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Targeting the trypanosome kinetochore with CLK1 protein kinase inhibitors

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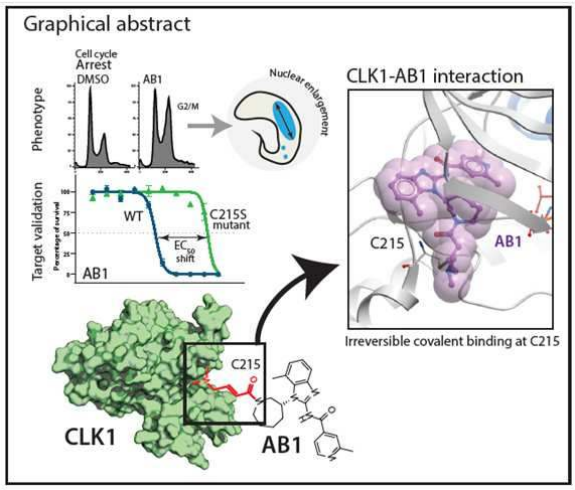

ABSTRACT

The kinetochore is a macromolecular structure that assembles on the centromeres of chromosomes and provides the major attachment point for spindle microtubules during mitosis. In *Trypanosoma brucei* the proteins that make up the kinetochore are highly divergent, with the inner kinetochore comprising at least 20 distinct and essential

proteins (KKT1-20) that include four protein kinases, CLK1 (KKT10), CLK2 (KKT19), KKT2 and KKT3. We report the identification and characterisation of the amidobenzimidazoles (AB) protein kinase inhibitors that have nanomolar potency against *T. brucei* bloodstream forms, *Leishmania* and *Trypanosoma cruzi*. Target deconvolution using a selection of 31 *T. brucei* mutants that over-express known essential protein kinases identified CLK1 as a primary target. Biochemical studies and the co-crystal structure of CLK1 in complex with AB1 show that the irreversible competitive inhibition of CLK1 is dependent on a Michael acceptor forming an irreversible bond with C215 in the ATP binding pocket, a residue that is not present in human CLK1, thereby providing selectivity. Chemical inhibition of CLK1 impairs inner kinetochore recruitment and compromises cell cycle progression, leading to cell death. This work highlights a unique drug target for trypanosomatid parasitic protozoa and a new chemical tool for investigating the function of their divergent kinetochores.

Main Text

The three kinetoplastid neglected tropical diseases; leishmaniasis, Chagas disease, and human African trypanosomiasis (HAT) remain a significant global health burden. Over a billion people living in endemic regions are at risk from these neglected diseases with an estimated 30,000 deaths annually (WHO)[1]. While there have been notable recent advances in disease control, current therapies have severe shortcomings and there is an urgent need for innovative treatments that are safe, efficacious and easy to administer. Some of the significant advances include the development of novel oral therapy for sleeping sickness[2,3] and identification of pre-clinical candidates for leishmaniasis[4–6]. In order to find additional growth inhibitors, a whole parasite based phenotypic screen was carried out using ~2.3 million compounds against bloodstream forms of *T. brucei*[4], which included the Novartis kinase focused inhibitor library. This led to the identification of AB0, an Amidobenzimidazole (Fig. 1a and Supplementary Figure 1a) with an EC_{50} of 290 nM in an *in vitro T. brucei* parasite growth inhibition assay (Fig. 1a).

Detailed structure activity relationship (SAR) studies using ~250 close analogs obtained from the Novartis compound archive and focused medicinal chemistry efforts were carried out. The growth inhibition activity of compounds against *T. brucei* ranged 3 log orders with many compounds showing < 100 nM potency. Results also showed that the analogs having Michael

acceptor chemical functionality are critical for potent activity and afford higher lipophilic efficiency (LipE >3) required for more favourable drug-like properties (Fig. 1b, Supplementary Table 1). SAR analysis also led to the identification of more potent bioactive compounds, including AB1 (EC_{50} of 72 nM against *T. brucei*), with a >100-fold cytotoxicity window (Fig. 1a). AB1 not only showed potent activity against *T. b. gambiense* and *T. b. rhodesiense*, the causative agents of sleeping sickness but also had pan-kinetoplastid activity against *T. cruzi* (aetiology for Chagas disease), *Leishmania mexicana* (cutaneous leishmaniasis) *and L. donovani* (visceral leishmaniasis) suggesting that the molecular target is conserved across trypanosomatids (Fig. 1a). Compound AB1 was cidal to *T. brucei* showing concentration and time dependent kill, further it also exhibited relapse free cidality in wash-off assays suggesting ability to achieve sterile cure (Extended data 1b, c). In vitro wash-off assays are presumed to be predictive of compounds' ability to achieve relapse free cure in the mouse model of infection. AB1 had better solubility and *in vitro* pharmacokinetic properties with moderate to low clearance in mice, rat and human microsomes (Fig. 1a), allowing us to evaluate the ability to cure *T. brucei* infection in mouse models.

In vivo efficacy of AB1 was tested in mice with haemolymphatic and central nervous system (CNS) *T. brucei* infections using both STIB795 and GVR35 optimised bioluminescence HAT mouse models[7]. A dose dependent cure was observed with AB1, resulting in relapse free cure with a daily oral dosage of 50 mg/kg for four consecutive days in the STIB795 haemolymphatic mouse model of infection (Fig. 1c). However, despite a 4 log reduction in parasitaemia observed in a pleomorphic GVR35 strain chronic (CNS) model of infection, the parasitemia relapsed, most likely due to poor access of AB1 to parasites in the brain (Extended data 2a,b). Although AB1 showed good brain partitioning (measured brain to plasma ratio was 0.5), due to high brain tissue binding (>99%), the free fraction available for acting against the parasites in the brain was negligible, leading to poor efficacy (Extended data 2c,d). Further medicinal chemistry optimization is required to find compounds with better pharmacokinetic properties in order to achieve cure in the CNS model. The mouse efficacy studies using AB1 showed *in vivo* chemical validation of AB series compounds as promising anti-trypanosomatid candidates.

AB1 is a known covalent inhibitor of mutant human epidermal growth factor receptor (hEGFR) kinase, a well-studied target for non-small cell lung cancer (Fig 1a)[8]. Whilst hEGFR belongs to the tyrosine kinase family, the *T. brucei* genome lacks members of the receptor-linked or cytosolic tyrosine kinase families[9]. A major challenge for phenotypic hits

is to identify the molecular target that is responsible for that effect. Since AB series compounds were hypothesized to target protein kinases, we generated inducible *T. brucei* gain-of function mutants of individual protein kinases to screen for resistance to AB1 (Fig. 2a). Twenty-nine essential protein kinases that had been assessed previously by RNAi as having specific RNAi induced cell cycle defects in bloodstream forms (BSF)[10] were analysed. In our screen, only one protein kinase, CLK1, showed a significant 6.5-fold increase in resistance compared with its uninduced control (Fig. 2b). In general over-expression of protein kinases show moderate resistance to their inhibitors. Wyllie and co-workers showed that co-over expression of CYC9 and wild type CRK12 in *L. donovani* resulted in 3-fold shift in cellular EC_{50} against the compound **5** (DDD853651/GSK3186899), validating CRK12 as the target[5]. Over-expression of PI4K (phosphatidylinositol 4-kinase) in *Plasmodium falciparum* also rendered moderate 3-fold shift in EC_{50} against imidazopyrazine compounds, confirming the on-target effect[11].

In mammals, CLK1 belongs to the Clk (Cdc2-like kinase) family implicated in RNA splicing control and consists of at least four members[12]. In *T. brucei*, CLK1 is a kinetochore component essential for mitosis and has been proposed to be one of the potential targets for the fungal antibiotic hypothemycin[13–15]. As treatment with AB1 resulted in a G2/M cell cycle arrest (Fig. 2c-e) with most of the treated cells having an enlarged nucleus (Fig. 2d) similar to CLK1 RNAi knockdown (Extended data 3a-c), we tested if overexpression of CLK1 would confer resistance to drug-induced G2/M cell cycle arrest. Indeed, parasites overexpressing CLK1 had a normal cell cycle profile after treatment with AB1, in comparison to the parental cell line and DMSO uninduced control, which were G2/M arrested after treatment (Fig. 2c). CLK1 overexpression impairs parasite fitness without affecting cell cycle progression, suggesting that *T. brucei* tightly regulates CLK1 expression (Extended data 3d). In addition, AB1 attenuates CLK1 toxicity caused by over-expression, providing further evidence that CLK1 is the compound's primary target (Extended data 3e, f).

We expressed recombinant TbCLK1 and human CLK1 and tested ~230 compounds from the AB series to determine the biochemical SAR. Apparent IC_{50} was determined after 10 min post-incubation with the enzyme. A strong correlation was observed between inhibition of the *T. brucei* CLK1 enzyme and cellular activity (Pearson' correlation $r = 0.68$), supporting the chemical validation of CLK1 as the molecular target for the amidobenzimidazole series of compounds (Fig. 3a, Supplementary Table 1). The majority of the compounds showed greater selectivity against TbCLK1 compared to hCLK1 (Fig. 3b, Supplementary Table 1). AB1

inhibited TbCLK1 with an apparent IC_{50} of 10 nM, with 90-fold selectivity observed over hCLK1 (Fig. 3b). *T. brucei* also has another protein kinase, CLK2, which is closely related to CLK1, with 92% overall sequence identity and 100% identity in the protein kinase domain. CLK2 is less abundant than CLK1 in bloodstream forms (Extended data 4a, b). AB1 would be expected to inhibit the kinase activity of CLK2, but this is unlikely to contribute to killing in bloodstream forms of the parasite as CLK2-specific RNAi suggests the protein is not essential[13].

