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The human Aurora kinase inhibitor danusertib is a lead compound for anti-trypanosomal drug discovery via target repurposing

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Abstract

New drugs for neglected tropical diseases such as human African trypanosomiasis (HAT) are needed, yet drug discovery efforts are not often focused on this area due to cost. Target repurposing, achieved by the matching of essential parasite enzymes to those human enzymes that have been successfully inhibited by small molecule drugs, provides an attractive means by which new drug optimization programs can be pragmatically initiated. In this report we describe our results in repurposing an established class of human Aurora kinase inhibitors, typified by danusertib (**1**), which we have observed to be an inhibitor of trypanosomal Aurora kinase 1 (TbAUK1) and effective in parasite killing *in vitro*. Informed by homology modeling and docking, a series of analogs of **1** were prepared that explored the scope of the chemotype and provided a nearly 25-fold improvement in cellular selectivity for parasite cells over human cells.

Keywords

Aurora kinase; *Trypanosoma brucei*; TbAUK1; cell cycle; neglected disease; target repurposing; sleeping sickness

1. Introduction

The neglected tropical disease (NTD) human African trypanosomiasis (HAT) is caused by the insect-borne protozoan parasite *Trypanosoma brucei*, resulting in an estimated 50,000

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Supporting Information. A tabulation of all the TbAUK1 inhibitors and screening data is publically available as a shared data set at www.collaborativedrugdiscovery.com. The Supporting Info includes the analytical characterization of additional library compounds, representative ¹H NMR spectra, including a temperature study for compound **5a** to confirm rotameric peaks, full tabulation of the single concentration screening data (including database registry numbers), and additional molecular modeling and docking details.

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new infections per year [1]. The disease progresses through two stages: Stage I, the bloodstream form; and Stage II, where the parasites have invaded the central nervous system. It is in this later stage that patients begin to show the trademark symptoms of sleeping sickness: lethargy, sleep disorder, and coma. HAT is invariably fatal when not treated. Although there are drugs that can effectively and safely treat HAT in the bloodstream infections, most patients are diagnosed when symptoms appear in Stage II. The current treatments for Stage II disease are melarsoprol, a toxic arsenical agent that itself has a 5% mortality rate, and eflornithine, which has toxic side effects and requires an extensive dosing regimen spread out over 14 days. Although a nifurtimox-eflornithine combination therapy decreases the dosing time and toxicity [2, 3], an acute need persists for safe, inexpensive and convenient therapeutics.

HAT and other NTDs primarily affect the destitute in developing nations. Consequently, the pharmaceutical industry has been slow to invest in the highly resource-intensive drug discovery programs necessary for the development of new therapies. As a result, the identification of new drug candidates remains a bottleneck in the process. The costly process of drug discovery can be accelerated, and costs reduced, by “target repurposing,” where essential enzyme targets in infectious agents are matched with human homologs that have been pursued for other indications [4]. This can accelerate drug discovery by informing new anti-infective programs with the wealth of medicinal chemistry, structural biology, and molecular and cellular biology knowledge that has accumulated over the years in pursuit of inhibitors for the human targets. In fact, eflornithine as a treatment for sleeping sickness was uncovered using this approach [5–7], and recent repurposing efforts have been reported for antimalarial [8] and antitrypanosomal [9] phosphodiesterase (PDE) and phosphoinositide-3-kinase (PI3K) inhibitors [10].

Drug repurposing programs can develop from target-based screens, or phenotype driven screens. For example, while eflornithine and PDE inhibitors have validated targets in parasites, the PI3K inhibitors are potent antiparasitic compounds whose specific mechanism of action is not yet known. In general, phenotype-driven drug discovery programs have resulted in the majority of new chemical entities introduced [11]. The observation is perhaps surprising in this ongoing era of target-based drug discovery, however it reflects the challenges and complexities of pivotal biological targets and pathways. Consequently, phenotype-based optimization is frequently employed in human systems, even in cases where much target/pathway knowledge exists. Thus, one would expect that phenotype based optimization would be most fruitful in studies of pathogens where their biological pathways are not as well understood. This expectation has been borne out in the fact that 70% of first-in-class anti-infective agents launched between 1999–2008 were discovered via phenotype driven approaches [11]! Further, these programs are enhanced when starting from established chemical classes with prior knowledge matching chemotype to cellular phenotype.

We have begun studying inhibitors of the trypanosomal cell cycle, starting with established mammalian Aurora kinase inhibitors. Aurora kinases are druggable targets that have been pursued for the discovery of new antineoplastic agents [12]. As an integral enzyme in cell division, these enzymes control events including mitotic spindle assembly, chromosomal separation, and cytokinesis. Since Aurora kinases are essential cell cycle mediating enzymes and are implicated in malignancy [13], they have been pursued as important targets in drug discovery, resulting in a wide variety of inhibitors of Aurora that have successfully moved into clinical trials [14]. An evaluation of the *T. brucei* kinome identified three Aurora kinase paralogs [15]. RNAi revealed that TbAUK1, but not TbAUK2 or TbAUK3, was required for mitotic progression [16]. Loss of TbAUK1 inhibits nuclear division, cytokinesis and growth in cultured infectious bloodstream forms (BF) and insect stage procyclic forms [17].

