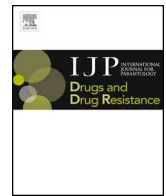




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New life for an old drug: *In vitro* and *in vivo* effects of the anthelmintic drug niclosamide against *Toxoplasma gondii* RH strain

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ABSTRACT

Toxoplasma gondii is the causative agent of toxoplasmosis and causes serious public health problems. However, the current treatment drugs have many limitations, such as serious side effects. Niclosamide is a salicylanilide drug commonly used to treat worm infections. Herein, the effectiveness of niclosamide for the treatment of *T. gondii* infection was demonstrated. This study was to evaluate the *in vitro* and *in vivo* activities of niclosamide against *T. gondii* and to explore its mechanism of action. The *in vitro* cytotoxicity of niclosamide on human foreskin fibroblast cells was evaluated by MTT test. Niclosamide displayed low host toxicity and its 50% inhibitory concentration was 8.3 $\mu\text{g}/\text{mL}$. The *in vitro* anti-proliferation and anti-invasion effects of niclosamide on *T. gondii* were determined by quantitative PCR and Giemsa staining. Niclosamide also inhibited *T. gondii* tachyzoite proliferation, with a 50% effective concentration of 45.3 ng/mL, and reduced the invasion of cells by tachyzoites (17.8% for the parasite control versus 1.9% for the niclosamide group treated with 100 ng/mL). A model was established by infecting BALB/c mice with the virulent RH strain of *T. gondii* and used to determine the *in vivo* effects of niclosamide on acute infection. The mice infected with tachyzoites and treated with 160, 200 or 240 mg/kg-bw niclosamide for 7 days exhibited 20%, 40% and 50% survival, respectively. In addition, niclosamide reduced the parasite burden in the blood and tissues of acutely infected mice, and niclosamide induced decreases in the mitochondrial membrane potential ($\Delta\Psi_m$) and adenosine triphosphate (ATP) levels in extracellular tachyzoites, as assessed by laser confocal microscopy and a multilabel reader. These findings indicated that the mechanism of action of niclosamide might be associated with *T. gondii* mitochondria oxidative phosphorylation. In conclusion, our results support the efficacy of niclosamide as a potential compound for the treatment of *T. gondii* infection.

1. Introduction

Toxoplasma gondii is a globally ubiquitous zoonotic apicomplexan parasite that can infect humans and other warm-blooded animals (Weiss and Kim, 2013). Felines are the definitive hosts of *T. gondii*, but the parasite can live for part of its lifecycle in a wide range of animal species, including humans, and thus adversely affects human health and disrupts animal husbandry (Dubey, 2004). Although *T. gondii* infection rarely causes severe symptoms in healthy humans and most other hosts (Dubey and Jones, 2008), infection during pregnancy can result in

vertical transmission of the organism, which imposes severe consequences in the foetus, including impaired visual acuity, mental retardation, intracranial calcifications, hydrocephalus, foetal malformations and even death (Fallahi et al., 2018). Moreover, immunocompromised individuals infected with *T. gondii* are at risk for developing life-threatening toxoplasmic encephalitis from primary or recrudescence infection (Machala et al., 2015).

The treatment and prevention of toxoplasmosis has long depended on drugs, such as azithromycin, atovaquone, pyrimethamine, clindamycin, and sulfadiazine, many of which have been used for several

Abbreviations: Human foreskin fibroblasts, HFFs; 50% effective concentration, EC_{50} ; 50% inhibitory concentration, IC_{50} ; 50% lethal dose, LD_{50} ; adenosine triphosphate, ATP; mitochondrial membrane potential, $\Delta\Psi_m$

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years. The combination of pyrimethamine and sulfadiazine is the gold standard for the treatment of toxoplasmosis. However, these drugs are not completely effective in eradicating encysted bradyzoites and treating congenital toxoplasmosis, and their use is also limited by their side effects (de la Torre et al., 2011). Sulfadiazine has been associated with bone marrow arrest and hypersensitive reactions (Bosch-Driessen et al., 2002; Guaraldo et al., 2018), and pyrimethamine can cause agranulocytosis and megaloblastic anaemia without any overt toxicity (Rothova et al., 1993). In addition, atovaquone has been linked to conditions such as myasthenia gravis and exerts other adverse effects (Haile and Flaherty, 1993). Thus, safe and effective drugs for the treatment of toxoplasmosis are urgently needed.

Continuous efforts have been made to develop drugs for the treatment of toxoplasmosis. However, drug development is an expensive and lengthy process (Hoelder et al., 2012). In an attempt to accelerate the process of drug discovery, older drugs are making a comeback by being developed for new activities. Niclosamide, a salicylanilide drug, has been approved by the US Food and Drug Administration (FDA) for the treatment of tapeworm infections in humans at a single 2 g dose or two 1 g doses given an hour apart for many years (Katz, 1986). In the past several years, mounting evidence has indicated that niclosamide is a multifunctional drug for the treatment of various diseases, including cancers, bacterial and viral infections, metabolic syndrome and artery constriction, which suggests that it can potentially be developed as a novel treatment for more than just helminthic disease (Imperi et al., 2013; Tao et al., 2014; Xu et al., 2016; Kim et al., 2017; Li et al., 2017; Chen et al., 2018). Most recently, Fomovska et al. (2012) indicated the promising activity of salicylanilide inhibitors against *T. gondii*; however, the anti-*T. gondii* activity of niclosamide *in vivo* has not been directly investigated, and the mechanism of action remains unknown. Herein, we investigated the new activity of the old anthelmintic drug niclosamide against the *T. gondii* RH strain. Specifically, the *in vitro* effect of niclosamide on repeated cycles of *T. gondii* infection was assessed. A mouse model of acute *T. gondii* infection was constructed to demonstrate the activity of niclosamide *in vivo*, and the underlying mechanism of action was also preliminarily explored.

