Tehran University of Medical Sciences Publication
http:// tums.ac.ir

Iranian J Parasitol

Open access Journal at
http:// ijpa.tums.ac.ir

Iranian Society of Parasitology
http:// isp.tums.ac.ir

Original Article

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

***Qasem ASGARI [1], Hossein KESHAVARZ [2], Mostafa REZAEIAN [2], Hossein SADEGHPOUR [3,4], Ramin MIRI [3,4], Mohammad Hossein MOTAZEDIAN [1]**

1. *Dept. of Parasitology and Mycology, Shiraz University of Medical Sciences, Shiraz, Iran*
2. *Dept. of Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran*
3. *Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
4. *Dept. of Medicinal Chemistry of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*

Received 12 Dec 2014
Accepted 23 Apr 2015

Keywords:
Toxoplasma gondii,
2-(naphthalene-2-ylthio)-1H-indole,
Viability,
Infectivity,
Immunity

***Correspondence**
Email:
asgarig@sums.ac.ir

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 LD_{50} 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 toxoplasmocidal 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 protozoon 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 chorioretinitis, hydrocephaly and microcephaly (4). The disease in HIV-positive patients is severe and it is estimated that 23% of them develop toxoplasmic 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.

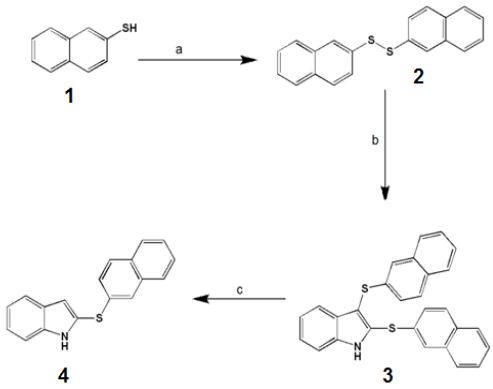

Fig. 1: Synthesis of 2-(naphthalene-2-ylthio)-1H-indole. The conditions are (a) NaI, H_2O_2, ethyl acetate 25-30° C; (b) 1: sulfuryl chloride, 1, 2-dichloroethane, 25-30° C, 2: indole, DMF, 25-30° C; (c) thiosalicyclic acid, TFA

Preparation of 1, 2-di (naphthalene-2-yl) disulfane

The compound was synthesized using previously reported procedure (16). To a stirred solution of naphthalene-2-thiol (1 mmol) in ethyl acetate (10 ml) was added NaI (1.5 mg, 0.01 mmol) and 30% H_2O_2 (0.11 ml, 1 mmol) and the mixture was stirred at room temperature for 0.5 h. The white precipitate was formed. After TLC showed complete conversion, the reaction was quenched by addition saturated

$Na_2S_2O_3$ 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 (Na_2SO_4) 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-ylthio)-1H-indole

2, 3-bis (naphthalene-2-ylthio)-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 assumed 0.22 M solution of sulfenyl chloride. This solution was added to a solution of 0.21 g of indole (1.8 mmol) in 10 ml of N, N-dimethylformamide (DMF) and stirred at room temperature for 2 h. The progress of the reaction was monitored by TLC. After completion of the reaction the mixture was concentrated under vacuum to remove 1, 2-dichloroethane, and the residue was partitioned between ethyl acetate and water. The crude product from the organic phase was purified on silica gel using petroleum ether and ethyl acetate as solvent system. The resultant was a white solid product with 83% yield (0.87 g).

M/Z (%): 433 (M^+, 50), 318 (5'), 274 (100'), 241 (8'), 115 (18'), 77 (6').

Preparation of 2-(naphthalen-2-ylthio)-1H-indole

2-(phenylthio)-1H-indole was prepared using selective desulfenylation of 2, 3-bis (naphthalene-2-ylthio)-1H-indole with some modifications. Briefly, to a mixture of 216 mg of 2, 3-bis (naphthalene-2-ylthio)-1H-indole (0.5 mmol) and 154 mg of thiosalicyclic acid (1 mmol) there was added 5 ml of trifluoroacetic acid (TFA). 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.