Additionally, TbAUK1 is required for infection in mice [18], and is susceptible to the Aurora kinase inhibitors hesperadin [18] and VX-680 [19]. Since hesperadin has not been advanced to human clinical trials, and the further development of VX-680 has been halted during Phase II clinical trials, we looked toward other chemotypes that are currently still under clinical development.

2. Results & Discussion

Inspired by the initial growth inhibition observed by hesperadin and VX-680, we decided to assess the pyrrolopyrazole danusertib (**1**, formerly PHA-739358) [20] and its predecessor analog PHA-680632 (**2**, Figure 1) [21]. This compound class is of particular interest since **1** is well advanced into clinical trials, is parallel-synthesis enabled, and its medicinal chemistry and structural biology profiles are well established. Thus, we hypothesized that this chemotype would afford an opportunity to explore rapidly the structure-activity relationships of the series.

We synthesized three additional analogs (Scheme 1, compounds **5a–c**) to compare simple replacements for the diethylphenyl urea headgroup of **2**. To verify that our lead compounds inhibit TbAUK1 activity, **1**, **2** and **5a** were each tested at 500 nM in an *in vitro* kinase assay. Since efforts to produce catalytically active recombinant TbAUK1 have proven fruitless, we resorted to the use of AU1-tagged TbAUK1, immunoprecipitated from trypanosome homogenates. Using this method, we have previously shown that hesperadin inhibits TbAUK1 at 200 nM to the level of a background kinase [18]. We wished to demonstrate that compounds **1** and **2** are able to lower kinase activity to the same background level as hesperadin (Figure 2). In this experiment, the AU1-tagged kinase was pulled down with anti-AU1 Sepharose and used to phosphorylate myelin basic protein (MBP). The upper panel of Figure 2 is an autoradiogram and the lower panel is a Coomassie stain of the same gel showing that each lane had an equivalent amount of MBP loaded on the gel. Hesperadin at 500 nM completely inhibited TbAUK1, and was used here to show background kinase activity in the pull-down assay. At 500 nM, compounds **1** and **2** inhibited TbAUK1 to the same background level as hesperadin, while **5a** did not.

We assessed growth inhibition of *T. brucei brucei* bloodstream form (BF) trypanosomes (90-13 strain) with the Cell Titer Blue[®] end point assay for the compounds shown in Table 1. Taken with the TbAUK1 kinase data above, these results demonstrate that **1** and **2** can completely inhibit TbAUK1 activity at 500 nM and cell growth with an effective concentration that inhibits cellular growth by 50% (EC₅₀) in a similar concentration range. Conversely, **5a** neither inhibits TbAUK1 activity nor has any significant effect on cell growth. Compounds **5b** and **5c** show an activity that approximates **2**.

For each of these compounds tested we measured inhibition of the acute myelogenous leukemia cell line MOLT-4 [22]. This cell line overexpresses Aurora kinases A and B when compared with uninduced peripheral blood mononuclear cells, and growth of this cell line has been shown to be selectively blocked by Aurora kinase inhibition [23]. MOLT-4 also shares with trypanosomes an ability to grow in suspension culture and circulate through the blood and lymph fluid.

Growth of MOLT-4 was indeed blocked by these inhibitors (Table 1). However, we note a range of selectivity between *T.b. brucei* and MOLT-4, providing confidence that the structural features that give rise to human and trypanosomal activity are not inextricably linked. This is an important finding, since the goal of our Aurora inhibitor repurposing project is to modify existing scaffolds to generate potent and selective inhibitors of trypanosome growth.

We sought to gain a better understanding of the differences in activity between these close-in danusertib analogs by development of a homology model of TbAUK1 based on the human [21] and mouse [24] Aurora A crystal structures (PDB ID 2BMC and 3D14, respectively). Inhibitors were docked to TbAUK1, and to human Aurora A in instances where there was no published ligand-enzyme crystal structure. When comparing the TbAUK model (Figure 3C) with the published Aurora A/1 complex (Figure 3A) [20] a similar ligand pose is observed, and most of the key ligand-protein contacts are retained. In both structures the pyrazinylphenyl tail is oriented towards the solvent, and the key H-bonding interactions between the pyrazolopyrrole core and the kinase hinge region are preserved. However, the head group region of **1** assumes a flipped orientation in the TbAUK1 binding site, placing the phenyl group into a hydrophobic pocket towards the N-terminus lobe (Figure 3C). This flip is likely driven by Met113 in TbAUK1, which corresponds to Thr217 in human Aurora A. The bulkier hydrophobic amino acid methionine side chain forces the phenyl group of danusertib up into the hydrophobic pocket. Notably, T217 mutation of human Aurora A confers resistance to MLN8054 [25], so this amino acid difference is likely to be an important consideration in the design of selective TbAUK1 inhibitors.

Based on this analysis, we suspected that the planar urea moiety of **2** is less able to accommodate the Thr-Met change in the protein, leading to the decrease in potency against *T. brucei* cultures, and a decrease in selectivity over MOLT-4 cells. This was supported by docking experiments: The headgroup of **2** (selectivity=0.06) is rigidly held against Met113, which is likely to lead to lower activity against the parasite enzyme (compare Figure 3B and C). Thus, noting this geometric difference between **1** and **2**, we hypothesized that the tetrahedral geometry of the carbon atom adjacent to the carbonyl group in the danusertib head group would enable the head group flip of danusertib analogs, and that lipophilic side chains at this position could also help drive potency and/or selectivity by fitting into the adjacent lipophilic pocket. The reduced potency of other planar substitutions (notably compounds **5a-c**) supports this contention (Table 1).

