In vitro screening of potential inhibitors targeting a putative Cryptosporidium transportin.

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BACKGROUND

Cryptosporidiosis is a gastrointestinal diarrheal disease caused by Cryptosporidium parasites, which are most frequently transmitted by oral ingestion of oocysts found in contaminated food or water. In infants, cryptosporidiosis has emerged in the last few decades as a major cause of diarrheal disease and death [1]. Currently, nitazoxanide is the only FDA-approved drug for cryptosporidiosis but shows limited efficacy. Thus, there is a need for developing new therapeutics. A previous study from our group identified a novel hypothetical protein, TU502HP, in the *Cryptosporidium hominis* genome [2] with similarity to human transportin 3 **(Fig. 1A).** In addition, a three-dimensional model of TU502HP [2] formerly subjected to a virtual screening of the ZINC database (https://zinc.docking.org/) identified putative inhibitors based on docking score and binding energy **(Fig. 1B-C and Table 1).**

OBJECTIVE. To evaluate TU502HP as potential therapeutic target for cryptosporidiosis, the current study performed an *in vitro* examination of the inhibitory properties of three top compounds previously identified by *in silico* molecular docking analysis [2].

Fig. 1. Preliminary characterization of *C. hominis* TU502HP

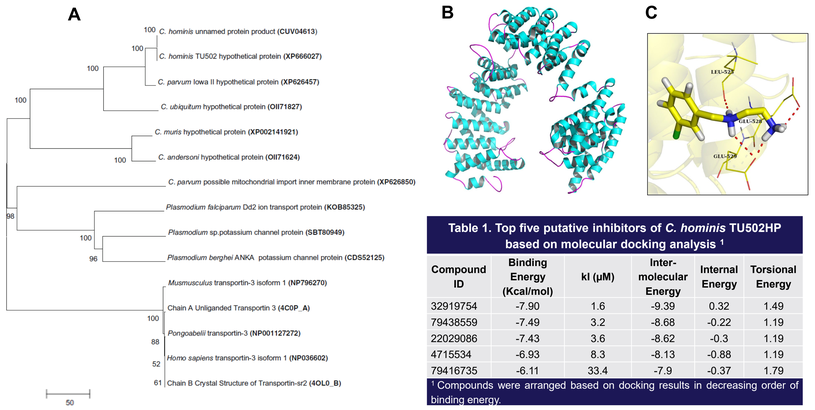

Table 1. Top five putative inhibitors of *C. hominis* TU502HP based on molecular docking analysis [1]

Compound ID	Binding Energy (Kcal/mol)	kI (μM)	Inter-molecular Energy	Internal Energy	Torsional Energy
32919754	-7.90	1.6	-9.39	0.32	1.49
79438559	-7.49	3.2	-8.68	-0.22	1.19
22029086	-7.43	3.6	-8.62	-0.3	1.19
4715534	-6.93	8.3	-8.13	-0.88	1.19
79416735	-6.11	33.4	-7.9	-0.37	1.79

[1] Compounds were arranged based on docking results in decreasing order of binding energy.

Fig. 1. Preliminary characterization of *C. hominis* TU502HP. **A,** phylogenetic analysis showing close relation to *C. parvum*, *C. ubiquitum*, *C. muris*, and *C. andersoni* hypothetical proteins. TU502HP is also closely related to human transportin 3 [2]. **B,** Three-dimensional model of *C. hominis* TU502HP showing secondary structures (α-helices, blue) and connecting loops (magenta). **C,** Molecular docking studies revealed the binding modes of compound 32919754 with TU502HP, giving insights of the key amino acid residues that are involved during the binding conformation [2]. Compound 32919754 has the highest binding energy **(Table 1),** and it forms four plausible hydrogen bonds with residues Leu 525, Ile 526, Glu 528, and Glu 529 of TU502HP [2].

