Chapter 14

Coupled Enzyme Activity and Thermal Shift Screening of the Maybridge Rule of 3 Fragment Library Against *Trypanosoma brucei* Choline Kinase; A Genetically Validated Drug Target

Louise L. Major, Helen Denton and Terry K. Smith

Additional information is available at the end of the chapter

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1. Introduction

Infectious diseases caused by parasitic protozoa affect approximately 15% of the global population, and more than 65% of the population in the Third and developing world, yet current drug therapies for protozoal infections are woefully inadequate. As protozoal infections take their toll predominantly in the developing world, market forces are insufficient to promote the development of novel anti-protozoal drugs. In 2000, only ca. 0.1% of global investment in health research was spent on drug discovery for tropical diseases [1].

One such neglected parasitic disease is Human African Trypanosomiasis (HAT) or African sleeping sickness, which is caused by the protozoan parasite *Trypanosoma brucei* and is transmitted by the bite of the Tsetse fly. The WHO estimates that HAT constitutes a serious health risk to 60 million people in sub-Saharan Africa, 300,000-500,000 of whom become infected each year, with an estimated 10,000 fatalities. The related disease in cattle, Cattle Trypanosomiasis or Nagana, also represents a major health concern due to its devastating economic, social and nutritional impact on African families, estimated by the WHO as an annual economic loss of ~US$ 4 billion. As such, the total burden of Trypanosomiasis translates into 1,598,000 Disability-Adjusted Life Years, this is on a par with big killers such as *Mycobacterium tuberculosis* and Malaria [2, 3].

Treatment of HAT is dependent upon four drugs: suramin, melarsoprol, pentamidine and eflornithine.These therapies are often toxic, difficult to administer and increasingly have an acquired drug resistance [4, 5].Developed before the 1950s suramin and melarsoprol are

used for chemotherapy of early stages of the disease, as is pentamidine. The arsenical melarsoprol is extremely toxic, with death for ~1 in 20 of cases and treatment failures as high as 30% in certain areas [4, 6]. Treatment of the second stage of the disease, where the parasites cross the blood-brain barrier and invade the central nervous system, is limited to melarsoprol and eflornithine [7].The WHO as a desperate measure recently introduced nifurtimox-eflornithine combination therapy for the treatment of HAT. This is despite nifurtimox, a drug often used to treat Chagas' disease (caused by the related protozoan the South American *Trypanosoma cruzi*), having low efficacy against HAT [8].

Hence there is an urgent need for new, more effective, less toxic, cheap and easy to administer therapeutic agents to treat African sleeping sickness and other closely related parasitic diseases, e.g. Chagas' disease and Leishmaniasis, whose current treatments suffer from similar limitations.

T. brucei is able to survive and multiply in the harsh environment of a mammalian hosts' bloodstream. This is due to the parasite's dense cell-surface coat of the glycosylphosphatidylinositol anchored variant surface glycoprotein (5 X 10^6 dimers/cell) [9-11], which protects the parasite in two ways. Firstly by acting as a diffusion barrier, such that complement is unable to reach and attack the plasma membrane of *T. brucei*. Secondly *T. brucei* is able to undergo antigenic variation, where by it is able to express a new variant surface glycoprotein from a repertoire of more than 1000 different genes, before the hosts' innate immune system is able to catch up [12, 13]. This antigenic variation is why a vaccine against this parasite is not a viable option as a therapy.

Phospholipids account for ~80% of total lipids in *T. brucei* with a significant proportion of these containing a choline-phosphate headgroup; phosphatidylcholine (PC) (~48%) and sphingomyelin (~15%) [14,15]. Sphingomyelin is made from PC via the sphingomyelin synthases transferring the choline-phosphate headgroup from PC to a ceramide lipid moiety [16]. These lipids contribute to the structural integrity of the membrane and in addition determine membrane fluidity and cell surface charge. Unsurprisingly, the biosynthesis and utilisation of these choline-containing molecules are implicated in a variety of cellular processes, including signaling, intracellular cellular protein sorting and transport [reviewed in 16]. Phosphocholine has been reported to be a required mitogen for DNA synthesis induced by growth factors [17]. Recently we have shown that the essential *T. brucei* neutral sphingomyelinase is actively involved in post Golgi sorting of the glycosylphosphatidylinositol anchored variant surface glycoprotein mentioned earlier [18].

Most eukaryotes have three alternative pathways by which PC can be synthesised [19 and reviewed in 20]. The first two pathways both involve three consecutive methylations of PE by S-adenosyl-L-methionemethyltransferases [20]. The PE can be derived from two alternative pathways, either from the concerted actions of the CDP-DAG dependantphosphatidylserine synthase and phosphatidylserine decarboxylase, or via the CDP-ethanolamine branch of the Kennedy pathway. This involves phosphorylation of ethanolamine by an ethanolamine kinase, its activation to CDP-ethanolamine by an ethanolamine-phosphate cytidyltransferase and its transfer to diacylglycerol by an ethanolamine phosphotransferase. The

presence of this branch of the Kennedy pathway was demonstrated in *T. brucei* [22], however only recently have the constituent enzymes been characterized [23].

The trypanosomal genomes have revealed that *T. brucei* does not contain homologues for any methyltransferase(s) required to convert PE to PC [24] (neither does *T. cruzi*, but *Leishmania* do). *Plasmodium falciparum* have an alternative single plant-like S-adenosyl-L-methionemethyltransferase [25-27], responsible for phosphoethanolamine conversion to phosphocholine, however there are no trypanosomatid homologues. This rather surprising absence of PE to PC methylation has been confirmed by *in vivo* labellings by ourselves and others [15, 22, 28].

The third alternative pathway for *de novo* synthesis of PC, and the only pathway by which *T. brucei* can *de novo* synthesise PC, utilises the CDP-choline branch of the Kennedy pathway [19, 29-33]. This involves the phosphorylation of choline by a choline kinase, its activation to CDP-choline by a choline-phosphate cytidyltransferase and its transfer to diacylglycerol by a choline phosphotransferase. Biochemical characterisation of the two choline/ethanolamine kinases involved in the initial steps of the Kennedy pathway show that unusually amongst eukaryotes only one of the kinases is able to phosphorylate choline [23].

Collectively this evidence of an absence of redundancy of *de novo* PC synthesis in *T. brucei*, compared with other organisms (including humans), suggests *T. brucei* has a vulnerability to inhibition of their only way to synthesise PC, i.e. the Kennedy pathway. Recently we have exploited this fact by genetically validating the only *T. brucei* choline kinase (*Tb*CK) as a drug target both in culture and in an animal model [34]. Chemical intervention of the *Tb*CK enzyme activity is likely to interfere with the parasite's biology in multiple ways and *Tb*CK is therefore of interest as a target for novel chemotherapeutics.

In this study we interrogate ~630 compounds of the Maybridge Rule of 3 Fragment Library for compounds that interact with, and inhibit *Tb*CK.The Maybridge Rule of 3 Fragment Library is a small collection of quantifiable diverse [35, 36], pharmacophoric rich, chemical entities that comply with the following criteria; MW ≤ 300, cLogP ≤ 3, H-Bond Acceptors ≤ 3, H-Bond Donors ≤ 3, Rotatable bonds (Flexibility Index) ≤ 3, Polar Surface Area ≤ 60 $Å^2$ and aqueous solubility ≥ 1 mM using LogS and high purity (≥ 95%). Comparisons between two different screening methods, a coupled enzyme activity assay and differential scanning fluorimetry,has allowed identification of compounds that interact and inhibit the *T. brucei* choline kinase, several of which possess selective trypanocidal activity.