2. Materials and methods

2.1. Cells

Human foreskin fibroblast (HFF) cells were kindly provided by the Stem Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 100 U/mL penicillin, 100 µg/mL streptomycin, 1% non-essential amino acids (NEAA), 1% GlutaMAX and 10% heat-inactivated foetal bovine serum (FBS) at 37 °C in a 5% CO₂ atmosphere.

2.2. Drugs

Niclosamide (FW 327/119) was purchased from Dr. Ehrenstorfer GmbH (Germany) at the highest purity available (> 98%). For the *in vitro* experiments, niclosamide was dissolved in dimethyl sulfoxide (DMSO, Sigma, USA) to obtain a concentration of 1.6 mg/mL and then diluted in DMEM containing 1% FBS to different concentrations. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/mL) was dissolved in DMEM containing 1% FBS and passed through a membrane filter with 0.22-µm pores. Azithromycin and sulfadiazine, which were used as positive drug controls in the *in vitro* experiments, were dissolved in DMEM with 1% FBS to final concentrations of 8.6 µg/mL and 0.4 µg/mL, respectively. For the *in vivo* experiments, niclosamide was suspended in solvent that contained phosphate-buffered saline (PBS), 0.5% carboxymethyl cellulose and 2% Tween 20 to final concentrations of 16, 20 and 24 mg/mL. The positive control drugs used in the *in vivo* experiments contained 10 mg/mL sulfadiazine, 5 mg/mL pyrimethamine and 1.5 mg/mL folic acid suspended in the above-

mentioned solvent. All the drugs were stored at 4 °C.

2.3. Animals

Eight-week-old inbred female BALB/c mice weighing 18–20 g were used in the acute infection experiments. All the mice were housed in cages under standard laboratory conditions (with an average ambient temperature of 20–25 °C) and provided drinking water and a regular mouse diet. The mice were acclimatized for one week prior to the start of experiments. All the animal care protocols and the environment utilized in this study were in strict accordance with the Guide for the Care and Use of Laboratory Animals of Lanzhou Institute of Husbandry and Pharmaceutical Sciences, China, and all efforts were made to minimize suffering.

2.4. Parasites

The tachyzoites used in our study were obtained from the virulent RH strain of *T. gondii*, which was generously donated by Dr. Xingquan Zhu, Lanzhou Veterinary Research Institute. The tachyzoites were maintained in HFF layers in DMEM supplemented with 1% FBS and collected by trypsinizing the cell monolayers and then centrifuging the suspensions for 20 min at 1500 × g. The tachyzoites in the pellet were released by forceful passage through a 27-gauge needle and filtered through polycarbonate membrane filters with 5-µm pores to separate the tachyzoites from the host cells. The tachyzoites were then centrifuged for another 20 min at 1500 × g, and the pellet was resuspended in DMEM containing 1% FBS. The number of tachyzoites was determined by counting with a haemocytometer under a light microscope. The infection experiments with *T. gondii* were performed under biosafety level 2 (BSL-2) conditions to ensure that the technicians and researchers who worked with this highly virulent strain of *T. gondii* (RH) were not infected.

2.5. Cell viability assay

The cytotoxicity of niclosamide to HFF cells was evaluated by an MTT assay. HFF cells in the exponential growth stage were seeded in 96-well plates and cultured in DMEM supplemented with 10% FBS at 1 × 10⁴ cells per well for 12 h to obtain a monolayer. The monolayer cells were washed and directly subjected to niclosamide at final concentrations of 0.125, 0.5, 2.0, 4.0, 8.0, and 16 µg/mL in DMEM with 1% FBS. As a control, monolayer cells were cultured without niclosamide. After incubation for 48 h, the culture supernatant was removed, 15 µL of MTT solution (5 mg/mL) in DMEM was directly added to each culture well, and the cells were incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. Formazan extraction was performed by incubation with DMSO (150 µL/well) at 37 °C for 10 min, and the absorbance of the plates at 570 nm was read using a Multiskan GO instrument (Thermo Fisher Scientific, MA, USA). The viabilities of the cells are expressed as percentages of the control value (defined as 100% survival). The 50% inhibitory concentration (IC₅₀) was calculated as the concentration that reduced HFF cell growth by an average of 50% in three replicates.

2.6. Anti-proliferation assay *in vitro*

The anti-proliferation effect of niclosamide on *T. gondii* was observed by Giemsa staining and determined by real-time quantitative PCR (qPCR). Confluent HFF monolayers in 6-well plates were infected with 2 × 10⁴ fresh RH tachyzoites per well and incubated for 6 h. The cell monolayers were washed twice with PBS to remove extracellular tachyzoites and then incubated with DMEM (1% FBS) containing various concentrations of niclosamide (30, 40, 50, 60, 70, 80, 90, and 100 ng/mL). As controls, the positive drugs azithromycin (8.6 µg/mL) and sulfadiazine (0.4 µg/mL) were added under identical conditions. Infected cells incubated with DMEM (1% FBS) in the absence of drug

were used the parasite control group. Uninfected cells cultured in DMEM (1% FBS) were used as the host cell control group. After 24 h of treatment, the cells were fixed with fresh 4% paraformaldehyde in PBS, stained with Giemsa stain, and observed by light microscopy.

