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A novel protein kinase is essential in bloodstream *Trypanosoma brucei*

Bryan C. Jensen^a, Nick Booster^{a,1}, Rama Subba Rao Vidadala^b, Dustin J. Maly^{b,c}, and Marilyn Parsons^{1,d,*}

^aCenter for Infectious Disease Research, formerly Seattle Biomedical Research Institute, 307 Westlake Ave. N., Seattle, WA 98109 USA

^bDept. of Chemistry, University of Washington, Seattle, WA 98195 USA

^cDept. of Biochemistry, University of Washington, Seattle, WA 98195 USA

^dDept. of Global Health, University of Washington, Seattle, WA 98195 USA

Abstract

Human African trypanosomiasis a fatal disease for which no vaccines exist and treatment regimens are difficult. Here, we evaluate a *Trypanosoma brucei* protein kinase, AEK1, as a potential drug target. Conditional knockouts confirmed *AEK1* essentiality in bloodstream forms. For chemical validation, we overcame the lack of AEK1 inhibitors by creating parasites expressing a single, functional analog-sensitive AEK1 allele. Analog treatment of mice infected with this strain delayed parasitemia and death, with one-third of animals showing no parasitemia. These studies validate AEK1 as a drug target and highlight the need for further understanding of its function.

Abstract

*Corresponding author. marilyn.parsons@cidresearch.org.

¹present address: Department of Entomology UC Davis, 1 Shields Ave Davis, CA 95616 USA

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Keywords

Drug target; Protein kinase; Trypanosome; AGC kinase; Analog-sensitive; In vivo

Trypanosoma brucei, the causative agent for human African trypanosomiasis (HAT, also known as African sleeping sickness), is a protozoan parasite transmitted by the bite of the tsetse fly. The fly injects parasites that rapidly differentiate into proliferative bloodstream form (BF) cells, causing disease and ultimately death (Kennedy, 2013). Development of an anti-HAT vaccine is not practical due to the renowned ability of the parasite to vary its outer surface coat (Horn, 2014; Mugnier et al., 2015). Early in infection, the parasite is predominantly found in the blood and lymphatic system, where it can be treated relatively effectively with few side-effects. However, due to its non-specific symptoms (e.g., fever, malaise, joint pain) stage 1 HAT frequently remains untreated or is misdiagnosed as malaria, allowing progression to stage 2. There, the parasite crosses the blood brain barrier. Patients in stage 2 HAT eventually drop into a coma and die unless treated. Once the infection has entered stage 2, only two drugs are approved for use: melarsoprol and eflornithine. Melarsoprol, an arsenical derivative, is effective against both *T. brucei* human-infective subspecies (*Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*) but is toxic, with 3-10% of patients receiving treatment suffering fatal encephalopathy (Kennedy, 2013). Eflornithine is effective against the more common *T. b. gambiense*, but is difficult to administer, requiring multiple daily intravenous treatments (Eperon et al., 2014). Recently a combination of eflornithine with nifurtimox, a drug approved for use against American trypanosomiasis, has shown promise in shortening the course of treatment (Kennedy, 2013; Eperon et al., 2014). Still, the combination therapy treatment remains complex and the prospect of drug-resistant parasites emerging in the field has created the need for additional, better therapeutic agents. Two new compounds, fexinidazole and SCYX-7158, show promise in clinical development (Brun et al., 2011; Eperon et al., 2014), although additional drugs will likely be needed to reach the goal of elimination of *T. b. gambiense* disease (Jones and Avery, 2015).

With the need to develop new drugs to treat HAT comes the need to identify new drug targets: essential proteins that can be inhibited by small bioavailable molecules. As a class, the protein kinases (PKs) meet the criterion of druggability by virtue of their ATP-binding pocket. Genome-wide analysis suggested that a significant number of *T. brucei* kinases are essential (Alsford et al., 2011), and a recent study in *T. brucei* found that 22% of the PKs (including canonical, atypical and pseudo PKs) are essential (Jones et al., 2014). Finally, despite the conserved overall architecture of the ATP binding site and millimolar concentration of ATP in most cells, over thirty drugs targeting PKs have been approved by the FDA for use in humans (Roskoski, Jr., 2015; Wu et al., 2016), mostly as cancer therapeutics.

