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Computer-aided Discovery of *Trypanosoma brucei* RNA-editing Terminal Uridylyl Transferase 2 Inhibitors

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Abstract

Human African trypanosomiasis (HAT) is a major health problem in sub-Saharan Africa caused by *Trypanosoma brucei* infection. Current HAT drugs are difficult to administer and not effective against all parasite species at different stages of the disease which indicates an unmet pharmaceutical need. TbRET2 is an indispensable enzyme for the parasite, and is targeted here using a computational approach that combines molecular dynamics simulations and virtual screening. The compounds prioritized are then tested in *T. brucei* via Alamar blue cell viability assays. This work identified 20 drug-like compounds which are candidates for further testing in the drug discovery process.

Keywords

TUTase; virtual screening; RET2; trypanosomes; *Trypanosoma brucei*; human African trypanosomiasis; sleeping sickness; HAT

Introduction

Human African trypanosomiasis (HAT), also known as sleeping sickness, is a major health problem threatening the lives of millions of people in sub-Saharan Africa.(1) The disease is caused by either of the two trypanosome subspecies *Trypanosoma brucei rhodesiense* or *Trypanosoma brucei gambiense* that are transmitted to humans by the tsetse flies.(1, 2) The majority of the cases is due to an infection by *Trypanosoma brucei gambiense*.(2) If untreated, HAT is fatal.

In the first stage of HAT, parasites live in blood and lymph as well as in the tissues of the host.(3, 4) Later, in the second stage, parasites transit the blood-brain barrier and infect the

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Conflict of Interest

We declare no conflicts of interest.

central nervous system of the host.(3, 4) Early diagnosis and treatment is desirable but typically HAT has reached the second stage at the time of diagnosis.(1)

Suramin and pentamidine are currently used to treat early phases of the disease.(3, 4) Although these two drugs are generally successful, they can cause undesirable side effects. For the treatment of the late neurological stages of HAT, melarsoprol and eflornithine are the only drugs currently available.(3, 4) These two drugs are very toxic and difficult to administer as well as having severe potential side effects that can even be fatal.(5) Currently, a combination therapy of nifurtimox and eflornithine is the first-line treatment against second-stage *T. brucei gambiense* cases of HAT.(6-9) However, eflornithine is effective only against *Trypanosome brucei gambiense*. Moreover, drug resistant trypanosomes are emerging which emphasizes the need for action to discover novel effective drugs against HAT.(10)

T. brucei is one of the kinetoplastid protozoa that have an unusual mitochondrial DNA, called kDNA, which consists of maxicircles and minicircles.(11, 12) The maxicircle DNA encodes for oxidative phosphorylation enzymes and mitochondrial rRNAs.(11, 12) However, most of the mRNAs encoded in the maxicircle DNA undergo extensive post-transcriptional editing before their translation. The editing process is performed by a dynamic, heterogenous multiprotein machinery which includes “the 20S editosome” that uses numerous guide RNAs (gRNA) encoded in the minicircles to specify the sites of editing and the final edited sequence.(11, 12) Recognition of the editing sites, which are sites of mRNA/gRNA duplex sequence mismatch, is followed by either deletion of uridylates (Us) by an exonuclease or insertion of Us by a terminal uridylyl transferase (TUTase) depending on the type of mismatch with the gRNA specifying the number of Us deleted or inserted.(11-13)

RNA editing terminal uridylyl transferase (TUTase) 2 of *T. brucei* (TbRET2) is an indispensable part of the editosome and has been shown to be essential for the viability of both the insect and the bloodstream forms of the parasite.(14, 15) To catalyze insertion of a U to the 3' RNA terminus, TbRET2 must bind RNA and UTP at the same time in its deep active site releasing a pyrophosphate group at the end of the reaction.(16, 17) A family of seven human TUTases have been identified recently with some sequence similarity to TbRET2.(18-23) However, not much is known about their structure due to the lack of data except for PAPD1 which has less than 20% sequence identity.(18) With its recently elucidated high-resolution crystal structure both in apo and UTP-bound forms, TbRET2 stands as a promising HAT drug target.(16)

In this work, we present 20 drug-like inhibitors of TbRET2 that we discovered by integrating Relaxed Complex Scheme (RCS) into our virtual screening protocol. Starting from a high resolution crystal structure (pdbID:2B51),(16) two copies of 50-ns molecular dynamics (MD) simulations were previously used to investigate the dynamics of UTP-bound TbRET2.(24) Using the resulting structures in conjunction with the RCS method, we predicted the most promising compounds for TbRET2 inhibition. The MD structural ensemble was significantly reduced by including only the most dominant 3 cluster centroids. Alamar Blue™ (AB) cell viability assays(25-28) confirmed 20 inhibitors with EC50 values

in the low μM range. We thus demonstrated that the use of receptor flexibility in the virtual screening process achieved an enrichment of the set of compounds to be tested, and led to discovery of several promising scaffolds as TbRET2 inhibitors.

