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Cyclic AMP effectors in African trypanosomes revealed by genome-scale RNAi library screening for resistance to the phosphodiesterase inhibitor Cpd A

Running Title:

cAMP effector genes in *T. brucei*

Authors:

Matthew K. Gould[1,4,*], Sabine Bachmaier[2,*], Juma A. M. Ali[1], Sam Alsford[3], Daniel N. A. Tagoe[1,5], Jane C. Munday[1,5], Achim C. Schnaufer[4], David Horn[3,$], Michael Boshart[2,#] and Harry P. de Koning[1,#]

Author Affiliations:

[1] Institute of Infection, Immunity & Inflammation, College of Medical, Veterinary & Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom

[2] Biocenter, Section Genetics, Ludwig-Maximilians-Universität München, 82152 Martinsried, Germany

[3] Faculty of Infectious & Tropical Diseases, London School of Hygiene & Tropical Medicine, London WC1E 7HT, United Kingdom

[4] Centre for Immunity, Infection & Evolution, Institute of Immunology & Infection Research, University of Edinburgh, Edinburgh EH9 3JT, United Kingdom

[5] Wellcome Trust Centre for Molecular Parasitology, University of Glasgow, Glasgow, UK

[$] Current address: Biological Chemistry & Drug Discovery, College of Life Sciences, University of Dundee, Dow Street, Dundee DD1 5EH, UK.

* These authors contributed equally.

[#] Joint corresponding authors.

E-mail:

Harry.De-Koning@gla.ac.uk

boshart@lmu.de

Abstract

One of the most promising new targets for trypanocidal drugs to emerge in recent years is the cAMP phosphodiesterase activity encoded by *TbrPDEB1* and *TbrPDEB2*. These genes were genetically confirmed as essential, and a high affinity inhibitor, Cpd A, displays potent anti-trypanosomal activity. To identify effectors of the elevated cAMP levels resulting from Cpd A action and, consequently, potential sites for adaptations giving resistance to PDE inhibitors, resistance to the drug was induced. Selection of mutagenised trypanosomes resulted in resistance to Cpd A, as well as cross-resistance to membrane-permeable cAMP analogues, but not to currently used trypanocidal drugs. Resistance was not due to changes in cAMP levels or in *PDEB* genes. A second approach, a genome-wide RNAi library screen, returned four genes giving resistance to Cpd A upon knockdown. Validation by independent RNAi strategies confirmed resistance to Cpd A and suggested a role for the identified cAMP Response Proteins (CARPs) in cAMP action. CARP1 is unique to kinetoplastid parasites, has predicted cyclic nucleotide binding-like domains, and RNAi repression resulted in over 100-fold resistance. CARP2 and CARP4 are hypothetical conserved proteins associated with the eukaryotic flagellar proteome or with flagellar function, with an orthologue of CARP4 implicated in human disease. CARP3 is a hypothetical protein, unique to *Trypanosoma*. *CARP1-4* likely represent components of a novel cAMP signaling pathway in the parasite. As cAMP metabolism is validated as a drug target in *T. brucei*, cAMP effectors highly divergent from the mammalian host, such as *CARP1*, lend themselves to further pharmacological development.

Introduction

Human African Trypanosomiasis (HAT or sleeping sickness) is a potentially lethal parasitic disease caused by two subspecies of *Trypanosoma brucei*: *T. b. rhodesiense* and *T. b. gambiense*, which have distinct geographical distributions (1). A third subspecies, *T. b. brucei*, is non-human infective but, alongside *T. vivax* and *T. congolense*, causes huge economic damage through the infection of domestic animals such as cattle, causing a disease known as Nagana or animal African trypanosomiasis (AAT) (2). *T. brucei* are transmitted to their mammalian hosts via the mouthparts of infected blood-sucking tsetse flies (3). Millions of people in sub-Saharan Africa are at risk of this infection, with over 175,000 cases reported between the years 2000 and 2009 across 25 countries (4); with an estimated three-fold under-reporting (5), as many as half a million people could actually have been infected. In the early stages of the infection (Stage I) the trypanosomes proliferate in the peripheral bloodstream and lymph, causing a relatively mild disease of intermittent fever and general malaise but the penetration of the parasite into the central nervous system (Stage II) causes severe neurological symptoms followed by coma and, almost invariably, death (6).

The treatment for Stage I HAT is pentamidine for the *T. b. gambiense* infection and suramin for *T. b. rhodesiense*, but since these compounds have at best minimal capacity to cross the blood-brain barrier, they are not suitable for treatment of the second stage of infection (7). Chemotherapies available for Stage II HAT are melarsoprol or eflornithine. Melarsoprol is a drug based on arsenic and can have very severe side-effects, with up to 5% of patients dying from reactive encephalopathy due to the drug (6). On top of the potential toxicity, resistance to melarsoprol appears to be increasing with treatment

failure rates as high as 37% in some regions (8). Current models describe the loss of one or more transporters including the TbAT1/P2 adenosine transporter (9), the high-affinity pentamidine transporter (HAPT) (10) and the aquaporin TbAQP2 (11, 12) as being involved in pentamidine/melarsoprol cross-resistance. Eflornithine is only effective against *T. b. gambiense* infections and is difficult to administer, requiring hospitalization and intravenous infusions every six hours for two weeks (7), although a recently introduced combination therapy of nifurtimox and eflornithine (NECT) has reduced the treatment burden (13). However, NECT is still not effective against *T. b. rhodesiense* and the need for more effective drugs with fewer side-effects and no cross-resistance is clearly urgent.

From mammals to protozoa and prokaryotes, cAMP generated by adenylate cyclases is an intracellular second messenger in cell signaling. The increase in cAMP concentration transduces the initial stimulus down the signaling cascade by activating or deactivating effector proteins, such as kinases. In *T. b. brucei*, a crucial role of cyclase activity, encoded by the most abundant *ESAG4* gene product and probably other members of the large family of adenylate cyclases, is to produce extracellular cAMP as part of the parasite's ability to subvert the host innate immunity upon infection (14).

The impact of changes in intracellular cAMP concentration on trypanosomes is evidenced by the severe phenotypes upon altered expression of enzymes involved in cAMP metabolism. Elevated cAMP is degraded to AMP by phosphodiesterases (PDEs) (15), of which there appear to be four distinct families in *T. b. brucei* (16, 17). Recently, cAMP-specific PDEs have been validated genetically and pharmacologically as excellent drug targets in the parasite (18–20). The combined activity of the two members of the

PDEB family was shown to be essential in bloodstream form trypanosomes. Simultaneous RNAi knock-down of both *PDEB* genes in *T. b. brucei* bloodstream forms generated an uncontrolled and sustained increase in cAMP concentration, resulting in cytokinesis defects producing multi-nuclear and multi-flagellated cells that eventually die (18). A similar impaired-cytokinesis phenotype is produced by repression of adenylate cyclase activity (21). This apparent paradox suggests that fine tuning of cAMP levels plays a role in regulation of cell division, with extreme or deregulated concentrations in either direction being detrimental (see discussion in (21)).

A phenotype similar to *PDEB* RNAi is also observed when bloodstream form trypanosomes are exposed to Cpd A, a compound that inhibits both TbrPDEB proteins with nanomolar affinity (19). Ongoing drug development work is exploiting unique structural differences between the trypanosomal PDEBs and the equivalent human PDEs in order to increase selectivity (22). The characterization of the first TbrPDE inhibitors also provided the first pharmacological tool to specifically manipulate cAMP levels in kinetoplastid parasites, and potentially identify downstream effectors. One promising approach to identify pathways involved in a drug's action is to study drug resistance mechanisms.