To obtain measurements of the *T. gondii* burden, the cells were infected with tachyzoites (2×10^4 per well) for 6 h, treated with niclosamide (30, 40, 50, 60, 70, 80, 90, or 100 ng/mL), azithromycin (8.6 $\mu\text{g/mL}$) or sulfadiazine (0.4 $\mu\text{g/mL}$) for 24 h, and washed twice with PBS. Infected or uninfected cells cultured in DMEM (1% FBS) were used as the parasite or host cell control group. The total genomic DNA from the cell samples was isolated using DNAiso Reagent (Takara), and the 529-bp repeat element of *T. gondii* was measured by qPCR with the following primers: oligonucleotides Tox-F (5'-AGG AGA GAT ATC AGG ACT GTA G-3'), Tox-R (5'-GCG TCG TCT CGT CTA GAT CG-3') and TaqMan probe Tox-TP1 (6-Fam CCG GCT TGG CTG CTT TTC CT BHQ1) (Homan et al., 2000). Amplification was performed in a final volume of 25 μL using a QuantStudio 6 Flex Real-Time PCR System (Life Technologies). Each reaction contained 12.5 μL of $2 \times$ Premix Ex Taq (Takara), 2 μL of template DNA, 8 μL of distilled water, 0.2 μM Tox-TP1 probe and 0.4 μM each primer. The following amplification conditions were applied: 10 min at 95 $^{\circ}\text{C}$; 40 cycles of 95 $^{\circ}\text{C}$ for 30 s (denaturation), 58 $^{\circ}\text{C}$ for 1 min (annealing), and 72 $^{\circ}\text{C}$ for 1 min (amplification); and a final extension at 72 $^{\circ}\text{C}$ for 10 min. The temperature was increased and decreased between steps at rates of 3.4 $^{\circ}\text{C/s}$ and 2.2 $^{\circ}\text{C/s}$, respectively. The number of tachyzoites in a sample was calculated from a standard curve ($Y = -3.585x + 34.994$; $R^2 = 0.9992$) constructed with serially diluted (3.45×10^6 to 34.5/mL) DNA samples from RH strain tachyzoites, and the 50% effective concentration (EC_{50}) was calculated. The dilution series containing tachyzoites was tested in triplicate, and no amplification product was observed in the host cell control wells. The qPCR assay and Giemsa staining of each experimental group were performed using independent triplicate replicates, respectively.

2.7. Anti-invasion test in vitro

HFF cells were seeded onto 6-well plates and cultured in DMEM with 10% FBS to obtain cell monolayers. The medium was replaced with fresh DMEM (1% FBS) containing niclosamide (30, 45, 60, 80, or 100 ng/mL), azithromycin (8.6 $\mu\text{g/mL}$), sulfadiazine (0.4 $\mu\text{g/mL}$), or no drugs (as a control), and 2×10^6 fresh tachyzoites per well were then inoculated into the confluent HFF monolayers. Two hours after infection, the anti-invasion capability of niclosamide was determined by Giemsa staining. The percentage of infected cells was determined by light microscopy. Triplicate independent experiments were conducted, and at least 20 randomly fields per well were counted with a $40 \times$ objective lens.

2.8. Anti-*T. gondii* activity of niclosamide in vivo

To evaluate the therapeutic effect on acute infection, we divided mice into 6 groups consisting of 10 mice each and injected 2×10^3 tachyzoites into each mouse by intraperitoneal injection. After 4 h, mice in the 5 treatment groups were treated with niclosamide (160, 200 or 240 mg/kg-bw), the positive drugs (100 mg/kg-bw sulfadiazine, 50 mg/kg-bw pyrimethamine or 15 mg/kg-bw folinic acid) or solvent, respectively (Si et al., 2018). The mice in the parasite control group were treated with only PBS. All drugs were administered by oral administration. The treatments were administered once a day for 7 consecutive days, and the mice were observed for 13 days after treatment cessation. The survival times of the infected mice and the numbers of deaths were monitored and recorded twice daily for 20 days.

In a preliminary experiment aiming to control the side effects of niclosamide and determine a safe dose, six groups of mice were orally treated with the drug dosages outlined above for 30 days, and no discernible clinical toxicity was observed with any of the tested drug doses during this time period.

2.9. Parasite load in mouse tissues and blood

To detect the effects of niclosamide on the parasite burden, 20 additional mice were divided into 2 groups (each consisting of 10 mice), intraperitoneally injected with 2×10^3 tachyzoites, and treated with niclosamide (240 mg/kg-bw) or PBS (parasite control group) by oral administration. The treatments were administered once a day for 7 consecutive days as described above. The body weight and body temperature of the mice were monitored daily. At the end of the observational period, the mice were anaesthetized through an intraperitoneal injection of pentobarbital sodium, and after blood was collected, the mice were humanely killed by cervical dislocation. The brain, liver, and spleen tissues of the mice were subsequently isolated and homogenized in PBS. To measure the parasite load, the total genomic DNA from the blood or tissue homogenates were isolated using blood DNA kits (D3392, Omega, Omega Bio-Tek, US) and tissue DNA kits (D3396, Omega, Omega Bio-Tek, US), respectively. The *T. gondii* 529-bp gene was detected by qPCR as described above. The number of parasites in the unknown samples was deduced from standard curves constructed using known numbers of tachyzoites added to uninfected liver, brain, or spleen tissues or blood.

2.10. Measurement of the adenosine triphosphate (ATP) level

The adenosine triphosphate (ATP) concentration in RH *T. gondii* tachyzoites was quantified using a luciferase-based enhanced ATP assay kit (S0027, Beyotime Institute of Biotechnology, China). Extracellular parasites (2×10^6 /per experimental group) collected as previously described were maintained in suspension with different concentrations of niclosamide (50, 100, or 200 ng/mL) in DMEM or without any drug (parasite control) for 8 h at 37 $^{\circ}\text{C}$. After incubation, the *T. gondii* tachyzoites were washed with PBS and immediately lysed on ice with 200 μL of lysis buffer. The lysates were then collected and centrifuged at 12,000 $\times g$ for 5 min, and 20 μL of the supernatants were added to wells of an opaque-walled 96-well microplate (Corning Inc., Corning, NY, USA) containing 100 μL of ATP detection working dilution in each well. The luminescence of each well was detected at 25 $^{\circ}\text{C}$ using a multilabel reader (EnSpire, PerkinElmer, USA). The ATP levels were calculated according to an ATP standard curve established by gradient dilution of the ATP standard supplied with the kit, and the results are expressed as μmol (μM). Three independent experiments were assessed in triplicate.