2. Experimental

2.1. Materials

All materials unless stated were purchased either from Sigma/Aldrich or Invitrogen. An in house Maybridge Rule of 3 Fragment Library kept in master plates at 200 mM in DMSO (100%), was transferred into working plates with compounds occupying the central 80 wells

of a 96-well plate, at 10 mM in 5% DMSO, allowing the two outside columns for positive and negative controls.

2.2. Recombinant expression and purification of *Tb*CK

Large-scale recombinant expression and purification of *Tb*CK was conducted using the construct pET-15bTEV-*Tb*CK in BL21 Rosetta (DE3) cells as described previously [23], except the cells were grown in tryptone phosphate broth [37], harvested by centrifugation at 3500 g for 20 min at 4°C and affinity purified with either a HisTrap™ FF crude column (enzyme activity assay) or a HisTALON Cartridge (thermal shift analysis).

Briefly, pelleted cells were suspended in buffer A (50 mMTris/HCl, pH 8.0, 300 mMNaCl and 10 mM imidazole) and lysed in the presence of DNase I by sonication. The lysate was cleared by centrifugation at 35000 g for 30 min at 4°C and applied to a 1 ml HisTrap™ FF crude column column (GE Healthcare) pre-loaded with Ni^{2+}. Unbound proteins were removed by washing the column with 15 column volumes of buffer A containing 32.5 mM imidazole and *Tb*CK was eluted with 250 mM imidazole in the same buffer. Using a PD10 column, *Tb*CK was buffer exchanged into 50 mMTris/HCl, pH 8.0, 300 mMNaCl, glycerol (15% w/v) and stored at -80°C.

Alternatively, pelleted cells were suspended in 50 mMTris/HCl, pH 8.0, 300 mMNaCl and 5 mM imidazole and lysed by sonication. The lysate was cleared by centrifugation at 35000 g for 30 min at 4°C and applied to a 1 ml HisTALON Cartridge (Clontech).Unbound proteins were removed by washing the column with 10 column volumes of loading buffer, *Tb*CK was eluted with 15 mM imidazole and a final clearing wash of 250 mM imidazole in the same buffer. Using a PD10 column, *Tb*CK was buffer exchanged into 50 mM HEPES pH 8.0, 300 mMNaCl and 15% glycerol prior to storage at -80°C.

Typical yields were > 10 mg per litre of bacterial culture,*Tb*CK was stable and freeze thawing did not lead to any significant loss of activity.

2.3. *T. brucei* choline kinase activity assay

High throughput screening of the Maybridge Rule of 3 Fragment Library was carried out at a final test concentration of 0.5 mM in 96-well plates (final assay volume 200 μl) using a spectrophotometric assay that has been described previously [23]. The screened library working plates consisted of compounds arrayed in 96 well plates at 10 mM in 5% DMSO; columns 1 and 12 contained 5% DMSO only. For high throughput screening, 10 μl from each well of the working plates was added to 110 μl of buffer containing 50 mM MOPS (pH 7.8), 150 mMKCl and 6 mM $MgCl_2$. 3 μg of purified *Tb*CK was added to each well in 30 μl of the same buffer and the plates were mixed and incubated for 5 min at room temperature. A further 30 μl of buffer containing PEP (1 mM final), ATP (0.5 mM final), NADH (0.5 mM final) and pyruvate kinase and lactate dehydrogenase(PK/LDH) (5 units/ml final) was added, and the reaction was started by addition of 30 μl choline (0.5 mM final) to rows 1-11, 30 μl buffer alone was added to row 12 (negative control) and this was used as an intra-plate control (background rate) in conjunction with row 1 (maximal rate). Following mixing the change in

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absorbance at 341 nm was monitored for 10 min at room temperature. For testing inhibition of the coupling enzymes (PK/LDH), standard buffer conditions were used but the assay contained 1 mM PEP, 0.1 mM ADP and 0.5 mM NADH. The PK/LDH was titrated to give a change in absorbance of approximately 0.05 absorbance units/min in the absence of inhibitor.

2.4. Differential scanning fluorimetry with *Tb*CK

Differential scanning fluorimetry was set up in 96 well PCR plates using a reaction volume of 100 μL. Samples contained 2.1 μM *Tb*CK, 6 mM $MgCl_2$, 50 mM HEPES pH 8.0, 80 mMNaCl, 5.25% glycerol (v/v) and 1.4 x Sypro Orange (Invitrogen), Maybridge Ro3 compounds were screened at 1 mM concentration with a final DMSO concentration of 0.5% (v/v).Two controls with eight repetitions per plate were used for the thermal shift experiments: 0.5% DMSO; 0.5 mM ATP, 0.5% DMSO.

Differential fluorimetric scans were performed in a realtime PCR machine (Stratagene Mx3005P with software MxPro v 4.01) using a temperature scan from 25°C to 95°C at 0.5°C min^{-1}.Data were then exported to Excel for analysis using "DSF analysis" modified from the template provided by Niesen et al. [38].T_m values were calculated by non-linear regression, fitting the Boltzmann equation to the denaturation curves using GraFit. *Tb*CK T_m, in the presence of 6 mM $MgCl_2$ and 0.5% DMSO, 41.21 ± 0.03°C (n > 60), T_m for *Tb*CK and 0.5 mM ATP = 44.46 ± 0.05°C (n > 60).

3. Results and discussion

Screening for inhibitors of the genetically validated drug target *Tb*CK is problematic due to the difficulty in following the reaction either continuously or directly. A direct choline kinase activity assay assessing the production of phosphocholine, utilising a modified method of Kim *et. al.* [39], using *Tb*CK and radiolabelled choline has been performed previously [23]. However this is not suitable for screening purposes, so choline kinase activity was measured by a spectrophotometric coupled assay (Figure 1). This coupled enzyme assay utilises regeneration of ATP from the ADP by-product of the choline kinase by pyruvate kinase, and subsequent oxidation of NADH as the resulting pyruvate is converted to lactate, by lactate dehydrogenase. This assay using coupled enzymes is also problematic, as a compound could potentially inhibit the coupled enzymes giving rise to a false positive.

An alternative approach for screening is differential scanning fluorimetry (Figure 2), allowing identification of compounds that interact with the *Tb*CK protein, either to stabilise or destabilise it, therefore influencing the protein's T_m (melting point) [38-40].

Initially *Tb*CK was subjected to differential scanning fluorimetry to ascertain if this approach was possible. Known components required for enzyme activity were tested to see if thermal shifts were observed.In the presence of 6 mM $MgCl_2$, a T_m of 41.2°C was obtained (Figure 1C, solid dark line). The addition of 0.5 mM ATP resulted in a > 3°C T_m shift for *Tb*CK(Figure 1C,

dashed-line). These encouraging results showed *Tb*CK was amenable to differential scanning fluorimetry and allowed validation of this screening method.It is worth noting the surprising low T_m of *Tb*CK, considering that these parasites live within the bloodstream of a mammalian host, i.e. 37°C, or higher with a fever. However, the presence of physiological

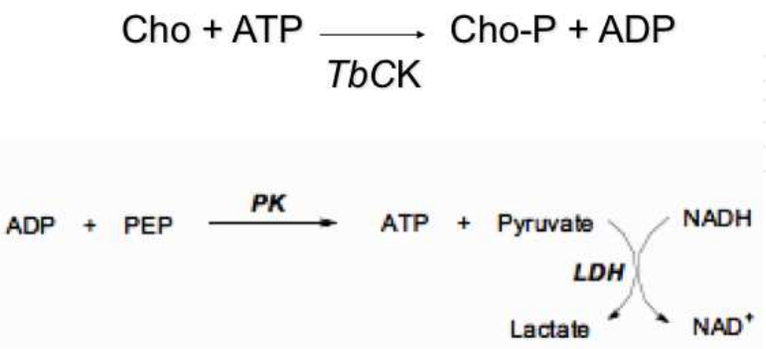

Figure 1. Schematic of the *Tb*CK reaction and coupled assay.