To further test this hypothesis, we designed a library of danusertib analogs that retained a tetrahedral geometry adjacent to the carbonyl group. With an eye towards preparation of this library using the chemistry shown in Scheme 2, a virtual library of analogs was enumerated using 208 arylacetic acids (**6**) that were available in pre-weighed quantities from a commercial vendor (ASDI, Inc). Those product molecules with molecular weight <500 and cLogP <5.0 were retained, and a diverse subset of 50 analogs was selected for docking into the TbAUK1 homology model. These docking experiments provided a prioritization by which the top twenty analogs would be synthesized first.

From the prioritized list, 19 compounds were successfully synthesized and each was tested at 1 and 10 μ M against two different *T. brucei* cell lines (Lister 427 90-13 and AnTat1.1A). Eleven compounds (**8-18**) showed greater than 60% inhibition at 1 μ M and were selected for a full dose-response analysis against the human infective *T. b. rhodesiense*, and MOLT-4 cells (Table 2 and Figure 4). The single concentration data for all 19 compounds and dose-response data for **8-18** against *T. b. brucei* 90-13 is tabulated in the Supporting Information.

Of note from these screening results is that, while compounds of improved potency over **1** have not yet been uncovered, an improved selectivity ratio for killing *T. b. rhodesiense* over MOLT-4 cells has been attained. Indeed, nearly 25-fold selectivity has been observed in the case of compound **8**; eight other analogs display selectivity >1.0 over MOLT-4 cells.

The docked pose of **8**, the most selective compound, is shown in Figure 3C. The head group of this compound attains a flipped conformation similar to that observed with danusertib, but

is extended a little deeper into the lipophilic pocket. In this case, the naphthyl head group provides lipophilic interactions with the top of the binding pocket, where it also encounters Lys58 of TbAUK1 in a geometry that could allow a favorable π -cation interaction [26]. In contrast, an adverse steric interaction of the naphthylene group of **8** is apparent upon docking to human Aurora A (Figure 3B), a result that is confirmed with a significantly reduced Glide docking score (−6.469) compared to **1** (−10.29).

Besides providing a framework for explanation of the selectivity observations of this compound series, the homology model has proved to be useful for biasing compound library designs. The rank-ordering of compounds by the docking experiments was aligned with the observed potency against trypanosome cells, and the docking scores showed a good correlation ($R^2=0.75$ for *T. brucei rhodesiense* and 0.72 for *T. brucei brucei*) with the cellular potency values (Supporting Information). This supports the hypothesis that the cellular growth inhibition phenotype is, at least in part, mediated by TbAUK1 inhibition.

3. Conclusions

We report that danusertib (**1**) inhibits both TbAUK1 kinase activity and growth of three different trypanosome strains, including human infective *T.b. rhodesiense*. Notably, the EC_{50} value we report for *T.b. rhodesiense* (150 nM) is consistent with the mean EC_{50} averaged from 15 different cancer cell lines (123 nM) [27]. Because danusertib is an optimized human Aurora inhibitor, it is not surprising that the structural changes we made decreased potency against mammalian cells. However, while we also observe loss of potency against trypanosomes for analogs of **1**, the loss is not as severe as that observed for mammalian cells. Consequently, we observe an improved selectivity ratio ranging from 2.2 to 23.3-fold. Furthermore, since the molecular model of TbAUK1 provides a plausible structural basis for this improvement, we expect to be able to develop compounds with further improvements in potency and selectivity.

A key aspect of the target repurposing approach for neglected disease drug discovery is the opportunity to utilize the extensive historic research performed in the process of developing a clinical candidate. In this case, the chemical matter previously disclosed as human Aurora inhibitors do indeed show great promise as inhibitors of parasite cell growth. While we acknowledge that we have direct TbAUK1 inhibition data only for compounds **1**, **2** and **5a**, this has provided a starting point to initiate inhibitor optimization studies in the absence of conclusive trypanosome kinase inhibition data. This is of great importance, given that the functional pathways of the trypanosome kinome are only lightly understood at this point in time. However, by starting the program with human Aurora inhibitors and utilizing previous medicinal chemistry and structural biology information, we have been able to drive cellular selectivity away from a mammalian cell line, which is a key goal of this project.

In summary, we have reported an initial structure-activity relationship study of growth inhibitors of *Trypanosoma brucei* spp. that mediate their effect, in whole or in part, by TbAUK1. Besides a demonstration of the expedience provided by repurposing historical medicinal chemistry and structural biology knowledge of the homologous human enzymes, we have shown that compounds with improved selectivity for parasite killing over host cells can be designed based on the structure of the hypothesized enzyme target. Further optimization of this compound chemotype for potency against the parasites, continued selectivity over human cells, and central nervous system exposure will be reported in due course.