Interestingly, all compounds with a Michael acceptor inhibited TbCLK1 kinase activity but not others, strongly suggesting this functionality is an essential feature of the pharmacophore (Fig. 3a). In order to test the putative critical role played by the Michael acceptor, a compound similar to AB1, which has a saturated double bond at the Michael acceptor, was profiled in both enzymatic and cellular assays. This compound (AB2) was completely inactive in both TbCLK1 enzyme (apparent IC_{50} > 20 μM) and whole cell growth inhibition assays (Tbb EC_{50} > 50 μm), confirming the importance of the Michael acceptor for activity (Supplementary Table 1). The hEGFR inhibitors having Michael acceptors are known to covalently interact with an active site cysteine (C797) near the ATP binding domain[8,16].

In order to directly visualize the binding mode, structure of TbCLK1 kinase domain bound to AB1 was determined to 2.7Å (PDB 6Q2A) (Fig. 3c). At this resolution, the electron density map has continuous density between the side-chain of the thiol group of C215 and the Michael acceptor of the inhibitor (Fig. 3c, d), strongly indicating the formation of an irreversible covalent bond between them. Furthermore, AB1 forms direct hydrogen bonds with the backbone amide NH of Y212 and C215 and a salt bridge with D218 (Fig. 3c), consistent with the binding mode of AB1 when covalently attached to hEGFR[8]. While both hEGFR and TbCLK1 have cysteine in their active sites, hCLK1 lacks cysteine and instead has serine in the same position (Extended data 5a-c). This has led to achieving significant selectivity for most AB series compounds for TbCLK1 (Fig. 3b). Beyond the AB1 binding site, the TbCLK1 kinase domain exhibits an overall structure that is in line with a kinase in the active conformation, closed kinase lobes, a well-ordered activation loop, DLG-in and an intact salt bridge to αC-helix (Fig. 3c).

To investigate the covalent binding and interaction with C215, we co-incubated AB1 and AB2 with both wild type and C215A mutant of TbCLK1 and assessed the interaction using biophysical methods. As expected, incubation of AB1 with wild type TbCLK1 enzyme

resulted in a 12° C shift in denaturing temperature as seen by differential scanning fluorimetry (Fig. 4a and Extended data 6a). Similarly, mass spectrometric analysis also showed one main product per protein which has an average mass 475 Da higher than the unmodified protein (Extended data 6b). This was consistent with the addition of one molecule of AB1, which has a mass of 475 Da. No shift in the mass was seen for AB2, or in C215A mutant CLK1 (Extended data 6a). These results clearly show covalent interaction of the Michael acceptor with C215 of TbCLK1. In addition, TbCLK1 C215A is not inhibited by AB1, but is inhibited by the non-covalent pan-kinase inhibitor staurosporine (Fig. 4b). Multiple sequence alignment of CLK1 from *T. brucei*, *T. cruzi* and *L. mexicana* showed high sequence similarity and C215 was conserved across parasites, further suggesting the growth inhibition of these parasites by AB1 could be due to TbCLK1 inhibition (Extended data 4c).

Further, to assess the importance of *T. brucei* CLK1 C215 in AB1 binding in parasites, a recoded version of CLK1 that contained a Cys to Ala mutation at position 215 was expressed in the TbCLK1 RNAi line. In this cell line, RNAi induction depleted the wild type CLK1 mRNA but the recoded CLK1 C215A mRNA was not susceptible to RNAi-induced degradation and was expressed. This triggered a G2/M cell cycle arrest, suggesting this residue is important for CLK1 function and may mimic AB1 –TbCLK1 mediated inhibition. Since expression of the C215A mutation affected the fitness of the parasites, we attempted to express a C215S mutation, as human CLK1 has serine at position 215. Parasites expressing the TbCLK1 C215S mutation had a normal cell cycle profile (Extended data 7a, b) and were resistant to treatment with 5x EC_{50} of AB1 (Extended data 7c). Thus, we evaluated if the overexpression of the C215S mutation in the parental cell line increased this resistance, and indeed, this mutation conferred a > 60-fold EC_{50} shift of resistance against AB1 (Fig. 4c), which also significantly reduced the effect of AB1 in the parasite's growth and survival (Fig. 4d, e). Interestingly, we observed that AB1 treatment induced a mobility shift on CLK1, which likely corresponds to phosphorylation, whilst the C215S mutant protein remained unaltered (Extended data 7d). Together, these data demonstrate target engagement in the parasite and inhibition of CLK1 with AB1.

TbCLK1 (KKT10) is a core component of the inner kinetochore. In other organisms, the kinetochore supports directional movement of chromosomes into microtubules to ensure faithful chromosome segregation[17]. Some of the *T. brucei* kinetochore components have been recently described, and grouped according to their patterns of expression/localization through the cell cycle[14,18]. The significant divergence between components of the human and parasite

kinetochores underpins its potential druggability. Although depletion of TbCLK1 has been associated with the presence of lagging chromosomes during mitosis in procyclic cells[14] the causal link of TbCLK1 to this process has yet to be established. To investigate this further *T. brucei* bloodstream form cells were synchronised by cell sorting[19] to give 2C and 4C nuclear DNA content populations and then allowed to enter the cell cycle in the presence or absence of AB1 (Fig 2e and Extended data 8 a-c). Inhibition of TbCLK1 in the 2C population (G1-phase) synchronously progressing through the cell cycle led to arrest of cells in late metaphase with a 2K1N configuration (defined as cells having 2 kinetoplasts (2K) and 1 nucleus (1N)). By contrast, inhibition of TbCLK1 in the 4C population synchronously progressing through the cell cycle induced an arrest in the late anaphase (2K2N population). These data are consistent with inhibition of TbCLK1 causing a disruption in kinetochore function during metaphase and exit from anaphase into cytokinesis. To confirm this, we tagged KKT2, a canonical centromere kinetochore protein, with mNeonGreen and assessed its localisation in the presence and absence of AB1. Similar to previous observations in procyclic form cells[14,18], we observed in bloodstream form cells that KKT2 is constitutively expressed until anaphase. Treatment with either 2 x EC_{50} of AB1 for 24 hr or 5 x EC_{50} of AB1 for 6 hr caused dispersal of the defined foci of the kinetochore within the nucleus (Fig. 5a). This change of KKT2 pattern was also observed after depletion of CLK1 by RNAi (Fig. 5b). These data demonstrate that inhibition of CLK1 by AB1 causes impairment of kinetochore function thereby defining a consequence of AB1 treatment.

Discussion

Our studies indicate that the principal mechanism of action for the amidobenzimidazole series is inhibition of the trypanosome CLK1 protein kinase. In support of this, we show for over 230 compounds that a strong correlation exists between inhibition of recombinant TbCLK1 and killing of the parasite. Only those compounds that have a Michael acceptor and are predicted to form a covalent bond with C215 of CLK1 are active and the X-ray structure, differential scanning fluorimetry and mass spectrum chromatograms clearly demonstrate the covalent linkage for AB1. We also show that over-expression of CLK1 or the CLK1 C215S mutant provide 6.5-fold or >60-fold resistance of the parasite to AB1, respectively, demonstrating target engagement. AB1 treatment of bloodstream form trypanosomes lead to chromosome mis-segregation and cell cycle arrest, consistent with inhibition of CLK1, which is a kinetochore kinase (KKT10). Generation of parasites resistant to compounds and the identification of mutations in target genes is a well-established method for target

deconvolution[4,5]. However, we failed to generate parasites resistant to AB1, even after a year and half under compound selection. This might be explained if AB1 has an element of polypharmacology and one potential secondary target is CLK2, which is an active enzyme[20] and has an identical protein kinase domain to CLK1, thus inhibited by AB1. In procyclic form trypanosomes CLK1 and CLK2 are functionally redundant[21], whilst in bloodstream form parasites CLK1 is essential, but CLK2 is not essential[15,22]. An explanation for this arises from our finding that CLK1 is more highly expressed than CLK2 in bloodstream forms, such that inhibition of CLK1 activity leads to chromosome mis-segregation and cell cycle arrest, phenotypes consistent with inhibition of a kinetochore kinase (KKT10).

We propose that inhibition of CLK1 activity with AB1 leads to impaired kinetochore function and irreversible arrest in metaphase leading to cell death. A combination of growth inhibition screening, target identification and characterization resulted in the chemical validation of CLK1 as a unique pan-kinetoplastid drug target. In recent years six covalent inhibitors of protein kinases have been successfully approved for cancer treatment[23], underlining the possibility of developing covalent inhibitors into drugs. The combination of a distinct mechanism of action targeting the kinetochore, and a new series of antitrypanosomatid AB compounds, which have reduced chances of acquiring resistance in the clinic, add to the proteasome and CRK12 inhibitors already entering clinical trials[4,5,24,25]. One of the key challenges for the AB series of compounds to progress to the clinic is to improve kinase selectivity and the co-crystal structure determined in the current study will significantly help in structure based medicinal chemistry optimization to resolve this issue. The current efforts of CLK1 target validation also open up opportunities for focused structure-based drug design using fragment screens in order to obtain non-covalent inhibitors that have pan-kinetoplastid growth inhibition properties. In addition, the high level of sequence divergence of trypanosome kinetochore complex proteins from humans [14,26] make the kinetochore a high value target.