T. brucei choline kinase (*Tb*CK) catalyses the ATP dependent phosphorylation of choline, the ADP is converted back to ATP by pyruvate kinase (PK), which converts phosphoenolpyruvate (PEP) to pyruvate in the process. The resulting pyruvate is reduced to lactate by the NADH dependent lactate dehydrogenase (LDH). The resulting conversion of NADH to NAD^+ is monitored, by measuring the reduction in absorbance at 341 nM.

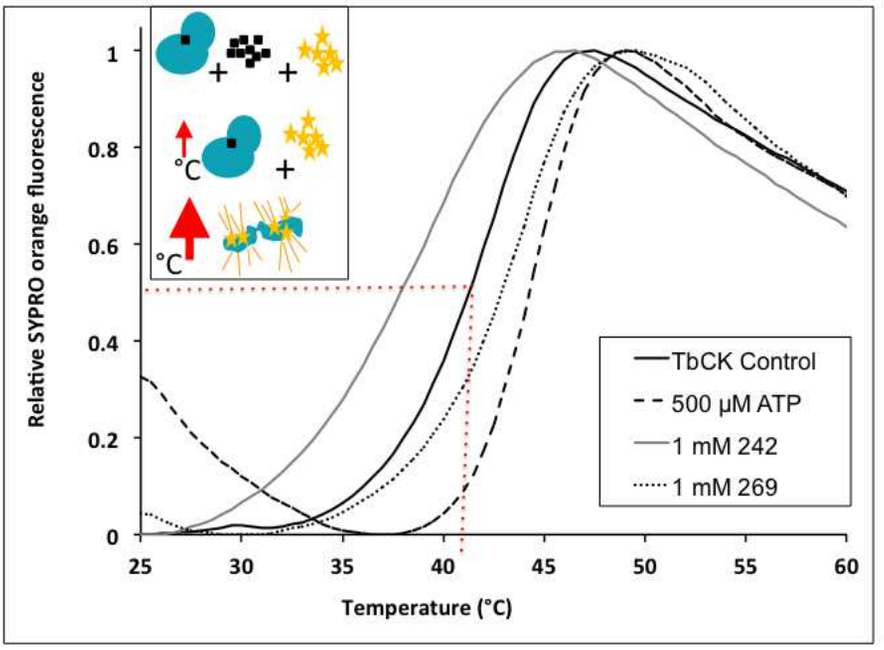

Figure 2. Thermal shift assay; typical differential fluorimetry scans of TbCK.

Differential fluorimetric scans were performed and analysed as described in Experimental. *Tb*CK + DMSO (control) solid dark line, *Tb*CK + 0.5 μM ATP (positive control) dashed line, *Tb*CK + 1 mM compound 242, solid light line, *Tb*CK + 1 mM compound 269, dotted line.T_m of *Tb*CK in the presence of 0.5%DMSO is 41.21 ± 0.03°C (control); T_m of *Tb*CK and 0.5 mM ATP is 44.46 ± 0.05°C (positive control). Insert: schematic representation of the thermal shift assay.A protein will unfold exposing hydrophobic domains as it is denatured due to the increasing temperature. Dyes such as sypro orange (star) are able to bind to these exposed hydrophobic areas giving rise to fluorescence. A plot of this increased fluorescence versus temperature allows determination of T_m (melting point) of the protein. If a compound (squares) is able to interact with the protein it may alter the protein's T_m and thus a library of compounds can be screened to see if they stabilise (increase in T_m) or destabilise (decrease in T_m) the target protein.

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The respective controls in both assay types allowed Z-factors to be determined for all of the plates screened (Figure 3). Both the coupled enzyme activity assay and the thermal shift analysis showed Z-factors to be above 0.5 for all plates, except for plate 5 for the thermal shift assay (but still above 0.45), this is indicative of good reliable assays, with meaningful results [41].

The MayBridge Rule of 3 Fragment Library was distributed over 9 plates (80 compounds per plate) providing space for adequate positive and negative controls, allowing Z-factors to be determined. This was done for each plate for both the choline kinase assay (+) and thermal shift analysis (x). A Z-factor above 0.4 is acceptable and validates the data on that plate as being reliable.

The ~630 compounds from the MayBridge Rule of 3 Fragment Library were assessed for their ability to inhibit the *Tb*CK coupled enzyme activity assay at a single concentration of 0.5 mM (Figure 4A). At this relative high concentration only 9 of the compounds (1.4%) showed > 70% inhibition. These primary hits were retested in triplicate at 0.5 mM (Table 1), 2 of the 9(compounds 320 and 635) were confirmed as being false positives, while the remaining 7 were confirmed to show good inhibition (80-100%) against the *Tb*CK coupled en-

zyme activity assay. These 7 compounds were then tested against just the coupled enzymes, some inhibition was observed for some of the compounds, but this was insufficient to account for the strong inhibition against the *Tb*CK, thus these 7 compounds were believed to show true *Tb*CK inhibition.

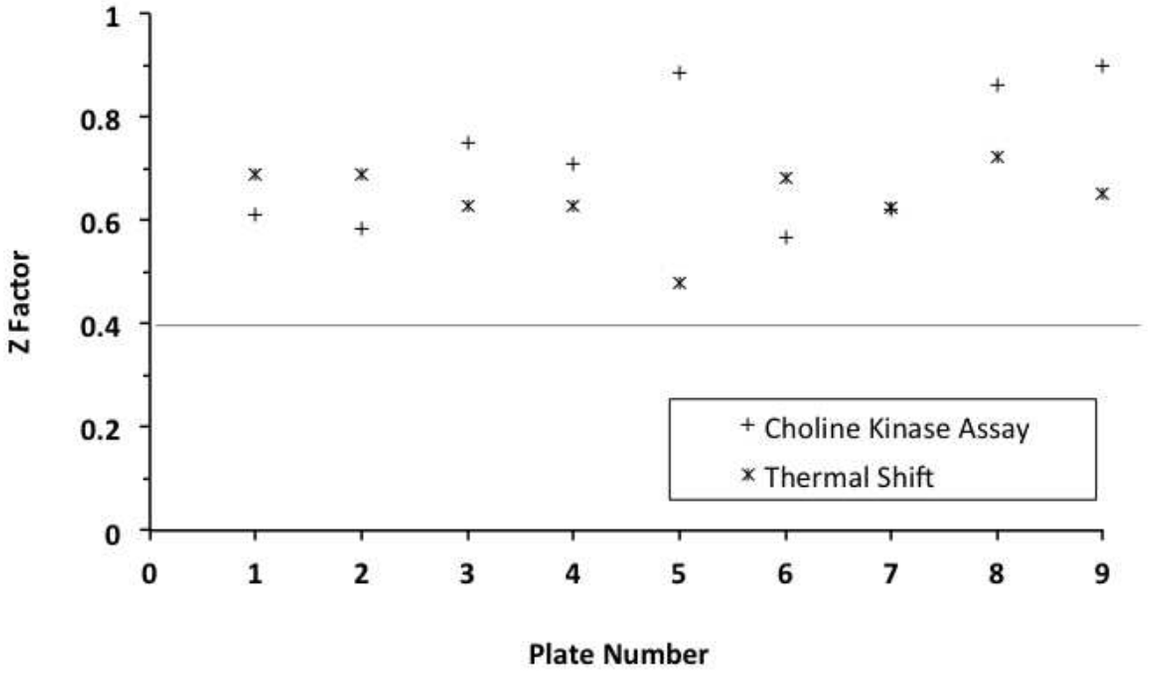

Figure 3. Quality control (Z factors) for the *Tb*CK thermal shift analysis and coupled enzyme activity assay.