At the back of the ATP-binding pocket of almost all PKs is a bulky hydrophobic residue, termed the gatekeeper residue, which limits the size of ATP-competitive ligands that can occupy this site. While a few human and *T. brucei* PKs have residues as small as threonine at this location, the large majority of PKs utilize methionine, leucine or phenylalanine. Although this is not always the case (Oh et al., 2007; Lopez et al., 2014), some PKs tolerate mutation of the gatekeeper residue to small glycine or alanine residues, which enlarges the size of the ATP-binding site (Johnson et al., 2008; Hengeveld et al., 2012; Lourido et al., 2012; Lozano-Nunez et al., 2013). This creates an analog-sensitive PK (as-PK) which can be specifically inhibited by ATP-competitive inhibitors that contain bulky side chains (called bumped kinase inhibitors, BKIs), unlike the wild-type (WT) PK (Liu et al., 1999). Replacement of the WT alleles with as-alleles creates cells that can be treated with BKIs that have previously been shown to be cell-permeant and for which some pharmacokinetic data may be available, thereby avoiding laborious medicinal chemistry for early stage studies of target validation. This chemical genetic approach is similar to therapeutic application of drugs, since the PK is present but inactivated with an ATP-competitive inhibitor, rather than absent as with genetic approaches such as RNA interference (RNAi) or conditional knockouts (cKO). To our knowledge this approach has been successfully applied in one instance thus far to study the cellular function of a kinetoplastid PK, the polo-like kinase of *T. brucei* (Lozano-Nunez et al., 2013), but has not yet been used for in vivo studies of parasite infection.

We decided to apply the as-PK/BKI approach to the PK encoded by Tb927.3.2440 (GenBank accession no **XP_843838**), which was recently described as essential in an RNAi study (Jones et al., 2014), but which has not been examined in detail. Tb927.3.2440 encodes a 46 kDa protein that belongs to the AGC family of PKs and hence we dubbed it AGC essential kinase 1 (AEK1). Within its catalytic domain, it shows ~36% identity to mammalian kinase AKT3 (also known as γ or protein kinase B γ), but it lacks the phosphoinositide binding domain of AKTs (Supplementary Fig. S1). Similar to many other AGC kinases, AEK1 possesses a hydrophobic motif near its C-terminus. Ribosome profiling data indicates that Tb927.3.2440 is well expressed, being in the top 23rd percentile for protein production in BF, with two-fold higher expression in procyclic cells (Jensen et al., 2014). The predicted protein has orthologues in *Trypanosoma cruzi* and *Leishmania major*, which show 85% and 78% amino acid identity across the kinase domain, respectively (Supplementary Fig. S1).

We independently corroborated previous RNAi experiments indicating that *AEK1* is an essential gene by generating a cKO in single marker Lister 427 strain BF (Wirtz et al., 1999). Fragments containing the 5' and 3' untranslated regions were fused via PCR to selectable markers as previously described (Merritt and Stuart, 2013). Then, the WT coding sequence cloned into the plasmid pLEW-3V5-PAC (Flaspohler et al., 2010) was introduced, allowing for tetracycline (Tet, Sigma-Aldrich, USA) inducible expression of AEK1 with three C-terminal V5 tags. The parasites were transferred into medium containing Tet and transfected with the PCR fragment to replace the second endogenous allele. Following selection, PCR analysis verified that both endogenous copies of *AEK1* were deleted and that the only remaining allele was the tagged Tet-regulated WT allele (Supplementary Fig. S2).

