Triazole-based compound as the candidate to develop a novel medicines to treat toxoplasmosis

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Running Head: \textit{s-Triazole as a novel medicines to treat toxoplasmosis}

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Abstract

This letter reports anti-Toxoplasma gondii activity of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione. The compound displayed significant and reproducible anti-parasitic effects at non-toxic concentrations for the host cells, with experimentally determined IC₅₀ value at least thirty time better than the one of known chemotherapeutic sulfadiazine. Purine nucleoside phosphorylase was defined as probable target for anti-Toxoplasma activity of tested compound. These results provide the foundation for the future work to develop a new class of medicines to better treat toxoplasmosis.

Keywords: s-triazoles, anti-Toxoplasma gondii activity, toxicity
Standard chemotherapy for the treatment of *Toxoplasma* infections relies on the inhibition of folate metabolism. The protocol recommends synergistic combination of diaminopyrimidines with sulfonamides, supplemented with folinic acid to mitigate the toxic effects of pyrimethamine on bone marrow. For patients with sensitivity to sulfonamines, macrolides and lincosamides are a second class of medications with anti-*Toxoplasma* activity. The third class of anti-*Toxoplasma* drugs, that are only occasionally used as a potential substitute, comprises the electron-transport inhibitors such as atovaquone [1]. In all of these situations, drug resistance, high cost, limited efficacy and side effects of these drugs often result in discontinuation of therapy [2, 3, 4, 5]. Therefore, new agents with better activity profiles and less expensive are needed. One possible class of drugs are *s*-triazole derivatives and in this letter we present newly found triazole-based candidate to develop novel medicines for more effective treatment of toxoplasmosis.

The search for agents that are potent and selective against *Toxoplasma* continues in several laboratories. Numerous inhibitors with activities in the nanomolar range with no appreciable *in vitro* toxicity to human cells were identified. Examples are pyrimidines, oryzalines, thiazolidinones, berberines, tryptanthrines, thiocyanates, bisphosphonates [6, 7]. Our attention has been focused on the role of *s*-triazoles series as potential new toxoplasmosis therapeutics. We found that 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (1) showed potent and reproducible anti-parasitic effect with no appreciable toxicity to human cells while 4-ethyl-3-(4-methyl-1,2,3-thiadiazol-5-yl)-1,2,4-triazole-5-thione (2) was inactive.

Procedure for synthesis of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (1) and 4-ethyl-3-(4-methyl-1,2,3-thiadiazol-5-yl)-1,2,4-triazole-5-thione (2), the effects of tested compounds and sulfadiazine on the viability of L929 (ATTC® No. CCL-1™) and HeLa (ATTC® No. CCL-2™) cells, inhibition of *Toxoplasma* (RH strain, ATTC® No. 50174™) growth were described elsewhere [8]. The effect of tested compounds on the intensity of *T. gondii* growth were described elsewhere [8].
proliferation [%] was measured using 2 methods: incorporation of \([^{3}H]\) uracil (Fig. 1, I) to the *T. gondii* DNA [9] and quantitative real-time PCR (qRT-PCR) (Fig. 1, II.) [8]. IC_{50} [\mu g/mL] - represents the concentration of tested compounds that was required for 50% of *T. gondii* proliferation inhibition on used cell lines. The cytotoxic effect of tested compound on the mouse L929 fibroblasts [%] (Fig. 2) was measured using the MTT assay according to the international standards: ISO 10993-5:2009(E). To calculate the reduction of cells viability compared to the untreated blank, the Equation was used: 

\[\% = \left[100 \times \frac{\text{OD}_{570 \text{ Sample}}}{\text{OD}_{570 \text{ Blank}}} \right]\]

where \(\text{OD}_{570 \text{ Sample}}\) and \(\text{OD}_{570 \text{ Blank}}\) are the mean value of the measured optical density of the tested samples/blank OD_{570} (the mean value of the measured optical density of the untreated cells). CC_{30} [\mu g/mL] - represents the concentration of tested compounds that was required for 30% cytotoxic effect of tested compounds on L929 cells. Selectivity refers to the ratio of the CC_{50} value for L929 fibroblasts to the IC_{50} value for *T. gondii* RH measured by incorporation of \([^{3}H]\) uracil (Fig. 2, I) or qRT-PCR (Fig. 2, II.) assay. CC_{30} and IC_{50} values were determined based on the plotted curves using GraphPad Prism program (version 6.04). The results of experiments were shown as a mean arithmetic values from 6-18 repeats (2-6 experiments). FlexX program was used as implemented in the LeadIT software package. The receptor for FlexX was prepared using default setting and details are described in [8].