It is worth noting the lack of false positives arising from significant inhibition of either of the coupling enzymes, pyruvate kinase and lactate dehydrogenase this is encouraging when screening other ATP utilizing enzymes.

Thermal shift analysis of *Tb*CK with the ~630 compounds from the MayBridge Rule of 3 Fragment Library showed that the vast majority of compounds had little or no affect on the T_m of *Tb*CK (Figure 4B). Relatively few compounds showed an increase in T_m (stabilisation), and only a handful of these showing an increase in T_m> 1°C, i.e. compound 269 (Figure 2, dotted line), this was rather surprising given that ATP stabilises *Tb*CK by > 3°C. Significantly more compounds showed a destabilisation affect, with 3 compounds having > 10°C decrease in the Tm, i.e. compound 242 (Figure 2, solid light line). Most of the compounds observed in this screen that show significant destabilisation of *Tb*CK, do not cause similar destabilisation affects with other enzymes that we have screened in a similar manner, the only exceptions are compounds 68 (2-aminothiophene-3-carbonitrile) and 565 (4-(2-amino-1,3-thiazol-4-yl)phenol) (Table 1).

Several drug discovery style studies have shown that an increase in the thermal stability of a protein is proportional to the concentration and affinity of the ligand to the protein in keeping with the equilibrium associated with ligand-protein binding [38, 41-44]. On

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those occasions where this interaction destabilizes a protein, i.e. lowering T_m, a thermodynamic model has been proposed which explains the how the same ligand can stabilise and destabilise different proteins [42]. While the same protein may be stabilized and destabilized by very similar ligands, this was exquisitely demonstrated by the changes in thermal stability of Acyl-CoA thioesterase, upon incubation with either CoA (destabilise) or Acyl-CoA (stabilise) [45].

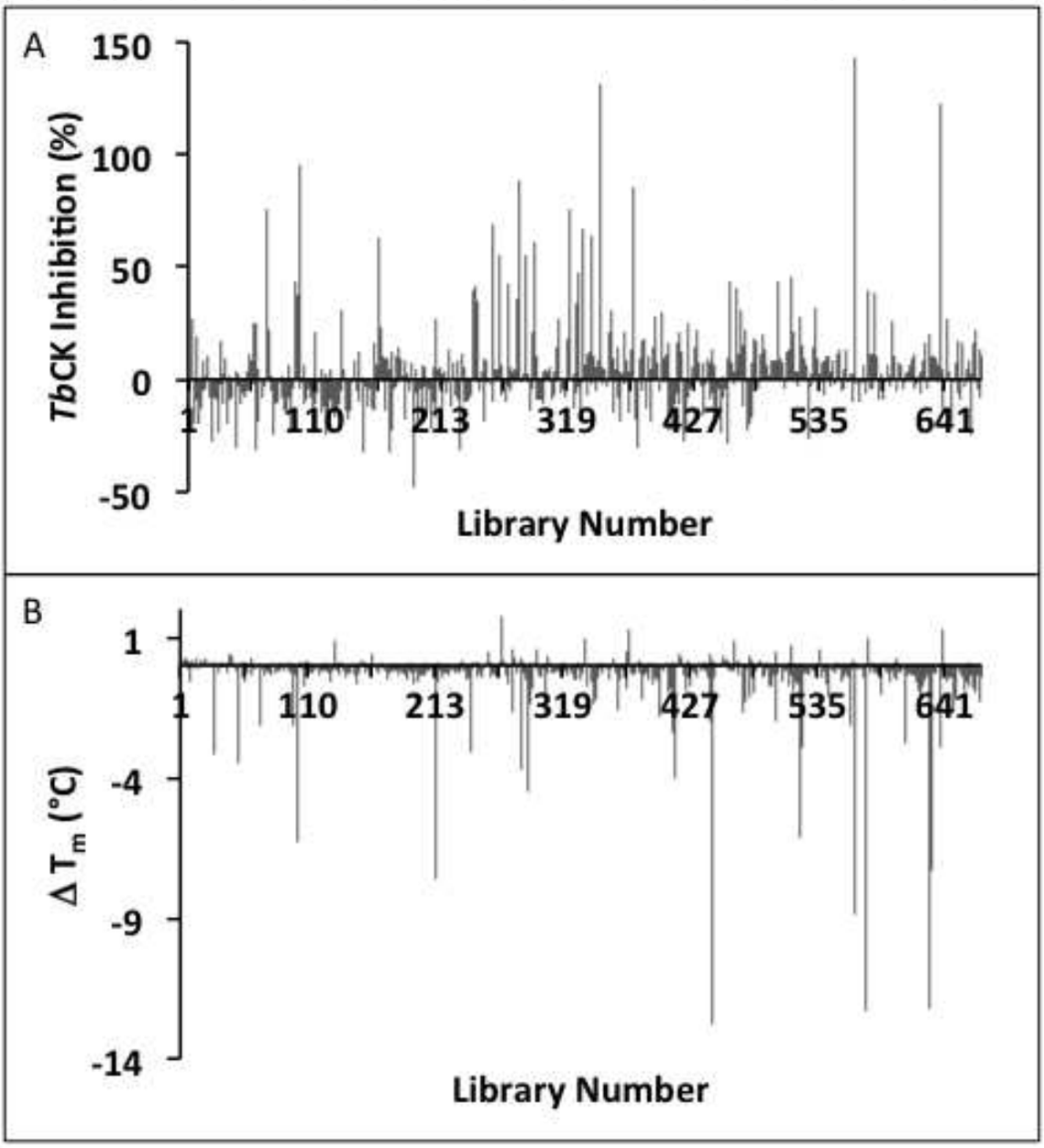
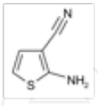
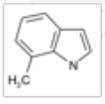
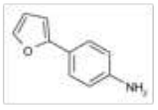
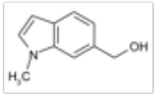
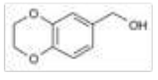
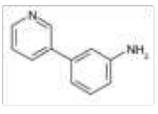
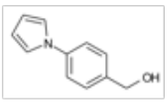
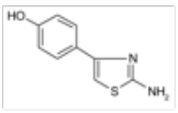
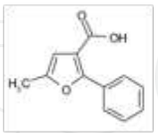


Figure 4. A) Percentage inhibition of *Tb*CK enzyme activity assay for each of the compounds tested. B) Observed thermal shifts of *Tb*CK for each of the compounds tested