Methods and Materials

Molecular Dynamics Simulations

For this work, two copies of 50 ns molecular dynamics (MD) simulations of UTP-bound TbRET2 are utilized. The details of MD preparation and simulation protocol are described elsewhere.(24) Snapshots of the system extracted at every picosecond were used to obtain a combined trajectory of 10,000 frames.

RMSD-based Clustering

Clustering of 10,000-frame MD trajectory of UTP-bound TbRET2 was performed using the gromos algorithm(29) with GROMACS4.0.5 analysis software.(30) After superimposing the protein with respect to Ca atoms to remove overall translation and rotation, the trajectory was clustered with respect to the coordinates of all atoms in the TbRET2 active site as described in Demir *et al.* (24) After trying different RMSD cutoff values, an optimum RMSD cutoff of 1.55 Å was used for clustering which resulted in 27 clusters. Most-populated 11 clusters spanned % 97.27 of all 10,000 frames. Population percentages of top 5 clusters were %26.22, %21.99, %21.74, %9.35, %4.26, respectively.

The Relaxed Complex Scheme (RCS)

Autodock Vina(31) was used for all docking experiments, and the lowest docking score computed is listed for each compound. We docked to the most-populated 3 cluster centroids for initial compound selection, and used the top 11 cluster centroids for the Relaxed Complex Scheme to calculate binding affinities. Ensemble-averaged binding affinities were computed as the weighted-average of docking scores into the most-populated 11 cluster centroids using cluster population percentages as weights.

ADME Filters for Drug-likeness

We filtered compounds for strict adherence to Lipinski's Rule of 5,(32, 33) Jorgensen's Rule of 3(34) and $\log P_{\text{oct/wat}} < 3$. Schrodinger's Quikprop module(35) was used to predict and filter for these ADME properties.

Similarity Search

A similarity search over the full NCI database for the 3 low nanomolar hits using %90 Tanimoto similarity as the criteria found 33 compounds which were rank-ordered according to their ensemble-based binding affinities using our RCS protocol.

Whole-cell Parasite Assays

Compounds were obtained from the NCI/DTP Open Chemical Repository (<http://dtp.cancer.gov>). Stock solutions were made in 100% DMSO and fresh aliquots were generated. Among the twenty four received compounds, seventeen were easily dissolved, six

needed 4 hours agitation at room temperature (RT) to obtain complete solubility and one 324623 could not be dissolved despite extensive agitation at RT and incubation at 37°C, and thus was not tested.

An Alamar Blue™ (AB) Viability Assay(25-28) was performed in a 96-well plate format to screen for drug sensitivity of *Trypanosoma brucei brucei* Bloodstream Form 427. Parasites at 2×10³ cells / ml were incubated with different drug concentrations (two-fold serial dilutions) at 200µl final volume of HMI-9 with 10% FBS / well for 48-hrs at 37°C and 5% CO₂. 20µl of AlamarBlue (Invitrogen) was then added to each well and cells were incubated for an additional 4-hrs under the same conditions. DMSO never exceeded 0.08% for these assays. Fluorescence signal was quantified using an SpectraMax M2 fluorescence plate reader (Molecular Devices) (Excitation 544nm – Emission 590nm). Background levels of fluorescence, representing complete parasite death, were determined with wells that contained no parasites and the maximum levels of fluorescence were determined with wells that contained parasites with no drug added. The EC₅₀ values were determined using the GraphPad Prism5 software (Log (inhibitor) vs. response – Variable slope (4 parameters)). Compounds were tested with three different parasite cultures (three biological replicates). The value of each biological replicate is the average of three replicates made in the same plate (three technical replicates).

An initial screening using a narrow range of concentrations from 8µM to 0.5µM was performed to select compounds with EC₅₀ values < 4µM using one replicate. Based on results from the initial test, compounds **D1**, **D2** and **D3** (Table 1) were selected for additional screening. For the 3 active similar compounds, we performed multiple biological replicates only if the initial EC₅₀s obtained were lower than 4µM.