EXPERIMENTAL APPROACH

- Oocysts of *C. parvum* IOWA (Cryptosporidium Production Laboratory, University of Arizona) and *C. hominis* (Dr. Saul Tzipori, Tufts University) were resuspended in excystation buffer at 1 x 10^7 per ml and incubated for 1 h at 37ºC. Excysted sporozoites were washed twice in PBS at 800 g.
- Hct-8 cells (ATCC® CCL-244™) growing in 8-well chamber slides were inoculated with 2 x 10^6 sporozoites per well. Cultures were incubated with increasing concentrations of inhibitor **(Table 2)** at 37ºC/5% CO^2 in RPMI 1640 medium supplemented with 1 mM sodium pyruvate and 10% FBS.
- Immunofluorescence assay (IFA) of infected Hct-8 was performed after 48 h as described [3]. IFA included a rabbit polyclonal antibody to intracellular stages of Cryptosporidium (Dr. Saul Tzipori, Tufts University) followed by Alexa 488-conjugated goat anti-rabbit secondary antibody.
- Cells were visualized under 160X magnification and microscopic images were captured using a digital camera and the Zen Imaging Software (Zeiss, Oberkochen, Germany). Automatic counting of parasite infective foci from captured images was performed with ImageJ https://ij.imjoy.io/ [4].
- Data from automated counts were collected from three experiments. In each experiment, automated counts were averaged from five microscopic images captured under 160X. Data were subsequently analyzed using GraphPad Prism 8 to calculate means and SEM. Where indicated, results were subjected to analysis of variance followed by the Dunnett's multiple-comparison test. A *P* value of <0.05 was used to determine statistical significance.

Fig. 2. Experimental Approach

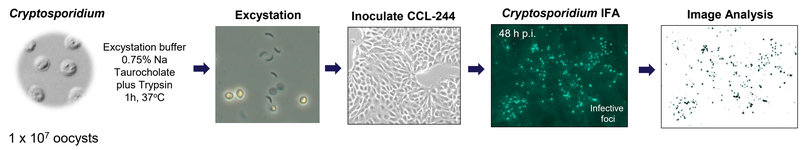

Fig. 2. Experimental approach used in the evaluation of *C. hominis* TU502HP as potential therapeutic target by *in vitro* examination of the inhibitory properties of compounds identified in an *in silico* virtual screening study [2].

Table 2. Compounds used in this study

Zinc ID/ Compound	Mwt	Score (Kcal/mol)	xLogP	H-bond donors	H-bond acceptors	Rot-Bond	Contact	Link and Compound Name
32919754	186.686	-57.47	0.96	5	2	4	2	https://zinc12.docking.org/substance/32919754 N-(3-Chlorobenzyl) ethane-1,2-diamine
79438559	221.348	-56.96	1.56	3	3	4	2	https://zinc12.docking.org/substance/79438559 Piperidin-2-ylmethyl-(1-pyridin-2-yl-ethyl)-amine hydrochloride
4715534	226.727	-56.37	3.49	3	2	3	2	https://zinc12.docking.org/substance/47155534 4-chloro-2-[(cyclopentylamino) methyl]phenol

Compounds were purchased from Biosynth and reconstituted in DMSO. Nitazoxanide was used as a positive control (not shown).

