

# Triazole-Based Compound as a Candidate To Develop Novel Medicines To Treat Toxoplasmosis

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This article reports anti-*Toxoplasma gondii* activity of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione. The compound displayed significant and reproducible antiparasitic effects at nontoxic concentrations for the host cells, with an experimentally determined 50% inhibitory concentration (IC<sub>50</sub>) at least 30 times better than that of the known chemotherapeutic agent sulfadiazine. Purine nucleoside phosphorylase was defined as the probable target for anti-*Toxoplasma* activity of the tested compound. These results provide the foundation for future work to develop a new class of medicines to better treat toxoplasmosis.

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 sulfonamides, macrolides and lincosamides are a second class of medications with anti-*Toxoplasma* activity. The third class of anti-*Toxoplasma* drugs, which are only occasionally used as a potential substitute, comprises 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 (2–5). Therefore, new agents with better activity profiles and that are less expensive are needed. One possible class of drugs are *s*-triazole derivatives, and in this article, we present a 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 have been identified. Examples are pyrimidines, oryzalines, thiazolidinones, berberines, tryptanthrines, thiocyanates, and bisphosphonates (6, 7). Our attention has been focused on the role of *s*-triazole series as potential new toxoplasmosis therapeutics. We found that 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (compound 1) showed a potent and reproducible antiparasitic 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 (compound 2) was inactive.

The procedure for synthesis of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (compound 1) and 4-ethyl-3-(4-methyl-1,2,3-thiadiazol-5-yl)-1,2,4-triazole-5-thione (compound 2), the effects of tested compounds and sulfadiazine on the viability of L929 (ATCC no. CCL-1) and HeLa (ATCC no. CCL-2) cells, and inhibition of *Toxoplasma* (RH strain; ATCC no. 50174) growth were described elsewhere (8). The effect of tested compounds on the intensity of *Toxoplasma gondii* proliferation (%) was measured by 2 methods: incorporation of [<sup>3</sup>H]uracil (Fig. 1) into the *T. gondii* DNA (9) and quantitative real-time PCR (qRT-PCR) (Fig. 1) (8). The 50% inhibitory concentration (IC<sub>50</sub> [μg/ml]) represents the concentration of tested compounds that was required for inhibition of 50% of *T. gondii* proliferation on the cell lines used. The

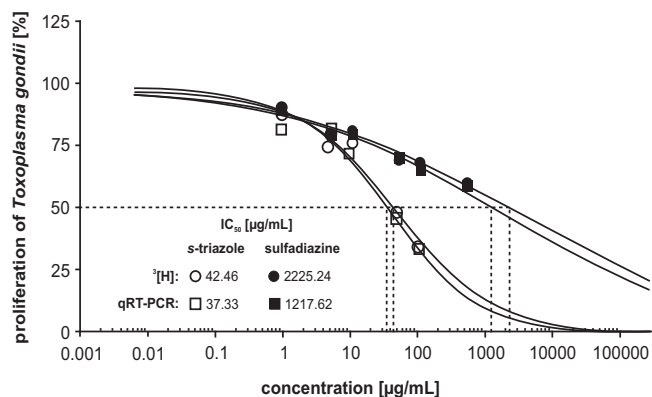


FIG 1 Intensity of *T. gondii* proliferation (percentage) on the L929 host cells in the presence of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (○ and □) and sulfadiazine (● and ■) with determined IC<sub>50</sub>s by 2 methods: incorporation of [<sup>3</sup>H]uracil (○ and ●) and qRT-PCR (□ and ■).

cytotoxic effect of the tested compound on the mouse L929 fibroblasts (percentage of viable cells) (Fig. 2) was measured using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay according to the international standard ISO 10993-5:2009(E). To calculate the reduction of cell viability compared to viability in the untreated blank, the following equation was used: % viability = 100 × (sample OD<sub>570</sub>/blank OD<sub>570</sub>), where sample OD<sub>570</sub> is the mean value of the measured optical density at 570 nm of the tested samples and “blank OD<sub>570</sub>” is the mean value of the measured OD<sub>570</sub> of the untreated cells. The 30% cytotoxic concentration (CC<sub>30</sub> [μg/ml]) represents the concentration of tested compounds that was required for a 30% cytotoxic effect of the tested compounds on L929 cells. Selectivity refers to the ratio of the CC<sub>50</sub> value for L929 fibroblasts to the IC<sub>50</sub> for *T.*

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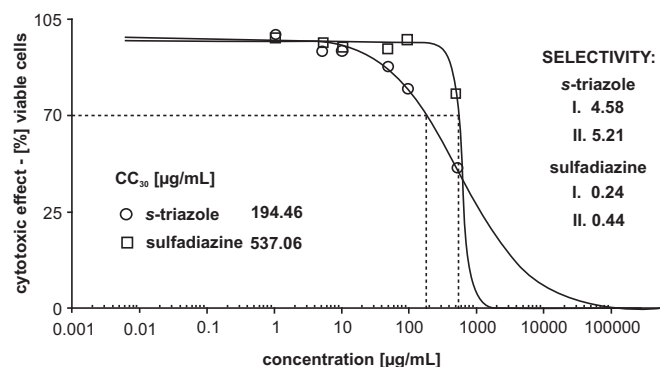
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**FIG 2** Cytotoxic effect of 3-(thiophen-2-yl)-1,2,4-triazole-5-thione (○) and sulfadiazine (□) on L929 mouse fibroblasts compared to selectivity ( $CC_{30}/IC_{50}$ ) for both *s*-triazole and sulfadiazine by 2 methods: incorporation of  $^3\text{H}$ uracil (I) and qRT-PCR (II).