2.11. Detection of mitochondrial membrane potential ($\Delta\Psi\text{m}$)

The changes in the mitochondrial membrane potential ($\Delta\Psi\text{m}$) of extracellular *T. gondii* tachyzoites treated with niclosamide were evaluated using a JC-1 assay kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's instructions. JC-1 (lipophilic cation 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a cationic and lipophilic mitochondrial vital dye that becomes concentrated in the mitochondria in response to $\Delta\Psi\text{m}$. The dye exists as a monomer at low $\Delta\Psi\text{m}$ (emission wavelength of 530 nm, green fluorescence) but forms J-aggregates at high $\Delta\Psi\text{m}$ (emission wavelength of 590 nm, red fluorescence). Thus, the change in the fluorescence colour of JC-1 is considered an indicator of the mitochondrial state.

Fresh tachyzoites (2×10^6 /per experimental group) collected as previously described were incubated with different concentrations of niclosamide (50, 100, or 200 ng/mL) in DMEM for 8 h at 37 $^{\circ}\text{C}$. The *T. gondii* tachyzoites in the positive and negative parasite control groups were incubated in only DMEM with no drug, and after 8 h of incubation, the negative parasite control samples were further incubated for 20 min with 10 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), a protonophore that can cause $\Delta\Psi\text{m}$ depletion. Subsequently, all the samples were centrifuged at 1500 $\times g$ for 10 min, and the tachyzoites were then incubated in 1 mL of a staining solution containing the potentiometric probe JC-1 for 20 min at 37 $^{\circ}\text{C}$ in the dark. The samples

were then rinsed twice with buffer and centrifuged at $1500 \times g$ for 10 min. The samples were then suspended in 200 μL of buffer solution, and the fluorescence intensity was then observed by laser confocal microscopy. The ratio of the JC-1 red to green fluorescence intensity was assessed using a multilabel reader (EnSpire, Perkin Elmer, USA).

2.12. Statistical analyses

The data were plotted using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). The results are presented as the means \pm standard deviations (S.Ds.) of data from at least three independent experiments. The calculated IC_{50} of niclosamide on HFF cell growth and the calculated EC_{50} of niclosamide on tachyzoite growth inhibition were plotted as functions of the drug concentration through a nonlinear curve analysis using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The *in vitro* results of the anti-invasion and anti-proliferation tests as well as the ATP and $\Delta\Psi\text{m}$ levels in the parasite control and treatment groups were analysed by one-way analysis of variance (ANOVA) using SPSS. The *in vivo* results of the survival rate and parasite burden in the drug treatment and parasite control groups, as well as the body weight and body temperature, were also analysed by ANOVA using SPSS. Differences were considered statistically significant at a P value ≤ 0.01 .

3. Results

3.1. Cytotoxicity activity

A cytotoxicity assay using an HFF cell line was performed to evaluate the effects of niclosamide on cell viability. Niclosamide had no effect on the growth of HFF cells at concentrations below 0.125 $\mu\text{g}/\text{mL}$ (Fig. 1). The results showed that niclosamide dose-dependently inhibited cell growth, with an IC_{50} value of 8.3 $\mu\text{g}/\text{mL}$. The final concentrations of DMSO in the cultures did not exceed 1% (v/v) and had no effect on cell proliferation. This assay established a safe dose range for subsequent tests.

3.2. Anti-proliferation activity

The ability of niclosamide to inhibit the intracellular replication of

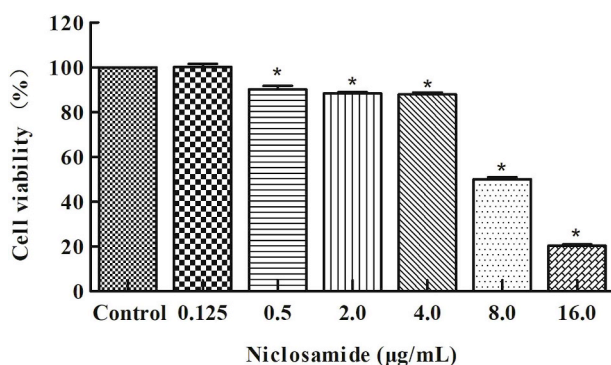


Fig. 1. Niclosamide cytotoxicity in HFF cells. HFF monolayer cells were cultured in the absence (control) or presence of niclosamide (0.125, 0.5, 2.0, 4.0, 8.0, or 16 $\mu\text{g}/\text{mL}$) for 48 h. Then, 15 μL of MTT solution (5 mg/mL) in DMEM was added, and the cells were incubated for 4 h at 37 $^{\circ}\text{C}$ in a 5% CO_2 atmosphere. Formazan crystals were extracted by incubation with DMSO (150 $\mu\text{L}/\text{well}$) at 37 $^{\circ}\text{C}$ for 10 min, and the absorbance of the 96-well plate at 570 nm was read using a Multiskan GO instrument. All the data are presented as the means \pm standard deviations (S.Ds.), and the experiments were performed in triplicate. * $P \leq 0.01$ compared with the control.

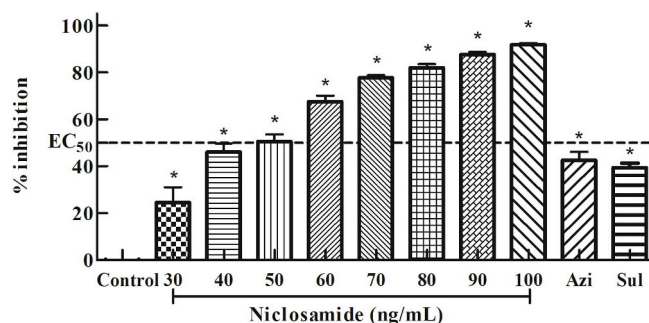


Fig. 2. Anti-proliferation effect of niclosamide treatment on *T. gondii* infection in HFF cells as determined by qPCR. HFF cells were infected with 2×10^4 *T. gondii* for 6 h and then treated with niclosamide (30, 40, 50, 60, 70, 80, 90, or 100 ng/mL), azithromycin (8.6 $\mu\text{g}/\text{mL}$), sulfadiazine (0.4 $\mu\text{g}/\text{mL}$) or without drug (parasite control). After 24 h of treatment, the amount of *T. gondii* DNA in each group was measured by targeting the 529-bp repeat element of the *T. gondii* genome using qPCR. The percent inhibition of tachyzoite proliferation in each group was compared with that in the parasite control group. The data are presented as the means \pm S.Ds. of three independent experiments. * $P \leq 0.01$ compared with the parasite control.