Contributions

J.C.M, T.T.D and S.P.S.R planned the studies. Compounds synthesis, compound docking, PK assays, and library screening by H.X.Y.K, D.P, Y.L.C, S.B.L, S.W, F.S and J.J; HAT animal studies by E.M, R.R, M.P.B and M.K; generation of individual protein kinase cell

lines by E.B and M.S; Compound target deconvolution, TbCLK1 functional characterisation, immunofluorescences and data analysis were performed by M.S and J.B.T.C.; protein recombinant production, kinase assays and mass spectrometry by E.F, De.P, C.B-L and M.S; Crystallization and structure determination by E.O, X.M.;CLK1 mutants plasmids designed and prepared by M.S, Da.P and R.M; J.C.M, S.P.S.R, and M.S prepared and wrote the manuscript. All authors reviewed, edited and approved the paper. J.C.M, S.P.S.R, M.P.B and T.T.D obtained funding.

Data availability

All the source data used for generating Figures 1-5 and Extended Data Figures 1-8 have been provided as source data. Any other data that support the findings of this study are available from the corresponding author upon request. *Trypanosoma brucei* CLK1 kinase domain in complex with covalent aminobenzimidazole inhibitor AB1 is annotated in PDB with an accession number 6Q2A.

Competing interests

The authors declare no competing interests, except that some authors have shares in Novartis.

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METHODS

Cellular assays

A kinase focused inhibitor library (containing approximately 10,000 compounds) was screened at a single concentration of 10 μM for the ability of compounds to inhibit growth of *T. b. brucei* Lister 427. This resulted in 2264 compounds showing > 50% growth inhibition. Chemi-informatic analysis of hits resulted in ~200 clusters, representatives of these were subjected to 10-point dose-response growth inhibition assays against bloodstream form of *T. b. brucei* Lister 427. Further removal of non-favourable chemical structures such as

281 nitrofuranes, cytotoxicity profiling using the HepG2 cell line (selectivity index of 10) and
282 favourable physico-chemical properties (polar surface area <100; molecular weight <500 and
283 lipophilicity <4.5) led to identification of an amidobenzimidazole scaffold (AB0) for further
284 follow up.

285

286 Bloodstream form of *Trypanosoma brucei brucei* Lister 427, *T. b. gambiense* STIB930 and
287 *T. b. rhodesiense* STIB900 were cultured in HMI-9 medium as described elsewhere[4].
288 Other kinetoplastid parasites *Leishmania donovani* HU3 and *L. mexicana*
289 (MNYC/BZ/62/M379) were cultured in RPMI 1640 media as described earlier [4,27]. The *T.*
290 *cruzi* Tulahuen parasites constitutively expressing *Escherichia coli* β-galactosidase[28] were
291 maintained in tissue culture as an infection in NIH 3T3 fibroblast cells[4]. NIH 3T3 and
292 human epithelial (HepG2) cells were obtained from ATCC and grown in RPMI media
293 (Life Technologies).

294 All other transgenic *T. b. brucei* parasites used in this study were derived from
295 monomorphic *T. b. brucei* 2T1 bloodstream forms[29] and were cultured in HMI-11 [HMI-9
296 (GIBCO) containing 10% v/v foetal bovine serum (GIBCO), Pen/Strep solution (penicillin
297 20 U ml^{-1}, streptomycin 20 mg ml^{-1})] at 37 °C/5% CO_2 in vented flasks. Selective
298 antibiotics were used as follows: 5 μg ml^{-1} blasticidin or hygromycin and 2.5 μg
299 ml^{-1} phleomycin or G418. RNAi or overexpression was induced *in vitro* with tetracycline
300 (Sigma Aldrich) in 70% ethanol at 1 μg ml^{-1}. Endogenous Ty, mNeonGreen or myc-
301 overexpression tagging were performed using the pPOTv6 vector[30] and pRPa[29], respectively.
302 The generation of inducible TbCLK1 RNAi was generated as previously described [13]. All
303 primers are listed in Supplementary methods Table 1.

304

305 All cell based assays were performed as described before [4,31]. Briefly, bloodstream form of *T.*
306 *b. brucei Lister* 427, *T. b. rhodesiense* and *T. b. gambiense* parasites were incubated with
307 varying concentration of compounds for 48 hr. Cell viability was assessed by measuring ATP
308 levels using CellTiter-Glo reagent (Promega) and 50% growth inhibition (EC_{50}) was
309 calculated using sigmoidal dose response curves. For both intracellular *T. cruzi* amastigotes
310 and *L. donovani* HU3 axenic amastigotes growth inhibition was measured as described
311 earlier[4]. Intracellular *L. mexicana* amastigote growth inhibition was assessed by microscopy.
312 Briefly, after primary peritoneal mouse macrophages were infected with late-log-phase
313 promastigotes at an infection ratio of 10:1; non-internalized parasites were removed by

washing the plates with PBS, and cells were cultured with different drug concentrations for 96 hr. Determination of intracellular parasite numbers were done by fixing the cells in methanol and then stained with DAPI .Cytotoxicity (CC_{50}) was also measured against mouse fibroblast NIH 3T3 and HepG2 cell lines by incubating compounds for 4 days[4].

Single protein kinase overexpression lines (Supplementary methods table 1) were generated by transfecting *T. brucei brucei* 2T1 cells with a tetracycline inducible overexpression plasmid linearized with *Asc*I restriction enzyme. Each overexpression construct contains the open reading frame (ORF) of a single protein kinase in the plasmid pGL2220 (pRPa-iMYCx)[32]. This plasmid integrates at the tagged rRNA spacer (single locus) of 2T1 *T. brucei.*

Protein kinase overexpression cell lines were adjusted to 2×10^5 cells ml^{-1} and induced for 18 hr by the addition of tetracycline to a final concentration of 1 μg ml^{-1} in 70% ethanol. To establish the EC_{50}, the protein kinase overexpression cell lines and parental control 2T1 were treated with two-fold increasing concentrations of compounds (with similar DMSO increasing concentration as control). Cell viability was measured at 48 hr with a POLARstar Omega plate reader spectrophotometer; the determination of cell viability was carried out by the established colorimetric technique using Alamar Blue (0.49 mM resazurin in phosphate-buffered saline (PBS)), in a 96-well plate format spectrophotometric assay which measures the ability of living cells to reduce resazurin. We used pentadimine isethionate (Sigma Aldrich) as a positive control. Fluorescence emission was detected using a CLARIOstar ® reader (BMG LABTECH; excitation filter at 540 nm and emissions filter at 590 nm). Fitting of dose-response curves and IC_{50}/EC_{50} determination were normalized as percentage of inhibition based on controls. Hesperadin was obtained from ApexBio Technology.

Mouse infection models for *T. brucei*.

The *T. brucei* strain STIB795 acute mouse model mimics the first stage of African trypanosomiasis[33]. Six female NMRI mice were used per experimental group, divided into two cages (A and B). Each mouse was inoculated by intraperitoneal injection with 10^4 bloodstream forms of STIB795, respectively. Heparinized blood from a donor mouse with approximately 5 x 10^6 ml^{-1} parasitaemia was suspended in phosphate saline glucose (PSG) to obtain a trypanosome suspension of 1×10^5 ml^{-1}. Each mouse was injected with 0.25 ml. Compounds were formulated in 0.5% Tween80 in 0.5% methylcellulose. Compound treatment was initiated 3 days post-infection and administered orally on four consecutive

days in a volume of 0.1 ml/10 g. Three mice served as infected-untreated controls. They were not injected with the vehicle alone since we have established in our labs that these vehicles do not affect parasitaemia nor the mice. Blood samples were taken after the 4th treatment. From the mice in cage A, -1 h, 2 h and 8 h after the 4th treatment, 20 uL of blood each were taken from the tail vein and from mice in cage B 1 h, 4 h, and 24 h after the 4th treatment. Parasitaemia was monitored microscopically by tail blood examination twice a week until 31 days post-infection. Mice were considered cured when there was no parasitaemia relapse detected in the tail blood over the 30-day observation period. *In vivo* efficacy studies in mice were conducted at the Swiss Tropical and Public Health Institute (Basel) (License number 2813) according to the rules and regulations for the protection of animal rights ("Tierschutzverordnung") of the Swiss "Bundesamt für Veterinärwesen". They were approved by the veterinary office of Canton Basel-Stadt, Switzerland.

Plasmids

Recoded *CLK1* was synthesised by Eurofins Genomics. The recoded CLK1 sequence ($CLK1^R$) codes for the same amino acid sequence as *CLK1* but only shares 95.06% nucleotide identity. All segments of identity between *CLK1* and $CLK1^R$ are less than 20 base pairs long. $CLK1^R$ was inserted by Gibson assembly® (New England Biolabs) into the plasmid pGL2492 using *Xba*I and *Bam*HI restriction sites, generating pGL2832. This plasmid is designed to constitutively express CLK1 from the tubulin locus, with the addition of a C-terminal 6x HA tag. To express the cysteine 215 mutants, the cysteine 215 was changed to serine or alanine by mutating pGL2832, carrying the coding sequence for *CLK1*, using site directed mutagenic PCR (Primer sequences in Supplementary methods). Similarly, C215S mutation was introduced into original CLK1 OE plasmid, to generate CLK1 C215S OE (NITD001 plasmid) into the parental 2T1 cell line.

Immunofluorescence, cell cycle analysis and cell sorting

Cells treated for 6 hr with compounds or DMSO were centrifuged at 1400 g for 10 min before washing twice with TDB-glucose at room temperature. Suspensions were centrifuged at 1000 g for 5 min and pipetted into 6-well microscope slides and dried at RT. Cells were fixed with 25µl 2% paraformaldehyde diluted in PBS and incubated at room temperature for 5 min. Cells were washed in PBS to remove paraformaldehyde prior to washing twice more with PBS and permeabilized with 0.05% NP40 for 10 min. Cells were washed twice in PBS

and dried at RT. Mounting media with DAPI was added to each well with a coverslip. Slides were kept at 4 ^{o}C before viewing using a Zeiss LSM 880 with Airyscan on an Axio Observer.Z1 invert confocal microscope.