In medium without Tet *aeK1* cKO parasite numbers were reduced by 24 h (compared to the +Tet condition), and by 48 h most of them had lysed (Fig. 1A,B), showing a somewhat stronger phenotype than seen by RNAi (Jones et al., 2014). The rapid onset of the growth defect suggests that AEK1 protein may be less stable than most trypanosome proteins, or that the expressed levels of the transgene are barely over that required for cell growth. By 24 h after Tet withdrawal, the parasites had only 3% of the tagged protein compared with controls (Fig. 1C). The drop in the levels of AEK1 was accompanied by cytologic abnormalities at 24 h as summarized in Fig. 1D (images are provided in Supplementary Fig. S3). In the normal cell cycle, the *T. brucei* mitochondrial DNA network (the kinetoplast, K) divides before the nucleus (N), yielding the progression of 1N1K to 1N2K to 2N2K parasites. At 24 h, approximately 25% of recognizable parasites had abnormal numbers of nuclei and/or kinetoplasts, with the most common defect being the presence of more than two nuclei. Additionally some cells lacked kinetoplasts (1N0K, known as zoids) or had a single kinetoplast but two nuclei (2N1K). These findings were consistent with defective cytokinesis following loss of AEK1, as was proposed previously (Jones et al., 2014), which may be a direct or indirect effect. Flow cytometric analysis of DNA content demonstrated a decreased ratio of G1 (2C) to G2/M (4C) phase parasites at 24 h after knockdown. Debris and particles with low DNA content had increased by 24 h and by 48 h very few parasites with G1 or G2/M DNA content were observed. This finding indicated that degradation of DNA had occurred, most likely during the process of cell death, although a small proportion may have been the result of nuclear mis-segregation yielding zoids.

We next wished to develop parasite strains that would allow pharmacological validation of AEK1 using a BKI. We began by testing whether the mutant protein could rescue the cKO, since as noted above gatekeeper mutants are often inactive or require second-site suppressor mutations to function, as is the case with *T. brucei* polo-like kinase (Lozano-Nunez et al., 2013). Starting with the cKO, we constitutively expressed (from the β -tubulin locus) another copy of AEK1 in which the gatekeeper site was mutated to a small amino acid (M138G and M138A). Additionally, we made strains in which the constitutive copy was WT or had a mutation in the essential lysine in subdomain II (K90M) that should inactivate the protein. As expected the WT copy allowed growth under restrictive conditions (-Tet, Fig. 2A), but the K90M mutant did not (Supplementary Fig. S4). Both the glycine and alanine gatekeeper mutations of AEK1 allowed parasite growth that was indistinguishable from WT AEK1, whether or not Tet was present (Fig. 2A, Supplementary Fig. S4). We tested four BKIs: NA-PP1 (Bishop et al., 1999) (Cayman Chemical, USA), NM-PP1 (Bishop et al., 1999)

(MedChem Express, USA), 1294 (Johnson et al., 2012) and 1553 (unpublished results)) for their effects on growth of parasites expressing WT AEK1 (structures and EC₅₀s are given in Supplementary Fig. S5). Preliminary experiments showed that the BKIs reduced growth of the cKO lines under conditions where only the gatekeeper mutant was expressed.

Compound 1294 was selected for further study because some pharmacokinetic and toxicity data in mice were available (Doggett et al., 2014), indicating that it could be suitable for experiments examining *T. brucei* infections. We focused on the comparison of cKO parasites that constitutively expressed either WT AEK1 or the M138G mutant using a 48 h growth assay after Tet removal (Fig. 2B, Supplementary Fig. S6). The cKO parasites expressing WT AEK1 were not affected by the compound 1294 up to 4 μM. In contrast, the growth of BF expressing AEK1(M138G) alone was affected by the drug, with an EC₅₀ of approximately 1 μM. No cellular abnormalities were seen at 8 h (approximately one cell cycle), but at 16 h >45% of parasites showed cytologic abnormalities similar to those observed in the *aeK1* cKO, with the most common abnormal phenotypes being more than three nuclei and 2N1K configurations (Fig. 2C). Flow cytometric analysis showed a decrease in G1 cells in populations expressing AEK1(M138G) or AEK1(M138A) that were treated with compound 1294, with a stronger increase in the relative abundance of higher DNA-content cells for the latter (Supplementary Fig. S7)). The reason for this difference is not known, although it could reflect the longer time in culture for AEK1(M138G). Taken together, these data provide in vitro chemical validation of AEK1 as a drug discovery target.