4. Materials and Methods

4.1. Chemistry

Unless otherwise noted, reagents were obtained from Sigma-Aldrich, Inc. (St. Louis, MO), and used as received. Reaction solvents were purified by passage through alumina columns on a purification system manufactured by Innovative Technology (Newburyport, MA). NMR spectra were obtained on Varian NMR systems, operating at 400 MHz or 500 MHz for ^1H acquisitions. LCMS analysis was performed using a Waters Alliance reverse-phase HPLC, with single-wavelength UV-visible detector and LCT Premier time-of-flight mass spectrometer (electrospray ionization). All newly synthesized compounds were deemed >95% pure by LCMS analysis prior to submission for biological testing. Published methods were employed for preparation of danusertib (**1**) [20], PHA-680632 (**2**) [21], **3** [21], and **5b** [21].

4.1.1. Ethyl 5-benzoyl-3-(4-(4-methylpiperazin-1-yl)benzamido)-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (4a)—To 0.015 g of **3** (0.034 mmol) was added pyridine (2.346 mL, 29.1 mmol) and benzoyl chloride (7.95 μL , 0.069 mmol). The reaction was stirred for 16 hours. The reaction was concentrated and the crude product was purified via silica gel chromatography, eluting with 0–8% MeOH in DCM to give **4a** (Yield: 21%). ^1H NMR (500 MHz, methanol- d_4) δ 7.89 and 7.78 (2d, J = 8.79 Hz, 2H, rotamers), 7.59–7.32 (m, 2H), 7.49–7.55 (m, 3H), 7.06 and 6.95 (2d, J = 8.79 Hz, 2H, rotamers), 4.95–4.82 (m, 4H), 4.50 (q, J = 7.00 Hz, 2H), 3.34–3.41 (m, 4H), 2.57–2.64 (m, 4H), 2.32 and 2.38 (2s, 3H, rotamers), 1.27 and 1.27 (2t, J = 7.32, 9.28 Hz, 3H, rotamers). LCMS found 503.01, $[\text{M}+\text{H}]^+$.

4.1.2. N-(5-Benzoyl-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (5a)—A solution of **4a** (5.80 mg, 0.012 mmol) in MeOH (3 mL) and Et_3N (0.3 mL) was stirred at 30 °C for 4 hours. The reaction was concentrated. The residue was dissolved twice in diethyl ether and concentrated to produce a white solid, which was triturated with a mixture of EtOAc (3 mL) and ether (0.3 mL) to give **5a** (Yield: 99%). ^1H NMR (500 MHz, d_6 -DMSO) δ 7.82 (7.91 minor) (d, J = 8.5 Hz, 2H), 7.6–7.54 (m, 2H), 7.49–7.45 (m, 3H), 6.92 (6.98 minor) (d, J = 9.0 Hz, 2H), 4.69–4.52 (m, 4H), 3.25–3.23 (3.29–3.27 minor) (m, 4H), 2.41–2.39 (2.44–2.43 minor) (m, 4H), 2.20 (2.21 minor) (s, 3H). The sets of amide rotamer signals that were doubled at room temperature and listed as minor were shown to coalesce at 140 °C. LCMS found 431.01, $[\text{M}+\text{H}]^+$.

4.1.3. Ethyl 3-(4-(4-methylpiperazin-1-yl)benzamido)-5-tosyl-5,6-dihydropyrrolo[3,4-c]pyrazole-1(4H)-carboxylate (4c)—To 0.013 g of **3** (0.029 mmol) was added pyridine (2 mL), and then tosyl chloride (5.57 mg, 0.029 mmol). The reaction mixture was stirred at rt for 16 h. The solvent was concentrated and the crude product was purified via silica gel chromatography, eluting with 0–10% MeOH in DCM to give **4c** (Yield: 23%). ^1H NMR (500 MHz, d_4 -CD $_3$ OD) δ 7.83 (d, J = 8.5 Hz, 2H), 7.80 (d, J = 8.75 Hz, 2H), 7.42 (d, J = 8.5 Hz, 2H), 7.00 (d, J = 8.75 Hz, 2H), 4.65–4.63 (m, 4H), 4.44 (q, 2H), 3.39–3.37 (m, 4H), 2.62–2.60 (m, 4H), 2.41 (s, 3H), 2.36 (s, 3H), 1.41 (t, J = 7.25 Hz, 3H). LCMS found 553.01, $[\text{M}+\text{H}]^+$.

4.1.4. 4-(4-Methylpiperazin-1-yl)-N-(5-tosyl-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (5c) [28]—A solution of **4c** (3.7 mg, 6.7 μmol) in MeOH (1 mL) and Et_3N (0.1 mL) was stirred at 30 °C for 4 hours. The solvent was concentrated. The residue was dissolved twice in diethyl ether and concentrated to produce a white solid, which was triturated with a mixture of EtOAc (3 mL) and ether (0.3 mL) to give **5c** (Yield: 93%). ^1H NMR (500 MHz, d_4 -CD $_3$ OD) δ 7.83 (d, J = 9.0 Hz, 2H), 7.88 (d, J = 8.5 Hz, 2H),

7.41 (d, J = 8.5 Hz, 2H), 7.01 (d, J = 9.0 Hz, 2H), 4.62-4.35 (m, 4H), 3.39-3.37 (m, 4H), 2.64-2.62 (m, 4H), 2.41 (s, 3H), 2.37 (s, 3H). LCMS found 481.01, [M+H]⁺.