For cell cycle analysis, bloodstream form *T. brucei* cell lines were incubated or not for 6 hr with AB compounds at a final concentration of 2X and 5X the individual EC_{50} value for each compound (averaged from viability assays). Control cultures were treated with 0.5µl DMSO but no AB compound. Cultures were pelleted and cells were collected and washed once in *Trypanosoma* dilution buffer (TDB) supplemented with 5 mM of EDTA and resuspended in 70% methanol. Cells were centrifuged at 1400 g for 10 min to remove methanol and washed once in TDB 1x with 5mM EDTA. Cells were resuspended in 1ml TDB 1x with 5mM EDTA, 10µg ml^{-1} of propidium iodide and 10µl of RNase A. Cell suspensions in 1.5 ml tubes were wrapped in foil to avoid bleaching by light. Cells were incubated for 30 min at 37°C in the dark until FACS analysis. Cells were analysed for FACS using a Beckman Coulter CyAn ADP flow cytometer (excitation; 535, emission; 617).

In the cell cycle analysis, CLK1 OE was induced during 18 hr with tetracycline (1 µg ml^{-1}) and later treated with 4X the individual EC_{50} value for each compound for 4 hr (maintaining tetracycline induction), and finally collected for flow cytometry as above.

Parasite cell sorting was conducted as described previously [19]. Briefly, cell lines were harvested during exponential growth by centrifugation for 10 min at room temperature. The parasite pellet was then resuspended at a concentration of 1×10^6 cells ml^{-1} in HMI-9 medium supplemented with 2% FCS and 10 µg ml^{-1} penicillin/streptomycin. Vybrant DyeCycle Violet (Molecular Probes, Invitrogen) was added to a final concentration of 1 µg ml^{-1}and the cell suspension incubated for 30 min at 37 °C, the tube being protected from light by wrapping in aluminium foil. The samples were then centrifuged and resuspended back in the staining media prior to sorting on a MoFlo XDP Sorter (Beckman Coulter Life Sciences). During and after the sorting, the samples were cooled to below 20 °C to limit cell metabolic activity. The dye was excited using a 407 nm Violet laser and emission detected via a 450/40 bandpass filter. Live parasites were gated based on FSC/SSC profiles, and the gates were set up to collect only the 2C fraction (G0/G1 cells) and 4C fraction (G2, mitotic and post-mitotic cells), to ensure efficient discrimination and selection of these cell-cycle stages.

Protein analysis

Biochemical assays for human EGFR (Epidermal growth factor receptor) and BTK (Bruton's tyrosine kinase) were carried out using a homogenous time-resolved fluorescence (HTRF) assay as described previously [8] .

For enzyme purification, full-length TbCLK1 (Q382U0) CDS was cloned in pET28a PreSc-His and pET24-MBP-TEV vector respectively. Human CLK1 (hCLK1; P49759) was obtained from Promega. Recombinant expression was carried out by lactose autoinduction in Terrific Broth containing 0.4% glycerol, 0.05% glucose, 0.05% lactose, 0.05% arabinose and buffered by 100 mM sodium phosphate (pH 7.0). In brief, 0.7 L of this media (in a 2.8 L Fernbach flask) was inoculated at 0.1 OD600 with an overnight Luria Broth culture and shaken at 37 ˚C and 250 rpm for 2.5 hr. Then, temperature was lowered to 18 ˚C and the culture was allowed to grow and induced overnight and harvested 20-24 hr later. Cells were pelleted and stored at -80 ˚C prior to purification. Cell lysis was done by sonication in an ice bath (20 sec ON/OFF, 3 min active sonication at 70-110 watts power) in 40 mL equilibration buffer (25 mM HEPES pH 7.5 300 mM NaCl 5% glycerol 0.5 mM TCEP) and the clarified lysate is purified by IMAC on a 5 mL HisTrap column (GE Healthcare). The IMAC elution was further purified by sizing on a 300 mL Superdex 200 prep grade column (GE Healthcare) packed in a 2.6 cm diameter housing. Included volume fractions were pooled and analysed by SDS-PAGE or LC-MS.

Preliminary characterization of the TbCLK1 enzyme was carried out and the Km for ATP and MBP (Myelin basic protein, dephosphorylated, Sigma-Aldrich Catalogue no: 13-110, LOT: 3107375) substrate was found to be 9.3 ± 3.6 μM and 0.065 ± 0.02 mg ml^{-1}, respectively. TbCLK1 and hCLK1 enzyme activity assays were performed in white 384 well, solid bottom, Small Volume™ plates (GREINER). The assay buffer contained 40 mM Tris (pH 7.5), 20 mM $MgCl_2$, 0.1mg/ml BSA and 2 mM DTT. Each enzyme (TbCLK1 = 3 nM; hCLK1 = 50 nM) was first incubated with different compound serial dilutions or DMSO control during 10 min and then ATP (10 μM) and MBP substrate (0.1 mg ml^{-1}) mixture was added to initiate the reaction. After 30 min reaction at room temperature, the ADP-Glo reagent and detection solution were added following the technical manual of ADP-Glo™ kinase assay kit (Promega). The luminescence was measured on CLARIOstar BMG LABTECH microplate reader. In all the assays, staurosporine was used as positive control of inhibition. Apparent IC_{50} values were plotted using GraphPad Prism software. The co-crystal

structure of TbCLK1 with AB1 showed specific covalent interaction with cysteine 215. It may be possible that all AB series compounds with Michael acceptors have covalent inhibition of TbCLK1. Since biochemical SAR analysis for all AB series compounds was carried out beyond 10 min incubation with rCLK1, IC_{50}s are represented as apparent IC_{50}s. The AB1 compound showed time dependent inhibition of rCLK1 wherein IC_{50} plateaued around 20 min.

For Western blotting parasites were washed with trypanosome dilution buffer (TDB) supplemented with 20 mM glucose. After centrifugation, the samples were resuspended in the RIPA buffer (New England Biolabs) supplemented with protease and phosphatase inhibitors obtained from Promega and Roche Life Science respectively. All samples were quantified by *Bradford protein* assay (Bio-Rad), 25 µg of protein was loaded, resolved in a 4-20% NuPAGE Bis-Tris gel (Invitrogen) in NuPAGE MOPS running buffer and transferred onto Hybond-C nitrocellulose membranes (GE Healthcare) at 350 mA for 2 hr or, for high molecular weight proteins, overnight at 4 ^{o}C.

After transfer, membranes were washed once in 1x TBST (tris-*buffered* saline (TBS), 0.01% Tween-20 (Sigma Aldrich)) for 10 min then incubated for 1 hr in blocking solution (1x TBST, 5% BSA) or, if required, overnight at 4 ^{o}C. Next, the membrane was rinsed for 10 min in 1X TBST and placed in blocking buffer containing the required primary antisera for 1 hr at room temperature or overnight at 4 ^{o}C. The membrane was then washed 3 times with TBST and placed in blocking solution containing the appropriate fluorescent secondary antisera for 1 hr. A list of antibodies is provided in Supplementary Methods.

Determination of solubility, plasma protein binding, brain tissue binding and microsomal clearance.

Solubility of AB0 and AB1 compounds were determined in a high-throughput thermodynamic solubility assay as described previously [34] . Plasma protein binding was determined for AB1 using mouse blood [34], whilst brain tissue binding was determined using rat brain tissues. Intrinsic metabolic clearance of AB0 and AB1 were determined in mouse, rat and human liver microsomes using the compound depletion approach and LC-MS/MS quantification [35].

Crystallization, data collection, and structure determination.

The TbCLK1/AB1 complex was formed by incubating TbCLK1 kinase domain (V117-M465) with 1mM AB1 at room temperature for 2 hours. The excess AB1 was removed by dialysis overnight in buffer containing 25mM HEPES pH 7.5, 250mM NaCl, 0.5mM TCEP. The TbCLK1 kinase domain/AB1 complex was concentrated to 15 mg/ml for crystallization trials. Crystallization experiments were carried out at 4°C using the sitting-drop vapor diffusion setup. Crystals of the TbCLK1 kinase domain/AB1 complex grew out of a well containing 0.1M HEPES pH 7.5, 1.6M Ammonium Sulfate, 2% w/v Peg 1000. Crystals were cryo-protected in reservoir solution supplemented with 20% glycerol and flash-cooled in liquid nitrogen for data collection. Datasets were collected under cryogenic conditions (100K) at the Advanced Light Source (ALS) beamline 5.0.2. All data were processed using Xia2[36], employing XDS[37] for data integration and AIMLESS[38] for scaling. Molecular replacement were carried out using PHASER[39] with coordinates from human CLK1 crystal structure (PDB ID 6FT8)[40]. Structure refinement was carried out in PHENIX[41] alternated with manual fitting in Coot[42]. Data collection and structure refinement statistics are included in Extended data Table 1.