We moved on to in vivo mouse studies, by testing the effect of 1294 on the course of infections with the BF cKO expressing AEK1(M138G) in the presence or absence of the Tet analog doxycycline (Dox, Sigma-Aldrich). Mice were infected with 5×10^4 parasites, and 21 h later began a 4 day treatment protocol with 1294 (10 mg/kg) or vehicle alone delivered twice each day by i.p. injection. We monitored the blood parasitemia and euthanized mice when the parasitemia reached 10⁸/ml. Both time to detection of parasitemia (Fig. 2D, Supplementary Fig. S7) and to euthanasia (Fig. 2E) were delayed for the test group, i.e., those mice infected with parasites expressing the M138G allele only (-Dox condition) and treated with compound 1294. Controls included mice infected with parasites expressing WT AEK1 (+ Dox condition) and treated with 1294, and mice receiving the vehicle only, whether or not Dox was present. On average, within 3 days, mice in these control groups had detectable parasitemias, showing that the BKI was not inhibiting parasite growth indiscriminately (Fig. 2D, Supplementary Fig. S8). In contrast, one-third of the mice in the test group showed no parasitemia throughout the 21 day experiment, and the remainder were delayed, with parasitemia appearing on average at day 11. Similar effects were seen on the progression of disease. Excluding the mice in which 1294 prevented detectable parasitemia, mice infected with parasites expressing only AEK1(M138G) and treated with 1294 survived more than twice as long as the other groups of mice (Fig. 2E). We rescued parasites from two of the 1294-treated mice that succumbed to infection in the -Dox condition, when no WT allele should be expressed. The parasites were still susceptible to in vitro treatment with 1294, indicating that parasite survival was not a result of evolved resistance or loss of regulation of the WT transgene, but rather shortcomings in the treatment itself. This is not surprising given the rather high in vitro EC₅₀.

Taken together, the genetic and chemical validation make a strong case for further exploration of AEK1 as a drug target for HAT. The high level of conservation of AEK1 in other pathogenic trypanosomatids suggests that compounds could be effective for multiple disease agents. With these results, it becomes important to identify the pathways in which AEK1 participates, as they may offer additional opportunities to disrupt parasite functions. For example, similar to many AGC PKs, specific phosphorylation events may be required to activate AEK1, which contains several phosphoserines (Nett et al., 2009; Urbaniak et al., 2012). The activating PKs could be potential therapeutic targets. Downstream substrates may be identified by taking advantage of the as-alleles created here in specific labeling strategies (Hengeveld et al., 2012; Rothenberg et al., 2016). Additionally, such as-alleles allow for near instantaneous loss of activity in a population of cells, rather than gradual loss of protein, potentially allowing primary effects of AEK1 inhibition to be studied as was done with *T. brucei* polo-like kinase (Lozano-Nunez et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Genetic validation of *Trypanosoma brucei* protein kinase AEK1
- Lack of inhibitors was overcome by creating an analog-sensitive mutant
- *Trypanosoma brucei* expressing an analog-sensitive mutant allowed chemical validation in vitro and in vivo

Fig. 1. Depletion of *Trypanosoma brucei* AEK1 mRNA leads to growth and cytologic defects. (A) Growth curve of the *aek1* conditional knockout (cKO) in the induced (+tetracycline (Tet)) and uninduced (-Tet) condition. Bloodstream forms from cultures grown in Tet were pelleted, washed twice in medium without Tet, and seeded into medium with or without Tet. Particle counts were performed daily using a Coulter Counter and include live cells plus dead cells that are relatively intact. The percentage of particles that were morphologically intact cells as judged by microscopic analysis is given for the -Tet condition. For the control condition, this number was always >95%. (B) Phase-contrast images of parasites over the time course. At 24 h rounded abnormal forms with detached flagella were present although