In this study two parallel approaches were used to identify possible modes of resistance to the TbrPDEB inhibitor Cpd A. The first attempted to generate resistance by gradually increasing concentrations of the compound in chemically mutagenised bloodstream form cultures, followed by characterization of the surviving cell lines. The second exploited a whole genome RNA interference screen for genes which confer resistance to Cpd A when knocked down. Four candidate genes were identified that were

125 necessary to mediate the lethal drug action of PDE inhibitors and consequently are
126 associated with reduced Cpd A sensitivity when knocked down by RNAi. This represents
127 an important advance, as downstream effector proteins of cAMP signaling have not been
128 previously characterized in trypanosomes. We propose that the newly identified genes
129 required for Cpd A sensitivity encode the first *bona fide* cAMP effector proteins
130 identified in *T. b. brucei*.
131

132 **Materials and Methods**

133 **Trypanosome strains and culturing.** Bloodstream forms of *Trypanosoma brucei*
134 *brucei* strain Lister 427 were grown at 37 ºC in a 5% CO_2 atmosphere in HMI-9 medium
135 (23) supplemented with 10% foetal bovine serum (FBS). The Cpd A resistant R0.8 line
136 was derived from wild type *T. b. brucei* strain Lister 427 and cultured under the same
137 conditions as the wild type except that 0.4 µM Cpd A was added to the medium to
138 maintain drug pressure. Before assaying, R0.8 trypanosomes were grown in medium
139 without Cpd A for at least 6 days (approximately 18 generations). The RNAi cell lines
140 based on MiTat 1.2 13-90 (24) were kept under selection with 2.5 µg/ml geneticin, 5
141 µg/ml hygromycin and 1 µg/ml phleomycin; 0.1 µg/ml puromycin was added to the
142 RNAi cell lines bearing a tagged *CARP* allele.

143 **RNAi construct generation and transfection.** RNAi fragments were amplified
144 from genomic DNA of *T. b. brucei* strain Lister 427 and cloned into the p2T7-177-BLE
145 vector (25) via BamHI and HindIII (or XhoI in the case of *CARP1*) restriction sites. The
146 RNAi target regions were chosen as follows: Tb427tmp.01.7890: 541 bp, pos. 1254-
147 1794; Tb427tmp.52.0004: 383 bp, pos 528-910; Tb427.07.5340: 422 bp, pos. 781-1202;

148 Tb927.3.1040/60 (the TREU927 reference strain was used here, since the respective
149 sequence in strain Lister 427 is not fully sequenced): 755 bp, pos. 835 (Tb927.3.1040) –
150 395 (Tb927.3.1060); see also Fig. 4 for schematic representations of targeting regions.
151 Primer sequences are available upon request. Electroporation and selection procedures
152 were performed as described (26).

153 **Tagging of CARP proteins.** *In situ* tagging of *CARP1*, *CARP3* and *CARP4* was
154 performed on pMOTag vectors using a PCR-based strategy (27). CARP1 and CARP4
155 were fused to a C-terminal 3xHA tag and CARP3 to a single Ty1-tag using the vectors
156 pMOTag2H or pMOTag2T, respectively (derivatives of the pMOTag2 vector series with
157 puromycin resistance cassette (27)). Primers were designed according to the published
158 protocol with stretches of 60 to 80 nucleotides homologous to the 3' end of the ORF or
159 the beginning of the 3'UTR, respectively. CARP1 was independently tagged with a
160 4xTy1 tag at the N-terminus using the vector p3077 (derivative of pN-PTP (28); kindly
161 provided by S. Kramer, Würzburg). An N-terminal fragment of the *CARP1* ORF (pos. 1-
162 780) was cloned into the vector p3077 via HindIII and EcoRV restriction sites. The
163 construct was linearized with SwaI for transfection. Tagging of CARP2 was based on the
164 vector p3074 (derivative of pC-PTP (28); kindly provided by S. Kramer, Würzburg)
165 fusing a 4xTy1-tag to the C-terminus of the protein. The *CARP2* ORF was cloned into the
166 vector p3074 via BamHI and SwaI restriction sites. After exchange of the resistance
167 cassette from neomycin to puromycin via BstBI and NdeI restriction sites, the construct
168 was linearized with XhoI for transfection. All primer sequences are available upon
169 request.

Test compounds. Cpd A and Cpd B were synthesized and generously provided by Geert-Jan Sterk, Mercachem, the Netherlands. Dipyridamole, etazolate, dibutyryl cAMP, 8-bromo-cAMP, 8-(4-cholorophenylthio)-cAMP (8-CPT-cAMP), pentamidine, phenylarsine oxide and diminazene were obtained from Sigma Aldrich and Fluka; melarsen oxide was a gift from Sanofi-Aventis; suramin was a gift from Brian Cover (University of Kent at Canterbury); nifurtimox and eflornithine were gifts from Mike Barrett (University of Glasgow); and cymelarsan a gift from Mike Turner (University of Glasgow). Stock solutions of all compounds were made up in dimethyl sulphoxide (DMSO), with the solvent never exceeding 0.5% (v/v) under experimental conditions.

Induction of resistance to Cpd A. Methylmethanesulfonate (MMS; Sigma) was added to a 50 ml culture of *T. b. brucei* strain Lister 427 wild type trypanosomes in late logarithmic growth phase to give a final concentration of 0.001% (v/v) and incubated at 37 ºC and 5% CO_2 for 1 hour. Subsequently, the culture was centrifuged at room temperature (610 × *g*, 10 min) and the supernatant carefully removed and discarded in 1 M NaOH (to deactivate the mutagen). The cell pellet was resuspended in fresh medium and washed twice by centrifugation as above. After the final wash the pellet was resuspended in 50 ml medium and incubated at 37 ºC, 5% CO_2. During this incubation approximately 95% of the trypanosomes died due to exposure to MMS. The remaining trypanosomes, some of which will have been mutagenized, proliferated. Once the surviving culture reached the late logarithmic phase of growth the cells were washed once, as above, and resuspended in fresh medium containing 0.1 µM Cpd A, at a cell density of 2.5×10^4 cells/ml. The mutagenized trypanosomes were added to multiple 24-well plates and incubated at 37 ºC, 5% CO_2. Cell viability was checked by light

193 microscopy every 24 hours for 5 days. Once the trypanosomes in a well reached the late
194 logarithmic phase of growth they were passaged into 3 wells of a new 24-well plate with
195 fresh medium: one containing Cpd A at the screening concentration, another at 2 × the
196 screening concentration and a third being a no drug control. The cultures were thus
197 continuously maintained under gradually increasing (doubling), sub-lethal concentrations
198 of Cpd A.