Differential Scanning Fluorimetry (DSF) for CLK1

Scanning fluorimetry (DSF) was performed on an Applied Biosystems Viia 7 RT PCR instrument, and data was analyzed using Protein Thermal Shift Software version 1.3 (Applied Biosystems/Thermo Fisher). Recombinant *Trypanosoma brucei* CLK1 wild-type or C215A protein was diluted to 5 μM in 25 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) pH 7.5 300 mM NaCl 5% glycerol and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP). Compounds in DMSO or DMSO control was added to a final concentration of 2 mM DMSO and 50 uM compound. SYPRO Orange (Invitrogen/Thermo Fisher) was pre-diluted to 100x in the protein buffer and added to a final concentration of 10x. 70 ul of each condition was prepared. After 30 min of incubation, 3x 20 ul was aliquoted bubble-free into a 384 well PCR plate and covered with RT PCR compatible film, then centrifuged 10 min at 2000 rpm at room temperature, and read in the RT PCR instrument with the appropriate filters. The thermal shift program consisted of 3 minutes at 25 ˚C followed by a 25-95 ˚C ramp at 0.03 ˚C/second and 5 seconds at 95 ˚C.

General Statistics.

All statistical analysis was performed using GraphPad Prism 8 (http://www.graphpad.com/scientific-software/prism/). The appropriate tests were conducted and are as detailed in the corresponding figure legends.

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Figure legends

Fig. 1 Pan kinetoplastid activity of amidobenzimidazole (AB) series.

(a) Structure, anti-kinetoplastid parasite activity, cytotoxicity, tyrosine kinase inhibition and *in vitro* pharmacokinetics of AB0 and AB1. $EC_{50,}$ and CC_{50} represent half-maximum growth inhibition concentration. hEGFR: human epidermal growth factor receptor enzyme. BTK: Bruton's tyrosine kinase. IC_{50} represents half-maximum enzyme inhibition concentration. All the cellular and biochemical data presented were generated with minimum of n=2 or more independent biological replicates (each biological replicate had n=2 technical replicates). All physicochemical and in vitro pharmacokinetics experiments were carried out with at least n = 4 technical replicates (except for rat microsomal clearance for AB0, which had n =2 technical replicates).

(b) Structure activity relationship of amidobenzimidazoles. Each dot represents one compound. Compounds with the Michael acceptor (orange circles) were more potent against *T. b. brucei* (Tbb pEC_{50}) and had better lipophilic efficiency (clogP, calculated octanol-water partition coefficient) compared to non-Michael acceptor compounds (blue circles). AB0 (dark blue) and AB1 (dark orange). The Tbb pEC_{50} data are the negative logarithm of 50% growth inhibition of bloodstream form of *T. b. brucei* (minimum n = 2 biological replicates). The compounds on the left side of the two slanted dotted lines represent lipophilic ligand efficiency (LipE) of 3 and 4. The compounds above two horizontal dotted lines have EC_{50} of 1000 and 100 nM against bloodstream form *T. b. brucei.*

(c) *In vivo* activity of AB1 in a haemolymphatic mouse model for human African trypanosomiasis (untreated control group, mice = 4; all the other experimental groups, mice = 6). Each group of mice were infected with *T. b. brucei* and 3 days post infection, mice were treated with varying concentration of AB1 for 4 days once daily and monitored for relapse in parasitemia over a period of 31 days. Cure plot (Kaplan–Meier plot) showing percentage of animals cured over time are shown. Note the dose dependent activity of AB1, achieving complete cure at 50 mg kg^{-1} once daily dose (in violet).

Fig. 2 Identification of CLK1 as the molecular target for the AB series.

(a) Inducible overexpression (OE) of essential protein kinases (PKs) and target deconvolution approach for the AB series. Schematic representation of the experimental workflow: 6-myc OE plasmids, each containing a specific protein kinase tagged with six myc epitopes, were individually transfected into 2T1 bloodstream

form *T. b. brucei* (VSG221 expressing, Tagged, clone 1). Viability of induced individual *T. b. brucei* OE lines after treatment was assessed by measuring the conversion of resazurin (Alamar blue) to resorufin.

(b) AB1 half-maximal effective concentration (EC_{50}) was analysed from twenty-nine essential individual protein kinase over-expression cell lines (dark grey circles). The graph represents EC_{50} fold change over the parental cell line (*T. b. brucei* 2T1 cell line, DMSO) (light grey shade box), where overexpression of CLK1 confers resistance to AB1. The grey bar represents the EC_{50} average of all cell lines over control. Box error bars represent the library mean with 95% of confidence interval of two biological replicates of the library and CLK1 (n = 4 biological replicates). P-value was calculated using two-tailed Student's t-tests comparing CLK1 OE with parental cell line where *** p-value = 5×10^{-5}.

(c) CLK1 over-expression confers resistance to AB1-induced cell cycle arrest. CLK1 over-expression was induced or not with tetracycline for 18 h, and cells then incubated for 6 h with 5x AB1 EC_{50} (dark grey bars); the 2T1 cell line was a parental control. Cell cycle distribution was determined by flow cytometry. Left: dotted line represents (basal) G2/M untreated average and *** represents a p-value =1.2×10^{-4} (2T1 untreated vs AB1), p-value = 5.8×10^{-4} (CLK1 OE uninduced vs AB1), and not signficant (ns) p-value = 0.02 (CLK1 OE induced vs AB1). P-value was calculated using two-tailed Student's t-test. Error bars represent mean ± SEM of three biological replicates, Right: representative cell cycle profile histogram of cells stained with propidium iodide showing G2/M cell cycle accumulation (arrow).

(d) Violin plot of average length (μm) of the nuclei from parasites treated (blue) or not (grey) with AB1 (n=160 2K1N parasites, *** p-value = 3.9×10^{-26}). Dashed line represents the mean value, and dotted lines indicate both quartiles (25^{th} & 75^{th}). P-value was calculated using unpaired two-tailed Student's t-test. Right: Example of a cell stained with DAPI (cyan) from each condition, where N= nucleus, K= Kinetoplast.

(e) Cell cycle analysis of synchronized *T. b. brucei* after treatment with AB1. Top: Kinetoplast/nucleus configuration of 2C and 4C synchronized parasites, treated or not with 5x EC_{50} AB1 was quantified by DAPI staining at the indicated points (n=200 cells). K = kDNA, N = nucleus, D = duplicating kDNA. Bottom: Schematic representation of *T. brucei* kinetoplast/nucleus configuration through the cell cycle.

Fig. 3 Mechanism of CLK1 inhibition by AB1

(a) Positive correlation between inhibition of recombinant *T. brucei* CLK1 enzyme (apparent pIC_{50}) and growth inhibition (pEC_{50}) of *T. b. brucei* bloodstream form parasites with AB series compounds (n=260; Pearson correlation coefficient (r) = 0.68. Presence (orange) or absence (blue) of Michael acceptor pharmacophore is shown, highlighting AB1 (dark orange) and AB0 (dark blue). Black dash line represents equimolar potency and grey dash line represents 10-fold higher potency for CLK1 pIC_{50} compared to Tbb pEC_{50}.

(b) Lack of correlation between inhibition of recombinant human CLK1 (hCLK1) and recombinant *T. brucei* CLK1 enzyme (apparent pIC_{50}) with AB compound series (n=228; Pearson correlation coefficient (r) = 0.03). Majority of compounds showed > 10-fold selectivity against TbCLK1 compared to hCLK1. Presence (orange) or

absence (blue) of Michael acceptor pharmacophore is shown, highlighting AB1 (dark orange) and AB0 (dark blue). Grey dash line represents equimolar potency and black dash line represents 10-fold higher potency for CLK1 pIC_{50} compared to hCLK1 pIC_{50}.

(c) Crystal structure of the *T. brucei* CLK1 kinase domain (V117-M465) in a covalent thioether bond with AB1 at C215 (PDB: 6Q2A). The CLK1 kinase domain is shown in grey, with a blue αC helix, and the side chains of the DFG motif (DLG in CLK1) shown as sticks. Inhibitor AB1 is shown as ball and sticks with carbon coloured purple, oxygen coloured red and nitrogen coloured blue. A semi-transparent representation of AB1 van der Waals surfaces are shown in magenta. Inset depicts the detailed view of the interactions of AB1. Hydrogen bonds and salt bridge are shown as dotted blue line. MAP-like insertion is showed in orange.

(d) Omit 2Fo-Fc map of AB1/C215 at 2.6Å resolution contoured at 2σ. Hydrogen bonds and salt bridge between the AB1 molecule and *T. brucei* bCLK1 are drawn as blue lines. Covalent bond indicated by yellow arrow.

Fig. 4 CLK1 is the primary target for AB1 in bloodstream form trypanosomes

(a) AB1 binding to CLK1, as probed via differential scanning fluorimetry (DSF). Thermal unfolding of CLK1 is monitored by SYPRO Orange. CLK1 WT (left) and CLK1 C215A (right) recombinant protein in the presence of AB0 (green), AB1 (red) or DMSO (blue) as a reference. Data from one representative experiment, out of three independent experiment with similar results, are shown. Note the shift in Tm (melting temperature of protein) for CLK1 in presence of AB1 compound.

(b) Expression of *T. brucei* CLK1 C215A mutant is resistant to AB1. Dose-response curves of apparent AB1 and staurosporine IC_{50} of recombinant WT *T. brucei* CLK1 and C215A mutant. Response to 3-fold serial dilutions of each compound were assessed as described in methods. Each data point represents the mean of two technical replicates.

(c) Overexpression of CLK1 C215S mutant confers > 60-fold EC_{50} shift to AB1. CLK1 C215S mutation (green) was overexpressed in the parental *T. b. brucei* 2T1 cell line and AB1 half-maximal effective concentration (EC_{50}) was determined after 72 h and compared with *T. b. brucei* 2T1 WT parental cell line (blue). Data represent mean ± SEM of three independent biological experiments. Inset: Tetracycline inducible over-expression of CLK1-myc was assessed for three clones by western blot, using an anti-myc antibody. EF1α was used as the loading control.

