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PROTOZOOLOGY - ORIGINAL PAPER

Triclosan inhibits the growth of *Neospora caninum* in vitro and in vivo

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Abstract

Neospora caninum is an apicomplexan parasite considered one of the main causes of abortion in cattle worldwide; thus, there is an urgent need to develop novel therapeutic agents to control the neosporosis. Enoyl acyl carrier protein reductase (ENR) is a key enzyme of the type II fatty acid synthesis pathway (FAS II), which is essential for apicomplexan parasite survival. The antimicrobial agent triclosan has been shown to be a very potent inhibitor of ENR. In this study, we identified an *E. coli* ENR-like protein in *N. caninum*. Multiple sequence alignment showed all the requisite features of ENR existed in this protein, so we named this protein NcENR. Swiss-Model analysis showed NcENR interacts with triclosan. We observed that ENR is localized in the apicoplast, a plastid-like organelle. Similar to the potent inhibition of triclosan on other apicomplexa parasites, this compound markedly inhibits the growth of *N. caninum* at low concentrations. Further research showed that triclosan attenuated the invasion ability and proliferation ability of *N. caninum* at low concentrations. The results from in vivo studies in the mouse showed that triclosan attenuated the virulence of *N. caninum* in mice mildly and reduced the parasite burden in the brain significantly. Taken together, triclosan inhibits the growth of *N. caninum* both in vitro and in vivo at low concentrations.

Keywords *Neospora Caninum* · Type II fatty acid synthesis pathway · Enoyl acyl carrier protein reductase · Triclosan

Background

Neospora caninum is an obligate intracellular protozoan parasite belonging to the phylum apicomplexa and is a cause of abortions and neonatal mortalities in cattle worldwide, resulting in significant economic losses (Dubey and Schares 2011; Dubey et al. 2007; Reichel et al. 2013). Therefore, there is an urgent need to develop new medicines for the treatment of neospora infections and protect cattle from abortions and vertical transmissions.

Several biochemical pathways located in the apicomplexa apicoplast, a relict plastid-like organelle, are the pursued targets for the development of agents against apicomplexa infections (Chakraborty 2016; Shears et al. 2015). The fatty acid biosynthesis pathway is a particularly attractive target, as the enzymes catalyzing this process in apicomplexa and mammalian are structurally different (Goodman and Mcfadden 2007; Jolly et al. 2006). In mammalian cells, the enzymes involved in this process are multifunctional proteins, by which the cell accomplishes fatty acid synthesis and each step is carried out by different domains of these large proteins. This is classified as a type I fatty acid synthesis pathway. In apicomplexa parasites, however, type II fatty acid synthesis pathway is used,

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and each step of fatty acid synthesis is catalyzed by different enzymes, which is quite disparate to the single multifunctional enzyme of the mammalian system. These differences have already been exploited by some inhibitors that selectively inhibit *Plasmodium falciparum* and *Toxoplasma gondii* rather than host cell (Mcleod et al. 2001; Surolia and Surolia 2001).

In apicomplexa parasite *Toxoplasma gondii*, mutation of the acyl carrier protein (ACP), a key molecule of the FAS II pathway, affects the apicoplast biogenesis and results in the death of the parasite, suggesting the FAS II pathway is important for the survival of the parasite (Jolly et al. 2006). However, this pathway is not indispensable for *Plasmodium* at the bloodstream and mosquito stages, although it is essential for the liver stage that is required for the establishment of infection in the mammalian host (Vaughan et al. 2010; Yu et al. 2008).

Enoyl acyl carrier protein reductase (ENR) catalyzes the NAD $(P)^+$-dependent reduction of a trans-2,3 enoyl moiety into a saturated acyl chain, which is the final step of the FAS II pathway, controlling the rate of fatty acid elongation rounds (Heath and Rock 1995). Some studies on ENR which was inhibited by compounds, such as the diazaborines (Baldock et al. 1996; Turnowsky et al. 1989) and triclosan (El-Zawawy et al. 2015; Mcleod et al. 2001; Surolia and Surolia 2001), have identified the enzyme as an optimum target for the development of new agents. In particular, triclosan, which is used in many consumer products, including soaps and toothpaste, is an extremely potent ENR inhibitor (Ward et al. 1999). Existing research shows triclosan inhibits several apicomplexa protozoa efficiently, such as *Plasmodium falciparum*, *Toxoplasma gondii* (Mcleod et al. 2001), *Babesia* (Sabine et al. 2003), *Eimeria tenella* (Lu et al. 2007), and *Trypanosoma brucei* (Paul et al. 2004).

