Western blotting, VSG-decrease and morphological studies. (A) Western blot analysis detecting dihydroliponamide dehydrogenase (DHLADH) after 16 h in BF medium. While the bloodstream form control (BF) contains 26% DHLADH protein in comparison to the procyclic forms (PC), the amount within the troglitazone-treated cells is up regulated to 43%. The 60.7 kDa and 47.4 kDa lanes of the marker are shown. For quantification of the differential integrated density (DID) GelScan V5.1 was used. DID's of treated and non-treated cells were correlated to the PC-DID. (B) Decrease of VSG visualized by Coomassie-staining. Samples were taken after 16 h in BF medium (first lane) and after 8 h, 24 h and 48 h in TF medium (second to fourth lane). Lanes 1–4: control cells; lanes 5–8: troglitazone treated cells; M: protein marker containing proteins with 40, 50, 60, 70, 80, 90, 100 and 120 kDa respectively. (C) Immunofluorescence for visualizing VSG-decrease after 16 h in BFM (first column), 8 h, 24 h and 48 h in transformation medium, counterstained with bisbenzimide. (1–4) control cells, (5–8) troglitazone treated cells, (bar = 10 μm).

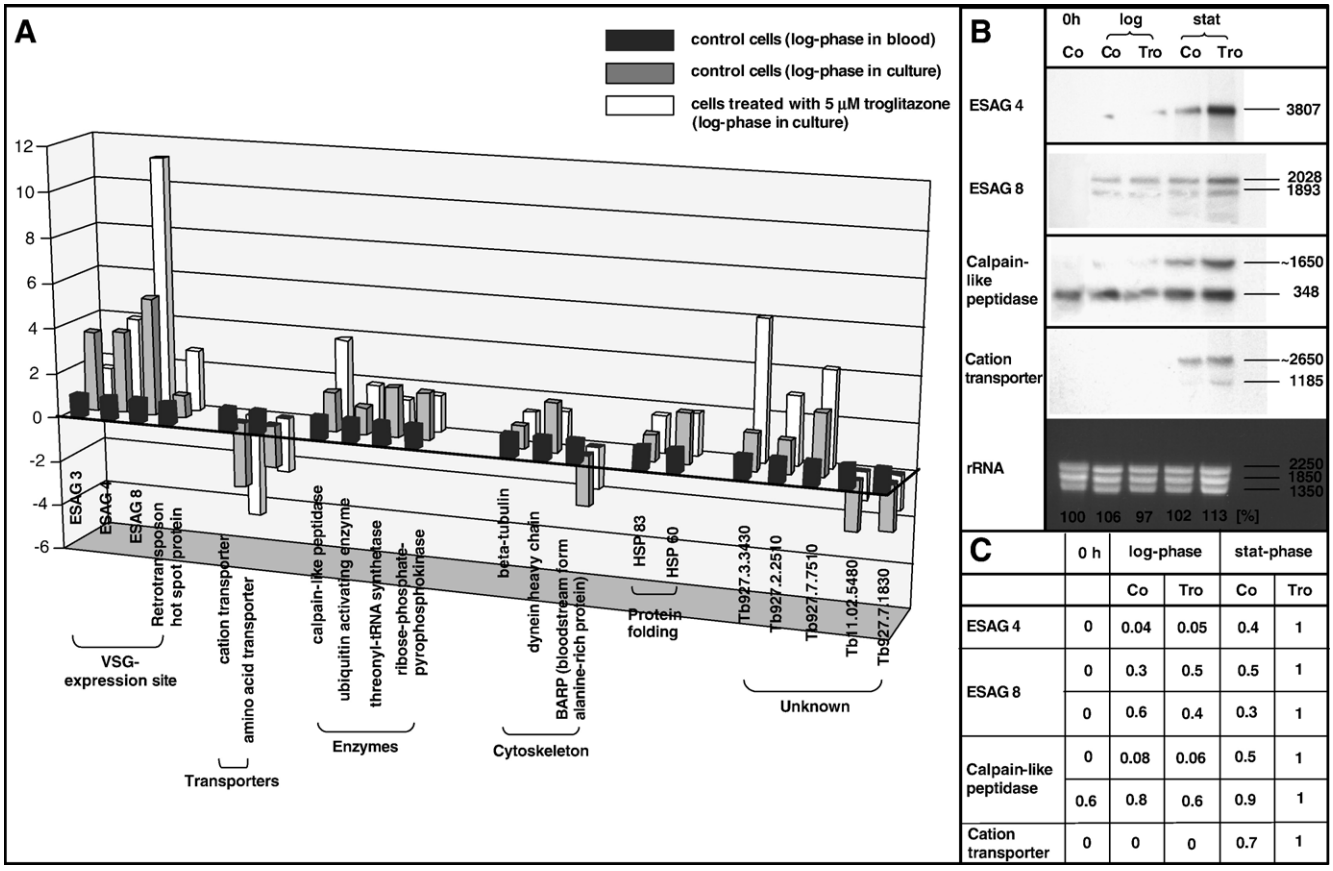
therefore performed additional morphological studies using the pleomorphic strain AnTat1.1.