(d) Overexpression of CLK1 C215S mutant impairs the parasite growth effect of AB1 treatment. Parasites overexpressing CLK1 C215S (green) and parental *T. b. brucei* 2T1 cell line (blue) were treated with 5x (line) or 10x AB1 EC_{50} (dashed line) during 48 h. Mean ± SEM (n=3) is shown. Data represent the mean of the percentage of cell growth relative to time=0 h (1.5×10^4 parasites ml^{-1}). P values were calculated using a two-tailed Student's t-test comparing with parental control where *** represent the P-values of 24 h (p-value = 5.4×10^{-4}), 48 h (p-value = 3.4×10^{-4}), and 72 h (p-value = 3.3×10^{-4}) after treatment with 5x AB1 EC_{50}, and 24 h (p-value = 9.9×10^{-7}), 48 h (p-value = 1.7×10^{-3}), and 72 h (p-value = 1.6×10^{-3}) for 10x AB1 EC_{50} treatment.

(e) Percentage of survival of parasites exposed to 5-150x AB1 EC_{50} fold range. Parental *T. b. brucei* 2T1 control (blue) and CLK1 C215S mutant (green) (2×10^3 parasites ml^{-1}) were exposed for 72 h to 5, 15, 50, and 150-fold AB1 EC_{50}. Survival was calculated by using CellTiter-Glo® luminescent cell viability assay. Data represent the mean ± SEM of percentage of survival. (C215S mutant n=3, Parental 2T1 n=4).

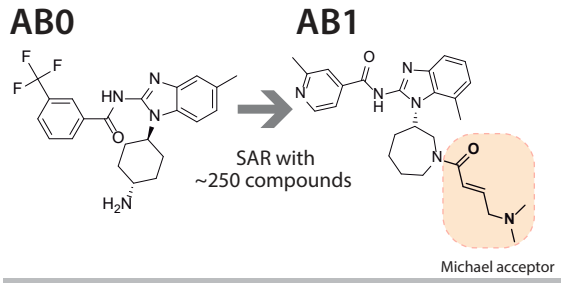
Fig. 5 CLK1 inhibition impairs inner kinetochore dynamics.

(a) Localization of kinetochore protein KKT2 after CLK1 inhibition by AB1. Parasites were incubated or not for 24 h with 2x EC_{50} (upper panel) or 6 h with 5 x EC_{50} AB1 (lower panel). Representative fluorescence micrographs, showing bloodstream form parasites endogenously expressing N-terminal mNeonGreen (mNG) tagged KKT2. Cells in metaphase and anaphase are shown. Cells were counterstained with DAPI to visualize DNA (cyan). The right panel shows the Nomarsky (DIC) corresponding images. Upper right panel shows cell cycle progression after treatment with 2x EC_{50} AB1 for 72 h. Data are representative from one of three independent biological replicates with similar results.

(b) Localization of KKT2 after CLK1 depletion by RNAi. Representative fluorescence micrographs, showing 24 h induction of CLK1 RNAi in bloodstream form parasites endogenously expressing N-terminal mNeonGreen (mNG) labelled KKT2, compared with not induced control cells in metaphase and anaphase are shown. Cells were counterstained with DAPI to visualize DNA (cyan). The right panel shows the Nomarsky (DIC) corresponding images. Data are representative from one of three independent biological replicates with similar results.

Figure 1

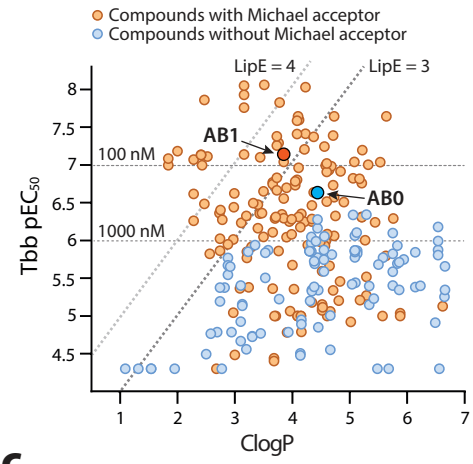
a



	AB0 Mean ± SEM	AB1 Mean ± SEM
Cellular activity [µM]		
T. brucei brucei▲ EC_{50}	**0.29** ± 0.014	**0.072** ± 0.006
T. b. gambiense▲ EC_{50}	**0.37** ± 0.072	**0.057** ± 0.013
T. b. rhodesiense▲ EC_{50}	-	**0.033** ± 0.009
T. cruzi▲▲ EC_{50}	**1.27** ± 0.052	**0.044** ± 0.017
L. donovani▲▲▲ EC_{50}	-	**0.600** ± 0.078
L. mexicana▲▲ EC_{50}	-	**1.564** ± 0.045
NIH 3T3 fibroblast CC_{50}	**4.38** ± 0.79	**9.660** ± 2.240
HepG2 CC_{50}	**2.35** ± 0.24	**9.060** ± 0.830
Tyrosine Kinase activity [µM]		
hEGFR IC_{50}	**0.3***	**0.002** ± 0.0005
BTK IC_{50}	-	**0.002** ± 0.0025
Physicochemical and *in vitro* Pharmacokinetics		
Solubility [µM]	**32,85** ± 0.01	**900** ± 8.165
Plasma protein binding [%]	-	**94.1** ± 0.100
Brain tissue binding [µM]	-	**>99**
Microsomal clearance (mice/rat/human) [µl/min/mg]	**42.4**±1.19/**184***/**27**±20.1	**65.5**±3.2/**22.2**±3.9/**42.4**±15.6

▲Bloodstream form, ▲▲Intracellular amastigote, ▲▲▲Axenic amastigote.
*Data obtained from two technical replicates

b



c

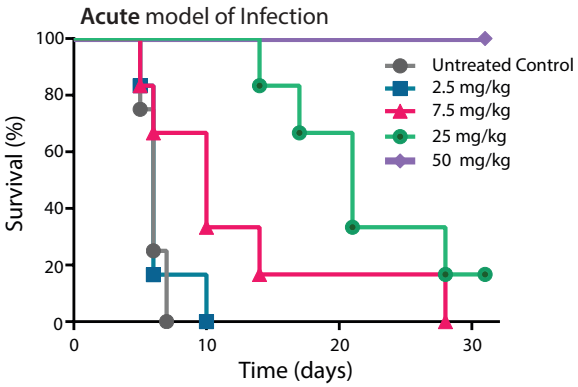
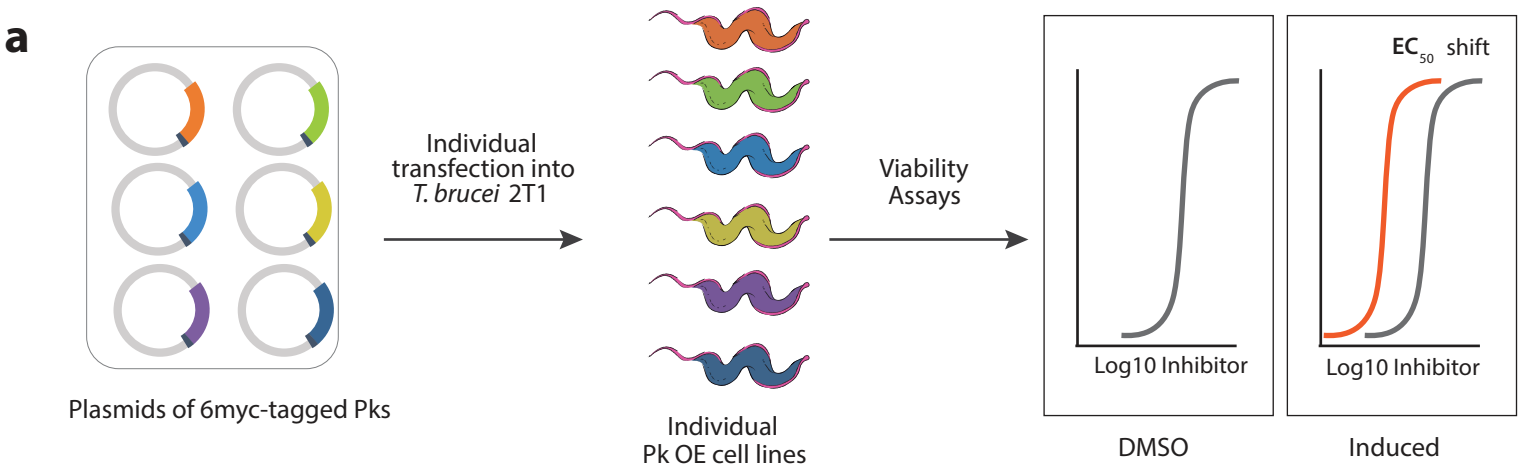
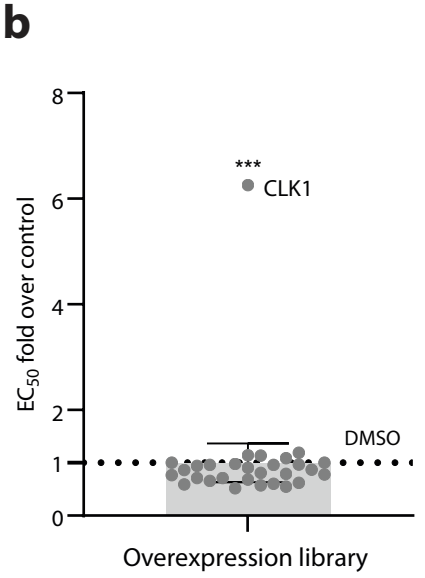
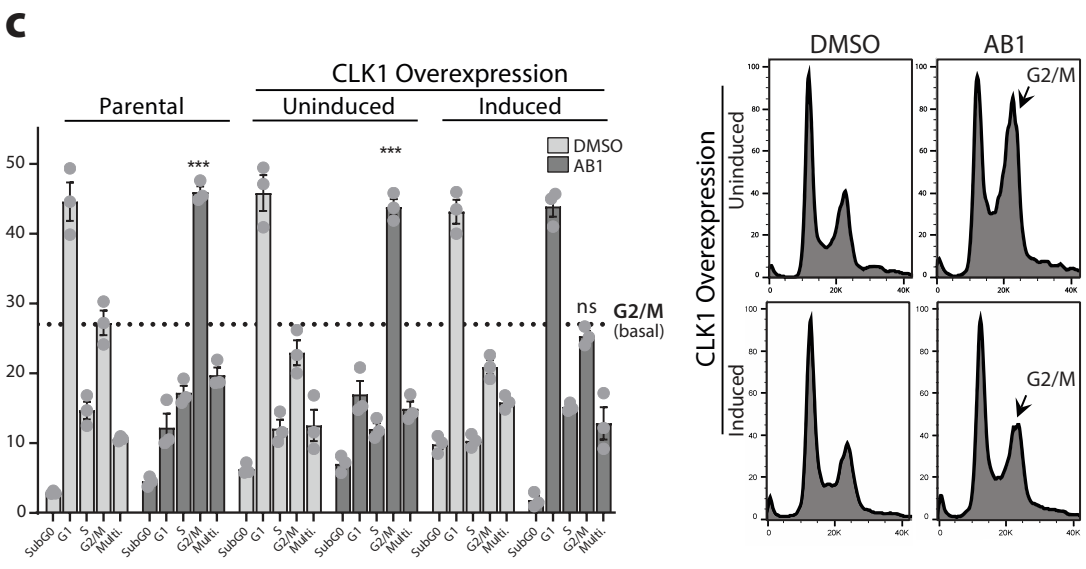
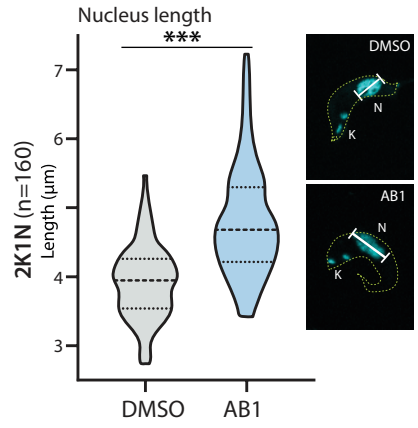