199 **Dose-response cell viability assay.** The efficacies of test compounds against
200 various cell lines of *T. b. brucei* strain Lister 427 were determined using a modified
201 version of the Alamar Blue® assay described previously (29, 30). Briefly, test
202 compounds were doubly diluted in white-bottomed 96-well plates (Greiner) with standard
203 culture medium. An equal volume (100 µl) of bloodstream form trypanosomes in
204 medium was added to each well to give a final cell density of 1×10^5 trypanosomes/ml.
205 The plates were incubated for 48 hours at 37 ºC, 5% CO_2, after which 20 µl of 0.5 mM
206 resazurin sodium salt (Sigma) in phosphate-buffered saline (PBS) was added to each well,
207 followed by a further 24-hour incubation under the same conditions. RNAi lines were
208 induced with 1 µg/ml tetracycline (Tet; Sigma) 24 hours prior to plating in test compound
209 dilutions, and Tet was included until the end of the experiment.

210 Following the final incubation, fluorescence was measured using a FLUOstar
211 OPTIMA fluorimeter (BMG Labtech) with excitation and emission filters of 544 nm and
212 590 nm, respectively. Data were analyzed using GraphPad Prism software and EC_{50}
213 values (Effective Concentration that inhibits 50% of maximal growth) were derived from
214 sigmoidal dose-response curves with variable slopes. EC_{50} values reported in this

manuscript are the averages of at least three independent experiments, except for DFMO (Eflornithine) where n = 2.

Quantification of intracellular cAMP concentration. The intracellular concentration of cAMP in bloodstream form *T. b. brucei* cell lines, upon incubation with various phosphodiesterase inhibitors, was measured as described previously (19) using the Direct Cyclic AMP Enzyme Immunoassay kit (Assay Designs). Samples were taken in duplicate and all assays were conducted independently at least three times.

PCR and sequencing of selected genes. Clonal cultures of the parental wild type *T. b. brucei* Lister 427 strain and the Cpd A resistant R0.8 line were derived by limiting serial dilution, with that of the R0.8 cell line conducted under selective pressure of 0.4 µM Cpd A; genomic DNA was extracted from each clonal cell line as described (31). The proofreading polymerase KOD (Novagen) was used to amplify the genes using standard reaction conditions. Once the cycles were completed 1 U GoTaq DNA polymerase (Promega) was added to each reaction mix and incubated at 72 °C for 10 min to add adenine nucleotide overhangs to the amplification products. The amplicons were then separated by electrophoresis on a 1% (w/v) agarose gel, excised, gel-purified, ligated into the pGEMT-easy vector (Promega), and used to transform *E. coli* JM109 bacteria (Stratagene). Single bacterial colonies picked from selective agar plates were grown in 5 ml LB, after which the plasmid DNA was extracted and purified using a mini-prep kit (Qiagen). BigDye® Sanger sequencing (Eurofins-MWG-Operon) was carried out with T7 and SP6 primers and internal primers. Each of the four genes identified by the RNAi library screen (*CARP1-4*) were also sequenced in the parental wild type *T. b. brucei*

Lister 427 and R0.8 cell lines in a similar fashion. All primer sequences are available upon request.

Genome-wide RNA interference screen for resistance to Cpd A. Detailed descriptions of the *T. b. brucei* RNA library and approaches to screening have been published previously (32–34) and were followed with only minor modifications. Briefly: a whole genome RNAi library in bloodstream form *T. b. brucei* strain Lister 427 was induced with 1 µg/ml Tet 24 hours prior to the addition of 30 nM Cpd A. While under Cpd A selection, RNAi induction was maintained throughout; upon passage to fresh medium the total number of cells transferred was never below 5×10^6, in order to maintain library complexity. Growth was monitored daily by haemocytometer and cell density was adjusted as required with fresh medium containing Cpd A and Tet. The inducibility of resistance to Cpd A due to RNAi induction was assessed by monitoring growth for 72 hours in the presence and absence of 1 µg/ml Tet and/or 60 nM Cpd A. The RNAi target DNA fragments were amplified from the genomic DNA, sequenced and identified as described previously (32).

Western blot analysis. Lysates of 4×10^6 cells were separated on 10% polyacrylamide gels and blotted onto an Immobilon-FL PVDF membrane (Millipore). Immunodetection of tagged CARP proteins was performed with anti-HA (mouse monoclonal antibody, clone 12CA5, kindly provided by E. Kremmer, Helmholtz Center Munich) or BB2 (Ty1 epitope; (35)) antibodies in a 1:1000 dilution. PFR-A/C detected by the monoclonal antibody L13D6 (dilution 1:2500; (36)) was used as internal loading control. Infrared detection was performed using an IRDye 800CW goat anti-mouse IgG (H+L) secondary antibody (1:5000) and the Odyssey™ IR fluorescence scanning system

(both from LI-COR). Signals of tagged CARP proteins were normalized to the PFR-A/C loading control after automatic subtraction of the background values (Median Left/Right method) using the Odyssey software (LI-COR).

***CARP* gene transcript level analysis.** cDNA was generated by reverse transcription (iScript cDNA synthesis Kit, Bio-Rad) of RNA isolated (NucleoSpin® RNA II, Macherey-Nagel) from MiTat 1.2 Lister 427 or the derived Cpd A-resistant R0.8 cell line treated or not with 0.1 µM Cpd A for 2 hours. Relative expression levels of *CARP* messenger RNAs were determined by quantitative real-time PCR (FAST SYBR® Green Master Mix, Applied Biosystems; CFX96TM Real-Time PCR Detection System, Bio-Rad) using the following cycling parameters: [5 min 95°C; 40 × (30 s 95°C, 30 s 60°C)]. TERT was used as reference gene (37). The primers sequences are available on request.

Results

Selection for resistance to Cpd A. Cpd A (Fig. 1A), a tetrahydrophthalazinone, has been demonstrated previously to be a highly potent inhibitor of cAMP-specific phosphodiesterase B (PDEB) enzymes in *T. b. brucei* (19). Incubation with low concentrations of Cpd A results in sustained elevation of intracellular cAMP, ultimately leading to cell death and validating PDEs as novel drug targets for potential chemotherapies against Human African Trypanosomiasis (HAT) as well as animal infections (19). In order to further dissect the mode of action of Cpd A in *T. b. brucei*, as well as to identify potential modes of resistance to tetrahydrophthalazinones, cells resistant to Cpd A were selected. Bloodstream form trypanosomes were exposed to the

283 chemical mutagen MMS to generate a heterogeneous mutated population. The culture
284 was then exposed to a normally lethal concentration of Cpd A (0.1 μM) and the surviving
285 trypanosomes continuously cultured in gradually increasing concentrations of the PDE
286 inhibitor. After 2 months of culturing, the maximum tolerated concentration of Cpd A
287 was above 0.8 μM; a clonal cell line was obtained by limiting dilution and termed R0.8.
288 The resistance phenotype was stable: it remained unaltered after 3 months of continuous
289 culture in Cpd A-free medium and also after storage in liquid nitrogen and subsequent
290 thawing, as assessed by re-exposure to 0.8 μM Cpd A (data not shown).
291 **Resistance and cross-resistance characterization of the R0.8 cell line.** To more
292 precisely quantify the degree of resistance to Cpd A acquired by the R0.8 trypanosomes,
293 *in vitro* efficacy assays were carried out. The EC_{50} value for Cpd A had increased >17-
294 fold compared to the parental *T. b. b.* Lister 427 wild type strain, from 0.08 ± 0.01 μM to
295 1.37 ± 0.19 μM (Fig. 1C; Table 1). Significant cross-resistance was displayed to another
296 tetrahydrophthalazinone PDE inhibitor designated Cpd B (for structure see Fig. 1B),
297 showing a 9.7-fold increase in EC_{50} value (Fig. 1C; Table 1). Conversely, no cross-
298 resistance was observed with the mammalian PDE inhibitor dipyridamole (Table 1).
299 However, the R0.8 cell line did display significant cross-resistance to the membrane
300 permeable cAMP analogues dibutyryl-cAMP and 8-bromo-cAMP, with 7.2 and 4.2-fold
301 increases to their EC_{50} values, respectively, compared to the parental Lister 427 strain
302 (Fig. 1C; Table 1). Conversely, no significantly different sensitivity was observed for 8-
303 (4-chlorophenylthio)-cAMP (8-CPT-cAMP) (Table 1). Nor did we observe any
304 significant differences in the EC_{50} values of the trypanocidal drugs used as controls,
305 including the diamidines diminazene and pentamidine, the arsenicals cymelarsan and

phenylarsine oxide, or to the nitroheterocycle nifurtimox. A slight but statistically significant increase in sensitivity to suramin was observed for the R0.8 cell line (Table 1).

