**Original Article**

**Anti-Toxoplasma Activity of 2-(Naphthalene-2-ylthiol)-1H-Indole**

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**Abstract**

**Background:** This study was undertaken to evaluate the viability, infectivity and immunity of *Toxoplasma gondii* tachyzoites exposed to 2-(naphthalene-2-ylthio)-1H-indole.

**Methods:** Tachyzoites of RH strain were incubated in various concentrations of 2-(naphthalene-2-ylthio)-1H-indole (25–800μM) for 1.5 hours. Then, they were stained by PI and analyzed by Fluorescence-activated cell sorting (FACS). To evaluate the infectivity, the tachyzoites exposed to the different concentrations of the compound were inoculated to 10 BALB/c mice groups. For Control, parasites exposed to DMSO (0.2% v/v) were also intraperitoneally inoculated into two groups of mice. The immunity of the exposed tachyzoites was evaluated by inoculation of the naïve parasite to the survived mice.

**Results:** The LD₅₀ of 2-(naphthalene-2-ylthio)-1H-indole was 57 μmol. The longevity of mice was dose dependent. Five mice out of group 400μmol and 3 out of group 800μmol showed immunization to the parasite.

**Conclusion:** Our findings demonstrated the toxoplasmodial activity of the compound. The presence of a well-organized transporter mechanism for indole compounds within the parasite in conjunction with several effective mechanisms of these compounds on *Toxoplasma* viability would open a window for production of new drugs and vaccines.
Introduction

Toxoplasma gondii is an intracellular protozoan that is distributed throughout the world. This parasite can infect nucleated cells of birds and mammals, including humans (1). In man, symptoms of toxoplasmosis are usually mild and including fever, malaise and lymphadenopathy (2, 3) but during pregnancy, may lead to abortion and severe CNS abnormalities in fetus such as choroiditis, hydrocephaly and microcephaly (4). The disease in HIV-positive patients is severe and it is estimated that 23% of them develop toxoplastic encephalitis (5).

In immune compromised individuals such as those suffering from cancer or autoimmune disease and those undergoing transplantation are at an increased risk of toxoplasmosis. Eye involvement may also happen and lead to loss of vision in both the acquired and congenital forms of the disease (2, 6).

A combination of pyrimethamine and sulfadiazine is the standard therapeutic regimen for the treatment of toxoplasmosis (2). These medications inhibit essential enzymes in biosynthesis pathway of pyrimidine in T. gondii (7) while treatment with these drugs for a long period may result into megaloblastic anemia or myelosuppression. Folate deficiency and myelotoxicity, neutropenia and teratogenic effects were reported after use of these drugs (8, 9). Toxoplasmosis in pregnant women is usually treated by administration of spiramycin to decrease the risk of fetal transmission even the drug has side effects such as skin rashes, itching, abnormal bruising, and uncommon gastrointestinal bleeding (10, 11), but spiramycin regimen not able to completely eradicate the parasite (12).

Tryptophan was an essential amino acid for intracellular proliferation of the parasite (13). Tryptophan is found in most proteins and has an indole functional group. The parasitostatic effect of IFN-γ was shown to result from starvation of T. gondii for tryptophan (14). The indole compounds, as inhibitors of nucleoside triphosphate hydrolase (NTPase), prevent the tachyzoite replication in vitro (15).

Therefore, the present study was performed to evaluate the direct effect of 2-(naphthalene-2-ylthio)-1H-indole on viability and infectivity of Toxoplasma tachyzoites and the acquired immunity from the tachyzoites exposed to this compound.

Materials and Methods

Synthesis of 2-(naphthalene-2-ylthio)-1H-indole

2-(naphthalene-2-ylthio)-1H-indole was prepared as described in Fig. 1.

![Fig. 1: Synthesis of 2-(naphthalene-2-ylthio)-1H-indole. The conditions are (a) NaI, H2O2, ethyl acetate 25-30° C; (b) 1: sulfuryl chloride, 1, 2-dichloroethane, 25-30° C, 2: indole, DMF, 25-30° C; (c) thiosalicyclic acid, TFA](http://ijpa.tums.ac.ir)
Na₂S₂O₃ solution (15 ml) and the mixture was extracted with ethyl acetate (3 × 50 ml). The combined organic layers were washed twice with 20 ml water and brine then dried over anhydrous sodium sulfate (Na₂SO₄) and concentrated. The solvent was evaporated, and the residue was purified by silica gel column chromatography in a solvent system containing petroleum ether and ethyl acetate and 1,2-di(naphthalene-2-yl) disulfane was isolated in a white solid form (90%, mp: 143-144°C).