4.1.5. General procedure for library synthesis—To the various carboxylic acids (obtained from ASDI, Inc., 0.076 mmol, 1 equiv.) was added dry DCM (0.5 mL), oxalyl chloride (0.076 mmol, 1 equiv.), and then 2 drops of DMF. The reactions were placed in the shaker and reacted overnight at ambient temperature for 24 hours. A solution of the hydrochloride salt of **3** (0.048 mmol, 1 equiv.) in 1.5 mL of DMF was added, followed by DIEA (0.240 mmol, 5 equiv.). The vials were agitated in the shaker for 24 hours at ambient temperature. The solvent was removed in a Genevac HT24 centrifugal evaporator, and the crude intermediates were carried forward without purification. To each of the above crude products was added 10% TEA in MeOH (2 mL). The reaction vials were then placed in the shaker at 33 °C overnight, and the solvent was evaporated. All the crude products were purified via preparative HPLC to >95% purity, and the desired products in the yields as described below.

4.1.5.1. 4-(4-Methylpiperazin-1-yl)-N-(5-(2-(naphthalen-1-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (8) (Yield: 30%): ¹H NMR (500 MHz, *d*₆-DMSO) δ 10.65 (s, 1H), 7.90-7.84 (m, 5H), 7.77 (d, J = 8.5 Hz, 1H), 7.48-7.43 (m, 3H), 6.99 (d, J = 9.5 Hz, 2H), 4.80-4.47 (m, 4H), 4.10-4.02 (m, 2H), 3.92 (d, J = 10.5 Hz, 2H), 3.15 (m, 2H), 2.56 (m, 4H), 2.31 (s, 3H). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 169.5, 169.5, 133.3, 133.0, 131.8, 129.3, 128.2, 127.7, 127.5, 127.4, 126.0, 125.5, 113.5, 53.9, 48.6, 46.3, 42.1, 40.1. LCMS found 495.01, [M+H]⁺.

4.1.5.2. 4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2,3,6-trifluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (9), (Yield: 62%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.70 (s, 1H), 7.93-7.89 (m, 2H), 7.50-7.38 (m, 1H), 7.18-7.10 (m, 1H), 7.01 (d, J = 8.4 Hz, 2H), 6.56 (s, 1H), 4.86-4.45 (m, 4H), 3.87 (s, 3H), 3.40-3.30 (m, 4H), 2.70-2.63 (m, 4H), 2.38 (s, 3H). LCMS found 499.01, [M+H]⁺.

4.1.5.3. N-(5-(2-Methoxy-2-phenylacetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (10), (Yield: 81%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.67 (d, J = 9.6 Hz, 1H), 7.89 (d, J = 12.0 Hz, 2H), 7.44-7.32 (m, 5H), 7.01 (d, J = 12.0 Hz, 2H), 5.10 (d, J = 12.0 Hz, 1H), 4.82-4.39 (m, 4H), 3.40-3.35 (m, 4H), 3.31 (d, J = 3.2 Hz, 3H), 2.80-2.65 (m, 4H), 2.48 (s, 3H). LCMS found 475.01, [M+H]⁺.

4.1.5.4. N-(5-(2-(3-Chlorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (11), (Yield: 27%): ¹H NMR (500 MHz, *d*₆-DMSO) δ 10.70 (s, 1H), 7.92-7.89 (m, 2H), 7.35-7.21 (m, 4H), 7.01 (d, J = 9.0 Hz, 2H), 4.76-4.44 (m, 4H), 3.76 (d, J = 14.0 Hz, 2H), 3.37 (m, 4H), 2.72 (m, 4H), 2.42 (s, 3H). LCMS found 479.01, [M+H]⁺.

4.1.5.5. N-(5-(3-Methyl-2-phenylbutanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (12), (Yield: 67%): ¹H NMR (500 MHz, *d*₄-methanol) δ 7.90-7.86 (m, 2H), 7.46-7.40 (m, 2H), 7.35-7.30 (m, 2H), 7.28-7.22 (m, 1H), 7.04 (t, J = 8.5 Hz, 2H), 5.03 (d, J = 12.0 Hz, 1H), 4.95-4.44 (m, 4H), 3.50-3.40 (m, 4H), 2.86-2.81 (m, 4H), 2.53 (s, 3H), 2.46-2.40 (m, 1H), 1.08 (d, J = 7.0 Hz, 3H), 0.71 (t, J = 4.5 Hz, 3H). ¹³C NMR (126 MHz, *d*₆-DMSO) δ 172.4, 172.4, 153.6, 139.6, 139.6, 130.0, 129.9, 129.2, 129.1, 129.1, 127.5, 114.2, 57.4, 57.1, 54.6, 46.9, 45.6, 32.6, 32.4, 22.2, 22.1. LCMS found 487.01, [M+H]⁺.

4.1.5.6. 4-(4-Methylpiperazin-1-yl)-N-(5-(2-(p-tolyl)propanoyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (13), (Yield: 68%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.62 (d, J = 9.6 Hz, 1H), 7.89-7.85 (m, 2H), 7.21-7.17 (m, 2H), 7.13-7.10 (m, 2H), 7.70-6.97 (m, 2H), 4.82-4.77 (m, 1H), 4.48-3.88 (m, 4H), 3.40-3.30 (m, 4H), 2.61-2.55 (m, 4H), 2.32 (s, 3H), 2.23 (s, 3H), 1.30 (d, J = 6.8 Hz, 3H). LCMS found 473.01, [M+H]⁺.