^{1}H-NMR (500MHz, $CDCl_3$): δ (ppm) 8.1 (br s, 1H), 7.78-7.8 (1H), 7.74 (s, 1H), 7.7 (s, 1H), 7.67-7.69 (m, 2H), 7.43-7.49 (2H), 7.32 (m, 1H), 7.32 (m, 1H), 7.29-7.32 (m, 1H), 7.25-7.28 (m, 1H), 7.18 (m, 1H), 6.9 (m, 1H). 275 (M^+, 100), 243 (20'), 215 (8'), 77 (8'). IR (KBr).

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 10^6 parasites in the mice, by repeated flushings in the peritoneal cavity using phosphate buffered saline (PBS) at a pH of

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 800g. The pellet, enriched with parasite tachyzoites, was recovered with PBS and used in all experiments (19).

Extracellular viability assay

2-(naphthalen-e2-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:

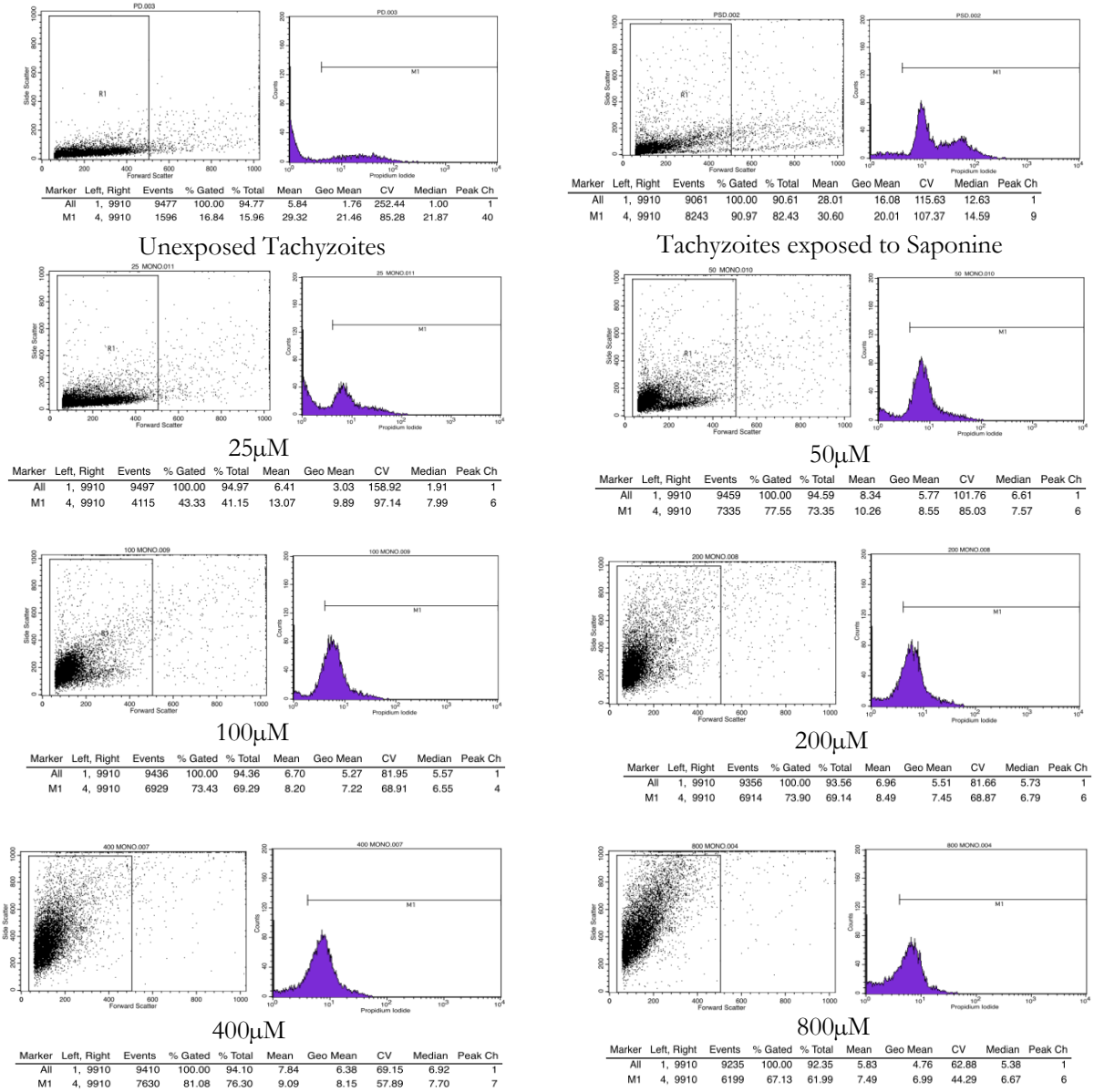

Fig 2: Flowcytometry analyses of unexposed *Toxoplasma* tachyzoites and *Toxoplasma* tachyzoites exposed to saponine and different doses of 2-(naphthalene-2-ylthio)-1H-indole