Purity Tests

Twelve of the twenty drug-like compounds which had the lowest EC₅₀ values were tested for purity. An Agilent 1260 HPLC system coupled with a Thermo LCQdeca mass spectrometer was employed for LC-UV-MS analysis using positive ion mode electrospray ionization (ESI) as the ion source. A Shiseido MGII C-18 column (2.0 mm ID × 100 mm length, 3 µm particle size) was used for LC separation with flow rate of 0.30 ml/min. 2.5% acetonitrile in water with 0.1% formic acid was used as mobile phase A, and pure acetonitrile with 0.1% formic acid was used as mobile phase B. Details for the LC gradient settings are as follows: Increased from 10% B to 95% B in 10 minutes, held at 95% B for 5 minutes, then returned to 10% B in 1 minute, and held at 5% B for 6 minutes. The UV detection wavelength was set at 254 nm.

Results and Discussion

Control Docking

In the control docking of UTP into the TbRET2 crystal structure (pdbID: 2B51), Autodock Vina predicted the most favorable binding pose as depicted in Figure 1A with a binding affinity of -8.6 kcal/mol. The binding poses of uridine and ribose in the crystal structure were reproduced well in the control docking experiment, however a large heavy-atom RMSD of 5.04 Å was still computed for UTP due to the deviation in the flexible

triphosphate tail. Excluding the terminal two phosphates from the RMSD calculation reduced the heavy-atom RMSD of UTP to 3.79 Å.

Initial Set of Recommended Compounds

For the initial virtual screening of National Cancer Institute Diversity Set 2 (NCIDS2), we used the static TbRET2 crystal structure (pdbID:2B51)(16) to dock the 1324 compounds (1541 entries including stereomers) according to their predicted binding affinity. To incorporate receptor flexibility into our virtual screening protocol, we also docked the NCIDS2 compounds into the most-populated 3 cluster centroids of UTP-bound TbRET2 MD simulations depicted in Figure 1B, and rank-ordered the compounds based on their docking scores. For each cluster centroid, 100 compounds with the best docking scores were selected and filtered for the ADME properties detailed in the Methods section.

There are two main binding sites in TbRET2 active site: one for UTP and one for the terminal RNA nucleotide. In order to target both binding sites, we performed virtual screening once in the absence of UTP to target the UTP binding site, and another in the presence of UTP to target the terminal RNA nucleotide binding site. Filtering the top scoring 100 ligands for each of the 3 cluster centroids left 14 unique compounds (16 entries) for the UTP binding site, and 15 unique compounds (18 entries) for the terminal RNA nucleotide binding site. When combined, these two sets form a set of 24 unique compounds. The binding affinities of these 24 compounds were refined using our Relaxed Complex Scheme and recommended for experimental testing (Table S1).

Low-Micromolar Inhibitors Found in Round 1

One compound in the recommended set, NCI compound 324623, was not tested since it was not soluble. Among the 23 tested, 3 compounds (**D1**, **D2** and **D3** in Table 1) had EC₅₀ values lower than or equal to 4 µM corresponding to 13% hit rate. Of the three, compounds **D1** and **D2** were predicted to bind favorably to both binding sites while compound **D3** was predicted to bind favorably to the UTP binding site.

If we had selected the same number of compounds to test based on the crystal structure docking score ranks, 7 out of 14 compounds for the UTP binding site and 7 out of 16 compounds for the terminal RNA nucleotide binding site would not have been tested. Also, among the 3 compounds with the lowest EC₅₀ values, compound **D3** would not have been tested based on crystal structure docking ranks indicating the enrichment achieved by incorporating active site flexibility.

Second Set of Recommended Compounds

We performed a second virtual screening with NCI Plated compounds (a combined set of the NCI's Mechanistic Set, Natural Products Set, COMBO Set, and Oncology Approved Set). Our ADME filters selected only 307 compounds out of 805 total. After docking the pre-filtered compounds into the most-populated 3 cluster centroids, we selected 39 compounds with binding affinities lower or equal to -8.2 kcal/ mol for the RNA nucleotide binding site, and 52 compounds with binding affinities lower or equal to -8.6 kcal/ mol for

the UTP binding site. A combined set of 60 compounds was selected for experimental testing.