4.1.5.7. N-(5-(2-(3,5-Dimethylphenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (14), (Yield: 75%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.69(s, 1H), 7.88 (d, J = 8.4 Hz, 2H), 6.98 (d, J = 8.4 Hz, 2H), 6.87-6.84 (m, 3H), 4.72-4.43 (m, 4H), 3.62 (d, J = 8.00 Hz, 2H), 3.32-3.29 (m, 4H), 2.53-2.48 (m, 4H), 2.27 (s, 3H), 2.23 (s, 6H). LCMS found 473.01, [M+H]⁺.

4.1.5.8. N-(5-(2-(2,5-Dimethylphenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (15), (Yield: 74%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.70 (s, 1H), 7.90 (d, J = 8.8 Hz, 2H), 7.04-6.99 (m, 3H), 6.94-6.92 (m, 2H), 6.56 (s, 1H), 4.76-4.46 (m, 4H), 3.66 (d, J = 4.0 Hz, 2H), 3.40-3.30 (m, 4H), 2.70-2.60 (m, 4H), 2.38 (s, 3H), 2.22 (s, 3H), 2.16 (s, 3H). LCMS found 473.01, [M+H]⁺.

4.1.5.9. 4-(4-Methylpiperazin-1-yl)-N-(5-(2-(2,3,5-trifluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)benzamide (16), (Yield: 73%): ¹H NMR (500 MHz, *d*₄-methanol) δ 7.89-7.86 (m, 2H), 7.12-7.07 (m, 1H), 7.05-7.02 (m, 2H), 7.0-6.95 (m, 1H), 4.93-4.59 (m, 4H), 3.91 (d, J = 5.5 Hz, 2H), 3.42-3.40 (m, 4H), 2.77-2.73 (m, 4H), 2.46 (s, 3H). LCMS found 499.01, [M+H]⁺.

4.1.5.10. N-(5-(2-(2-Methyl-1H-indol-3-yl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (17), (Yield: 70%): ¹H NMR (400 MHz, *d*₆-DMSO) δ 10.84 (d, J = 8.0 Hz, 1H), 10.68 (d, J = 6.4 Hz, 1H), 7.89 (m, 2H), 7.46 (d, J = 8.0 Hz, 1H), 7.23-7.20 (m, 1H), 7.01-6.88 (m, 4H), 4.76-4.41 (m, 4H), 3.71 (s, 2H), 3.33-3.30 (m, 4H), 2.61-2.56 (m, 4H), 2.36 (d, J = 16.0 Hz, 3H), 2.31 (s, 3H). LCMS found 498.01, [M+H]⁺.

4.1.5.11. N-(5-(2-(3,5-Difluorophenyl)acetyl)-1,4,5,6-tetrahydropyrrolo[3,4-c]pyrazol-3-yl)-4-(4-methylpiperazin-1-yl)benzamide (18), (Yield: 56%): ¹H NMR (500 MHz, *d*₄-methanol) δ 7.89 (d, J = 9.0 Hz, 2H), 7.07-7.05 (m, 2H), 6.96-6.93 (m, 2H), 6.86-6.82 (m, 1H), 4.85-4.56 (m, 4H), 3.82 (s, 2H), 3.54-3.52 (m, 4H), 3.17-3.13 (m, 4H), 2.75 (s, 3H). LCMS found 481.01, [M+H]⁺.

4.2. Computational chemistry

4.2.1. Virtual Library Design—A virtual library of analogs was enumerated using Pipeline Pilot (Figure S1, based on 208 arylacetic acids that were available in pre-weighed quantities from a commercial vendor (ASDI, Inc). Those product molecules with molecular weight >500 and cLogP > 5.0 were removed, and SciTegic extended connectivity fingerprints (FCFP₆) were calculated for the set of molecules. A subset of 50 maximally diverse analogs was selected for docking in the TbAUK1 homology model.

4.2.2. Homology modeling—The protein sequence of TbAUK1 (Tb11.01.0330) was searched against the PDB (<http://www.rcsb.org/>) using PSI-BLAST [29]. Models were built for a portion of the catalytic domain (residues 28–219). TbAUK1 shares 43% sequence identity with human Aurora A and 41% sequence identity with human Aurora B, with 88% sequence coverage by these crystal structures. Homology modeling of the kinase catalytic domain was performed with the YASARA suite of programs [30, 31]. The final obtained

model was a hybrid based on human Aurora A crystal structure (PDB ID 2bmc) [21] and a mouse Aurora A X-ray crystal structure (PDB ID 3d14) [24] as templates. The resulting hybrid model was refined by energy minimization for 500 picoseconds using an explicit solvent molecular dynamics simulation with a YAMBER3 force field in YASARA [32]. The refinement consists of a short steepest descent minimization to remove the largest intermolecular and intramolecular clashes, then a second steepest descent minimization with all potential energy terms, followed by a simulated annealing procedure. The quality of the model was examined using PROCHECK [33] and MolProbity [34] and was found to be of sufficiently good quality (see Supplementary Material).

