

## THERAPEUTIC EFFICACY OF RESIQUIMOD ON TREATING CHRONIC TOXOPLASMOSIS IN EXPERIMENTAL INFECTED MICE

By

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### Abstract

Toxoplasmosis is a zoonotic protozoan infectious disease affects people worldwide with a high rate of morbidity and mortality. The incomplete efficacy of the approved drugs for the chronic *Toxoplasma gondii* infections endangers one-third of the global humans and animals with reactivation. This is due to the risky complications chronic toxoplasmosis causes.

This study evaluated the TLRs 7/8 agonist, Resiquimod<sup>®</sup> for treating chronic toxoplasmosis in BALB/c mice infected with the ME49 strain of *T. gondii* as compared the traditionally used Pyrimethamine<sup>®</sup> & Sulphadiazine<sup>®</sup>. This was judged by the number of brain cysts, the histopathological degrees in the brain and retina, expression of the apoptotic marker, caspase-3 compared to negative and positive controls.

The results showed a statistically significant decrease in the number of brain cysts in resiquimod-treated mice correlated with the histopathological improvement of brain and retina and apoptosis.

**Keywords:** *T. gondii*, Mice, Pyrimethamine, Sulphadiazine TLR7/8 agonist, Resiquimod.

### Introduction

*Toxoplasma gondii* is a zoonotic protozoan parasite of worldwide geographical and zoological distribution (Etewa *et al*, 2021). In Egypt, it has been reported in asymptomatic blood donors (Elsheikha *et al*, 2009), child-bearing women (Saleh *et al*, 2014), man, and animals (Abbas *et al*, 2020), with cats; definitive hosts passing infective oocysts (Al-Kappany *et al*, 2010). Man is infected with *T. gondii* either acquired or congenital (El Sharazy *et al*, 2023), and by nosocomial infection as occupational disease (Abdel-Motagaly *et al*, 2017).

Toxoplasmosis is usually asymptomatic in immunocompetent people, but it may progress to encephalitis, brain abscesses, or even death in immunocompromised ones (Rostami *et al*, 2014). Also, studies linked between latent toxoplasmosis and neuropsychiatric disorders; such as schizophrenia, obsessive-compulsive disorder, bipolar disorder, anxiety, and Parkinsonism (Sutterland *et al*, 2015) and infertility (Shiadeh *et al*, 2016). Congenitally infection has the risk of ocular and neurological sequelae as epilepsy, seizures, hydrocephalus, microcephaly, or mental ret-

ardation if they avoid spontaneous abortion, preterm, or even stillbirth or neonatal death (Robert-Gangneux and Dardé, 2012). Ocular lesions don't develop until after birth, but 20 to 80% of infected individuals develop them by adulthood, it may reactivate, each time doing greater harm to the retina and may end by blindness (WHO, 2015).

Drugs; as piramycin, azithromycin, atovaquone, pyrimethamine, sulfadiazine, and trimethoprim-sulfamethoxazole were frequently used to treat acute toxoplasmosis (Dard *et al*, 2018). As the first line of treatment, pyrimethamine & sulfadiazine was advised. However, this combination has several hematological adverse effects, including elevated serum liver enzymes and creatinine levels as well as allergic reactions and the development of parasites that are resistant to treatment (Denkers 2010). Also, these medications, whether taken for therapeutic or prophylactic purposes, only treat acute toxoplasmosis and are ineffective in cases of chronic infection (Hamie *et al*, 2021). As a result, it is urgently necessary to develop a new strategy to eradicate the parasite's cyst form and thereby treat the chronic infection.

Toll-like receptors are innate immune receptors which play a significant role in the resistance to *T. gondii* infections. When TLRs identified the parasite-derived pathogens-associated molecular patterns (PAMPs), they activate regulatory factors which control the transcription and expression of proinflammatory factors, interferons, and chemokines. Also, they initiated innate and adaptive immune responses targeting the parasitic infection (Pu *et al*, 2021).