**Preparation of 2, 3-bis (naphthalene-2-yithio)-1H-indole**

2, 3-bis (naphthalene-2-yithio)-1H-indole was prepared using slightly modified known procedure (17). In brief, 0.30 g of sulfuryl chloride (0.18 ml, 2.2 mmol) was added to a solution of 0.77 g of 1, 2-di (naphthalene-2-yl) disulfane (2.42 mmol) in 18 ml of 1, 2-dichloroethane at room temperature. The resulting red solution was stirred for 30 min, giving an anhydrous sodium sulfate and finally the solution (15 ml) and the mixture was refluxed for 1 h. On completion of the reaction, monitored by TLC, the TFA was evaporated off and the residue was diluted with ethyl acetate and washed twice with 1 N NaOH and then three times with water. The organic layer then dried over anhydrous sodium sulfate and finally the solvent was evaporated off. The residue was purified by preparative TLC on silica gel using petroleum ether and ethyl acetate as solvent systems resulted into preparation of 86 mg of 2-phenylthio)-1H-indole (63%) formed as a white solid product with a mp of 103-105°C.

**Animals**

Seventy of 6-8 week-inbred BALB/c mice (weight 22-25 gr) provided from Pasteur Institute, Tehran, Iran were enrolled. The animals were kept at 22°C and 40-50% relative humidity and had access to standard food and water ad libitum at the Laboratory Animal Center of Shiraz University of Medical Sciences, Shiraz, Iran. During the experiments from May to June 2012, animals were housed in cages and maintained under controlled conditions. The experiments were undertaken based on guidelines for laboratory animals and Ethical Committee of Shiraz University of Medical Sciences (18).

**Parasites**

The virulent RH strain of *T. gondii* was obtained from Tehran University of Medical Sciences, Tehran, Iran. Tachyzoites of the RH strain of *T. gondii* were maintained by serial intraperitoneal passages in BALB/c inbred mice. Tachyzoites were collected 72 hours after inoculation of 10⁶ parasites in the mice, by repeated flushings in the peritoneal cavity using phosphate buffered saline (PBS) at a pH of

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7.2. Then, the tachyzoites were harvested and centrifuged for 5 min at 200 g at room temperature to remove peritoneal cells and cellular debris. The supernatant was collected and centrifuged for 10 min at 800 g. The pellet, enriched with parasite tachyzoites, was recovered with PBS and used in all experiments (19).

Extracellular viability assay

2-(naphthalene-2-ylthio)-1H-indole (Fig. 1) was dissolved in DMSO to obtain a final concentration of 10 mM. The final concentration of DMSO did not exceed 0.2 % v/v. Various concentrations (25-800 μM) of 2-(naphthalene-2-ylthio)-1H-indole were then prepared as follows:

Fig 2: Flowcytometry analyses of unexposed Toxoplasma tachyzoites and Toxoplasma tachyzoites exposed to saponine and different doses of 2-(naphthalene-2-ylthio)-1H-indole

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2.5-80μl of the final concentration was added to 920-997.5μl of suspension that contained 2x10⁶ tachyzoites per ml of PBS. The tachyzoites were incubated with either DMSO (0.2% v/v) as control or the diluted compounds for 1.5 h at 4°C. The tachyzoites were collected in Eppendorf tubes and incubated for 30 min at 4°C with 50 μg/ml propidium iodide (PI) (Sigma Company, USA). After incubation, the parasites were kept on ice until analysis. Positive controls for PI staining were acquired by incubating parasites in the presence of 0.2% saponin (19).

The cell suspension was transferred into polystyrene flow cytometry tubes (BD Falcon, USA). Data analysis was performed using FACS Calibur flow cytometer (Becton-Dickinson, San Jose, USA) and Cell Quest Pro software. A total of 10000 or 30000 events were acquired in the region that had been previously established as corresponding to the parasites.

**Tachyzoite infectivity in animals**

A total of 2x10⁶ tachyzoites exposed to the concentrations of the compound mentioned above were intraperitoneally inoculated in ten mice of each group. For the control, parasites exposed to DMSO (0.2% v/v) were also intraperitoneally inoculated in another group of mice.