2.5-80µl of the final concentration was added to 920-997.5µl of suspension that contained $2x10^6$ 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 flowcytometry 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 $2x10^6$ 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 10^6 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 microcopically.

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 IC_{50} 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).

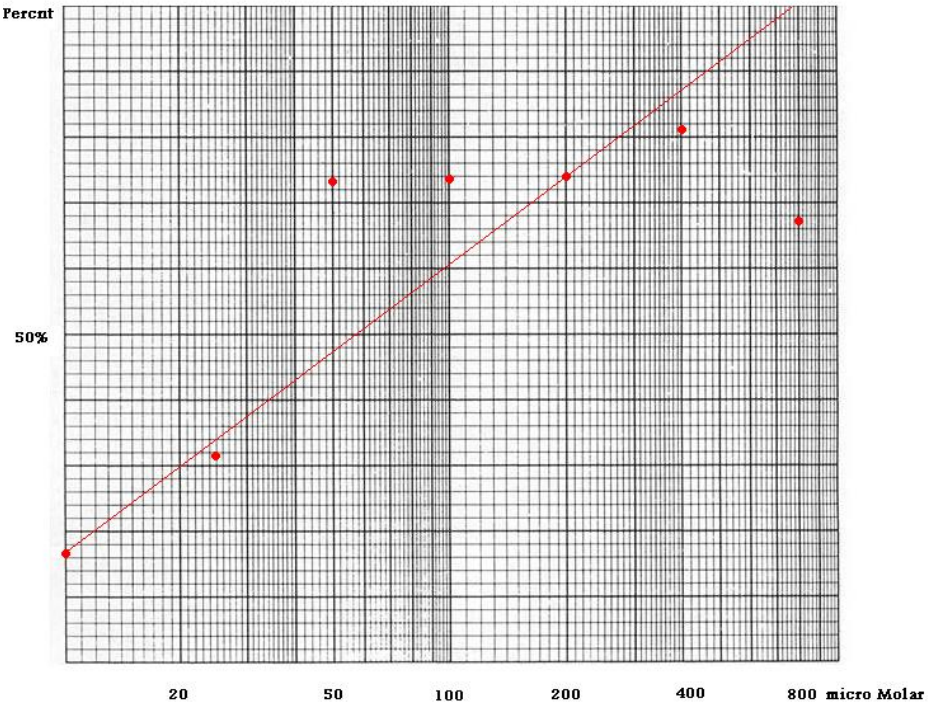

Fig. 3: The mortality of *Toxoplasma* tachyzoites after 1.5 hours exposure to different doses of 2-(naphthalene-2-ylthio)-1H-indole

Table1: The mean of life duration (days) of mice groups inoculated by *Toxoplasma* tachyzoites exposed to 2-(naphthalene-2-ylthio)-1H-indole

	DMSO	2-(naphthalene-2-ylthio)-1H-indole					
	0.2%	25µM	50µM	100µM	200µM	400µM	800µM
Total number of mice (70)	**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 endured 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 IC_{50}'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 brassicae* (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).

Indolenaphthyridinones 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 prokaryotic 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 Freunds 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.

Acknowledgment

We would like to thank the Office of Vice-Chancellor for Research of the Tehran University of Medical Sciences, Tehran, Iran for financial support of this project. We express our appreciation to the staff of the Laboratory Animal Center, the Medicinal and Natural Products Chemistry Research Center and Central Laboratory of Shiraz University of Medical Sciences, Shiraz, Iran for development of the experimental studies. The authors declare that there is no conflict of interests.