Intracellular cAMP metabolism in the R0.8 strain. The intracellular concentration of cAMP was monitored over time on incubation with various concentrations of Cpd A in the resistant R0.8 cell line and its parental *T. b. brucei* wild type strain Lister 427 (Fig. 2A & B). No significant difference (2-tailed, paired Student's T-test) in the steady state level of cAMP (i.e. the no drug controls) was detected between the two cell lines over three hours of observation. The addition of Cpd A resulted in a rapid increase in the intracellular cAMP concentration within 20 minutes in both strains and again no statistical differences between strains were observed at any of the Cpd A concentrations used or at any of the time-points sampled (Fig. 2A & B). Cpd B also significantly raised the intracellular cAMP concentration compared to the no drug control, with identical increases in both cell lines (Fig. 2C). The intracellular cAMP levels induced with Cpd B are ~10-fold lower than upon Cpd A treatment at the same concentration, as expected from >10-fold lower affinity to target (IC_{50} for recombinant TbrPDEB is 3.98 nM for CpdA and 50.12 nM for CpdB; G. J. Sterk, personal communication). The mammalian PDE inhibitor etazolate had no effect on cAMP levels in both cell lines. The ORFs of both *TbrPDEB* genes were cloned from R0.8 and wild type cells and sequenced, including the predicted untranslated regions (UTR). For *TbrPDEB1*, the wild type parental strain contained two distinct alleles, with polymorphisms at positions 738, 1362 and 1602 of the ORF (Fig. S1). The R0.8 strain appears to be homozygous, with all 9 plasmid clones having an identical sequence to that of allele B of the wild type. 28 allelic polymorphisms were identified in the ORF of

329 *TbrPDEB2*, of which 27 are located in four clusters in the GAF-A domain (38) and one
330 in the catalytic domain (base-pair 2365; Fig. S1) resulting in an amino acid change
331 (codon 789; Cys in allele A; Ser in allele B). Both alleles were present in the R0.8 line,
332 however, only the cysteine residue was present in each at codon 789. Thus, while some
333 allelic recombination events appear to have occurred in the R0.8 cell line, no
334 polymorphisms of either *TbrPDEB* gene were identified that were present only in the
335 R0.8 strain. This is consistent with the unchanged basal and PDE inhibitor-induced
336 cAMP concentrations in the R0.8 strain.

337 **An RNAi screen identifies genes involved in sensitivity to Cpd A.** In order to
338 identify genes for cAMP effector proteins (e.g. components of a signaling cascade) rather
339 than cAMP metabolism, that confer sensitivity to Cpd A, a whole genome RNAi screen
340 was carried out. The bloodstream form RNAi library generated and described previously
341 (32–34) was induced with tetracycline (Tet) for 24 hours before selection with 30 nM
342 Cpd A. Four days of selection resulted in only a slight decrease in the growth rate of the
343 Cpd A-exposed Tet-induced culture, compared to the Tet-induced control without Cpd A
344 (Fig. 3A). Therefore, the selective concentration was increased to 60 nM Cpd A.
345 Subsequently, the population doubling time increased to over 24 hours between days 5
346 and 11, and later returned to around 8 hours (similar to control). Fifteen days after the
347 initial selection with Cpd A, genomic DNA was extracted from the +Tet/+Cpd A culture
348 of surviving trypanosomes for PCR cloning of RNAi target fragments. At the same time-
349 point the effect of RNAi induction on population resistance to Cpd A was analyzed (Fig.
350 3B). After 72 hours growth in fresh medium without Tet, cell density in the culture
351 treated with 60 nM Cpd A (-Tet/+Cpd A) was 19% of the untreated culture (-Tet/-Cpd A).

In Tet-induced cultures, growth in the presence of 60 nM Cpd A (+Tet/+Cpd A) was 56% of that of the untreated control. Thus, resistance to Cpd A in the selected population is, at least in part, due to induction of RNAi.

PCR amplification of the RNAi target fragments from the resistant population gave several products, comprising at least eight discreet visible bands following gel electrophoresis (Fig. 3C). Five contiguous regions of the gel were excised and the DNA was purified and cloned in *E. coli*. Multiple clones from each excised region, representing all the different RNAi target fragment sizes, were sequenced and mapped to the reference genome (39) using TriTrypDB (40). Ten distinct RNAi target fragments were obtained from the 24 clones sequenced, representing all eight bands in the agarose gel (Table 2 & Fig. 3C). Three ORFs were identified by multiple, independent RNAi target fragments, and one by a single RNAi fragment; the genes were designated *CARP1-4* for cAMP Response Protein 1 - 4 and their identifications are listed in Table 2.

One of the genes knocked down in the Cpd A-resistant cultures was Tb427tmp.01.7890 (*CARP1*; Tb927.11.16210 in *T. b. brucei* reference strain TREU 927), encoding a 705 amino acid protein containing two apparently intact and one partial cyclic AMP binding-like domains (Fig. 4) that is conserved in synteny in each of the kinetoplastid genomes sequenced. No close orthologues were identified in other organisms, but cyclic nucleotide dependent kinases and ion channels appear to be the most closely related proteins outside the Kinetoplastida.

CARP2 (Tb427tmp.52.0004; Tb927.11.12860 in TREU 927) codes for a hypothetical protein of 302 amino acids, but a downstream alternative start codon may produce a shorter protein of 235 amino acids (41). This corresponds to the ORF length of

375 the majority of *CARP2* homologues that are well conserved across the Kinetoplastida
376 (>82% amino acid identity in all *Trypanosoma* spp. and >59% identity in *Leishmania*
377 spp.) and many other species including humans (47.7% identity). The apparent molecular
378 mass of the C-terminally tagged *T. b. brucei* protein (see Western blot in Fig. 5B) shows
379 that the first ATG is in fact used and that the trypanosomal CARP2 carries an N-terminal
380 extension. There is no known function, and no recognizable functional domains could be
381 identified in any of the homologues. It has been detected in proteomes of *T. b. brucei*
382 flagellum (42) and of cytoskeletal and plasma membrane fractions (43), as well as in an
383 *in silico* predicted proteome of the flagellar and basal body of *Chlamydomonas*
384 *reinhardtii* (44, 45).
385 *CARP3* (Tb427.07.5340; Tb927.7.5340 in TREU 927) encodes a hypothetical
386 protein of 498 amino acids with orthologues only in *Trypanosoma spp* and strains. A
387 BLASTP search identified the putative stibogluconate resistance gene family in
388 *Leishmania spp.* as the closest homologue outside trypanosomes (*L. braziliensis* LBRM-
389 _31_1110; 20.4% identity); amplification of this gene family in *L. tarentolae* resulted in
390 resistance to antimony containing drugs (46). The protein was found in the plasma
391 membrane-enriched fractions of bloodstream *T. b. brucei* (43) and in mitochondrial
392 fractions of procyclic trypanosomes (47) and is possibly palmitoylated (48). At the N-
393 terminal end of the protein a weak TPR-like domain (tetratricopeptide repeat) signature is
394 detected. TPR domains can mediate protein-protein interactions such as dimerization and
395 the assembly of multiprotein complexes (49).
396 The fourth ORF identified from the RNAi target fragments, *CARP4*
397 (Tb927.3.1040/60), is a hypothetical gene that spans three automatically annotated ORFs