If the mice died, liver touch smears were prepared and stained by Giemsa and observed under light microscopy for detection of the parasite.

**Immunity in animals**

After one month, if the mice survived, the animals were inoculated with 10⁶ of naïve tachyzoites of the parasites. Crush and impression smears were prepared from the liver, spleen and brain tissues of the live mice one month after the inoculation to detect either tachyzoites or tissue cysts microscopically.

**Data analysis**

Data were analyzed by SPSS software (version 11.5, Chicago, IL, USA) using Mann-Whitney non-parametric test. A P<0.05 was considered statistically significant.

**Results**

According to the flowcytometry findings, approximately 85% of the *Toxoplasma* tachyzoites obtained from peritoneal passages could survive. Apoptosis or mortality was seen in 91% of tachyzoites that were exposed to 0.2% saponin and stained by PI (Fig.2). Flow cytometry analyses of different concentration (25-800 μM) of 2-(naphthalene-2-ylthio)-1H-indole on *Toxoplasma* tachyzoites viability was demonstrated in Fig. 3. The IC₅₀ of the compound was 57 μM.

The result of infectivity test on tachyzoites exposed to 25 and 50 μM of 2-(naphthalene-2-ylthio)-1H-indole and DMSO (0.2 %v/v) revealed that all mice died. The longevity of mice was directly correlated with the concentration of the compound. Moreover, the number of live mice increased when the concentration of the compound raised (Table 1).

The findings of immunity evaluation in survived mice inoculated with tachyzoites exposed to 100-800μM of the compound and were re-inoculated after 1 month with the intact parasites showed that 8 mice [5 out of the group 400 μmol and 3 out of the group 800μM of 2-(naphthalene-2-ylthio)-1H-indole] had immunization against the parasite (Table 1).
Fig. 3: The mortality of *Toxoplasma* tachyzoites after 1.5 hours exposure to different doses of 2-(naphthalene-2-ylthio)-1H-indole.

Table 1: The mean of life duration (days) of mice groups inoculated by *Toxoplasma* tachyzoites exposed to 2-(naphthalene-2-ylthio)-1H-indole.

|                  | DMSO 0.2% (10) | 25µM (10) | 50µM (10) | 100µM (10) | 200µM (10) | 400µM (10) | 800µM (10) |
|------------------|----------------|-----------|-----------|------------|------------|------------|------------|
| Total number of mice | 10             | 10        | 10        | 10         | 10         | 10         | 10         |
| Longevity mean of mice (days) | 5.4            | 7         | 7.2       | 8 deaths with a mean of 7 days | 5 deaths with a mean of 10 days | 3 deaths with a mean of 10.5 days | 2 deaths with a mean of 11 days |
| Number of survived mice | ------       | ------    | ------    | 2          | 5          | 7          | 8          |
| Number of immunized mice | ------      | ------    | ------    | ------     | 5          | 3          |            |

Discussion

Treatment of toxoplasmosis is difficult due to toxic effects of available drugs and the fact that reinfection may rapidly occur. Tissue cysts of *Toxoplasma* are usually resistant to commonly used drugs including pyrimethamine, sulfadiazine, and atovaquone, either alone or in combination. The cyst wall can protect the parasite from host immune system and act as a barrier for antiparasitic compounds (20). Therefore, introducing new anti-toxoplasma drugs and vaccines seems essential.

Tryptophan was shown as an essential amino acid for survival and proliferation of the...
parasite (13). Suzuki (2002) showed that depletion of intracellular tryptophan might happen by indoleamine 2, 3-dioxygenase (IDO) pathway as IFN-γ controls the intracellular replication of *T. gondii* tachyzoites in various types of human cells (21). The local tryptophan-depleted microenvironments are created by macrophages that have a unique tryptophan high-affinity importing system. Using the highly specific and effective transportation, macrophages are able to import and then degrade tryptophan even at very low concentrations of its exogenous amino acid (22).

2-(naphthalene-2-ylthio)-1H-indole with an indole group is considered as a competitive molecule for tryptophan. In this study, tachyzoites exposed to the molecules were intracellular and ended in parasitophorous vacuoles (PV) of the host cell. The PV membrane is considered as a permeable structure with a size-prohibiting limit of ~1,300 Da (23). *Toxoplasma* is auxotrophic for tryptophan and purine molecules (13, 14, 24), so these pores may be used in receipt of these molecules by the host cytosolic ATP.