References

1. Dubey JP. History of the discovery of the life cycle of *Toxoplasma gondii*. Int J Parasitol.2009; 39: 877-882.
2. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004; 363: 1965-1976.
3. Remington JS, Mcleod R, Thulliez P. In Infection Diseases of the Fetus and Newborn Infant ed. Philadelphia: Elsevier Saunders; 2006.
4. Joynson DHM, Wreghitt TG. In Toxoplasmosis: A comprehensive clinical guide, ed. Cambridge: Cambridge University Press; 2001.
5. Oksenhendler E, Charreau I, Tournerie C, Azihary M, Carbon C, Aboulker JP. *Toxoplasma gondii* infection in advanced HIV infection. AIDS.1994; 8: 483-487.
6. Pereira-Chioccola VL, Vidal JE, Su C. *Toxoplasma gondii* infection and cerebral toxoplasmosis in HIV-infected patients. Future Microbiol. 2009; 4: 1363-1379.
7. Anderson AC. Targeting DHFR in parasitic protozoa. Drug Discov Today.2005; 10: 121-128.
8. Mori T, Kato J, and Okamoto S. Pancytopenia due to pyrimethamine triggered by transplant-associated microangiopathy after allogeneic bone marrow transplantation. J Infect Chem. 2011; 17(6): 866–867.
9. Lipka B, Milewska-Bobula B, Filipek M. Monitoring of plasma concentration of pyrimethamine (PYR) in infants with

congenital *Toxoplasma gondii* infection own observations. Wiad Parazytol.2011; 57: 87-92.
10. Ostlere LS, Langtry JA, Staughton RC. Allergy to spiramycin during prophylactic treatment of fetal toxoplasmosis. Br Med J.1991; 302: 970.
11. Rubinstein E, Keller N. Spiramycin renaissance. J Antimicrob Chemother.1998; 42: 572-576.
12. Grujic J, Djurkovic-Djakovic O, Nikolic A, Klun I, Bobic B. Effectiveness of spiramycin in murine models of acute and chronic toxoplasmosis. Int J Antimicrob Agents. 2005; 25: 226-230.
13. Dai W, Pan H, Kwok O, Dubey JP. Human indoleamine 2, 3-dioxygenase inhibits *Toxoplasma gondii* growth in fibroblast cells. J Interferon Res.1994; 14: 313-317.
14. Pfefferkorn ER, Eckel M, Rebhun S. Interferon-gamma suppresses the growth of *Toxoplasma gondii* in human fibroblasts through starvation for tryptophan. Mol Biochem Parasitol. 2019; 86: 215-224.
15. Asai T, Takeuchi T, Diffenderfer J, Sibley LD. Identification of small-molecule inhibitors of nucleoside triphosphate hydrolase in *Toxoplasma gondii*. Antimicrob Agents Chemother. 2002; 46: 2393-2399.
16. Kirihara a, Asai Y, Ogawa S, Noguchi T, Hatano A, Hiraib Y. A Mild and Environmentally Benign Oxidation of Thiols to Disulfides. Synthesis.2007; 21: 3286-3289.
17. Hamel P, Zajac N, Atkinson JG, Girard Y. Nonreductive Desulfenylation of 3-Indolyl Sulfides. Improved Syntheses of 2-Substituted Indoles and 2-Indolyl Sulfides. J Organ Chem. 1994; 59: 6372-6377.
18. Akins CK, Panicker S, Cunningham CL.In Laboratory Animals in Research and Teaching: Ethics, Care, and Methods. Washington, DC: APA; 2004.
19. Asgari Q, Keshavarz H, Rezaeian M, Motazedian MH, Shojaee S, Mohebali M,Miri M. Direct Effect of Two Naphthalene-Sulfonyl-Indole Compounds on *Toxoplasma gondii*-Tachyzoite. J Parasitol Res. 2013; 2013: 1-8.
20. Di Cristina M, Marocco D, Galizi R, Proietti C, Spaccapelo R, Crisanti A. Temporal and spatial distribution of *Toxoplasma gondii* differentiation into Bradyzoites and tissue cyst formation in vivo. Infect Immun. 2008; 76: 3491-3501.
21. Suzuki M, Maghni K, Molet S, Shimbara A, Hamid QA, Martin JG. IFN-gamma secretion by CD8T cells inhibits allergen-induced airway eosinophilia but not late airway responses. J Allergy Clin Immunol. 2002; 109: 803-809.
22. Seymour RL, Ganapathy V, Mellor AL, Munn DH. A high-affinity, tryptophan-selective amino acid transport system in human macrophages. J Leukocyte Biol. 2006; 80: 1320-1327.
23. Schwab JC, Beckers CJ, Joiner KA. The parasitophorous vacuole membrane surrounding intracellular *Toxoplasma gondii* functions as a molecular sieve. Proc Natl Acad Sci U S A.1994; 91: 509-513.
24. Schwartzman JD, Pfefferkorn ER. *Toxoplasma gondii*: purine synthesis and salvage in mutant host cells and parasites. Exp Parasitol. 1982; 53: 77-86.
25. Sibley LD, Niesman IR, Asai T, Takeuchi T. *Toxoplasma gondii*: secretion of a potent nucleoside triphosphate hydrolase into the parasitophorous vacuole. Exp Parasitol. 1994; 79: 301-311.
26. Nakaar V, Samuel BU, Ngo EO, Joiner KA. Targeted reduction of nucleoside triphosphate hydrolase by antisense RNA inhibits *Toxoplasma gondii* proliferation. J Biol Chem. 1999; 274: 5083-5087.
27. Browne LM, Conn KL, Ayer WA, Tewari JP. The camalexins: new phytoalexins produced in the leaves of *Camelina sativa* (Cruciferae). Tetrehedron. 1991; 47: 3909-3914.
28. Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC. Phytoalexin Accumulation in Arabidopsis thaliana during the Hypersensitive Reaction to *Pseudomonas syringae* pv *syringae*. Plant Physiol. 1992; 98: 1304-1309.
29. Thomma BHJ, Nelissen I, Eggermont K, Broekaert WF. Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus *Alternaria brassicicola*. Plant J. 1999; 19: 163-171.
30. Glawischnig E. Camalexin. Phytochemistry. 2007; 68: 401-406.
31. Sellam A, Dongo A, Guillemette T, Hudhomme P, Simoneau P. Transcriptional responses to exposure to the *Brassica ceous* defence metabolites camalexin and allyl-isothiocyanate in the necrotrophic fungus