Following a mouse infection, parasitemia was monitored daily and parasites were isolated under sterile conditions from blood after the population consisted of more than 80% slender cells. Trypanosomes were transferred to *in vitro* culture and treated with troglitazone. After 16 h, the troglitazone treated cultures contained about 62% stumpy forms, as compared to 22% under control conditions (Fig. 9). This result is consistent with those obtained with the monomorphic strain.

Discussion

In contrast to previous reports [16,17], we could show differentiation of slender bloodstream forms into further developed forms in a monomorphic strain, without any obvious impairment of cellular functions. Bisbenzimid staining showed no increase of enlarged nuclei or multinucleated cells. Necrosis or loss of mitochondrial membrane potential was not detectable by FACS-analysis, and TEM, SEM or light microscopy showed no indication of cell damage. Using a monomorphic strain for the main part of this study had the advantage of easy to handle cultures and the benefit of sufficient amounts of cells. The disadvantage of less obvious morphological changes was overcome by the additional use of a pleomorphic strain. Troglitazone treated monomorphic parasites traversed all the steps in mitochondrial development which normally occur during differentiation [10], including upregulation of dihydroliponamide dehydrogenase, succinate dehydrogenase, cytochrome C transcripts and, additionally, 9S rRNA. Electron-microscopy affirmed these data, since mitochondria of troglitazone treated cells were clearly enlarged and further developed as those of control cells.

Transformation experiments showed that troglitazone treated cells were far more capable of developing to procyclic cells than control cells. 8 h after transfer to transformation medium, about one third of control cells showed an abnormal morphology, indicating that they had not left the slender stage during the previous incubation in bloodstream form medium. In contrast, troglitazone treated cells remained morphologically intact. It is no contradiction that the proportion (not the absolute number) of procyclic cells was similar in both cultures after 48 h, as only adapted cells can survive until then. Cell density within the treated culture was about 3–5-fold higher (depending on the experiment).



C

	0 h	log-phase		stat-phase	
		Co	Tro	Co	Tro
ESAG 4	0	0.04	0.05	0.4	1
ESAG 8	0	0.3	0.5	0.5	1
	0	0.6	0.4	0.3	1
Calpain-like peptidase	0	0.08	0.06	0.5	1
	0.6	0.8	0.6	0.9	1
Cation transporter	0	0	0	0.7	1

Fig. 8 – Microarray- and Northern blot analysis. (A) Microarray data. TotalRNA of cells incubated 14 h with or without 5 μM troglitazone was hybridisized against totalRNA-samples of cells directly out of the bloodstream. Differential fluorescence intensities were analyzed as previously described [38,39]. (B) Northern blots. As a control Northern blots for some transcripts were performed. In addition to the log-phase, treated and control cells were also harvested in the stationary phase in order to see a further regulation. The data also show an upregulation of the respective transcripts, especially ESAG4 and 8, confirming the microarray data. (C) The differential integrated density (DID) of each spot was quantified by GelScan V5.1. Afterwards the RNA loadings were equalized, the DID's of the spots with the highest density were set as 1.0 and the correspondent DID's of the other samples were correlated.