many cells were intact. By 48 h, although cell bodies were observed, few had the normal elongated structure, and many were apparently fragmented. All images are at the same magnification (scale bar = 5 μ m). (C). Western blot analysis showing rapid depletion of AEK1 after Tet withdrawal. The tagged complementing gene was detected with anti-V5 antibodies. As a loading control, the blots were also probed with rabbit anti-*T. brucei* CKII α antibodies. (D) Knockdown of AEK1 leads to cytologic abnormalities. Following DAPI staining, the numbers of nuclei and kinetoplasts per parasite were enumerated before and 24 h after Tet withdrawal. Abnormal configurations are designated as “other” and are detailed at right. At least 200 parasites that appeared intact were counted under each condition. Representative images with DAPI staining are provided in Supplementary Fig. S3. (E) Flow cytometric analysis shows a decreased ratio of G1 to G2/M parasites. Parasites were fixed in methanol, treated with RNase, and stained with propidium iodide (PI) as previously described (Gale, Jr. et al., 1994) prior to flow cytometry. Events (25,000) were evaluated and a combination of forward and side scatter was used to eliminate particles that were much smaller than cells. The positions of cells possessing 2C DNA content (diploid, G1), 4C (G2/M), 6C and 8C are marked. Cells with replicating DNA lie between these peaks, as would G2/M or multinucleate cells undergoing DNA degradation. The asterisk indicates a peak with much less than diploid DNA content. This peak includes cells lacking nuclei (zooids) and dead/dying cells in which the DNA was degraded.

Fig. 2. Chemical validation in *Trypanosoma brucei* using analog-sensitive AEK1 protein kinase. (A) Parasites expressing only the gatekeeper mutant AEK1 (M138G) exhibit normal growth. Bloodstream form (BF) lines were washed twice in medium lacking tetracycline (Tet) and resuspended in medium with or without Tet. The test group is the parasites expressing AEK1 (M138G) in the -Tet condition (no expression of a wild type (WT) allele). Other groups are positive controls. (B) Parasites expressing only the gatekeeper mutant of AEK1 (M138G) are sensitive to BKI 1294. BF *aeK1* conditional knockouts additionally constitutively expressing either WT AEK1 or AEK1 (M138G) were grown for several days in the absence of Tet prior to the addition of compound 1294. Particles were enumerated 48 h later, with the numbers being compared with the same BF strain in the absence of both Tet and bumped kinase inhibitor 1294 (set to 100%). Data is representative of three experiments. (C) Treatment of parasites expressing only AEK1 (M138G) results in cytologic abnormalities.

BF cKOs expressing AEK (M138G) were grown for several days in the absence of Tet before the addition of BKI ($t=0$). The numbers of DAPI-stained nuclei and kinetoplasts per parasite were enumerated at the times indicated ($n>100$ per time point). Abnormal configurations are designated as “other”; and the 16 h “other” data are detailed. (D) Treatment with compound 1294 delays parasitemia in mice infected with the gatekeeper mutant of AEK1. The cumulative fractions of mice that had microscopically detectable parasitemias by each day are graphed. Mice were infected with the cKO parasites under conditions in which both the WT and M138G alleles were expressed (+Dox) or in which only M138G was expressed (-Dox). Dox was providing in the drinking water for the +Dox groups. Starting 21 h later, treatment with vehicle alone (7% Tween 80, 3% ethanol in saline) or with 1294 (i.p. 10 mg/kg, twice per day) began and continued for 4 days. Parasitemia was monitored by a tail-prick daily on days 2-8 p.i. Thereafter, surviving mice were monitored on alternate days if no parasites were detected and daily when parasites were detected. Three mice infected with M138G parasites showed no parasitemia for the 21 days of the experiment. Data shown result from two experiments. The numbers of animals for each condition were: -Dox, 1294 ($n=9$); -Dox, vehicle ($n=6$); +Dox, 1294 ($n=7$); +Dox, vehicle ($n=3$). Detailed parasite counts are provided in Supplementary Fig. S8. (E) Treatment with compound 1294 enhances survival of mice infected with the gatekeeper mutant of AEK1. The fraction of mice surviving infection is shown for each day. The animals shown in Fig. 1D were monitored for parasitemia and euthanized by CO₂ asphyxiation under anesthesia when it reached 10⁸/ml. The three animals that never showed a parasitemia survived the 21 day experiment.