Library number[a]	CAS number[b]	Molecular Structure	Compound Name	*Tb*CK activity % inhibition at 500µM Mean ± SD (*n*=3)	*TbCK IC50 (uM)*	PK/LDH % inhibition at 500 uM mean (n=2)	*TbCK T_mShift*[c]	*T. brucei % survival*[d]
68	4651-82-5		2-aminothiophene-3-carbonitrile	101 ± 3	~758	40	-2.15 ± 0.08	10.3 ± 11
95	933-67-5		7-methyl-1H-indole	84 ± 4	~380	49	-2.16 ± 0.14	4.1 ± 5.2
257	59147-02-3		4-(2-furyl)aniline	80 ± 3	~234	63	0.46 ± 0.08	9.3 ± 8.5
278	199590-00-6		(1-methyl-1H-indol-6-yl)methanol	101 ± 2	25.45 ±1.16	28	0.26 ± 0.09	8.8 ± 10.8
320	39270-39-8		2,3-dihydro-1,4-benzodioxin-6-ylmethanol	6 ± 4	ND	ND	-0.17 ± 0.07	63.1 ± 9.5
346	57976-57-5		3-pyridin-3-ylaniline	100 ± 0	12.35 ± 0.64	68	-1.18 ± 0.06	15.6 ± 9.3
372	143426-51-1		[4-(1H-pyrrol-1-yl) phenyl] methanol	80 ± 1	109.7 ± 10.6	26	1.24 ± 0.04	27 ± 14
565	57634-55-6		4-(2-amino-1,3-thiazol-4-yl)phenol	100 ± 0	~120	85	-8.91 ± 0.49	28.7±19.3
635	64354-50-3		5-methyl-2-phenyl-3-furoic acid	23 ± 4	ND	ND	-2.94 ± 0.16	20.5 ± 7.4

[a] Arbitrary library number

[b] CAS numbers are unique identifiers assigned by the "Chemical Abstracts Service" to describe every chemical described in open access scientific literature.

[c] Tm shift in °C, observed for TbCK in the presence of compound (1mM), value is mean ± SD from the Boltzman curve fitting, see Experimental for details.

[d] Cytotoxicity studies, see Major and Smith 2011 for details, values are percentage of controls in the absence of compound, either mean ± SD (n=3) or mean ± SE (n=2), the latter being in bold.

Table 1. The compounds from the Maybridge Rule of 3 library that show >70% inhibition of TbCK.

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All of the compounds in the two data sets (the coupled enzyme activity assay and the thermal shift analysis), were compared to assess any correlation between the two very different methods. In other words looking for compounds that showed a significant change in T_m and a significant inhibition in *Tb*CK enzyme activity (Figure 5A). The vast majority of compounds showed little or no inhibition and little or no shift in T_m. Compounds showing < 40% inhibition of the enzyme activity were removed for clarification (Figure 5B), this highlighted that the majority of compounds that show *Tb*CK enzyme inhibition do not significantly alter the T_m of *Tb*CK. The exceptions are compound 565 with a decrease in T_m~9°C, and compound 68 and 95 with a decrease in T_m~2°C respectively, all show complete inhibition at 0.5 mM (Table 1). Twenty-one compounds showed > 40% enzyme inhibition, 9 of these (43%) displayed > 1°C change in *Tb*CK T_m. This is a substantial enrichment compared to the 7% of compounds with T_m shifts > 1°C observed for the entire library.

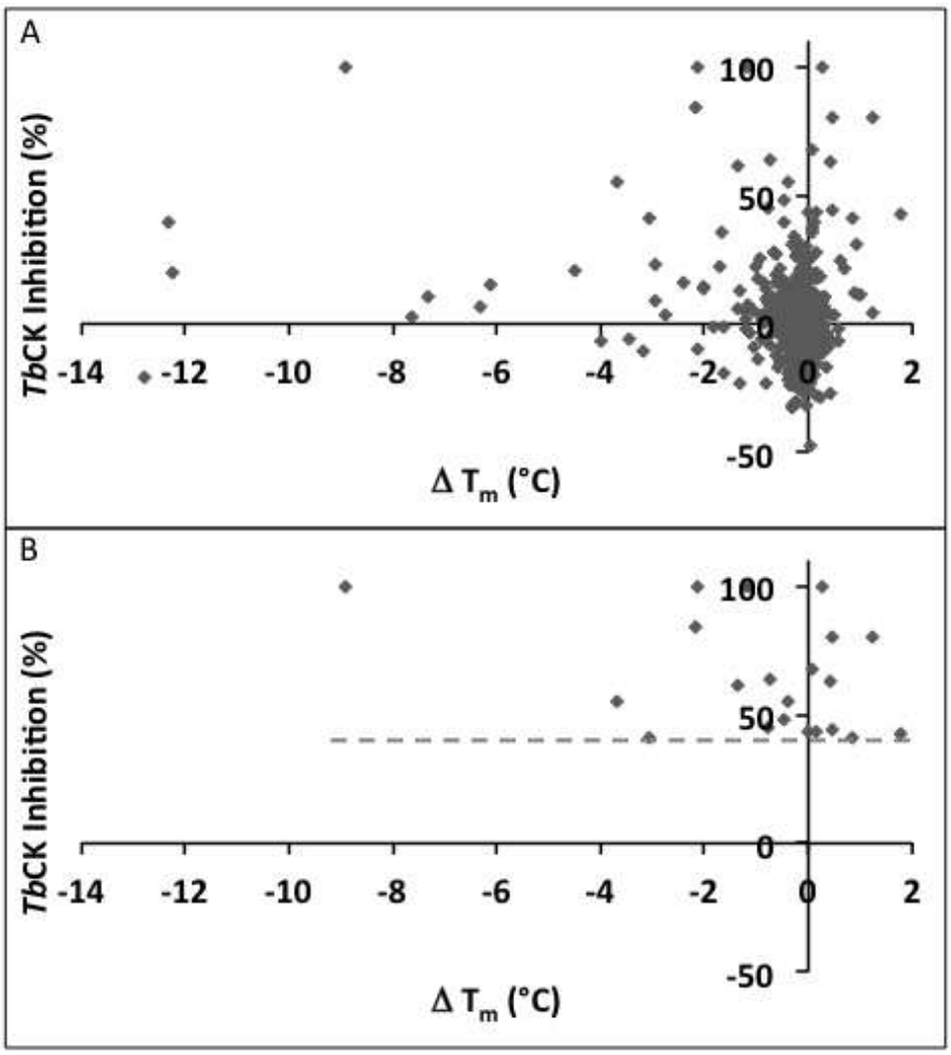

Figure 5. Scatter graph representation showing the correlation between the observed thermal shifts and percentage inhibition of *Tb*CK enzyme activity. A) All of the compounds from the MayBridge Rule of 3 Fragment Library tested. B) For those compounds with > 40% (dotted line) inhibition of *Tb*CK enzyme activity.

Of the compounds identified that alter the T_m of *Tb*CK by > 1°C, ~20% of them inhibit *Tb*CK enzyme activity by > 40%. This suggests that for *Tb*CK thermal shift analysis has allowed significant enrichment, but not total capture of the potential inhibitors of *Tb*CK. However, if a direct assay for a potential drug target was very problematic, prior thermal shift analysis could significantly streamline the number of compounds to be screened, thereby increasing the potential to identify lead compounds. Thermal shift analysis has the disadvantage that good inhibitors could be missed if they do not significantly alter the T_m of the protein.

This raises an interesting question, is it a viable option to target compounds that specifically destabilise an enzyme, causing a decrease in enzyme activity? One could argue this is exactly what pharmaceutical companies are focusing their research efforts upon, but with a slightly different approach. Some of their therapeutic targets rely on finding compounds that disrupt various interactions; hetero- or homo-oligomeric protein-protein, DNA-binding protein and RNA polymerases, many of these are associated with signaling events. Success stories include the identification of HDM2 antagonists associated with P53 activation [46], the identification of anti-cancer agent for the BCL-X_L protein-protein complex and several others, reviewed by Wells and McClendon [47] and more recently by Coyne and colleagues [48].