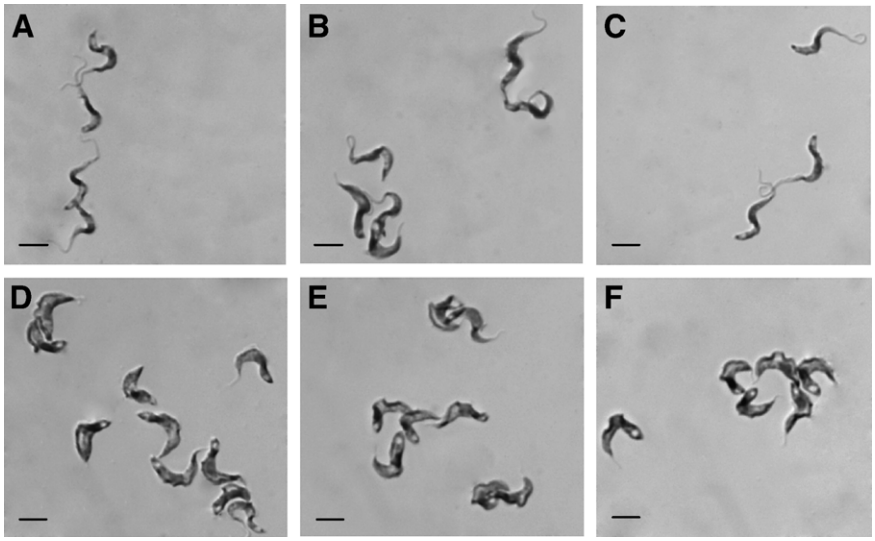


Fig. 9 – Morphology of pleomorphic cells of the strain AnTat1.1 after 16 h incubation in HMI-9 complete at 37 °C. (A–C) Control cells, (D–F) cells treated with 5 μM troglitazone (bar = 10 μm).

We also tested the transformation efficiency of cells treated with ciglitazone or rosiglitazone. Although it was elevated in comparison to control cells, it was lower than in troglitazone-treated parasites, as judged by the FACS data of the mitochondrial membrane potential and morphology observations by TEM (data not shown). Interestingly, treatment with 15-deoxy-PGJ_2 (a physiological ligand of PPARγ) resulted in the reverse effect, i.e. cells were forced to die faster in transformation media, consistent with our observations that prostaglandin treatment of trypanosomes led to apoptosis [19,44]. Additionally we used two high affinity antagonists, T0070907 [62] and SR-202 [63] to abolish the troglitazone effect. However adding these substances together with troglitazone revealed no influence on transformation potency (data not shown).

In order to verify the specificity of the observed effects on trypanosomes, we also tested different concentrations of α-tocopherol on bloodstream form cells. This antioxidant shares the lipophilic tail with troglitazone, i.e. the condensed ring system (Fig. 2). However, α-tocopherol had no effect on cell growth and proved to be virtually non toxic to trypanosomes, as determined by a cytotoxicity assay ($ED_{50} \geq 500$ μM). This suggests that the thiazolidinedione-residue of troglitazone is responsible for the decreased generation doubling time. Nevertheless, we failed in demonstrating a cell cycle arrest in G_1/G_0, which was considered a prerequisite for stumpy formation [3]. In fact, experimental and statistical evidence suggests that the onset of biochemical and morphological changes is initiated by a differentiation factor well before cell cycle arrest [16,64,65]. As discussed earlier, we rather consider slender to stumpy differentiation as a multi purpose process leading to intermediate forms pre-adapted to transformation in the fly's midgut and cell cycle arrested stumpy forms bound to undergo apoptosis as a mechanism to control cell density in the mammalian host [44].