Given the important role of ENR in the type II fatty acid synthesis pathway, we investigated the biological characteristics of NcENR and tested the effects of triclosan on the enzyme in vitro and in vivo. In our work, we identified ENR in *N. caninum* and observed the enzyme is localized in the apicoplast. The specific inhibitor of ENR, triclosan, was found to inhibit *N. caninum* through inhibition of its invasion and proliferation. In addition, triclosan inhibited *N. caninum* in vivo, attenuated the virulence in mice mildly, and reduced the parasite burden in the brain significantly. Together, the existence of ENR in *N. caninum* and the anti-neospora activity of triclosan provide attractive targets for the development of new agents targeting *N. caninum* infections.

Methods

Host cells and parasite culture

African green monkey kidney cell (Vero) and human foreskin fibroblast (HFF) (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 25 mM glucose and 4 mM glutamine and supplemented with 10% or 15% fetal bovine serum (FBS, Gibco, USA) as previously described (Hui et al. 2014). The *Neospora caninum* Nc-1 strains were maintained by continuous passages in Vero cells incubated at 37 °C and 5% CO_2.

Bioinformatics analysis of NcENR

The sequences of NcENR were obtained from ToxoDB (http://toxodb.org/toxo/). Batch CD-Search (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi) was used to analyze the domains of the protein. The multiple sequence alignment was analyzed by DNAMAN (Lynnon Biosoft, USA) and three-dimensional structure modeling was performed by Swiss-model (https://swissmodel.expasy.org/).

Cloning of NcENR and preparation of anti-rNcENR polyclonal antibody

The coding sequence of *N. caninum* ENR was cloned from cDNA of the Nc-1 strain and linked with the pET28a vector (New England Biolabs), producing a recombinant protein fused with His Tags. Then, the recombinant plasmid, pET28a-NcENR, was transformed into BL21 Star (DE3) cells (Invitrogen). Cells were cultured in LB medium at 37 °C to an optical density at 600 nm of 0.8 and the expression of recombinant protein was induced by 0.1 mM IPTG at 37 °C for 6 h in shaker flasks.

The recombinant protein NcENR (rNcENR) was purified through HisTrap FF purification columns (Novagen, Germany) and identified by reducing SDS-PAGE (H_2O, 30% acrylamide, 1.5 M Tris-HCl (pH 8.8), 10% SDS, 10% ammonium persulfate, TEMED). To produce polyclonal antibody against NcENR, BALB/c mice were immunized with the purified rNcENR (100 μg) mixed with the same volume of Freund's complete adjuvant (Sigma, USA) for the first time. Booster immunization was carried out by immune the mice with rNcENR (50 μg) mixed with the same volume of Freund's incomplete adjuvant (Sigma, USA) for twice after a 2-week interval. Anti-rNcENR polyclonal antibody was collected after 10 days of the last immunization.

Western blotting

Purified tachyzoites were lysed with the RIPA buffer (Beyotime, China) supplemented with protease inhibitors cocktail (Sigma, USA). After a 12% (w/v) SDS-PAGE, the protein strips were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA), blocked with 5% skim milk (BD Difco, USA) in PBS (w/v) for 1 h at 37 °C and then incubated with the mouse anti-NcENR

polyclonal antibody (prepared above) for 1 h at 37 °C. After washing with PBST (0.1% Tween-20) three times, the membranes were incubated with horseradish peroxidase (HRP)–labeled goat anti-mouse IgG (H+L) secondary antibody (1:5000) (Sigma, USA) for 1 h. Finally, the chemiluminescence (CoWin Biotech Co., Ltd., China) was adopted to visualize the protein bands.

Indirect immunofluorescence staining

Parasites cultured in HFF monolayers on coverslips were fixed with 4% formaldehyde, permeabilized with 0.25% Triton X-100 for 20 min, blocked with 3% bovine serum albumin (BSA) at 37 °C for 1 h, and then incubated with mouse anti-NcENR polyclonal antibody (1:50) (prepared above) or mouse anti-NcACP polyclonal antibody (1:100) (preserved in our lab) or rabbit anti-NcENR polyclonal antibody (1:100) (preserved in our lab) or rabbit anti-NcSRS2 polyclonal antibody (1:100) (preserved in our lab) at 37 °C for 1 h. After washing with PBS three times, the parasites were incubated with FITC-conjugated goat anti-mouse IgG (H+L) (1:50) (Sigma, USA) and Cy3-conjugated goat anti-rabbit IgG (H+L) (1:100) (Sigma, USA). Images were captured using a fluorescence microscope (IX71, Olympus, Japan) or Leica confocal microscope system (Leica, TCSSP52, Germany).