The techniques utilized to study the formation / disruption of protein-protein complexes are driven by high throughput drug discovery, including fragment based approaches, these include X-ray crystallography, NMR, dynamic light scattering, differential static light scattering, differential scanning fluorimetry [42-50].

In summary, destabilisation by a ligand could affect the oligomeric state of a protein, or in the case of a monomer disrupt intra-molecular interactions, i.e. between stacking α-helixes or β-sheets, causing partial unfolding and thus destabilisation.In the case of *Tb*CK, which we know exists as a dimer, one of several potential mechanisms of destabilisation could be disruption of the dimer interface, whereby a ligand is able to bind to freshly exposed hydrophobic surfaces on the protein, and this interaction allows further destabilisation of the monomer structure.

As it was clear that compounds that inhibit *Tb*CK enzyme activity do not necessarily show a significant increase or decrease in T_m, it was decided to compare inhibition of *Tb*CK enzyme activity with previously determined trypanocidal activities for the compounds [51]. From this comparison (Figure 6) a group of compounds above a threshold of > 70% inhibition of *Tb*CK showed significant trypanocidal activity (circled), suggesting a direct correlation.

Compounds from the May Bridge Rule of 3 Fragment Library with greater than 70% inhibition (dotted line) of the TbCK enzyme activity are circled and numbered. Numbers correspond to arbitrary compound library numbers; see Table 1 for chemical structures and extra data. *T. brucei* survival data was previously determined [51].

http://dx.doi.org/10.5772/52668

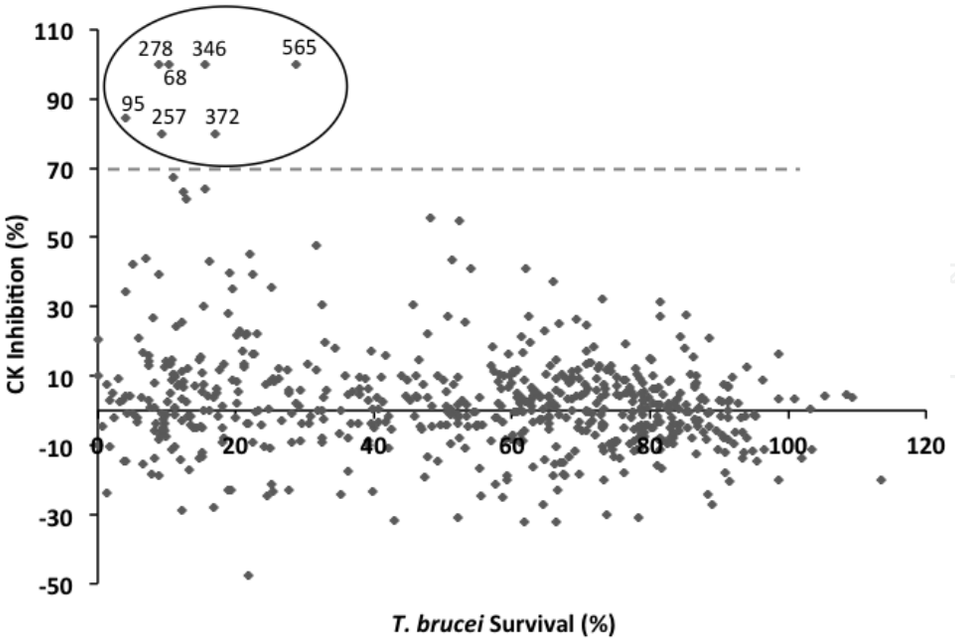

Figure 6. Correlation of the percentage inhibition of *Tb*CK enzyme activity assay and *T. brucei* survival.

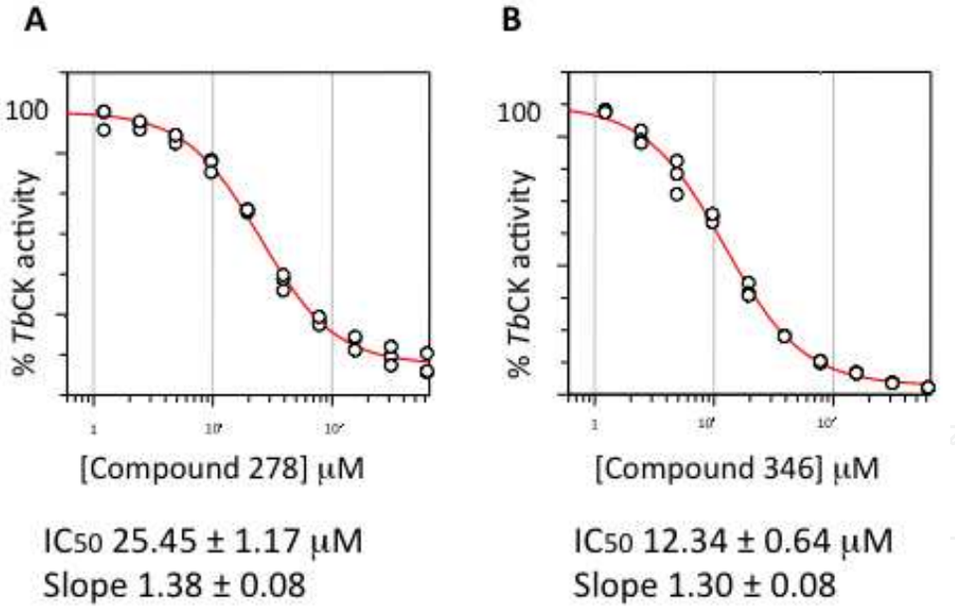

Figure 7. IC50 curves of the promising compounds (278 and 346) that show significant TbCK inhibition.

IC50 values were determined for these compounds (Table 1, Figure 7) ranging from 100s of μM to low μM. For example, compound 278 (1-methyl-(1H-indol-6-yl)methanol) has an IC50 value of 25.45 ± 1.16 μM however the selectivity index is not very good, while compound 346 (3-pyridin-3-ylaniline) has an IC50 value of 12.35 ± 0.64 μM with a high selectivity index.

One of the strengths of the Maybridge Rule of 3 Fragment Library is the chemical diversity, additionally a range of analogous structures can normally be found within it allowing initial structure activity relationships to be formulated. There are several close analogues of compound 278 (1-methyl-(1H-indol-6-yl)methanol), which highlight that the N-methyl indole moiety seems necessary to have any *Tb*CK inhibition and the methanol portion of the molecule can not be replaced by a carboxylic acid. Investigation of the CHEMBL database for similar compounds identified 1-Methyl-1H-pyrrolo[2,3-c]pyridine (CHEMBL594467) which was screened as one of a library of tricyclic and bicyclic analogues of indoloquinoline alkaloids against a variety of protozoan parasites. The analogue mentioned here showed weak trypanocidal activity (624 μM) against *Trypanosoma brucei rhodesiense*, but significantly better (37 μM) against *Plasmodium falciparum* [52].