Recently, an NTPase as essential enzyme for tachyzoite replication has been documented in the PV of the host cell that may be partly responsible for the salvage process (25, 26).

NTPases as new targets were shown to have the possibility for chemotherapeutic approaches against the disease. It seems that the enzyme is unique to the parasite and its activity appears to be imperative for the parasite's proliferation. Studies on modifications of the indole and phenol rings revealed that the compounds had modest IC₅₀'s in low µM ranges to the inhibit *T. gondii* NTPases and proliferate the tachyzoites (15). Our study showed that 2-(naphthalene-2-ylthio)-1H-indole was effective on viability of tachyzoites. These experiments were undertaken on exposed tachyzoites but not intracellular ones. It seems that the compound affects tachyzoites due to other mechanisms, which described former.

Camalexin (3-thiazol-2'-yl-indole) was first isolated from the leaves of *Camelina sativa* in response to an infection by *Alternaria brassicaceae* (27). Moreover, camalexin is synthesized and accumulated in high levels *Arabidopsis thaliana* after infection with an avirulent strain of *Pseudomonas syringae* (28). The indole ring of camalexin may be derived from indole-3-glycerol phosphate, an intermediate in tryptophan biosynthesis (29, 30).

Another indole compound derived from tryptophan, brassinin, is provided from plants. The results of the study confirmed antifungal effects of camalexin and brassinin at different developmental stages of both *Alternaria* species (31).

Indolnenaphthyridiones is introduced as inhibitors of bacterial enoyl-ACP reductases. Enoyl-ACP reductase (FabI) is considered as a key enzyme of type II fatty acid biosynthesis (FAS-II) pathway and a validated antimicrobial target (32). The fatty acid synthesis in apicoplast of *T. gondii* is essential for organelle biogenesis and parasite survival. Apicoplast pro-karyotic fatty acid synthesis is type II and has recently received particular attention. The FAS II pathway, a metabolic process fundamentally different from the analogous FAS I pathway in humans, was proposed as drug target (33, 34).

In our study, the viability of tachyzoites exposed to different concentrations of 2-(naphthalene-2-ylthio)-1H-indole was correlated to in vivo experiments. In mice, inoculation of exposed tachyzoites to high concentration of the compound resulted into more longevity and less mortality. Moreover, 8 out of 22 survived mice acquired immunity against the parasite.

A vaccine against *T. gondii* would be extremely valuable to protect against primary fetal infection and reactivation in immunocompromised individuals and it might reduce economical losses by preventing abortions in farm animals. Only a commercial vaccine (S-48) which is an attenuated live *T. gondii* tachyzoite vaccine has been successfully employed for
animal use. It cannot presently be carried out safely in human beings (35).

Eissa et al. demonstrated that a delayed death might be noticed in vaccinated mice using autoclaved tachyzoites of RH stain. Besides, a significant increase in splenic CD8+ T-lymphocytes and a significant decrease in parasite density and the pathological changes in the liver may be seen while the induced immunity may not efficient (36).

Wilkins et al. showed that in immunized ewes using a killed vaccine of disintegrated Toxoplasma tachyzoites with Freund's incomplete adjuvant, high levels of antibody were visible in vaccinated ewes whereas, no difference was noticed in fertility and lambing performance of the unvaccinated ewes (37).

Recently, gene-deficient attenuated strains were used instead of killed parasite. These stains due to the deletion of genes encoding proteins involved in host cell invasion process lead to a decrease in virulence and acted similar to avirulent strains (38). However, the replication rate in host cells did not decrease in comparison to the naïve stain (39).

In our study, flowcytometry revealed that all parasites were not killed ones. Although cell viability was evaluated by propidium iodide, it could not differentiate between apoptosis and necrosis of cells (40). According to our study, high concentrations of the compound may act as an apoptotic factor and the mice exposed to the tachyzoites may provide immunity.

Conclusion

The presence of a well-organized transporter mechanism for indole compounds within the parasite in conjunction with several effective mechanisms of these compounds on Toxoplasma viability would allow creation of an antagonist that may contain indole groups and enable researchers to open a window for production of new drugs and vaccines.

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Asgari et al.: Anti-Toxoplasma Activity of 2-(Naphthalene-2-Ylthiol)-…

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