Many investigations on different higher eukaryotic cells have been reported, showing an inhibition of proliferation and an induction of differentiation by thiazolidindiones, especially troglitazone [26,27,29]. Normally this substance class is most potent and selective to activate PPARγ [25], while the physiological ligands like linolenic acid, 9-hydroxyoctadecadienoic acid, 13-hydroxyoctadecadienoic acid or 15-deoxy-PGJ_2 show only moderate affinity to this protein [24,25,66]. Since the activation mechanism of this transcription factor is a complex interaction with the RXR-receptor, many cofactors [26] and corepressors [67], the major physiological role of PPARγ remains to be solved. Recently a number of PPARγ-independent effects of thiazolidinediones have been reported. These imply induction of apoptosis by inhibition of Bcl-xL/Bcl-2 functions [28], binding to newly discovered mitochondrial enzymes and inhibition of cell proliferation by ablation of cyclin D1 involving proteasome facilitated proteolysis [68,69] or by suppression of casein kinase 2 activity [70]. Troglitazone turned out to be the most potent of the thiazolidinediones, followed by ciglitazone and rosiglitazone. Interestingly, this is in reversed order to their respective affinities to PPARγ.

Our results show that the effect of the thiazolidinediones, especially troglitazone, on slender to stumpy differentiation is highly specific and depends on the shared hydrophilic residue of this substance class. It was assumed that trypanosomes show exclusively post-transcriptional regulation of gene expression, until the first DNA-binding protein complex was shown to have a transcription-activating function on *SL*-RNA synthesis [71,72]. The involvement of an ancient form of a PPARγ analog cannot be excluded anymore. However, since we could not find any gene with significant homology to PPARγ by database searches, we rather favor a PPARγ independent action of troglitazone on differentiation of *T. brucei*. It is tempting to speculate that thiazolidinediones share some structural homologies with the proposed stumpy induction factor (SIF), produced by the parasite.

To get further insights into the mechanisms involved in troglitazone induced differentiation and the still not fully discovered interplay of proteins involved in this process, we performed microarray analyses. The striking increase of ESAG8 transcripts is not due to a general upregulation of silent VSG expression sites after DNA damage [73], since there was a specific increase in ESAG8 and also ESAG4 transcripts, but not in other expression site associated genes. This is consistent with reports, showing a VSG-independent regulation of ESAG8 [56,74] and ESAG3, which was even downregulated by troglitazone treatment. Taken together, our results of troglitazone-induced differentiation and observations made by other groups (which are based on localization, structure, stage-specific expression and the low abundance of transcripts) [54], support the role of ESAG8 as a cell cycle regulator. Its sequence obtains high similarity with the Ras-interacting domain of the yeast adenylyl cylase and could thus play a role in cAMP-mediated differentiation. Beside, its ring-finger motif is indicative either for protein–protein- or protein–DNA-interaction. Hoek et al. [56] have reported the occurrence of several high molecular weight fractions containing ESAG8, one of them being the PUF1–ESAG8 complex. On the other hand, ESAG8 may act as an ubiquitin-ligase, which could lead to the degradation of cyclins and cyclin-dependent kinases [54,56].

The import of respiratory-chain proteins into the mitochondrion could be supported by both the upregulation of HSP60 and the troglitazone induced increase of the mitochondrial membrane potential [75], which is an important premise.

Further studies will trace these questions and will be focused on the expression pattern of proteins in comparison to their transcripts, because of the post-transcriptional and post-translational regulation in *T. brucei* [76]. This and investigations of functionally unknown proteins will be a helpful tool towards a better understanding of the slender to stumpy differentiation process and maybe will fit together the pieces of the puzzle.

Acknowledgments

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