4.2.3. Compound docking—The model TbAUK1 structures were prepared using the Maestro 9.1 protein preparation wizard (Schrodinger, LLC, 2010, New York, NY) before docking, bond orders were assigned and the orientation of hydroxyl groups, amide groups of the side chains of Asn and Gln, and the charge state of histidine residues were optimized. A restrained minimization of the protein structure was performed using the default constraint of 0.3 Å RMSD and the OPLS 2001 force field [35].

4.3. Biology

4.3.1. Cell Cultures [36]—The bloodstream form (BF) trypanosomes used in this study include: *Trypanosoma brucei rhodesiense* YTat1.1, a variant derived from UGANDA/60/TREU 164 (ETat 3) and kindly provided by C. Patton; *T.b.brucei* AnTat1.1A, kindly provided by E.Pays; and *T.b.brucei* Lister 427 strain 90-13 from G. A. M. Cross. The YTat1.1 and AnTat1.1A BF cells were culture adapted from rat blood in HMI-9 medium [36] with 10% FBS (Atlanta Biologicals) and 10% Serum Plus™ (SAFC Biosciences) at 37°C and 5% CO₂. The 90-13 cells were grown in HMI-9 medium supplemented with G418 (2.5 µg mL⁻¹) and hygromycin B (5 µg mL⁻¹). The human T lymphocytic cell line CRL-1582™ (MOLT-4, obtained from ATCC) was grown in RPMI 1640 medium with 20% FBS, penicillin (100 units mL⁻¹) and streptomycin (0.1 mg mL⁻¹) at 37°C and 5% CO₂. Cell densities for trypanosomes and mammalian cells were maintained in the range of 5×10⁴ to 1×10⁶ cells mL⁻¹.

4.3.2. Cell Viability Screen—Inhibitor sets were dissolved in DMSO and stored at -80°C as a 500x dilution series. The final concentrations ranged from 50 nM to 8 µM, and DMSO was constant at 0.2%. Each drug was tested at a minimum of 8 concentrations in triplicate. Log phase BF trypanosomes were counted with a hemocytometer and seeded at an initial density of 1×10⁵ cells mL⁻¹ in a volume of 1.5mL in a 24 well plate format. The cultures were incubated with the inhibitor sets for 4-doubling times (48 hr). At the end of this time, trypanosomes were concentrated by centrifugation, suspended in 100 µL of medium and transferred to 96-well flat bottom microtiter plates (Costar UV transparent plates). The end-point Cell Titre Blue® (CTB) assay was used to test for cell viability (Promega). CTB reagent (20 µL) was added to each well and the plates were incubated at 37°C and 5% CO₂ for 3 hours. Fluorescence was measured at 560_{Ex}/590_{Em} using the SPECTRAMax GEMINI XPS Microplate Spectrofluorometer (Molecular Devices). Background was calculated from wells containing CTB and media without cells, while 100% growth was calculated from cells with DMSO and without drugs. The background fluorescence was subtracted from all the data points. The EC₅₀ values were computed from the inhibition curves with OriginPro 8.5. The CTB end-point assay measures non-selective dehydrogenases, where the total activity is proportional to cell number. Because Aurora kinase inhibitors tend to be trypanostatic rather than trypanolytic [18], and because the non-dividing cells continue to grow in size and cell activity, the assay tends to overestimate values for EC₅₀ (i.e. it underestimates the potency of the drugs). The CTB end-point assay

was linear out to 180 min and at trypanosome cell densities in the range of 5×10^5 - 2×10^7 cells mL^{-1} .

The viability of MOLT-4 cells was also determined with the CTB assay. Cells were seeded at an initial density of 5×10^4 cells mL^{-1} in a 96 well plate format (100 μL /well). Following a 24 hr growth period, inhibitors were added. The inhibitor sets described above were diluted to 2x concentration in 1.5 ml of medium, and then 100 μl of the diluted compound was added to the cells. Depending on the drug, the 8 triplicate concentration points ranged between 50 nM to 30 μM . Cultures were treated for 4-doublings (4 days) and then transferred to 96-well flat bottom microtiter plates containing 20 μl of CTB. After a 3 hr incubation at 37°C, fluorescence was measured as described above.