Many studies investigated TLR agonists either as possible therapeutic agents or as vaccine adjuvants for infectious diseases or cancers (Farooq *et al*, 2021). TLR7/8 is located in the intracellular endosomes (Sana *et al*, 2022). Once activated, they trigger induction of a Th1 immune response to compete *T. gondii* infection (Sun *et al*, 2022).

Resiquimod is one of the imidazoquinoline derivatives (Dockrell and Kinghorn, 2001), a Toll-like receptor (TLR)-7 and TLR-8 agonist that, like imiquimod<sup>®</sup>, belongs to class of imidazoquinolines, small organic molecules with potent antiviral and anticancer activity (Farr *et al*, 2021). Resiquimod<sup>®</sup> (R848) is a potent Toll-like receptor 7, & 8 (TLR7/8), agonist for skin lesion treatment with antiviral and antitumor immune responses (Li *et al*, 2021).

The study aimed to investigate the TLRs 7/8 agonist, Resiquimod<sup>®</sup> for treating chronic toxoplasmosis induced by the ME-49 strain in experimentally infected mice.

### Materials and Methods

**Ethics consideration:** Mice were kept in the animal house of Theodor Bilharz Research Institute (TBRI) Giza, and given commercial pelleted food, in air conditioned animal house at 20 to 22°C. All these procedures were carried out according to ethical approval (IRP No. 7/2023PARA8).

**Parasite and preparation of infection:** The eight-week-old ME49 avirulent *T. gondii*-infected mice were euthanized, brains removed, into sterile PBS (1 mL PBS/brain), and then they were homogenized in a tissue homogenizer (Wheaton, IL, USA) in prepara-

tion for the infection. Cysts were counted by using a hemocytometer at a 400x magnification after homogenates were blended. The brain suspension was diluted to be 100cysts/ml. Each mouse was infected with 0.1ml contained 10 cysts by gavage (El-Kady *et al*, 2022).

**Animals and study design:** A total of 20 inbred pathogen-free male BALB/c mice (6-8 weeks, & 18-20g) were randomly divided into 4 groups. GI: 5 mice served as negative control. GII: 5 mice served as infected, non-treated or positive control. GIII: 5 infected mice treated with a combination of sulfadiazine (dose of 200mg/kg/day) and pyrimethamine (dose of 12.5mg/kg/day). GIV: 5 infected mice treated with Resiquimod.

**Treatment protocol:** Sulfadiazine<sup>®</sup> (Dohms Laboratories) and pyrimethamine<sup>®</sup> (Sigma Chemical Co., St. Louis, MO) were in a powder form and daily suspended in distilled water and given orally to each mouse via a feeding tube. Treatment started 42 days post infection (PI) and continued for ten days. Resiquimod<sup>®</sup> (Sigma Aldrich, USA) was given as a powder dissolved in endotoxin-free water (G-Biosciences, USA) at a concentration of 1mg/ml and administered intraperitoneally in doses of 2.5mg/kg/day, once every other day, on the day 21, treatment up to day 49 PI (Zahr *et al*, 2022). All mice were killed by decapitation on the 60<sup>th</sup> day PI. The removed eyeballs were preserved in 10% formalin. The brains after removal were split into 2 halves, one half was utilized to count cysts and the second one was processed for the histological examination.

**Evaluation of drugs' efficacy:** Brain tissue was washed, weighed, and homogenized in 1ml of sterile normal saline solution. On a clean slide, 0.1ml of homogenized brain was spread, allowed to air dry, and fixed in methanol. Slides were left to air dry, stained for 30-45minutes with Giemsa stain (Merck, Germany), washed with water, dried, and examined by microscopy to count cysts' number. Cyst count in brain was calculation by the following equation: Total cysts= Cysts

number in 0.1ml×10×2 (Nasr *et al*, 2020).