To assess inhibition of RH strain growth *in vitro*, tachyzoites were cultured with varying concentrations of the s-triazole (1) ranging from 1 to 500 \(\mu\)g/mL. Parasite growth inhibition in L929 cells by compound (1) and control drug sulfadiazine together with IC_{50} values are shown in Fig. 1. As it can be seen, compound (1) showed statistically significant anti-parasitic effect with IC_{50} value (42.46 or 37.33 \(\mu\)g/mL) which was at least 32.6-fold lower than the one observed for sulfadiazine (2225.24 or 1217.62 \(\mu\)g/mL), depending on which method was used for measuring the intensity of parasite proliferation (\([^{3}H]\) uracil incorporation or qRT-PCR, respectively). In case of HeLa cells IC_{50} value (58.5 \(\mu\)g/mL) was
also lower (30.6-fold) as compared with IC$_{50}$ value (1790.0 µg/mL) for sulfadiazine using
[^H] uracil incorporation assay. Compound (1) showed evident anti-Toxoplasma activity and
exhibited more than four times greater selectivity, as compared to the sulfadiazine (Fig. 2). It
should be mentioned, that drug susceptibility for sulfadiazine non-resistant RH strain depends
on the host cells used. Determined IC$_{50}$ values varied from 2.5 to 70 µg/mL for normal cell
lines (MRC-5, HFF and Vero) and from 600 to 1724 µg/mL for carcinoma Hep-2 or HeLa
cell lines [10, 11, 12].

Since the ideal antiparasitic agent would have no or low toxicity effects on host cells,
the effect of (1) for its ability to inhibit the growth of mouse fibroblasts (Fig. 2) and human
cervical cancer cell lines was also explored (data not shown). Toxicity was defined as the
highest dilution of test samples that causes 30% or greater destruction of cells. From a
comparison of the results on toxicity and anti-T. gondii activity tests, it can be clearly seen
that (1) inhibited the parasite growth at non-cytotoxic concentrations for host cells.

The last part of our study was dedicated to understanding the molecular basis of
inhibitory potency of (1) against Toxoplasma. The literature search identified six enzymes, i.e.
3MB8 - purine nucleoside phosphorylase [13], 1LII - adenosine kinase [14], DHRF -
dihydrofolate reductase [15], 4M84 - calmodulin-domain protein kinase-1 [16], 3AU9 -1-
(deoxy-D-xylulose-5-phosphate reductoisomerase [17], 2O2S - enoyl-acyl carrier reductase
[18] as reasonable targets for discovering anti-Toxoplasma agents. Based on the structures
deposited in the Protein Data Bank, we analyzed binding affinity of active (1) and inactive (2)
s-triazoles to the active sites of aforementioned enzymes. We have excluded the model of the
dihydrofolate reductase binding site (PDB ID 4EIL) because this enzyme is also present in
humans so treatment with DHRF inhibitors may induce a folate deficiency, which is possibly
responsible for severe hematological pathologies and embryopathies. The largest difference in
docking score has been observed for the enzyme model 3AU9, followed by 4M84, and 3MB8
Given the difference in bioactivity of (1) and (2) these three enzymes seem to be potential targets. In all cases the apparent main source of the differentiation is the size exclusion; active sites are tight and deeply buried in the protein making access for the bulkier compound much harder or impossible. In fact, the active site is so tight in the case of 3AU9 that it was not possible to dock inactive s-triazole (2) using hard docking protocol, and only after loosening Maximum Allowed Overlap Volume to 100 Å³ (i.e., soft docking) it became possible (Fig. 3.). Nearly no preference in binding to the active site was observed in case of 1LII while in case of 2O2S docking preference is reversed with strong binding of the inactive s-triazole (2) what was in clear disagreement with experimental observations. Out of the chosen three proteins the strongest binding is expected for 3AU9 and this enzyme seems to be the most probable target. The binding mode of the active ligand (1) in its active site is illustrated in Fig. 3.

5. References


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Fig. 1 Intensity of *T. gondii* proliferation [%] on the L929 host cells in the presence of 3-
(thiophen-2-yl)-1,2,4-triazole-5-thione (○, □) and sulfadiazine (●, ■) with determined IC$_{50}$
values using 2 methods: incorporation of $^3$H uracil (○, ●) and qRT-PCR (□, ■).

Fig. 2 Cytotoxic effect of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (○) and sulfadiazine (□) on
L929 mouse fibroblasts compared to selectivity (CC$_{30}$/IC$_{50}$) for both s-triazole and
sulfadiazine using 2 methods: incorporation of $^3$H uracil (I) and qRT-PCR (II).

Fig. 3. Scores of top poses of (1) and (2) docked to different proteins: 3AU9 - 1-deoxy-D-
xylulose 5-phosphate reductoisomerase, 4M84 – calmodulin-domain protein kinase 1, 3MB8 -
purine nucleoside phosphorylase, 1LII – adenosine kinase, 2O2S - enoyl-acyl carrier
reductase; * results in parenthesis are for soft docking conditions (see text). Schematic
representation of interactions of (1) in the active site of 1-deoxy-D-xylulose 5-phosphate
reductoisomerase and binding cavity with active superimposed on the native ligand.
<table>
<thead>
<tr>
<th>concentration [µg/mL]</th>
<th>cytotoxic effect [%] viable cells</th>
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</tr>
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<tr>
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**SELECTIVITY:**

- **s-triazole**
  - I. 0.24
  - II. 4.58
- **sulfadiazine**
  - I. 5.21
  - II. 0.44

**CC50 [µg/mL]**

- s-triazole: 194.46
- sulfadiazine: 537.06

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Purine nucleoside phosphorylase as probable target for anti-Toxoplasma activity of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione

<table>
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<tr>
<th>Compounds</th>
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<tbody>
<tr>
<td>2O2S</td>
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(-14.3) no docking

(-20.3)*

1) (2)