tachyzoites within HFF cells was evaluated. A Giemsa staining analysis with light microscopy revealed that the amount of tachyzoites decreased with increases in the concentration of niclosamide (30–100 ng/mL), as shown in Fig. S1 (A – E). Furthermore, the number of tachyzoites was detected by qPCR, and the qPCR results, which as shown in Fig. 2, revealed that niclosamide significantly ($P \leq 0.01$) inhibited the intracellular replication of *T. gondii* in a concentration-dependent manner at the concentration range of 30–100 ng/mL. The EC_{50} of niclosamide on tachyzoite growth inhibition was 45.3 ng/mL. In addition, the positive drug groups showed 42.6% (azithromycin) and 39.5% (sulfadiazine) inhibition, respectively.

3.3. Assessment of anti-*T. gondii* invasion

Two hours after infection, the niclosamide-mediated inhibition of invasion was measured as percentages of HFF cells, as shown in Fig. 3. Concentration-dependent reductions in tachyzoite invasion were also observed in the niclosamide-treated cell samples, and these decreases were significantly ($P \leq 0.01$) compared with the parasite control group

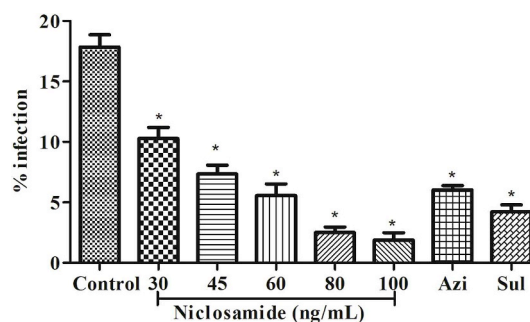


Fig. 3. Effects of niclosamide on *T. gondii* invasion. HFF cells were cultured in DMEM with niclosamide (30, 45, 60, 80, or 100 ng/mL), azithromycin (8.6 $\mu\text{g}/\text{mL}$), sulfadiazine (0.4 $\mu\text{g}/\text{mL}$) or no drug (parasite control) and infected with 2×10^6 *T. gondii* tachyzoites. Two hours after infection, the cells were stained with Giemsa stain to determine the anti-invasion capability of niclosamide. The percentage of infected cells in each group was determined by light microscopy. The independent experiments were assessed in triplicate, and at least 20 random fields per well were counted. The data are presented as the means \pm S.Ds. * $P \leq 0.01$ compared with the parasite control.

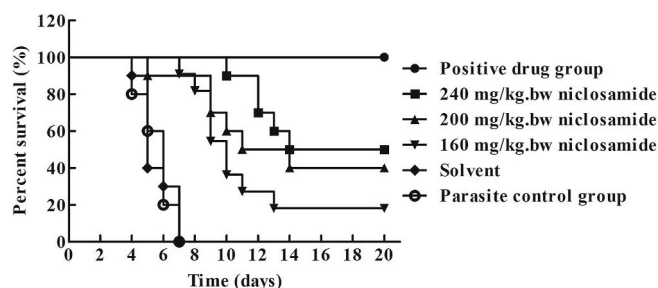


Fig. 4. Effect of niclosamide on the survival rate of acutely infected mice. All the mice were infected with 2×10^3 *T. gondii* tachyzoites and then treated with niclosamide (160, 200 or 240 mg/kg bw), positive drug (100 mg/kg bw sulfadiazine, 50 mg/kg bw pyrimethamine or 15 mg/kg bw folinic acid), solvent or PBS (parasite control) once daily for 7 days. The mice were then observed for an additional 13 days, and the survival times of the infected mice were recorded for 20 days.

(17.8%). The infection percentages of the positive drug groups were 4.2% (sulfadiazine) and 6.0% (azithromycin).

3.4. Survival rate of acutely infected mice treated with niclosamide

All the healthy mice treated with niclosamide and the positive control drugs survived, as shown in Fig. S2, which indicated that the test drugs did not result in the death of uninfected mice under our experimental conditions.

To determine the *in vivo* anti-*T. gondii* effect of niclosamide, we subsequently examined the survival rate of *T. gondii*-infected mice during a 20-day test period. All the infected mice in the parasite control and solvent-treated groups succumbed to infection by day 7, as shown in Fig. 4. After the 7-day period of continuous administration, niclosamide had a significant ($P \leq 0.01$) dose-dependent effect, as demonstrated by survival of 20%, 40% and 50% of the infected mice in the groups treated with doses of 160, 200 and 240 mg/kg bw, respectively. All the mice in the positive drug groups survived.

3.5. Parasite load in mouse tissues or blood after niclosamide treatment

To further evaluate the parasite load in the mice after drug treatment, blood, liver, spleen and brain samples from infected mice were assayed by qPCR, and the results are shown in Fig. 5A. Treatment with niclosamide significantly ($P \leq 0.01$) reduced the parasite load in the blood, brain, spleen and liver tissues compared with the parasite control group after PBS treatment. We also monitored the body weight and body temperature of each group during the experiment, as shown in Fig. 5B and C. Our findings indicated that the body temperature was significantly ($P \leq 0.01$) decreased in the parasite control group at day 7 compared with that at day 1, and the same trend was found for the body weight. However, no obvious changes in body weight or body temperature were found in the mice treated with niclosamide.