Figure 2


a
Plasmids of 6myc-tagged Pks
Individual transfection into T. brucei 2T1
Individual Pk OE cell lines
Viability Assays
Log10 Inhibitor
DMSO
EC50 shift
Log10 Inhibitor
Induced


b
EC50 fold over control
***
CLK1
DMSO
Overexpression library


c
CLK1 Overexpression
Parental
Uninduced
Induced
DMSO
AB1
***
ns
G2/M
(basal)
SubG0
G1
S
G2/M
Multi.
DMSO
AB1
CLK1 Overexpression
Uninduced
Induced
G2/M


d
Nucleus length
***
2K1N (n=160)
Length (µm)
DMSO
AB1
DMSO
AB1
N
K

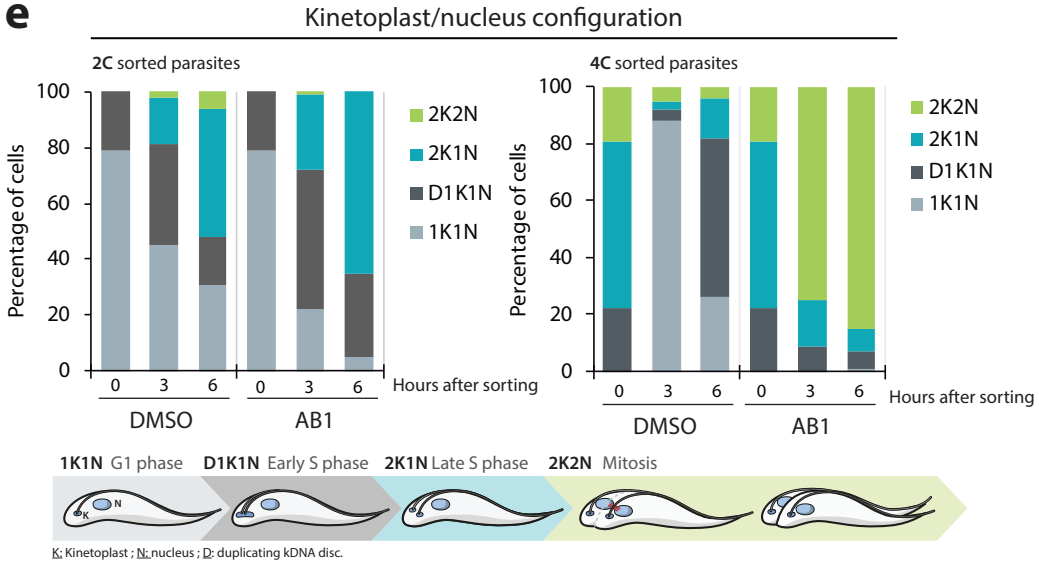

e
Kinetoplast/nucleus configuration
2C sorted parasites
4C sorted parasites
Percentage of cells
2K2N
2K1N
D1K1N
1K1N
Hours after sorting
DMSO
AB1
1K1N G1 phase
D1K1N Early S phase
2K1N Late S phase
2K2N Mitosis
K: Kinetoplast ; N: nucleus ; D: duplicating kDNA disc.

Figure 3

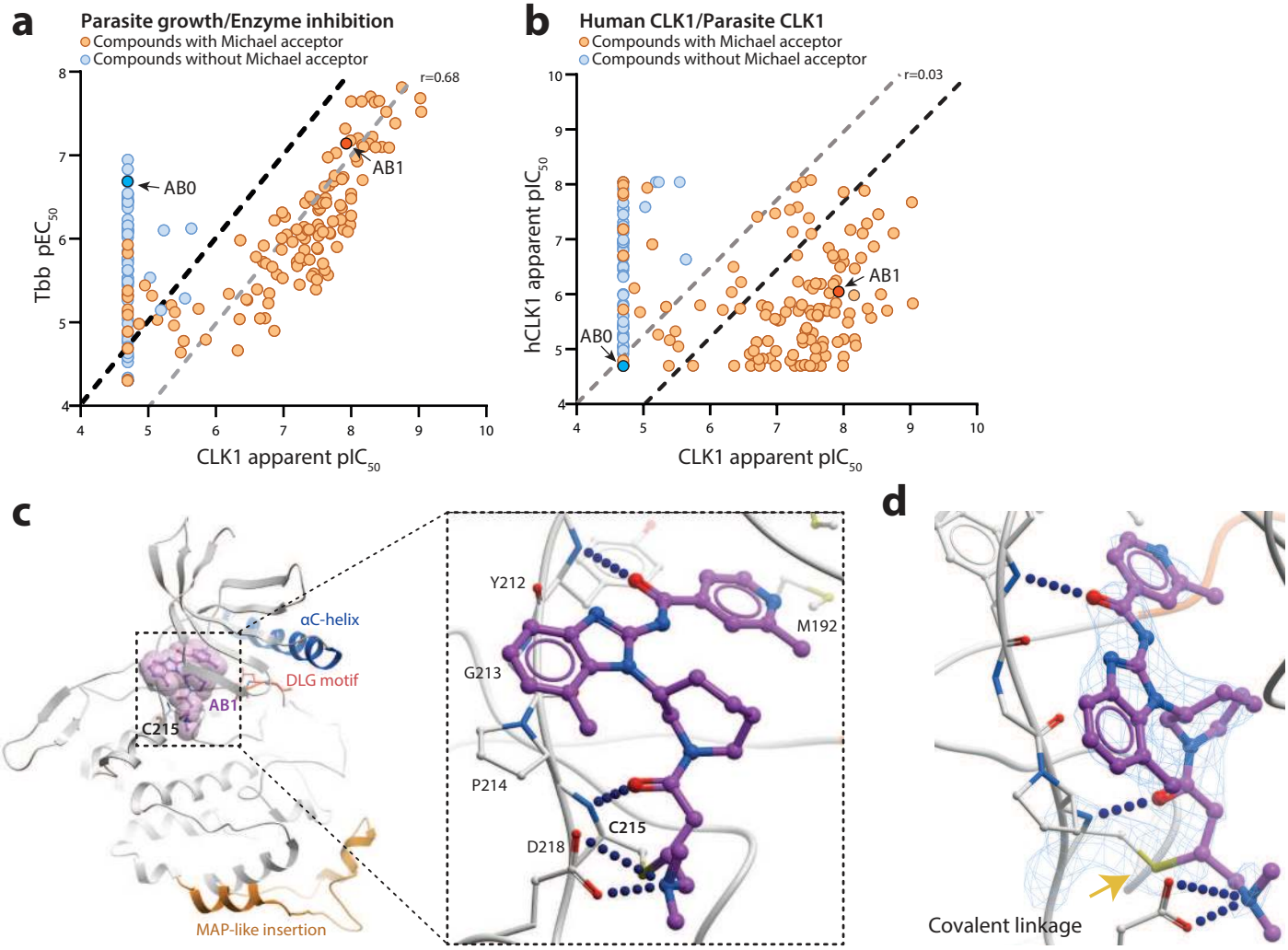

a
Parasite growth/Enzyme inhibition
Compounds with Michael acceptor
Compounds without Michael acceptor
r=0.68
AB0
AB1
Tbb pEC50
CLK1 apparent pIC50
b
Human CLK1/Parasite CLK1
Compounds with Michael acceptor
Compounds without Michael acceptor
r=0.03
AB0
AB1
hCLK1 apparent pIC50
CLK1 apparent pIC50
c
αC-helix
DLG motif
AB1
C215
MAP-like insertion
Y212
M192
G213
P214
C215
D218
d
Covalent linkage