4.3.3. Kinase Reactions—The kinase reaction was performed as described previously [18]. Briefly, procyclic form (PF) trypanosomes derived from AnTat1.1 (provided by E. Pays) were transformed with the constitutive expression vector pHD496 containing an NH₃-AU1 epitope tagged TbAUK1. Logarithmically growing cultures (1×10^8 cells total) were treated with the proteasome inhibitor MG132 (16 μM) for 3 hr to increase the cellular content of TbAUK1. The remaining steps were all performed at 4°C. The cells were washed twice in PBS with Dulbecco's salts (Gibco), and then incubated for 15 min in 400 μL of lysis buffer (50 mM Hepes, pH 7.4, 100 mM KCl, 25 mM NaF, 0.5% NP-40, 1 mM Na₃VO₄, 1 mM DTT and 100 nM okadaic acid) containing protease inhibitor cocktail (Sigma, P-8340). The lysate was centrifuged at $10,000 \times g$ for 15 min. A 50% slurry of Sephadex G-25 beads was prepared in lysis buffer and 80 μL was added to the supernatant. After 2 hr, the pre-cleared supernatant was collected and combined with 50 μL of a 50% slurry of AU1-conjugated beads. The beads had previously been washed once in 1X kinase buffer (20 mM Hepes, 150 mM KCl, 5 mM NaF, 5 mM MgCl₂, and 1 mM DTT, pH 7.4). The beads were incubated overnight at 4°C, washed, collected by centrifugation and suspended in 50 μL of kinase buffer. The beads were the source of kinase for the kinase reactions. Kinase reactions were in 50 μL volumes containing 10 μL of beads, 25 μL 2X kinase buffer and 160 μg of myelin basic protein (Sigma). Drugs were added in 2 μL DMSO and incubated for 5 min before the reaction was initiated with 8 μM ATP (4 μCi of [γ -³²P]ATP). After a 30 min incubation at 30°C, the reaction was stopped with the addition of 50 μl 2x Laemmli buffer and boiled. The products were separated by SDS-PAGE and gels were exposed to X-ray film for 24 hr.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HAT	human African trypanosomiasis
BF	bloodstream form <i>T. brucei</i>
cAMP	cyclic adenosine monophosphate

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HIGHLIGHTS

- We assessed a class of human Aurora kinase inhibitors against *Trypanosoma brucei*
- The human Aurora inhibitor danusertib inhibits TbAUK1 and *T. brucei* growth
- Analogs of danusertib were designed based on a TbAUK1 homology model
- New analogs show improved selectivity for killing *T. brucei* cells over host cells
- We show that target repurposing can provide a rapid start for NTD drug discovery

Figure 1.
Pyrazolopyrazole inhibitors of human Aurora kinases.

Figure 2.
Inhibition of kinase activity by compounds in the inhibitor set.

Figure 3.

Comparison of (A) the human Aurora A/danuseritib complex (PDB ID: 2J50, danuseritib colored in green); (B) The predicted conformation of **2** (colored in purple), **8** (colored in yellow), and danuseritib (colored in green), docked into human Aurora A. and (C) in the TbAUK1 model. The colors in (C) are shown for sidechain heteroatoms in Lys58 (blue) and Met113 (yellow).

Figure 4.

Dose response of **1**, **2**, **12** and **18** against *T.b rhodesiense* cultures. YTat1.1 BF cells were incubated for a 48 hr period with varying drug concentrations. Cell viability was monitored with the Cell Titer Blue® assay. Values plotted are the average $\pm$ std dev of 2–3 independent experiments, each done in triplicate.

^aReagents and conditions. a. benzoyl chloride, pyridine; b. phenylisocyanate, THF; c. *p*-toluenesulfonyl chloride, pyridine; d. 10% Et₃N, methanol.

Scheme 2.^a Synthesis of arylacetamide derivatives **8-18**.^a Reagents and conditions: (a) oxalyl chloride, CH_2Cl_2 , DMF. (b) **3**, DMF, DIEA. (c) 10% Et_3N , MeOH.

Table 1

Screening data summary of singleton analogs of **1** tested against *T. brucei brucei* and MOLT-4 cells.^a

Compd	<i>T. b. brucei</i> ^a EC ₅₀ ^d (μM)	MOLT-4 ^b EC ₅₀ (μM)	Selectivity MOLT-4/Tbb
1	0.6	0.15	0.25
2	4.0	0.22	0.06
5a	11.1	0.54	0.05
5b	6.1	3.55	0.58
5c	5.7	0.94	0.16

^a *T.b.b.* Lister 427 90-13.

^b MOLT-4 acute myelogenous leukemia cell line.

^d EC₅₀ values were calculated from inhibition curves at a minimum of 8 different drug concentrations tested in triplicate and using OriginPro 8.5 analysis software.

Table 2

Dose-response experiments on the parallel array of analogs of **1** tested against *T. b. rhodesiense* and MOLT-4 cells.

Compd	R ₁	Ar	T.b.r. ^b EC ₅₀ (μM) ^d	MOLT-4 ^c EC ₅₀ (μM) ^d	Selectivity MOLT/Tbr
1	OMe	phenyl	0.15	0.15	1.0
8	H	1-naphthyl	0.61	14.25	23.4
9	H	2,3,6-trifluorophenyl	0.32	2.22	6.9
10^a	OMe	phenyl	0.61	4.13	6.8
11	H	3-Cl-Ph	0.58	4.0	6.9
12^a	iPr	phenyl	0.4	2.5	6.3
13	Me	4-methylphenyl	0.86	5.48	6.4
14	H	3,5-dimethylphenyl	1.04	4.46	4.3
15	H	2,5-dimethylphenyl	1.2	2.65	2.2
16	H	2,3,5-trifluorophenyl	2	2.31	1.2
17	H	3-(2-methylindolyl)	1.2	1.16	1.0
18	H	3,5-difluorophenyl	0.91	0.63	0.7

^aIndicates compounds tested as racemate.

^b*T. brucei rhodesiense* YTAT1.1 strain.

^cMOLT-4 acute myelogenous leukemia cell line.

^dEC₅₀ values were calculated from inhibition curves at a minimum of 8 different drug concentrations tested in triplicate and using OriginPro 8.5 analysis software.