Low-Nanomolar Inhibitors Found in Round 2

Among the recommended set of 60 compounds, 9 compounds were not available from NCI, another 10 compounds (with NCI codes 11440, 40341, 118732, 157004, 314622, 623051, 634568, 5890, 292147, 634224) could not be tested due to solubility problems, and one compound (with NCI code 94600) was already tested in NCIDS2 screening. Out of the tested 40 compounds in the second round (Table S2), 12 compounds had EC50 values lower than 4 μ M corresponding to 30% hit rate. Three of these compounds (compounds **P1**, **P2**, and **P3** in Table 1) had EC50 values lower than 30 nM.

5 out of the 39 compounds for the terminal RNA binding site and 9 out of 52 compounds for the UTP binding site would not have been tested if we had used crystal structure docking rank-orders to select the same number of compounds. One of the three low-nanomolar hits, compound **P2**, would not have been tested if only crystal structure ranks were used to select compounds.

Similarity Search over the Full NCI Database

A similarity search over the full NCI database for the 3 low-nanomolar hits using %90 Tanimoto similarity as the criterion found 33 compounds which we docked into the top three TbRET2 cluster centroids, and ranked according to their binding score. 22 of these compounds were not available from NCI/DTP, and one of the delivered compounds (NCI code 609084) could not be tested due to insolubility leaving us with 10 compounds to test (Table S3). Experiments demonstrated that 5 of the compounds have EC50 values lower than 4 μ M (compounds **S1** through **S5** in Table 1). Notably, **S1** and **S2** had EC50 values of ~22 nM and ~50 nM, respectively.

Purity of Compounds

Among the 20 compounds reported in Table 1, the purity of twelve compounds with the lowest EC50 values were evaluated via LC-UV-MS analysis. The mass spectrometry data is presented in the Supplementary Info. The purity of all compounds except one is higher than 88% (Table 2).

Predicted Modes of Binding for the Top Three Hits

We identified the best scoring cluster centroids for each of the top 3 hits in the second round and analyzed the most favorable mode of binding predicted. In the predicted binding mode of compound **P2** to the UTP binding site (Figure 2A), part of the compound occupies the polar water pocket adjacent to the UTP binding site we identified in our previous work. (24) There are additional hydrogen-bonding interactions of the compound to the Asn 248 side chain and the Ser289 backbone. The predicted binding mode of compound **P1** to the UTP binding site also indicates occupation of the polar water pocket adjacent (Figure 2B). There was also very good shape and electrostatic complementarity observed between the compound and the pocket.

When the terminal RNA nucleotide binding site was targeted in the presence of UTP at the active site, the predicted binding poses for compounds **P3** and **P1** indicated the importance of π - π stacking interaction with the UTP nucleobase (Figure 3). There are additional hydrogen bonding interactions with Cys83 and Asp267 in the case of compound **P1**, and with Glu424 in the case of compound **P3** (Figure 3).

Overall, these predicted binding poses emphasize the potential use of adjacent water pocket when targeting the UTP binding site, and the importance of μ - μ stacking interaction with the UTP nucleobase when targeting the terminal RNA nucleotide binding site. The polar side chains in the region could also be used to improve binding affinities.

Further Considerations on the First Set of HAT Inhibitors

Drug discovery is a long and tedious process which must take into account many aspects for potency, selectivity and safety. Although a handful of compounds were discovered that kill the whole-cell *T. brucei* parasites, further assays with TbRET2 are required to validate their target. In addition, compound **P12** has a reactive alkene functional group that needs to be addressed for further use in the drug discovery process. Cytotoxicity towards human cells is also an important issue in the drug discovery process. For the NCI compounds in Table 1, the average EC50 values in the NCI/DTP website against 59 different human cancer cell lines are compiled and reported. Three compounds, namely **D2**, **P4** and **P11**, achieve more than 10 times selectivity against the parasites meeting the cytotoxicity criteria suggesting them as promising compounds for HAT drug discovery.(36) However, it is known that the sensitivity of cancer cells and healthy cells to compounds may differ, and thus these data can be taken as a rough estimate for cytotoxicity. Further biological analysis of the compounds described here is needed to evaluate and move these scaffolds further in the drug discovery process.