Alternaria brassicicola. Mol Plant Pathol. 2007; 8: 195-208.

32. Seefeld MA, Miller WH, Newlander KA, Burgess WJ, DeWolf WE, et al. Indole naphthyridinones as inhibitors of bacterial enoyl-ACP reductases FabI and FabK. J Medicin Chem. 2003; 46: 1627-1635.
33. Smith MA, Moon H, Chowrira G, Kunst L. Heterologous expression of a fatty acid hydroxylase gene in developing seeds of *Arabidopsis thaliana*. Planta. 2003; 217: 507-516.
34. Mazumdar J, EH W, Masek K, CA H, Striepen B. Apicoplast fatty acid synthesis is essential for organelle biogenesis and parasite survival in *Toxoplasma gondii*. Proc Natl Acad Sci USA. 2006;103:13192-13197.
35. Jongert E, Roberts CW, Gargano N, Forster-Waldl E, Petersen E. Vaccines against *Toxoplasma gondii*: challenges and opportunities. Mem Inst Oswaldo Cruz. 2009; 104: 252-266.
36. Eissa MM, El-Azzouni MZ, Mady RF, Fathy FM, Baddour NM. Initial characterization of an autoclaved *Toxoplasma* vaccine in mice. Exp Parasitol. 2012; 131: 310-316.
37. Wilkins MF, O'Connell E, Te Punga WA. Toxoplasmosis in sheep: Effect of a killed vaccine on lambing losses caused by experimental challenge with *Toxoplasma gondii*. N Z Vet J. 1987; 35: 31-34.
38. Moire N, Dion S, Lebrun M, Dubremetz JF, Dimier-Poisson I. Mic1-3KO tachyzoite a live attenuated vaccine candidate against toxoplasmosis derived from a type I strain shows features of type II strain. Exp Parasitol. 2009; 123: 111-117.
39. Cerede O, Dubremetz JF, Soete M, Deslee D, Vial H, et al. Synergistic role of micronemal proteins in *Toxoplasma gondii* virulence. J Exp Med. 2005; 201: 453-463.
40. Lecoeur H. Nuclear apoptosis detection by flow cytometry: influence of endogenous endonucleases. Exp Cell Res. 2002; 277: 1-14.