398 in release 5.0 of TriTrypDB (*T. b. brucei* TREU 927 strain; the respective sequence
399 segment of strain Lister 427 is annotated as incomplete). However, the middle 'ORF'
400 appears to be a sequence contaminant disrupting a single open reading frame
401 encompassing Tb927.3.1040 and Tb927.3.1060. The middle ORF is absent from all
402 RNAseq data on the TriTrypDB website and has no homologues or orthologues in any of
403 the other kinetoplastid genomes on the database. The full length Tb927.3.1040/60 ORF,
404 on the other hand, is conserved in synteny in all kinetoplastid genomes sequenced to date,
405 with amino acid identity of 53.6% in *L. major* and 96.3% in *T. b. gambiense*. The
406 combined Tb927.3.1040/60 ORF codes for a hypothetical protein of 779 amino acids and
407 is predicted to have three DM10 domains and one EF-hand domain located at the C-
408 terminal end (Fig. 4). BLASTP and domain architecture (NCBI CDART) searches
409 uncovered three other genes in *T. b. brucei* strain 927 containing the same domain
410 architecture (Tb927.11.1430, Tb927.5.2950 and Tb927.10.7690).

411 **All four *CARP* genes confer sensitivity to Cpd A.** Independent RNAi constructs
412 individually targeting each of the four genes identified by the RNAi screen were
413 generated and transfected into the *T. b. brucei* Lister 427 strain MiTat 1.2 13-90 cell line
414 for tetracycline-inducible expression. Where possible, specific RNAi target sequences
415 were chosen that do not overlap with the target sequences returned from the RNAi screen
416 (Fig. 4). For *CARP4,* a target fragment covering the central part of the combined ORF
417 Tb927.3.1040/60 was amplified from Lister 427 genomic DNA, sequenced and cloned
418 into the RNAi vector (25). This provided proof of a contiguous ORF in strain Lister 427
419 and a possible sequence assembly error and misannotation in that region of the reference
420 TREU 927 genome sequence. Growth of the parental and transfected uninduced or

induced (1 µg/ml tetracycline) trypanosomes was monitored over 120 h (Fig. 5A). *CARP1* RNAi resulted in a slight growth phenotype, which was noticeable in part without tetracycline induction, probably the result of 'leaky' RNAi repression.

To quantify the RNAi-mediated knockdown of CARP protein amounts, each *CARP* gene was tagged *in situ* in the respective RNAi clone for quantitative Western blot analysis of endogenous expression levels (Fig. 5B). RNAi induction for 24 hours caused a substantial reduction of the specific tagged CARP protein (Fig. 5B). The strongest repression was observed for CARP3 (to 5%), whereas only a 2- to 3-fold reduction of CARP1, CARP2 or CARP4 protein levels was detected. For CARP1, reliability of the quantification was confirmed by several independent cell lines *in situ* tagged at the N- or C-terminus using a 4xTy1 or 3xHA tag, respectively (Fig. S2). For selected clones (the ones shown in Fig. 5A) the EC_{50} for Cpd A was determined by the Alamar Blue cell viability assay with and without induction of RNAi. As controls, several trypanocidal drugs in use for therapy were included. No cross-resistance to pentamidine, suramin or DFMO (eflornithine) was observed for any of the clones upon *CARP* RNAi induction. In contrast, RNAi mediated knockdown of all *CARP* genes conferred significant resistance to Cpd A (Fig. 6). The degree of resistance to Cpd A was highest upon knockdown of *CARP1* (117-fold; $P<0.01$) and was 10.1-fold, 7.9-fold and 5.4-fold for knockdown of *CARP2*, *CARP3* and *CARP4*, respectively. The effect of the RNAi knockdown on sensitivity to lipophilic cAMP analogues was also investigated. *CARP1* knockdown resulted in 5.0- and 3.7-fold increases of the EC_{50} for 8-bromo-cAMP and to dibutyryl-cAMP, respectively. Similarly, *CARP2* knockdown also resulted in resistance to 8-

443 bromo-cAMP and dibutyryl-cAMP, but to the lesser extents of 2.2- and 1.9-fold,
444 respectively. For *CARP3* and *CARP4* the differences were not significant.

445 **Sequencing and transcript levels of candidate resistance genes in the R0.8 cell**
446 **line.** Each of the four *CARP* genes identified by the RNAi screen was PCR amplified
447 from the Cpd A resistant R0.8 cell line, cloned and sequenced for mutations in the ORF,
448 as well as in any predicted UTR regions. Multiple clones for each gene were sequenced
449 and aligned, however, no polymorphisms could be identified in the R0.8 strain that were
450 not present in at least one allele of the parental *T. b. brucei* Lister 427 wild type strain.
451 Similarly, qPCR data comparing transcripts of each of the four *CARP* genes in wild type
452 *versus* the R0.8 cell line showed no difference in transcript abundance, either in the
453 presence or absence of Cpd A (Fig. S3). In conclusion, the Cpd A resistance of the R0.8
454 line cannot be attributed to mutations in the identified *CARP* genes or to reduced *CARP*
455 transcript levels. Although protein expression remains to be investigated, it seems likely
456 that additional genes are involved in resistance of the R0.8 line to elevated cAMP.
457

458 **Discussion**

459 In order to exploit the full therapeutic potential of PDE inhibitors in the future, an
460 understanding of how resistance, if any, might arise in the field is essential. Moreover, a
461 full understanding of the action of any PDE-targeting trypanocides is hampered by the
462 almost complete absence of information about intracellular cAMP signaling in *T. b.*
463 *brucei* and related kinetoplastids, beyond characterization of families of adenylyl
464 cyclases (ACs) and PDEs (17). Two approaches were employed to investigate potential
465 modes of resistance: 1. mutagenesis and selection of cell lines resistant to the

466 trypanosomal PDE inhibitor Cpd A, followed by their characterization; 2. a whole
467 genome RNAi screen for drug efficacy determinants of Cpd A.