*gondii* RH measured by incorporation of  $^3\text{H}$ uracil (Fig. 2, I) or the 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 the experiments are shown as mean arithmetic values from 6 to 18 repeats (2 to 6 experiments). The FlexX program was used as implemented in the LeadIT software package. The receptor for FlexX was prepared using the default setting, and the details are described in reference 8.

To assess inhibition of RH strain growth *in vitro*, tachyzoites were cultured with various concentrations of the *s*-triazole (compound 1) ranging from 1 to 500  $\mu\text{g}/\text{ml}$ . Parasite growth inhibition in L929 cells by compound 1 and the control drug, sulfadiazine, together with the  $IC_{50}$ s is shown in Fig. 1. As can be seen, compound 1 showed a statistically significant antiparasitic effect by the  $IC_{50}$  (42.46 or 37.33  $\mu\text{g}/\text{ml}$ ), which was at least 32.6-fold lower than the one observed for sulfadiazine (2,225.24 or 1,217.62  $\mu\text{g}/\text{ml}$ ), depending on which method was used for measuring the intensity of parasite proliferation ( $^3\text{H}$ uracil incorporation or qRT-PCR, respectively). In the case of the HeLa cells, the  $IC_{50}$  (58.5  $\mu\text{g}/\text{ml}$ ) was also lower (30.6-fold) than that with  $IC_{50}$  (1,790.0  $\mu\text{g}/\text{ml}$ ) for sulfadiazine using the  $^3\text{H}$ uracil incorporation assay. Compound 1 showed evident anti-*Toxoplasma* activity and exhibited more than 4 $\times$  greater selectivity than sulfadiazine (Fig. 2). It should be mentioned that the drug susceptibility of the

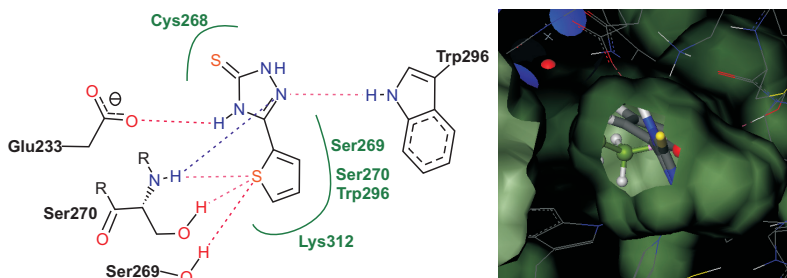
sulfadiazine-nonresistant RH strain depends on the host cells used. The determined  $IC_{50}$ s varied from 2.5 to 70  $\mu\text{g}/\text{ml}$  for normal cell lines (MRC-5, HFF, and Vero) and from 600 to 1,724  $\mu\text{g}/\text{ml}$  for the carcinoma Hep-2 and HeLa cell lines (10–12).

Since the ideal antiparasitic agent would have no toxic or low-toxicity effects on host cells, the effect of compound 1 in terms of 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 compound 1 inhibited the parasite growth at noncytotoxic concentrations for host cells.

The last part of our study was dedicated to understanding the molecular basis of inhibitory potency of compound 1 against *Toxoplasma*. The literature search identified six enzymes as reasonable targets for discovering anti-*Toxoplasma* agents: 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), and 2O2S (enoyl-acyl carrier reductase) (18). Based on the structures deposited in the Protein Data Bank, we analyzed the binding affinities of the active compound 1 and inactive compound 2 *s*-triazoles to the active sites of the aforementioned enzymes. We have excluded the model of the dihydrofolate reductase binding site (PDB ID no. 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 (Fig. 3). Given the difference in bioactivities of compounds 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 (compound 2) using the “hard” docking protocol, and only after loosening the “maximum allowed overlap volume” to 100  $\text{Å}^3$  (i.e., “soft” docking) did it become possible (Fig. 3). Nearly no preference in binding to the active site was observed in case of 1LII, while in case of 2O2S, the docking pref-

enzyme	Compounds	
	(1)	(2)
3AU9*	-21.3 (-20.3)*	no docking (-14.3)
4M84	-14.5	-10.4
3MB8	-18.4	-16.2
1LII	-12.7	-12.6
2O2S	-13.3	-20.2

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



**FIG 3** Scores of top poses of compounds 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 parentheses are for the soft docking conditions (see the text). Shown is a schematic representation of interactions of compound 1 in the active site of 1-deoxy-D-xylulose 5-phosphate reductoisomerase and the binding cavity with the active site superimposed on the native ligand.

erence is reversed, with strong binding of the inactive s-triazole (compound 2), which 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 (compound 1) in its active site is illustrated in Fig. 3.

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