Another analogue, 1-Methyl-1H-indole (CHEMBL19912) has been shown to interact with human intracellular adhesion molecules and highlights the importance of selectivity [53]. 1H-indol-5-yl-methanol (CHEMBL1650258) has previously been screened against *Leishmania* as a potential PTR1 inhibitor but was shown to be inactive at 500 μM [54]

For the relatively simple compound 346 (3-pyridin-3-ylaniline), there are several analogous structures in the library, including compound 262 (2-(1H-imidazo-1-yl)aniline) which shows ~55% *Tb*CK enzyme inhibition and is trypanocidal. Compound 347 (4-pyridin-3-ylaniline) is a structural isomer of 346 but shows no *Tb*CK enzyme inhibition and is not trypanocidal. The only related structure in the CHEMBL database was 3-(pyridin-3-yl)benzenaminium (CHEMBL1778131) which was shown to be a weakinhibitor of metallo-β−lactamase IMP-1 [55].

4. Conclusions

In this study, screening of a comparatively small fragment library by two different screening methods has allowed identification of several compounds that interact with and inhibit *Tb*CK, a genetically validated drug target against African sleeping sickness. Some of the inhibitory fragments were also selectively trypanocidal, considering these are relatively simple molecules with no optimization, finding low μM inhibitors is very encouraging. Moreover some of the morphological phenotypes of these trypanocidal compounds include cell-cycle arrests similar to those observed for the *Tb*CK conditional knockout grown under permissive conditions.

This study highlights that if faced with a drug target that is problematic to screen, prior thermal shift analysis could significantly triage the number of compounds to be screened, thereby significantly increasing the potential to identify lead compounds. This approach obviously has the limitation that potential inhibitors could be missed if they do not significantly alter the T_m of the protein.

Future follow up work with *Tb*CK will include expanding the structure activity relationship of our most promising hits identified by this study. Their trypanocidal mode of action will

be investigated by undertaking various *in vivo* biochemical phenotyping experiments to ascertain if they are inhibiting *Tb*CK, thus causing a lack of *de novo* PC synthesis, known to be essential for the parasite.

The ultimate goal is to identify new easy to make, affordable, easy to administer, drugs in the fight against African sleeping sickness and other closely related protozoan transmitted Third World diseases.

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Author details

Louise L. Major, Helen Denton and Terry K. Smith

Biomedical Sciences Research Centre, The North Haugh, The University, St. Andrews, Fife, Scotland, U.K.

References

[1] WHO web site: http://www.who.int/topics/trypanosomiasis_african/en/

[2] Steverding, D. Parasites & vectors 2010, 3, 15.

[3] Priotto, G.; Kasparian, S.; Mutombo, W.; Ngouama, D.; Ghorashian, S.; Arnold, U.; Ghabri, S.; Baudin, E.; Buard, V.; Kazadi-Kyanza, S.; Ilunga, M.; Mutangala, W.; Pohlig, G.; Schmid, C.; Karunakara, U.; Torreele, E.; Kande, V. Lancet 2009, 374, 56.

[4] Delespaux, V.; de Koning, H. P. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy 2007, 10, 30.

[5] Baker, N.; Alsford, S.; Horn, D. MolBiochemParasit 2011, 176, 55.

[6] Bouteille, B., O. Oukem, S. Bisser, and M. Dumas. 2003. Treatment perspectives for human African trypanosomiasis. Fundam. Clin. Pharmacol. 17:171

[7] Priotto, G., C. Fogg, M. Balasegaram, O. Erphas, A. Louga, F. Checchi, S. Ghabri, and P. Piola. 2006. Three drug combinations for late-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in Uganda. PLoSClin. Trials 1:e39

[8] Priotto, G., S. Kasparian, W. Mutombo, D. Ngouama, S. Ghorashian, U. Arnold, S. Ghabri, E. Baudin, V. Buard, S. Kazadi-Kyanza, M. Ilunga, W. Mutangala, G. Pohlig, C. Schmid, U. Karunakara, E. Torreele, and V. Kande. 2009. Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. Lancet 374:56-64

[9] M. A. Ferguson; G. A. Cross Myristylation of the membrane form of a *Trypanosoma brucei* variant surface glycoprotein. J. Biol. Chem. 1984, 259, 3011-3015.

[10] M. A. Ferguson; M. G. Low; G.A. Cross Glycosyl-sn-1,2-dimyristylphosphatidylinositol is covalently linked to *Trypanosoma brucei* variant surface glycoprotein. J. Biol. Chem. 1985, 260, 14547-14555.

[11] Ferguson, M.A.J., Brimacombe, J.S., Brown, J.R., Crossman, A., Dix, A., Field, R.A., Guther, M.L.S., Milne, K.G., Sharma, D.K. and Smith, T.K., (1999). The GPI biosynthetic pathway as a therapeutic target for African sleeping sickness. Biochim. Biophys. Acta 1455, 327-340.

[12] G. A. M. Cross Antigenic variation in trypanosomes: Secrets surface slowly. BioEssays 1996, 18, 283-287.

[13] N. Aitcheson; S. Talbot; J. Shapiro; K. Hughes; C. Adkin; T. Butt; K. Sheader; G. Rudenko VSG switching in *Trypanosoma brucei*: antigenic variation analysed using RNAi in the absence of immune selection. MolMicrobiol. 2005, 57, 1608-1622.

[14] Smith, T.K, &Bütikofer, P. (2010) Phospholipid biosynthesis in Trypansomabrucei. Mol Bio Para 172:66-79.

[15] Richmond, G.S,Gibellini, F., Young, S.A., Major, L., Denton, H. Lilley, A. and Smith T.K (2010) Lipidomic Analysis of bloodstream and procyclic form *Trypanosoma brucei* Parasitology 137 (9)|: 1357-1392.

[16] Young SA, Mina JG, Denny PW, Smith TK.(2012) Sphingolipid and ceramide homeostasis, potential therapeutic targets Biochem Res Int. 2012;2012:248135.

[17] Li, Z. and Vance, D.E. (2008) Phosphatidylcholine and choline homeostatis J Lipid Res. 49: 1187-1194.

[18] Young, S.A & Smith, T.K, (2010) The essential neutral sphingomyelinase is involved in the trafficking of the variant surface glycoprotein in the bloodstream form of *Trypanosoma brucei*. Molecular Microbiology 76(6): 1461-1482.

[19] Kennedy E.P., and Weiss, S.B. (1956) The function of cytidine coenzymes in the biosynthesis of phospholipids. J. Biol. Chem. 222:193-214

[20] Gibellini, F & Smith T.K (2010) Critical Review on the Kennedy Pathway for IUBMB Life 62: 414-428.

[21] Kanipes, M.I. and S.A. Henry, 1997 The phospholipid methyltransferases in yeast. BiochimBiophysActa. 1348(1-2): p. 134-41.

[22] Rifkin, M.R., C.A. Strobos, and A.H. Fairlamb, 1995 Specificity of ethanolamine transport and its further metabolism in *Trypanosoma brucei*. J Biol Chem. 270(27): p. 16160-6.

[23] Gibellini. F., Hunter W. N. & Smith, T.K, (2008) Biochemical characterisation of the initial steps of the Kennedy pathway in *Trypanosoma brucei*- the ethanolamine and choline kinases BJ 415 135-144.

[24] Choi, J.Y., W.E. Martin, R.C. Murphy, and D.R. Voelker, 2004 Phosphatidylcholine and N-methylated phospholipids are nonessential in Saccharomyces cerevisiae. J Biol Chem. 279(40): p. 42321-30.

[25] Pessi, G., J.Y. Choi, J.M. Reynolds, D.R. Voelker, and C.B. Mamoun, 2005 In vivo evidence for the specificity of Plasmodium falciparum phosphoethanolamine methyltransferase and its coupling to the Kennedy pathway. J Biol Chem. 280(13): p. 12461-6.