468 A substantial level of resistance to Cpd A was induced in *T. b. brucei*, resulting in
469 the R0.8 cell line. Resistance to Cpd A conferred cross-resistance to another
470 tetrahydrophthalazinone PDE inhibitor, Cpd B, identified in the same HT screen with
471 recombinant TbrPDEB. Not surprisingly, resistance to one PDE inhibitor gives resistance
472 to the entire inhibitor class; indeed, two additional related tetrahydrophthalazinone
473 compounds also showed similar cross-resistance profiles (data not shown). On exposure
474 to tetrahydrophtalazinones, the cAMP level in the wild-type and resistant R0.8
475 populations increase similarly, indicating that resistance is not caused by mutated PDEs
476 or adapted PDE expression. Thus, in the R0.8 cell line, resistance must be based on
477 tolerating high intracellular cAMP. This is also compatible with the cross-resistance
478 observed for the cAMP analogues dibutyryl cAMP and 8-bromo cAMP, and consistent
479 with the lack of mutations in the *PDEB* gene sequences in the R0.8 trypanosomes. The
480 absence of an effect of etazolate on cAMP levels in *T. b. brucei* shows that this
481 compound, previously reported to inhibit *T. b. brucei* PDEB1 (50), does not, in fact, act
482 as an effective PDE inhibitor on *T. b. brucei* cells.

483 Given that Cpd A is quite lipophilic, it is expected to diffuse rather than be
484 transported across the plasma membrane, so that uptake-related resistance is not possible,
485 in contrast to actively accumulated trypanocidal drug classes like the diamidines (51).
486 Importantly, no cross-resistance was observed with the current trypanosomiasis drugs,
487 including diamidines, arsenicals, suramin and nifurtimox, showing that PDE inhibitors
488 have a distinct mechanism of resistance. Thus, combinations with current drugs could

489 significantly delay the onset of treatment failures and/or improve the effectiveness of the
490 currently unsatisfactory armamentarium against HAT.

491 Surprisingly, the R0.8 line was not resistant to the cAMP analogue 8-CPT-cAMP,
492 which is widely used as a cAMP agonist in mammalian cells and induces cell cycle arrest
493 and stumpy stage development in *T. b. brucei* (52). However, it has been shown that
494 products of intracellular hydrolysis of 8-CPT-cAMP are responsible for growth inhibition,
495 by a cAMP-independent mechanism (53). The observed lack of cross-resistance to 8-
496 CPT-cAMP corroborates this. This analogue does not qualify as a cAMP agonist in
497 trypanosomes and hence the lack of cross-resistance is compatible with Cpd A resistance
498 resulting from changes in cAMP effector proteins.

499 RNAi library screening has proven to be a powerful approach for uncovering
500 novel genes involved in the mode of action of many of the current trypanocides (11, 32,
501 34) and consequently, candidates for changes associated with resistance. While the
502 specific target of Cpd A is the PDEB family of proteins (19), the targets of the resulting
503 increase in cAMP were unknown. In this study, RNAi library screening uncovered four
504 putative cAMP target or effector proteins. Although cAMP metabolism has been
505 validated as a drug target in trypanosomes (18, 19) and the signaling molecule has
506 important roles in cell division and cytokinesis (19, 21) this is the first time that cAMP
507 response proteins have been identified in this pathogen, showing the power of this
508 genomic approach.

509 Knock-down by RNAi of *CARP1* resulted in over 100-fold increases in EC_{50}
510 value for Cpd A. The prediction of cyclic nucleotide binding-like domains in CARP1 is
511 clearly suggestive of a pivotal part to play in the cAMP signaling cascade by this protein,

512 although cAMP binding will need to be experimentally verified. This is particularly
513 significant as all the cAMP effectors widely conserved among other organisms, either
514 have no detectable orthologues in the *T. b. brucei* genome (EPAC and cNMP-gated ion
515 channels), or are refractory to cAMP and have acquired a distinct mode of regulation
516 (PKA-like kinase; (54), S.B. and M.B. unpublished). CARP1 may thus be part of the first
517 second messenger signaling cascade to be delineated in kinetoplastids. We propose that
518 the CARP2-4 proteins, whose repression resulted in more moderate but still highly
519 significant Cpd A resistance, are likely to be part of the same signaling pathway as
520 CARP1 or even associated in a complex. CARP2 and CARP4 are both predicted as
521 conserved proteins in motile flagella of several organisms, along with the three other 3 ×
522 DM10 domain-containing proteins similar to CARP4 (55). This may link to the
523 cytokinesis phenotype resulting from aberrant cAMP levels (19, 21) since a crucial role
524 for the trypanosome flagellum in cytokinesis is well-documented (56). The localization of
525 TbrPDEB1 and B2 (18) and adenylate cyclases (57) to the flagellum is consistent with
526 this hypothesis. For CARP2 we provide the first functional assignment for this highly
527 conserved eukaryotic flagellar protein of previously unknown function. Interestingly, a
528 human homologue of CARP4, EFHC1, has been shown to be a component of axonemes
529 and cilia, with mutations in EFHC1 being implicated in juvenile myoclonic epilepsy (58,
530 59). This suggests that *T. b. brucei* may be an exciting model organism to further
531 investigate the functions of these critical, but poorly characterized, DM10 domain-
532 containing proteins.

533 In summary, resistance to PDE inhibitors by bloodstream form *T. b. brucei* can
534 occur and has been found downstream of the PDEs in the cAMP signaling cascade,

535 which is currently undefined in trypanosomes. However, four potential downstream
536 cAMP effector proteins are already reported here, and reduced expression of any one of
537 them by RNAi results in resistance to PDE inhibitors. While much work needs to be done
538 to fully characterize these proteins, they could potentially be the first *bona fide*
539 downstream cAMP effector proteins identified in *Trypanosoma brucei* and provide the
540 first step to mapping the downstream cAMP signaling cascade. As no mutations, or
541 changes in transcript level, in any of the four *CARP* genes could be detected in the
542 resistant R0.8 cell line, analysis of such lines may reveal additional components of that
543 pathway in the future. Finally, CARP1 may be a good drug target in its own right, as it is
544 specific to kinetoplastid parasites and appears to have cyclic nucleotide binding-like
545 pockets. The huge experience in the pharmaceutical industry in designing inhibitors and
546 activators for cNMP-binding proteins would be a distinct advantage in this case.
547

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791 **Figure Legends**

792 **FIG. 1.** (A & B) Chemical structures of two novel tetrahydrophthalazinone PDE
793 inhibitors, Cpd A (19) and Cpd B, with their IC_{50} values against recombinant TbrPDEB1
794 (G. J. Sterk, personal communication). (C) Representative dose-response curves of
795 trypanosome killing by both PDE inhibitors and two cell-permeable cAMP analogues
796 assayed against wild type bloodstream form *T. b. brucei* Lister 427 strain (solid lines,
797 filled symbols) and the Cpd A-resistant R0.8 strain (dashed lines; unfilled symbols). See
798 Table 1 for mean EC_{50} values.
799
800 **FIG. 2.** Intracellular cAMP concentrations elicited by Cpd A in wild type *T. b.*
801 *brucei* strain Lister 427 bloodstream form trypanosomes (A) and in the derived Cpd A
802 resistant R0.8 strain (B); the graphs shown are representative of three paired, independent
803 experiments. (C) Intracellular cAMP concentrations after incubation for 3 hours with 1
804 μM Cpd A, 1 μM Cpd B and 40 μM etazolate of Lister 427 wild type bloodstream form
805 trypanosomes (grey bars) or the derived CpdA resistant R0.8 cell line (unfilled bars);
806 error bars are SEM, $n \geq 3$.
807
808 **FIG. 3.** (A) Cumulative growth of an induced (1 μg/ml tet) whole genome RNAi
809 library in bloodstream form *T. b. brucei* Lister 427 strain trypanosomes in the presence
810 (squares) or absence (circles) of Cpd A. Initial concentration of Cpd A was 30 nM, which
811 was increased to 60 nM after 4 days. (B) Relative growth of the surviving RNAi library
812 trypanosome population after selection with Cpd A. Cells were grown for 72 hours in the
813 presence of 60 nM (+Cpd A) or absence of Cpd A (-Cpd A) with RNAi either induced