Conclusion

There is an urgent pharmaceutical need to develop novel drugs against HAT due to the emerging drug resistance in the relevant *T. brucei* parasites against current drugs that are already difficult to administer and cause many serious side effects. Indispensable parts of the multi-subunit RNA editing machinery in trypanosomes are considered potential drug targets, and there have been considerable efforts to achieve inhibition of such enzymes such as RNA ligases. (37-40) In this work, we present 20 compounds that are active against *T. brucei* which could be used as scaffolds for further drug design efforts. These compounds were discovered as potent TbRET2 inhibitors using a computational scheme that combines all-atom molecular dynamics simulations and virtual screening experiments. Such ensemble-based approaches taking receptor flexibility into account are promising for early drug discovery projects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Control docking result and receptor structures used for docking compound selection. A) Docked pose and crystal structure binding pose of UTP are shown in stick representation and ball-and-stick representation, respectively. Nitrogen, oxygen, carbon and phosphorus atoms are colored in blue, red, cyan and tan, respectively. B) Active sites of the most-populated 3 cluster centroids superimposed. UTP is also depicted in sticks and ball representation for reference. All the active site residues used in clustering are depicted in lines colored with respect to residue name.

Figure 2.

Predicted binding poses of top hits at the UTP binding site. Best-scoring binding mode for (A) compound **P2** and (B) compound **P1** are depicted in stick representation. The carbon, oxygen, nitrogen and hydrogen atoms of the hit compound are colored in orange, red, blue and white, respectively. The carbon, oxygen and nitrogen atoms of the receptor are colored in cyan, red and blue, respectively. Only the polar hydrogen atoms of the hit compounds are depicted while no receptor hydrogen atoms are depicted for clarity. The polar water pocket adjacent to UTP binding site is shown in surface representation.

Figure 3. Predicted binding poses of top hits at the terminal RNA nucleotide binding site. Best-scoring binding mode for (A) compound **P3** and (B) compound **P1** are depicted in stick representation. The color codes for the receptor and top hits are the same as in Figure 2. The carbon, nitrogen and oxygen atoms in the UTP are colored in green, blue and red, respectively.

Table 1

The low micromolar hits found in this study. BE: Predicted binding energy in kcal/mol.

Compd. ID	NCI No	Molecule	Crystal structure BE for RNA binding site	RCS BE for RNA binding site	Crystal structure BE for UTP binding site	RCS BE for UTP binding site	EC ₅₀ (uM)	NCI60 dose response (uM)
D1	94600		-8.8	-8.10	-9.5	-9.06	0.96	0.0381
D2	71881		-8.5	-8.14	-9.4	-9.10	2.29	100
D3	329249				-8.4	-8.92	4	3.44
P1	266535		-9.0	-8.39	-9.1	-8.61	0.013	0.0459
P2	132791				-8.6	-8.72	0.028	0.0648
P3	267461		-8.5	-8.28			0.026	0.158
P4	154020		-8.5	-7.93			0.285	8.42
P5	629971		-8.6	-8.24	-9.2	-9.16	0.905	0.052
P6	106193		-8.3	-7.73	-9.1	-8.50	2-4	2.95

Compd. ID	NCI No	Molecule	Crystal structure BE for RNA binding site	RCS BE for RNA binding site	Crystal structure BE for UTP binding site	RCS BE for UTP binding site	EC ₅₀ (uM)	NCI60 dose response (uM)
P7	643148		-9.1	-8.02	-9.8	-9.22	2-4	51.6
P8	637729				-9.1	-8.49	2-4	5.81
P9	196524		-6.6	-8.16	-9.1	-8.67	2-4	2.89
P10	335142		-8.9	-8.32	-9.1	-8.85	2-4	4.87
P11	634658				-9.1	-8.49	2-4	84
P12	668270		-8.7	-7.84	-9.1	-8.99	2-4	2.4
S1	126765		-7.8	-7.32	-8.9	-7.94	0.0226	0.0287
S2	137443		-8.8	-8.82	-8.8	-8.30	0.0506	0.0941

Compd. ID	NCI No	Molecule	Crystal structure BE for RNA binding site	RCS BE for RNA binding site	Crystal structure BE for UTP binding site	RCS BE for UTP binding site	EC ₅₀ (uM)	NCI60 dose response (uM)
S3	217307		-7.8	-7.09	-8.1	-7.91	1.1538	7.64
S4	344557		-9.0	-7.58	-8.3	-7.92	1.4513	NA
S5	349155		-8.3	-8.04	-8.9	-8.27	0.1462	0.184

Table 2

Purity of compounds.

Cmpd. ID	NCI No	Purity (%)
D1	94600	94.09
D2	71881	99.14
P1	266535	96.86
P2	132791	98.39
P3	267461	97.20
P4	154020	100.00
P5	629971	96.55
S1	126765	98.38
S2	137443	97.38
S3	217307	59.40
S4	344557	100.00
S5	349155	88.74