3.6. Effect of niclosamide on mitochondrial function

To explore whether the effects of niclosamide on *T. gondii* are associated with oxidative phosphorylation, the ATP content and $\Delta\Psi_m$ were measured. After 8 h of treatment with niclosamide, the ATP concentrations of the extracellular parasites in each group were measured (Fig. 6). Increases in the concentrations of niclosamide significantly ($P \leq 0.01$) decreased the levels of ATP compared to those in the parasite control group.

The changes in the $\Delta\Psi_m$ in each group, as assessed using the

fluorescent probe JC-1, are shown in Fig. 7. Under a confocal laser scanning microscope, the positive parasite control group showed intense red fluorescence, indicating a normal $\Delta\Psi_m$ (Fig. 7A), whereas intense green fluorescence was observed in the negative parasite control, indicating that the addition of CCCP resulted in complete loss of the $\Delta\Psi_m$ (Fig. 7B). In the niclosamide-treated groups, the red intensity decreased and the green intensity increased, the fluorescence intensity of the group treated with 200 ng/mL niclosamide group is shown in Fig. 7C. Moreover, the ratios of JC-1 red to green fluorescence intensities in the positive and negative parasite control groups were 2.5 and 1.8, respectively. Niclosamide treatment decreased the JC-1 red/green fluorescence intensity ratios compared with that of the positive parasite control group, as shown in Fig. 7D. These results indicated that niclosamide decreased the $\Delta\Psi_m$ of extracellular *T. gondii* tachyzoites.

4. Discussion

Niclosamide is an anthelmintic drug approved by the US FDA for the treatment of intestinal parasite infections. Additionally, niclosamide is the only molluscicide recommended by the World Health Organization (WHO) and has been widely used for the killing of *Oncomelania* and thus prevent and cure snail fever for many years (Goennert, 1961; Pearson and Hewlett, 1985). In the present study, niclosamide demonstrated activity against *T. gondii* RH strain tachyzoites. Niclosamide affects the intracellular replication and invasion of *T. gondii* tachyzoites in a concentration-dependent manner, as determined through *in vitro* anti-proliferation and anti-invasion assays. Furthermore, niclosamide showed low cytotoxicity in HFF cells, and the IC_{50} of niclosamide for HFF cells was 183-fold higher than the EC_{50} against *T. gondii*, which demonstrates that niclosamide has a high therapeutic index and that the use of niclosamide has a wide safety range. Accordingly, we can conclude that niclosamide presents excellent anti-*T. gondii* activity *in vitro*.

Based on the *in vitro* findings described above, we sought to determine whether niclosamide exerts anti-*T. gondii* effects on acute infections *in vivo*. To that end, a mouse model was established by infecting mice with the virulent RH strain of *T. gondii*. Before that, we treated healthy mice with the same doses of niclosamide, and all the mice survived. According to Andrews et al. (1982), the 50% lethal dose (LD_{50}) of niclosamide administered orally in female mice was 1500 mg/kg bw. The niclosamide doses used in this study were well below this threshold and did not result in the death of healthy mice under our experimental conditions. Furthermore, a previous study found that the administration of niclosamide as a single dose of 160 mg/kg or two doses at a 3-day interval showed 91.1% and 92.6% efficacy, respectively, against *paramphistomes* in the small intestine without any toxic effects (Rolfe and Boray, 1987). However, the administration of niclosamide at a dose of 240 mg/kg bw could only protect 50% of the acutely *T. gondii*-infected mice. This limited effect observed *in vivo* might be due to the poor solubility of niclosamide, which would substantially limit its oral absorption (Andrews et al., 1982). The plasma concentration-time data obtained from mice after the oral administration of 200 mg/kg niclosamide showed that the peak concentration (C_{max}) was 893.7 ng/mL at the peak time ($T_{max} = 15$ min), rapidly decreased at 30–45 min to a low plasma concentration of 50–40 ng/mL, and then decreased at a slower rate (Osada et al., 2011). Accordingly, the plasma concentration after the once-daily oral administration of 200 mg/kg niclosamide was too low, indicating that this drug *in vivo* could not be maintained at a level above the effective inhibition concentration (EC_{50}) over a 24-h period. *T. gondii* is an intracellular parasite, and the low plasma concentration of niclosamide might result in poor anti-parasite efficacy *in vivo*. However, the parasite burdens in the liver, spleen, brain or blood after niclosamide treatment were