814 (+Tet) or uninduced (-Tet). Growth is expressed as a percentage of that of the -Tet, -Cpd
815 A population. (C) Ethidium bromide stained agarose gel (1% w/v) of the genomic PCR
816 products representing the RNAi target fragments in the library constructs selected after
817 15 days in Cpd A (i.e. those fragments which are associated with resistance to Cpd A).
818 DNA ladder size markers on the left hand side of the figure are denoted in base-pairs.
819 Slices refer to the portion of the gel excised for cloning and sequencing.
820
821 **FIG. 4.** Maps of the genomic loci of the *CARP* genes, RNAi target fragments and
822 domain annotations. The sequence data are from tritrypdb.org, ORFs are indicated in
823 black. 'RNAi screen fragments' were identified as described in Fig. 3C; 'RNAi
824 confirmatory' are the target fragments designed for the experiments shown in Fig. 5 and
825 6. Domain architecture was analysed using SMART (smart.embl-heidelberg.de/) and
826 SUPERFAMILY (supfam.cs.bris.ac.uk). cAMP binding-domain-like: SSF51206; DM10:
827 SM000676; EF hand: SSF47473; TPR-like SCOP48452. Scale bar represents 100 bp.
828
829 **FIG. 5.** Independent RNAi targeting of identified *CARP* genes. **(A)** Cumulative
830 growth of *CARP* RNAi cell lines in the presence (+ Tet, empty circles) or absence (- Tet,
831 filled circles) of 1 µg/ml tetracycline. The parental 13-90 cell line was included as control
832 (filled triangles). The cells were counted and diluted daily in order to keep the cell
833 density below 8×10^5/ml. (B) Western Blot analysis of CARP protein expression in the
834 presence (+ Tet, 24h) or absence (- Tet) of 1 µg/ml tetracycline. CARP1 was tagged at
835 the N-terminus with a 4xTy1 tag, CARP2 with a C-terminal 4xTy1 tag, CARP3 with a C-
836 terminal Ty1 tag, and CARP4 with a C-terminal 3xHA tag. CARP protein levels were

normalized to PFR-A/C detected by the monoclonal antibody L13D6 (36) and set to 100% in the absence of Tet. The relative scan gain in the 800 nm channel was set to 1 for the CARP1 Western Blot and to 3 for the CARP2-4 Western Blots. Relative expression levels are indicated as percentage of the non-induced cultures.

FIG. 6. Validation of identified *CARP* genes for function in Cpd A susceptibility. EC_{50} values as determined by Alamar blue assay are presented as mean of three or more independent determinations. Uninduced EC_{50} values (hatched bars) were determined in parallel with induced (1 µg/ml Tet for 24h, solid bars) EC_{50} values. Significance of uninduced/induced differences was tested by a paired two-tailed Student's t-test as indicated: *, P<005; **, P<0.02; ***, P<0.01.

Tables

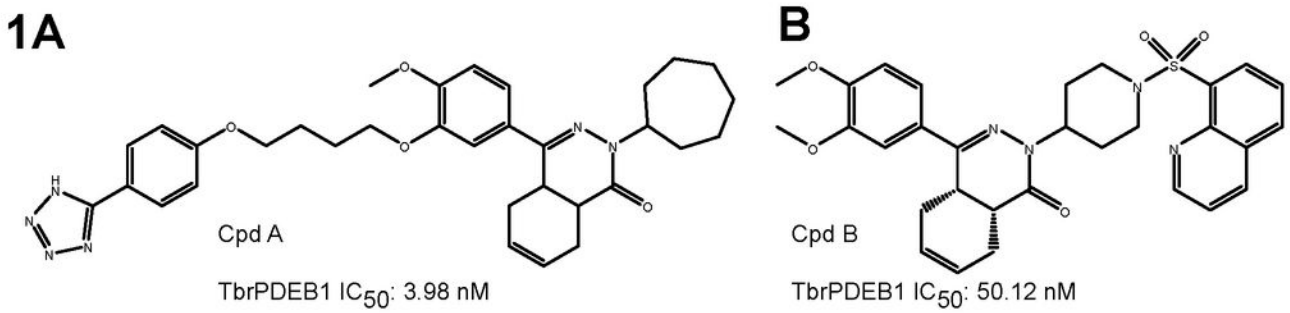
TABLE 1. Resistance and cross-resistance characterization of the R0.8 bloodstream form cell line, compared to the parental wild type *T. b. brucei* strain Lister 427.

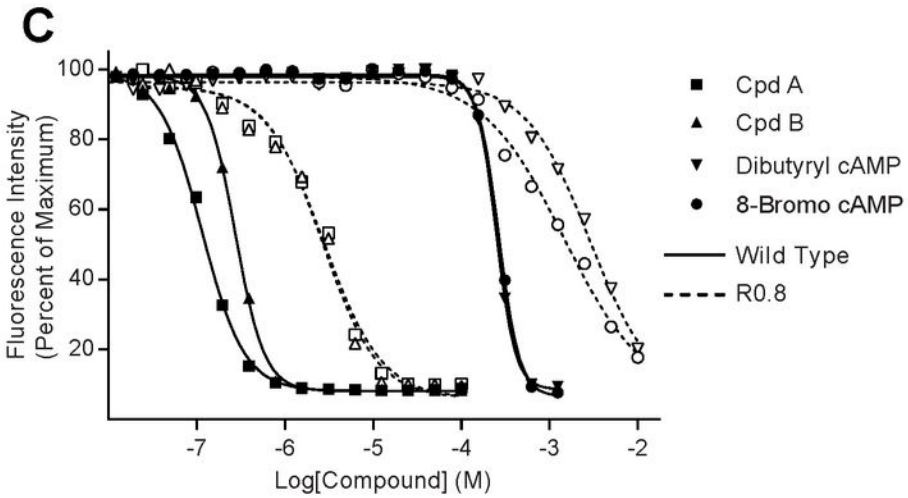
Compound	Average EC_{50} values (μM)		Resistance factor	P-value (different from Lister 427)
	Lister 427	R0.8		
PDE Inhibitors				
Cpd A	0.08 ± 0.01	1.4 ± 0.2	17.2	0.004
Cpd B	0.13 ± 0.03	1.28 ± 0.25	9.7	0.016
Dipyridamole	17.9 ± 2.7	9.2 ± 0.8	0.5	0.059
cAMP Analogues				
Dibutyryl cAMP	263 ± 13	1890 ± 314	7.2	0.011
8-Bromo-cAMP	271 ± 8	1133 ± 185	4.2	0.014
8-(4-chlorophenylthio)-cAMP	1.24 ± 0.4	0.25 ± 0.05	0.2	0.201
Known Trypanocides				
Suramin	0.0212 ± 0.0008	0.0156 ± 0.0005	0.7	0.001
Diminazene	0.022 ± 0.007	0.011 ± 0.001	0.5	0.133
Pentamidine	0.0016 ± 0.0004	0.0014 ± 0.0002	0.9	0.683
Cymelarsen	0.0038 ± 0.0004	0.0038 ± 0.0003	1.0	1.000
Phenylarsine Oxide	0.00083 ± 0.00006	0.00088 ± 0.00011	1.1	0.783
Nifurtimox	2.01 ± 0.24	1.61 ± 0.08	0.8	0.246