RESULTS

Fig. 3. Effects of putative TU502HP inhibitors on the growth of *C. parvum*

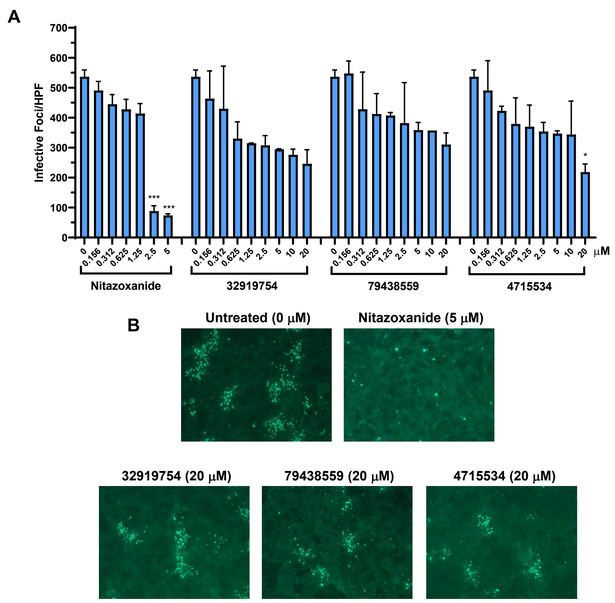

Fig. 3. Effects of putative TU502HP inhibitors on *C. parvum* growth. Hct-8 cells (ATCC® CCL-244™) growing in 8-well chamber slides were inoculated with *C. parvum* sporozoites and cultures were incubated with increasing concentrations of each inhibitor for 48 h. IFA was performed with a rabbit polyclonal antibody to intracellular stages of *Cryptosporidium* and microscopic images were captured under 160X magnification. Automatic counting of parasite infective foci was performed with ImageJ [4]. **A,** bar graphs represent means ± SEM of three experiments. *, *P*<0.05; ***, *P*<0.001 as compared to cultures incubated without compound (0 μm). **B,** fluorescence microscopy images corresponding to a representative experiment of three performed.

Fig. 4. Effects of putative TU502HP inhibitors on the growth of *C. hominis*

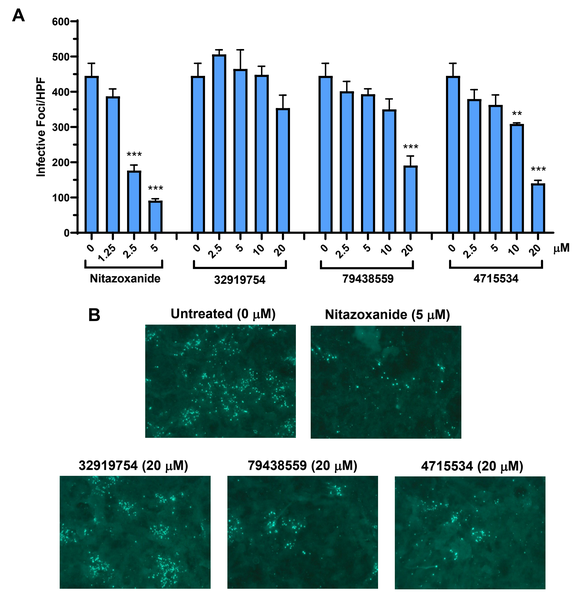

Fig. 4. Effects of putative TU502HP inhibitors on *C. hominis* growth. Hct-8 cells (ATCC® CCL-244™) growing in 8-well chamber slides were inoculated with *C. hominis* sporozoites and cultures were incubated with increasing concentrations of each inhibitor for 48 h. IFA was performed with a rabbit polyclonal antibody to intracellular stages of *Cryptosporidium* and microscopic images were captured under 160X magnification. Automatic counting of parasite infective foci was performed with ImageJ [4]. **A,** bar graphs represent means ± SEM of three experiments. **, *P*<0.01; ***, *P*<0.001 as compared to cultures incubated without compound (0 μm). **B,** fluorescence microscopy images corresponding to a representative experiment of three performed.

SUMMARY

- We report the *in vitro* inhibitory activities of three compounds targeting *C. hominis* TU502HP that had been previously identified by virtual screening [2].
- Only compound 4715534 used at 20 μM caused a >2-fold statistically significant decrease in *C. parvum* growth compared to untreated parasite cultures.
- In contrast, treatment of *C. hominis*-infected cultures with 20 μM of compounds 79438559 and 4715534 resulted in 2-fold and 3-fold reductions in parasite growth.
- None of the compounds tested performed at the same level as the nitazoxanide control which required ~10-fold lower concentrations to induce significant inhibition.
- Of note, compound 32919754 which was predicted to have the highest binding energy to TU502HP, underperformed in our assay compared to the two remaining putative inhibitors.
- The results warrant future studies to evaluate the absorption of these three compounds in infected cells, their potential synergistic effects with other anti-parasitics, and mechanisms of inhibition in *Cryptosporidium*.

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