Histopathological examination of brain and retina: Brain (cerebrum grey and white matter), and eye tissues of all mice were paraffinized and stained with hematoxylin and eosin (Abdel Wahab *et al*, 1989). Inflammation grade was determined by counting number of inflammatory foci as mild inflammation < 2 foci, moderate < 4 foci, severe < 6, & very severe > 6 (El-Kowrany *et al*, 2019).

Tissue apoptosis: Expression of the apoptotic marker, caspase-3 in brain and eye tissues was assessed by using anti-caspase-3 antibody (Abcam, USA) after El-Kady *et al*. (2022). Brown coloration of the cell membrane or cytoplasm identified positive staining. Histo score (H-score) was used to grade caspase-3 staining. Staining intensity was given a number from 0, 1+, 2+ & 3+ & multiplied by brown stained cells percent in each tissue (Fraser *et al*, 2003). A score of 0-300 was given to each specimen [ $1 \times (\% \text{ cells } 1+) + 2 \times (\% \text{ cells } 2+) + 3 \times (\% \text{ cells } 3+)$ ]

Statistical analysis: Data were expressed in Number (No), percentage (%) mean ( $\bar{x}$ ) and standard deviation (SD). The normality of data was tested by Shapiro-Wilkis test. The Kruskal Wallis test was used for comparison of quantitative variables between more than two groups of not normally distributed data with Tamhane's test as Post hoc test. Chi-square test ( $\chi^2$ ) was used to study the association between qualitative variables and whenever any of the expected cells were less than five, Fischer's exact test was used. Two-sided  $P < 0.05$  was considered statistically significant. Analysis was done by an IBM compatible personal computer with SPSS statistical package version 26 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armonk, NY: IBM Corp.).

### Results

The resiquimod-treated group had the least mean cyst count ( $123.80 \pm 17.55$ ), followed by the sulfadiazine & pyrimethamine-treated mice came in second ( $316.80 \pm 20.52$ ), with a significant difference between both groups.

Histopathological examination of brain tis-

sues showed improvement in both treated mice as compared with positive control one. Resiquimod-treated mice showed the least pathological inflammations (80% mild and 20% moderate). In sulfadiazine & pyrimethamine-treated mice inflammations were 60% moderate and 40% severe pathology with significant differences between both groups ( $P < 0.001$ ).

Besides, the lowest grade of retinal inflammation was in the resiquimod treated mice (100% mild), but pyrimethamine & sulfadiazine-treated mice showed (60% moderate inflammation and 20% mild).

Treatment with either resiquimod or sulfadiazine & pyrimethamine achieved a significant reduction of caspase-3 H-score as compared to positive control mice with the least score in resiquimod-treated ones in brain or retinal sections followed by pyrimethamine & sulfadiazine-treated mice and folic acid

Details were shown in figures (1, 2, 3, 4, 5, 6, 7, 7 8).

### Discussion

Generally speaking, the high seroprevalence of chronic toxoplasmosis in humans was identified as a significant risk factor for the brain illnesses, such as schizophrenia, Parkinson's disease, Alzheimer's disease and congenital hydrocephalus, microcephaly (Samojłowicz *et al*, 2019) as well as neurological manifestations in children (Wishahy *et al*, 1972). Pyrimethamine is commonly used in combination with sulfadiazine and folic acid (CDC, 2022).

Some of the TLRs agonists agents proved to be efficacious in preclinical models and have now entered clinical trials, as well as hold the potential to serve as a perfect target in the immunotherapies era (Farooq *et al*, 2021).

Resiquimod was influenced by other studies and displayed a synergistic effect in treating parasitic infection. Ahmad *et al*. (2010) in USA found that the vaccination with Smp80 (prime-boost approach) showed 49% reduction in adults *Schistosoma mansoni* burden with recombinant protein approach the

protection was found to be 50%. They concluded that the protective effects of Sm-p80 in both DNA prime-protein boost and recombinant protein immunization approaches in the murine model. Peine *et al.* (2014) reported that the FDA-approved resiquimod, in a liposomal formulation, has promising results in visceral leishmaniasis treatment. Also, resiquimod treated *Leishmania infantum chagasi* in mice (Craft *et al.*, 2014) and *Leishmania major* & *L. tropica* (El Hajj *et al.*, 2018), as well as a potent drug against the chronic toxoplasmosis and imiquimod TLRs 7/11/12 agonist, which elucidated its mechanism of action (Hamie *et al.*, 2021).