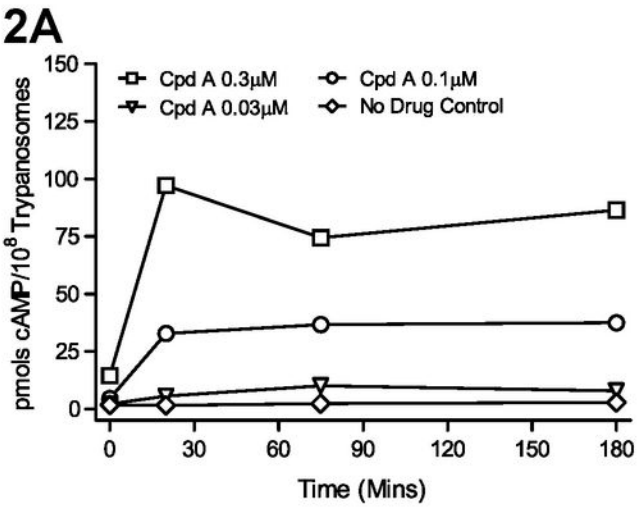
865 **TABLE 2.** Systematic gene IDs of RNAi target fragments selected with Cpd A.

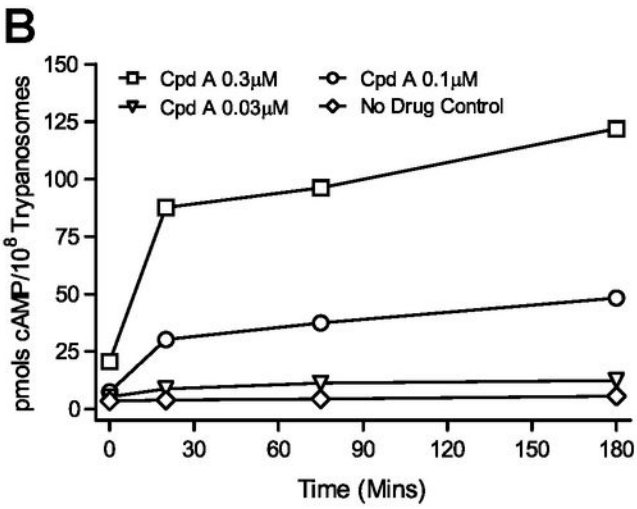
Gene name	Gene ID strain TREU 927	Gene ID strain Lister 427	Length (amino acids)	RNAi target fragments	
				number	sizes (bp)
CARP1	Tb927.11.16210	Tb427tmp.01.7890	705	3	446, 851, 1101
CARP2	Tb927.11.12860	Tb427tmp.52.0004	302	2	736, 1507
CARP3	Tb927.7.5340	Tb427.07.5340	498	4	386, 431, 532, 635
CARP4	Tb927.3.1040/60	Tb427.03.1040/60	779	1	780

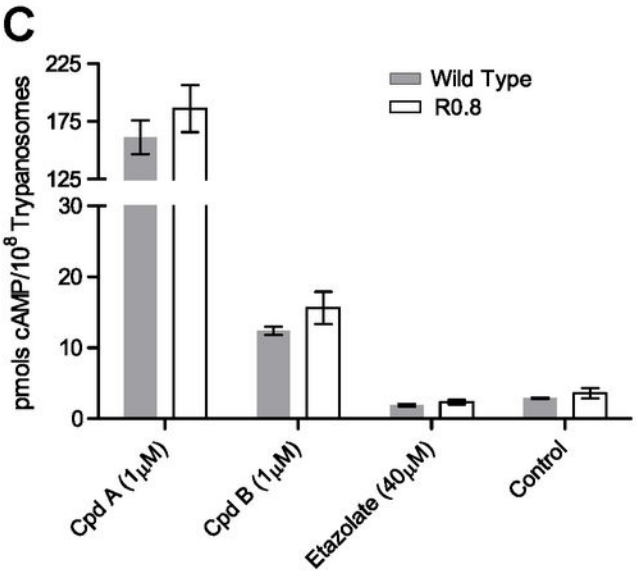
866

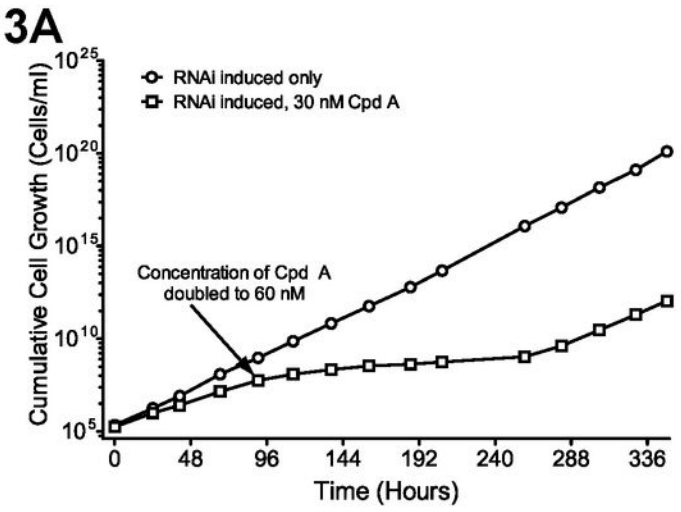


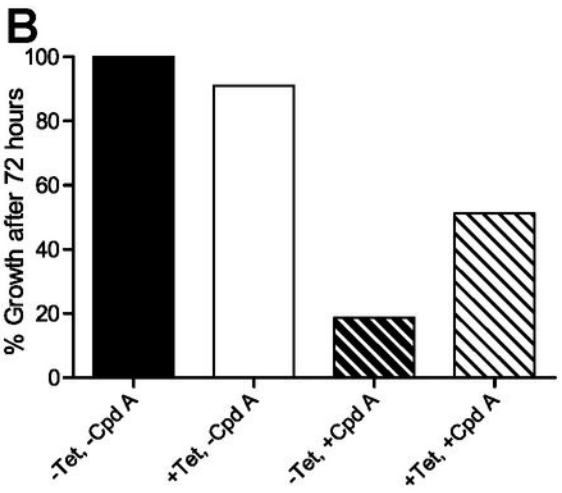


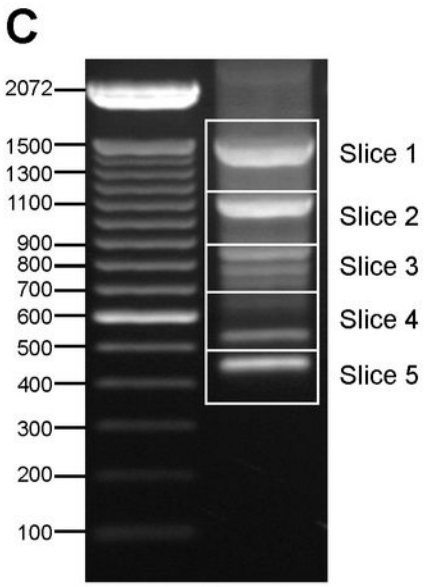












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