Figure 4

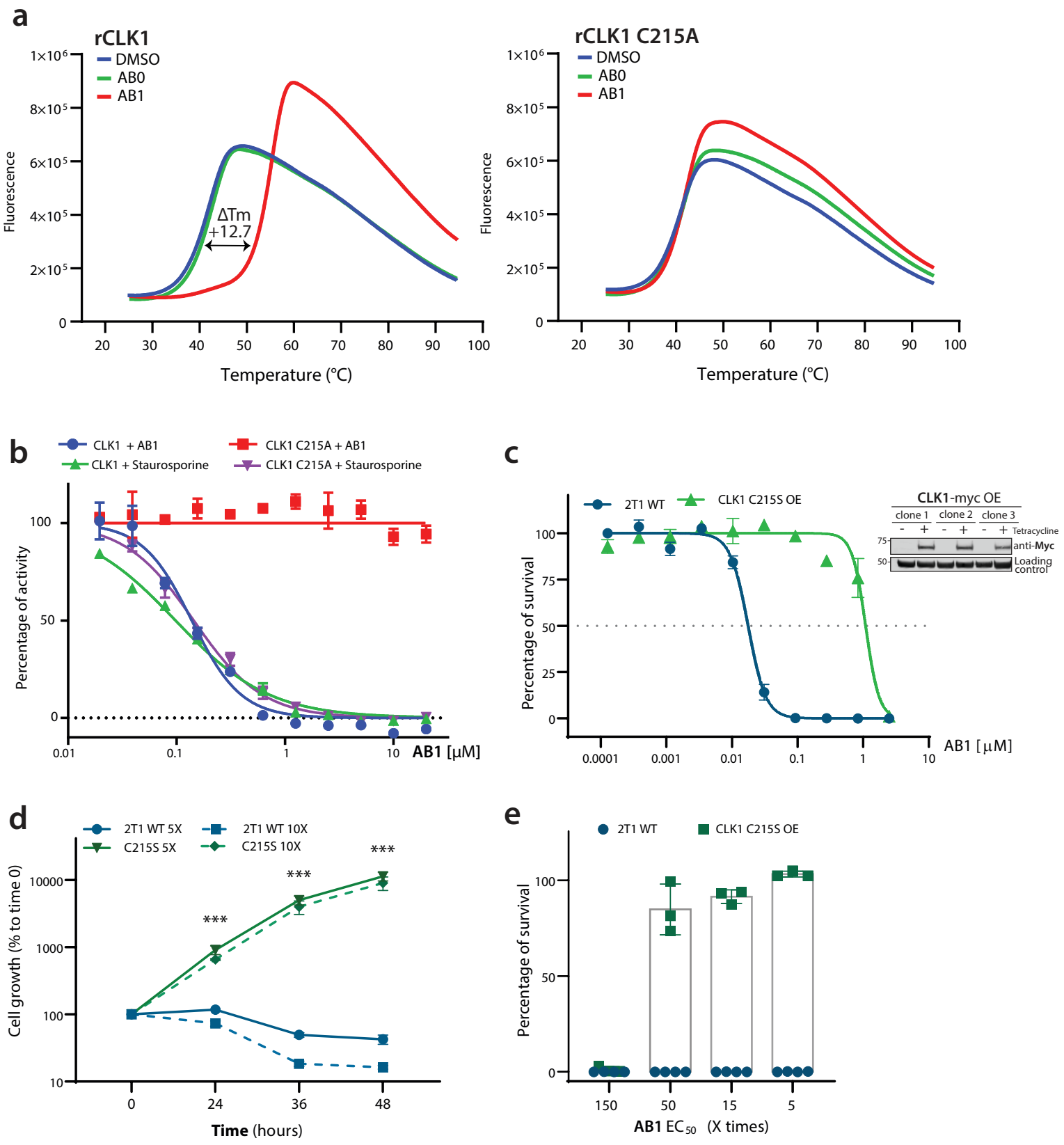
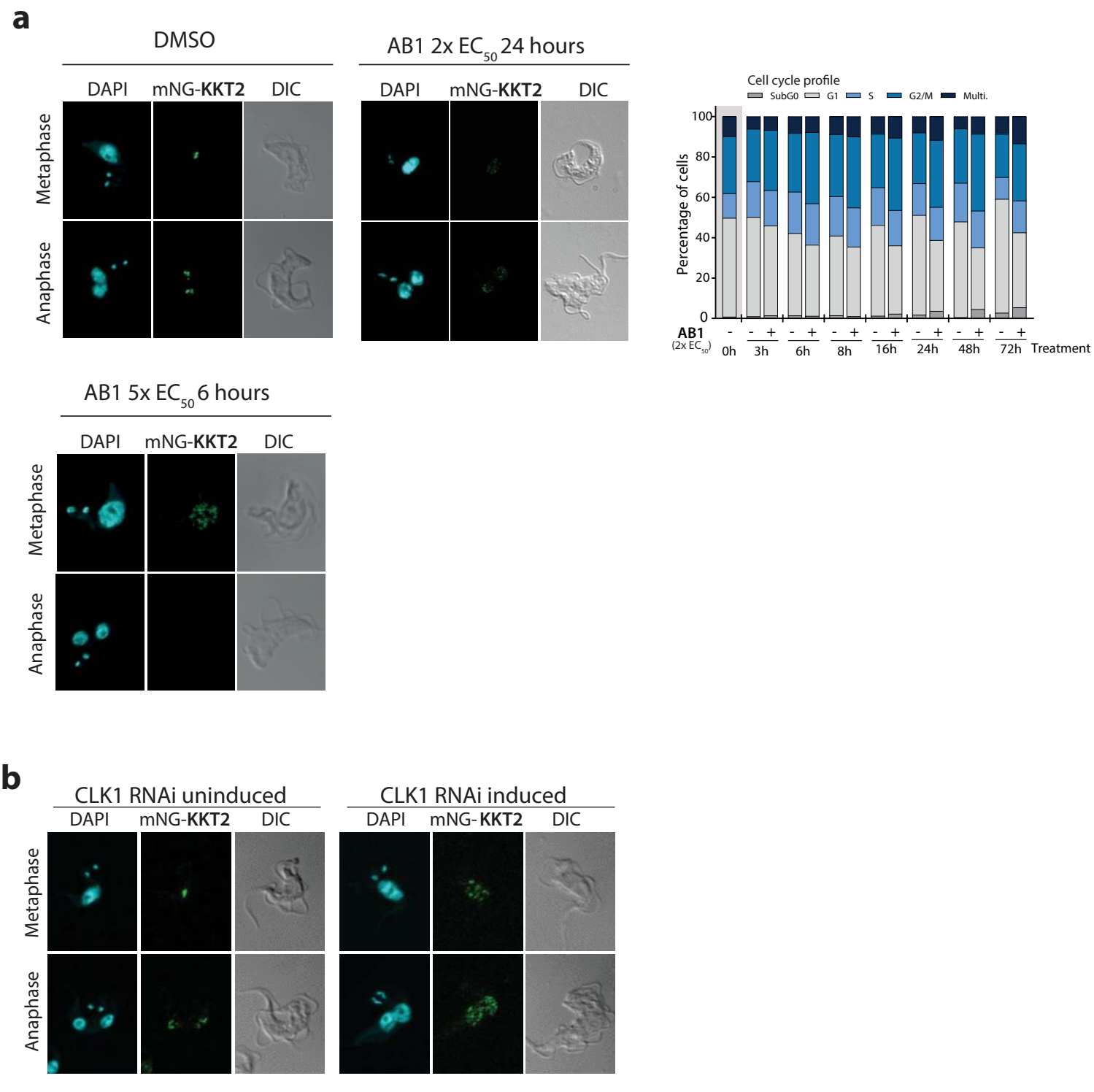

a
rCLK1
DMSO
AB0
AB1
ΔTm
+12.7
Fluorescence
Temperature (°C)
rCLK1 C215A
b
CLK1 + AB1
CLK1 C215A + AB1
CLK1 + Staurosporine
CLK1 C215A + Staurosporine
Percentage of activity
AB1 [µM]
c
2T1 WT
CLK1 C215S OE
CLK1-myc OE
clone 1
clone 2
clone 3
Tetracycline
anti-Myc
Loading control
Percentage of survival
AB1 [µM]
d
2T1 WT 5X
C215S 5X
2T1 WT 10X
C215S 10X
Cell growth (% to time 0)
Time (hours)
e
2T1 WT
CLK1 C215S OE
Percentage of survival
AB1 EC50 (X times)

Figure 5


a
DMSO
AB1 2x EC50 24 hours
DAPI
mNG-KKT2
DIC
Metaphase
Anaphase
Cell cycle profile
SubG0
G1
S
G2/M
Multi.
Percentage of cells
100
80
60
40
20
0
AB1
(2x EC50)
0h
3h
6h
8h
16h
24h
48h
72h
Treatment
AB1 5x EC50 6 hours
b
CLK1 RNAi uninduced
CLK1 RNAi induced