In the present study, the resiquimod-treated infected mice as compared to pyrimethamine & sulfadiazine treated ones significantly reduced number of brain cysts. This agreed with Tomai *et al.* (2007), they reported that resiquimod was safe and effective activating the local immune response. Also, this agreed with Yarovinsky (2014) in USA, they reported that Toll-like receptor (TLR)-dependent mechanisms were responsible for the *T. gondii* recognition and for induction of IFN $\gamma$  production by NK cells, as well as the emerging data about the TLR-independent mechanisms that led to IFN $\gamma$ -mediated to eliminate the infection. Besides, Xiao *et al.* (2018) in USA, reported that the chronic *T. gondii* cysts always present in the brain tissues and resiquimod targeted the immune checkpoint blockage of programmed cell death protein-1 pathway, and thus decreasing the T cell apoptosis, in lowering the number of brain tissue cysts.

The present study, as TLR11 was exclusively expressed in mice and only concentrated on 7 and 8 TLRs because they played a crucial part in human toxoplasmosis. This agreed with Andrade *et al.* (2013) in USA, they reported that the triple TLR7/TLR9/TLR11-deficient mice were highly susceptible to *T. gondii*, recapitulating the phenotype of 3d mice but, in man lacked functional TLR11 and TLR12 genes. They concluded that human cells produced high levels of

proinflammatory cytokines in response to *Toxoplasma* derived RNA and DNA, but not to its profiling, and supporting a more critical role for NAS-TLRs in human infection.

Dockrell and Kinghorn (2001) in United Kingdom reported that resiquimod can be administered topically, but also existed as an oral formulation. The range of potential infections for which these agents may have clinical utility included chronic hepatitis C virus infection and Kaposi's sarcoma. Franklin *et al.* (2011) in Brazil reported that pro-inflammatory cytokines excessive released by innate immune cells is an important component in malaria pathogenicity. They added that the nucleic acid sensing TLRs in pathogenesis interfered with the activity of these receptors proved to be a promising strategy preventing deleterious inflammatory responses mediating pathogenesis in cerebral malaria caused by *P. berghei*. However, Bhaghandani *et al.* (2021) in USA reported that resiquimod was more potent than imiquimod in inducing cytokine expression as antiviral and anti-skin cancer. They added that the role of synthetic TLR7/8 agonists in combination with established as well as emerging treatments was of utmost importance in cancer, and infectious diseases, as well as allergic and autoimmune conditions.

The present study showed that in positive control, *T. gondii* disrupted the blood-brain barrier, invaded brain tissue, significant oxidative stress, elevated nitric oxide levels, glial activation, and ultimately apoptosis. Also, retinal tissues showed severe retinitis and a clear infiltration of lymphocytes and macrophages, particularly in deep layers that disfigured and necrotized retino-choroiditis. This agreed with Nishi *et al.* (2019) in Brazil, who reported that rosuvastatin<sup>®</sup> didn't interfere with cerebral toxoplasmosis in chronic infected Swiss mice.

In the present study, the reduction in tissue cysts had an impact on the pathological degrees in the brain and retina. Resiquimod significantly reduced the inflammation and histological alterations caused by *T. gondii* infe-

ction as compared to other groups. It displayed decreased inflammatory cellular infiltrate, edema, a lack of red neurons, increased glial cells (gliosis), and decreased apoptosis. Also, the retinal tissue displayed nearly the normal structures. This agreed with Xiao *et al.* (2023), they hypothesized that preventing T lymphocyte markedly decreased the neuroinflammation driven on by *T. gondii*. This improvement in systemic immunity led to *Toxoplasma* clearance, which in turn improved the neuroinflammation, and immunostimulatory actions. This agreed with Craft *et al.* (2014), who reported that resiquimod generated a significant systemic immune activation by trafficking of leukocytes, including B cells, CD4+ and CD8+ T cells, dendritic cells, and macrophages, with potent preventive and therapeutic effects against visceral leishmaniasis infection.