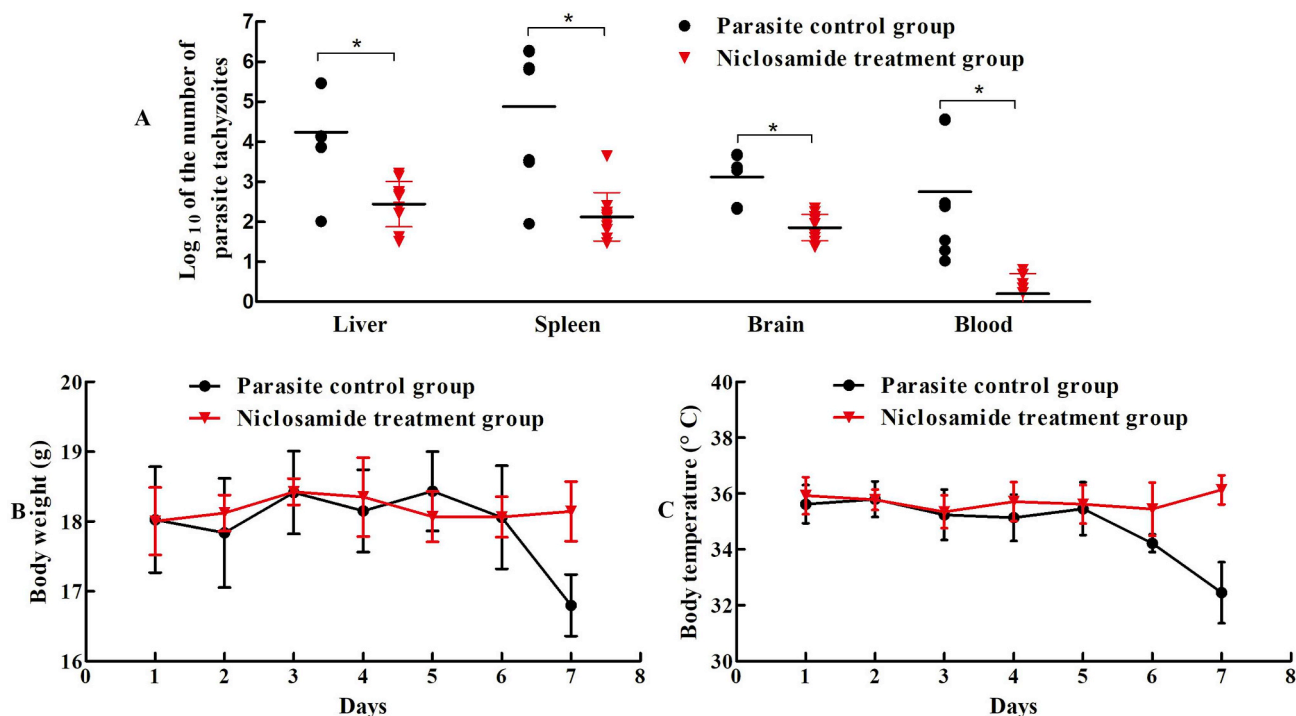


Fig. 5. Niclosamide treatment reduced the parasite burden in blood or tissue from the acutely infected mice. The mice were challenged intraperitoneally with 2×10^3 *T. gondii* tachyzoites, treated with niclosamide (240 mg/kg-bw) or PBS (parasite control) once daily for 7 days. The body weight and body temperature were detected once daily, and on day 7, the parasite loads in the blood, brain, liver and spleen tissues of the infected BALB/c mice were isolated and homogenized. Total genomic DNA was isolated, and the *T. gondii* 529-bp gene was detected by qPCR. The quantified parasite loads in the tissues and blood of mice are presented as the \log_{10} values of the numbers of tachyzoites per 20 mg of tissue or 50 μL of blood (A). The body weight (B) and body temperature (C) are presented as the means \pm S.Ds. * $P \leq 0.01$ compared with the parasite control.

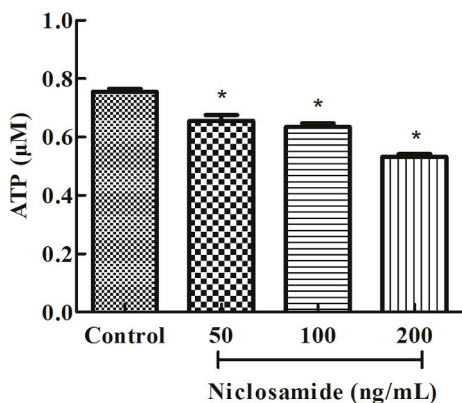


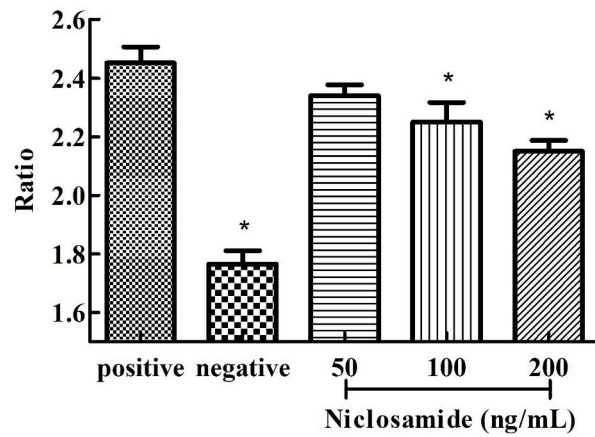
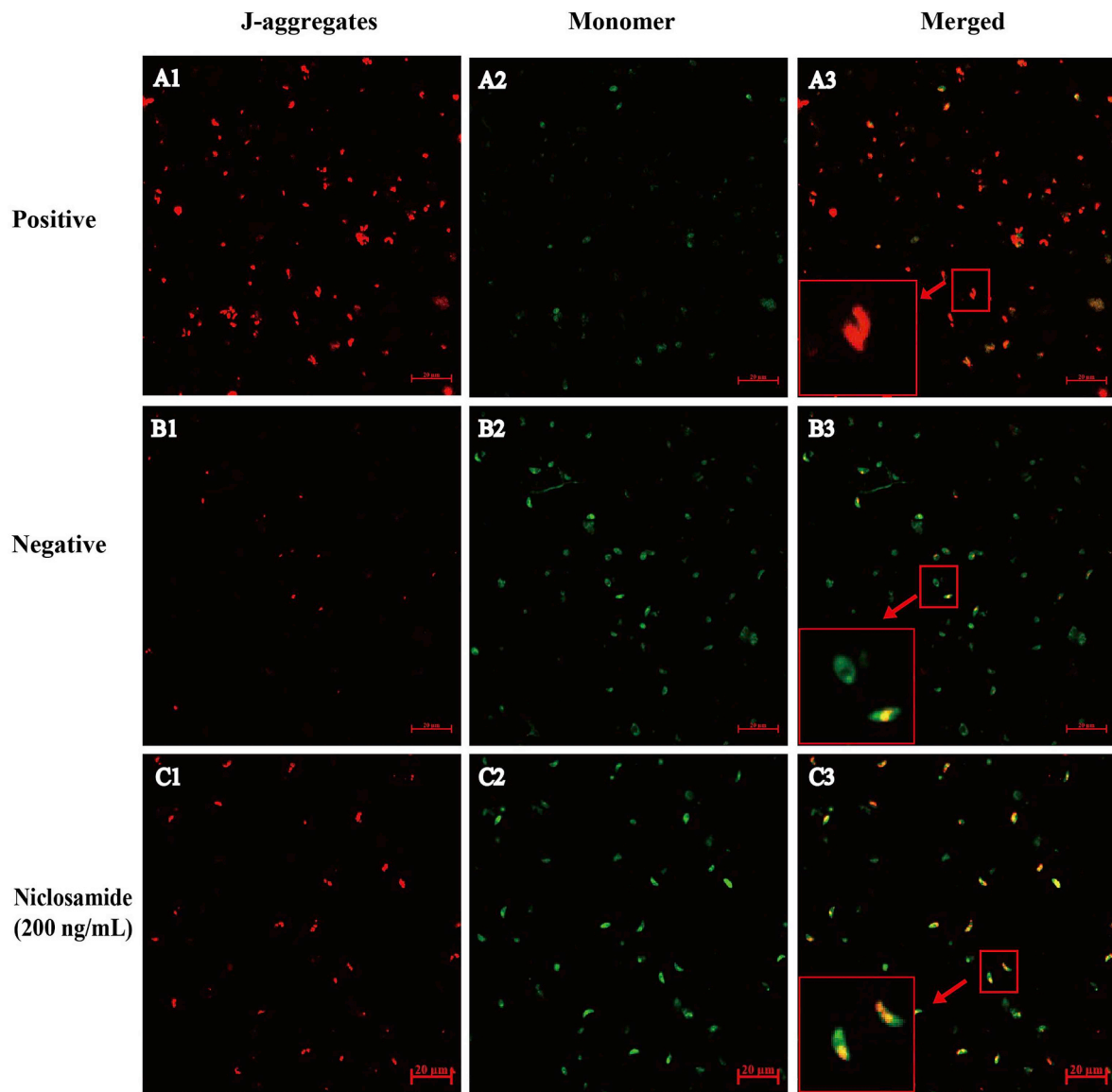
Fig. 6. Niclosamide induced a decrease in the concentration of ATP. Extracellular *T. gondii* tachyzoites ($2 \times 10^6/\text{mL}$) was incubated with niclosamide (50, 100, or 200 ng/mL) or without drug (parasite control) in DMEM for 8 h at 37°C . Then, *T. gondii* tachyzoites were lysed to determine the ATP levels using a multilabel reader. ATP levels were expressed as μM . Three independent experiments were performed in triplicate. The data are presented as the means \pm S.Ds. * $P \leq 0.01$ compared with the parasite control.