[26] Pessi, G., G. Kociubinski, and C.B. Mamoun, 2004 A pathway for phosphatidylcholine biosynthesis in Plasmodium falciparum involving phosphoethanolamine methylation. ProcNatlAcadSci U S A. 101(16): p. 6206-11.

[27] Witola, W.H., G. Pessi, K. El Bissati, J.M. Reynolds, and C.B. Mamoun, 2006 Localization of the phosphoethanolaminemethyltransferase of the human malaria parasite Plasmodium falciparum to the Golgi apparatus. J Biol Chem. 281(30): p. 21305-11.

[28] Cornell, RB and Northwood, I.C. (2000) Regulation of CTP:phosphocholinecytidyltransferase by amphitropism and relocalization. TIBS 25 441-447

[29] Jackowski, S. and P. Fagone, 2005 CTP: Phosphocholine cytidylyltransferase: paving the way from gene to membrane. J Biol Chem. 280(2): p. 853-6.

[30] Kent, C., 1997 CTP:phosphocholinecytidylyltransferase. BiochimBiophysActa. 1348(1-2): p. 79-90.

[31] Weinhold, P.A. and D.A. Feldman, 1992 Choline-phosphate cytidylyltransferase. Methods Enzymol. 209: p. 248-58.

[32] McMaster, C.R. and R.M. Bell, 1997 CDP-choline:1,2-diacylglycerol cholinephosphotransferase. BiochimBiophysActa. 1348(1-2): p. 100-10.

[33] Carman, G.M.andZeimetz, G.M (1996) Regulation of phospholipid biosynthesis in the yeast Saccharomyces cerevisiae. J. Biol. Chem. 271, 13293-13296.

[34] Simon Young, Federica Gibellini, Keith R. Mathews, William N. Hunter & Terry K. Smith (2012) In the mammalian bloodstream, *Trypanosoma brucei* is totally dependent upon its only choline kinase for survival. Pending submission.

[35] R. A. E. Carr, M. Congreve, C. W. Murray, and D. C. Rees, "Fragment-based lead discovery: leads by design," DrugDiscovery Today, vol. 10, no. 14, pp. 987–992, 2005.

[36] www.maybridge.com/images/pdfs/ro3frag.pdf

[37] J.T. Moore, A. Uppal, F. Maley, G.F. Maley (1993) Overcoming Inclusion Body Formation in a High-Level Expression System. Protein Expression and Purification, 4: 160-163

[38] Niesen, F. H., Berglund, H. and Vedadi, M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nature Protocols, 2, 2212-2221.

[39] Kim, K., Kim, K.H., Storey, M.K., Voelker, D.R. and Carman, G.M. (1999) Isolation and characterization of the *Saccharomyces cerevisiae* EKI1 gene encoding ethanolamine kinase. *JBiolChem* 274: 14857-14866

[40] M. C. Lo, A. Aulabaugh, G. Jin et al., "Evaluation of fluorescence-based thermal shift assays for hit identification in drug discovery," Analytical Biochemistry, vol. 332, no. 1, pp. 153–159, 2004.

[41] Zhang, J,H., Chang, T.D. and Oldenberg, K.R. A simple statistical parameters for use in evaluation and validation of high throughput screening assays 1999 J Biomol Screen 4: 67-73.

[42] Kopen, J. And Schneider, G. Comparison of fluorescence and light scattering based methods to assess formation and stability of protein-protein complexes 2011 J Structural Biology 175: 211-223.

[43] Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P, Salemme FR. High-density miniaturized thermal shift assays as a general strategy for drug discovery. J Biomol Screen. 2001 Dec;6(6):429-40.

[44] PiotrasCimmperman, LinaBaranauskienė, SimonaJachimovičiūtė, JelenaJachno, JolantaTorresan, VilmaMichailovienė, JurgitaMatulienė, JolantaSereikaitė, VladasBumelis, DaumantasMatulis

A Quantitative Model of Thermal Stabilization and Destabilization of Proteins by Ligands Biophysical Journal 2008 (Vol. 95, Issue 7, pp. 3222-3231)

[45] Marfori M, Kobe B, Forwood JK. Ligand-induced conformational changes within a hexameric Acyl-CoA thioesterase. J Biol Chem. 2011 Oct 14 ;286(41):35643-9

[46] Grasberger BL, Lu T, Schubert C, Parks DJ, Carver TE, Koblish HK, Cummings MD, LaFrance LV, Milkiewicz KL, Calvo RR, Maguire D, Lattanze J, Franks CF, Zhao S, Ramachandren K, Bylebyl GR, Zhang M, Manthey CL, Petrella EC, Pantoliano MW, Deckman IC, Spurlino JC, Maroney AC, Tomczuk BE, Molloy CJ, Bone RF Discovery and cocrystal structure of benzodiazepinedione HDM2 antagonists that activate p53 in cells.J Med Chem. 2005;48(4):909-12.

[47] Wells, J.A. and McClendon, C.L. Reaching for high-hanging fruit in drug discovery at protein-protein interfaces 2007 450: pp1001-1009

[48] Coyne A.G, Scott, D.E and Abell, C. Drugging challenging targets using fragment-based approaches 2010 Current opinions in Chemical Biology 14:299-307.

[49] Murray C.W. and Rees D.C. The rise of fragment-based drug discovery Nature Chemistry 2009 1:1187-192

[50] Shuker S.B., Hajduk P.J., Meadows R.P. and Fesik, S.W. Discovering high-affinity ligands for proteins SAR by NMR 1996 Science 274: 5292, pp 1531-1534.

[51] Louise L. Major & Terry K. Smith (2011) Screening of the MayBridge Rule of 3 Fragment Library for trypanocidal compounds that interact with the myo-inositol-3-phosphate synthase from *Trypanosoma brucei* Molecular Biology International Vol 2011, Article ID 389364,doi:10.4061/2011/389364).

[52] Van Baelen G, Hostyn S, Dhooghe L, Tapolcsanyi P, Matyus P, Lemiere G, Dommisse R, Kaiser M, Brun R, Cos P, Maes L, Hajos G, Riedl Z, Nagy I, Maes BU, Pieters LStructure-activity relationship of antiparasitic and cytotoxic indoloquinoline alkaloids, and their tricyclic and bicyclic analogues.Bioorg. Med. Chem. (2009) 17:7209-7217

[53] Liu G, Huth JR, Olejniczak ET, Mendoza R, DeVries P, Leitza S, Reilly EB, Okasinski GF, Fesik SW, von Geldern TW Novel p-arylthiocinnamides as antagonists of leukocyte function-associated antigen-1/intracellular adhesion molecule-1 interaction. 2. Mechanism of inhibition and structure-based improvement of pharmaceutical properties.J. Med. Chem. (2001) 44:1202-1210

[54] Stefania Ferrari, Federica Morandi, DomantasMotiejunas, Erika Nerini, Stefan Henrich, Rosaria Luciani, Alberto Venturelli, Sandra Lazzari, SamueleCalò, Shreedhara Gupta, Veronique Hannaert, Paul A. M. Michels, Rebecca C. Wade, and M. Paola Costi Journal of Medicinal Chemistry 201154 (1), 211-221

[55] Vella P, Hussein WM, Leung EW, Clayton D, Ollis DL, Mitic N, Schenk G, McGeary RP The identification of new metallo-BETA-lactamase inhibitor leads from fragment-based screening. Bioorg. Med. Chem. Lett. (2011) 21:3282-3285

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