In the present study, the brain, and retinal sections of resiquimod-treated, showed a significant reduction in the *T. gondii*-associated apoptosis that typically happened via the natural infection course and resulted in neurodegeneration. This raised resiquimod role in preventing mice apoptosis with *T. gondii* infection (Dincel and Atmaca 2021). Also, this agreed with El-kady *et al.* (2022), they linked the level of apoptosis to the *Toxoplasma* infection severity and tissue cysts quantity.

### Conclusion

The outcome data showed that Resiquimod<sup>®</sup> the immunotherapeutic drug reduced the cyst number brain tissues in chronic *Toxoplasma gondii* ME49 infected mice. Also, the drug improved the histopathological changes of the brain and retinal tissues as well as reduced apoptotic score in them.

The proved that the resiquimod could be a potential immunotherapeutic agent for treating chronic toxoplasmosis.

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#### Explanation of figures

Fig. 1: Column chart presentation of brain cyst counts in groups. Lowest cyst count detected in resiquimod-treated mice: Differences with other groups significant. N.B. columns with different letters refer to significant differences.

Fig. 2: Column chart presentations of brain pathology degree among groups: 80% of resiquimod-treated mice showed mild inflammation score. Fig. 3: H & E-stained brain sections of positive control mice. A: bradyzoites (green arrows), with stromal edema (x400). B: massive inflammation with perivascular cuffing with chronic inflammatory cells (green arrow) (x400). C: neuronal degeneration (red neurons) (green arrows) (x 200).

Fig. 4: H & E-stained brain sections of treated mice. A: Sulfadiazine & pyrimethamine treated mice showed moderate inflammation (green arrows) (x400). B: resiquimod-treated mice showed mild inflammation (circle) and degenerated cyst (green arrow) (x 400).

Fig. 5: Column chart presentation of retinal pathology degree. All (100%) resiquimod-treated mice showed mild inflammation.

Fig. 6: H & E-stained retinal sections of treated mice. A: infected control mice showed necrotizing retino-choroiditis with tissue necrosis (green arrow) and disfigurement of retina and choroid (x400) B: Infected untreated mice showed extensive inflammation (Retinitis) composed of many lymphocytes (green arrow) and few macrophages (yellow arrow) affecting deep layer of retina with retinal disfiguring (x400). C: Sulfadiazine + pyrimethamine treated mice showing moderate chronic inflammation (green circle) (x 400). D: resiquimod-treated mice showed nearly normal structure (x400).

Fig. 7: Brain apoptosis. A: column chart presentation of the mean H-score of caspase-3 expression in groups: Lowest degree of apoptosis detected in resiquimod-treated mice with significant differences with others. N.B. columns with different letters refer to significant differences. B: Positive control showed severe apoptotic changes by high caspase 3 expression (green arrows) (x400). C: Sulfadiazine + pyrimethamine treated mice showed moderate apoptotic changes by caspase 3 expression (green arrows) (x400). D: Brain section of resiquimod treated mice showed mild apoptotic changes by caspase 3 expression (green arrows) (x400).

Fig. 8: Retinal apoptosis. A: column chart presentation of mean H-score of caspase-3 expression among groups, Resiquimod-treated mice showed lowest apoptosis degree, differences significantly with other groups. N.B. columns with different letters refer to significant differences. B: Positive control showed severe apoptotic changes by high caspase 3 expression (green arrows) (x400). C: Sulfadiazine + pyrimethamine treated mice showed moderate apoptotic changes by caspase 3 expression (green arrows) (x400). Fig. 8d resiquimod treated mice showed mild apoptotic changes by caspase 3 expression (green arrows) (x400).