significantly decreased compared with those in the parasite control group, and the body temperature and weight were also not obviously changed in this groups. These results also indicated that niclosamide exerts an inhibitory effect on *T. gondii*, but additional work is needed to improve its solubility, absorption and systemic bioavailability.

Nonetheless, our results indicate that niclosamide can partially provide protection against death due to *T. gondii* infection, reducing the parasite burden in the blood and tissues of mice.

Previous studies identified niclosamide as an oxidative phosphorylation uncoupler that interferes with the energy metabolism of helminths by inhibiting ATP production (Katz, 1986; Frayha et al., 1997). In our present study, niclosamide treatment induced dose-dependent decreases in the ATP level of *T. gondii*, and ATP deficiency might lead to an energy shortage in tachyzoites and block *T. gondii* replication and invasion. In addition, niclosamide treatment reduced the $\Delta\Psi\text{m}$ in tachyzoites, which might interfere with *T. gondii* mitochondrial function. Therefore, these emerging lines of evidence supports the notion that the action of niclosamide against *T. gondii* is likely associated with oxidative phosphorylation of the mitochondria. However, the exact mechanism of action needs to be further explored.

In conclusion, our findings suggest that niclosamide exerts activity against *T. gondii* by inhibiting its replication and invasion *in vitro* in the absence of host toxicity. The *in vivo* experiments showed that niclosamide protected mice with acute toxoplasmosis from death to a certain extent and reduced the parasite burden in the blood and tissue. Niclosamide might also interfere with the energy metabolism in *T. gondii* mitochondria, as suggested by the decreased $\Delta\Psi\text{m}$ and ATP levels. Therefore, the anthelmintic drug niclosamide has potential to be developed into a novel and safe anti-*Toxoplasma* agent. Further investigations of niclosamide are needed to improve its absorption and systemic bioavailability, evaluate its efficacy against different *T. gondii* strains and explore its anti-*T. gondii* mechanism of action.



D

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Fig. 7. Niclosamide decreased the $\Delta\Psi_m$ of extracellular *T. gondii* tachyzoites. *T. gondii* tachyzoites (2×10^6 /per sample) were incubated with niclosamide (50, 100, or 200 ng/mL) or no drug (parasite positive or negative control) in DMEM for 8 h at 37 °C. The parasite negative control samples were then further incubated with CCCP to deplete the $\Delta\Psi_m$. Subsequently, the tachyzoites in all the groups were incubated with a staining solution containing the potentiometric probe JC-1 for 20 min at 37 °C in the dark. The fluorescence intensity was observed by laser confocal microscopy: positive parasite control group (A); negative parasite control group (B); and group treated with 200 ng/mL niclosamide (C). The JC-1 red/green fluorescence intensity ratio of each group was assessed using a multilabel reader (D). The results are presented as the means \pm S.Ds. from at least three independent experiments. * $P \leq 0.01$ compared with the positive parasite control group. Scale bars: 20 μm . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2018.12.004>.

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Author contributions

XS, XZZ, XKZ and BL revised the manuscript. HS evaluated the anti-proliferation activity of niclosamide *in vitro*. JYZ directed the project and reviewed the manuscript. JLZ supervised the experiments and wrote the manuscript. All the authors read and approved the final manuscript.

Declarations of interests

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The final article has been approved by all the authors.

Ethics approval and consent to participate

All experimental procedures were approved and performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Lanzhou Institute of Animal Science and Veterinary Pharmaceutics.

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