Giemsa stain

Freshly released tachyzoites were inoculated in confluent HFF cells; after 45 min, the extracellular parasites were washed with Hanks' balanced salt solution (HBSS; Gibco Inc., USA), and then the medium with 10 mM triclosan (dissolved in DMSO) or 0.1%DMSO (negative control) was added. The glass coverslips were taken out from the plates after 24-, 48-, and 72-h treatment, washed with PBS, and then fixed with methanol before staining with Giemsa (Sigma Inc., USA). Images were observed and captured by using a fluorescence microscope.

MTS assay

Vero cells were inoculated in 96-well plates (6×10^3 cells/well) first; after the cells had adhered on the wall, the freshly released *N. caninum* tachyzoites were inoculated into the plates (6×10^4 tachyzoites in 100 μl medium per well, MOI (multiplicity of infection) ≈ 10:1). After 6 h, the wells were washed and replaced with the RPMI 1640 medium. After 18 h, the medium was changed with RPMI 1640 supplemented with 2% FBS along with different concentrations of triclosan (final concentrations 1–16 μM) or 0.1% DMSO. After treatment for 24 h, anti-neospora activity and host cell cytotoxicity of triclosan were evaluated by MTS assay using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega Biotech, Beijing, China). The OD value was determined by a microplate reader (Model 680, Bio-Rad, Richmond, CA, USA) at a wavelength of 450 nm. Cell viability was shown as a percentage of the OD value of 0.1% DMSO control well. The 50% cytotoxicity concentration (CC50) expressed the concentration of triclosan reducing cell growth by 50%, and the 50% effective concentration (EC50) expressed the concentration of triclosan inhibiting *N. caninum*–induced cytopathic effect by 50%.

Invasion assay

Freshly released tachyzoites were harvested and treated with either 6 μM triclosan or 0.1% DMSO for 1 h. Then, approximately 1×10^6 parasites were inoculated in confluent HFF cells in 12-well plates (MOI ≈ 20:1). After 45 min, the extracellular parasites were removed by washing with PBS three times. After 24 h, indirect immunofluorescence assay (IFA) was performed. The number of cells and parasitophorous vacuoles (PVs) were counted from several random fields by a fluorescence microscope. The invasion efficiency was calculated by the number of PVs divided by the number of cells.

Proliferation assay

Freshly released tachyzoites were inoculated in HFF cells for 45 min to allow tachyzoite invasion, followed by washing with PBS and then treating with 10 μM triclosan or DMSO for 24 h; then, IFA was performed. The parasites were stained by rabbit anti-SRS2 polyclonal antibody (1:50, preserved in our lab) and then FITC-conjugated goat anti-rabbit IgG (1:50, Proteintech Group Inc., Chicago, IL, USA). The proliferation stages were calculated by counting the tachyzoite numbers of each PV (i.e., 1, 2, 4, or 8 tachyzoites) through a fluorescence microscope. One hundred PVs were calculated in each experiment.

The in vivo anti–*N. caninum* activity of triclosan

We determined the in vivo activity of the inhibitor with a 30-day suppressive test. BALB/c mice were intraperitoneally inoculated with 5×10^6 tachyzoites. On day 1, the treatment was started: triclosan dissolved in soybean oil (for injection) (M&C Gene Technology (Beijing) LTD) was administered subcutaneously every other day, over a period of 30 days. During this period, the clinical signs and mortality of the mice were monitored every 8 h. Meanwhile, we set the control group of mice that inoculated with 5×10^6 tachyzoites but not treated with triclosan, and the drug control group of mice that were only treated with triclosan. To determine the appropriate concentration of triclosan used in this assay, a pilot study has been performed previously. Briefly, mice inoculated with 8×10^6 tachyzoites (absolute lethal dose)

were treated with triclosan in dose schedules of 0, 5, 10, 15, and 20 mg/kg every other day and recorded their clinical signs and mortality over a period of 30 days. The minimal effective and clinically safe dose was chosen to perform the experimental design. The parasites burden in the mouse brains were monitored at the 30th day as described previously (Ma et al. 2017a; Ma et al. 2017b).

Statistical analysis

The statistical analysis of all the data was performed using GraphPad Prism 5 v. 5.01 (San Diego, CA, USA). EC50 and CC50 were calculated using non-linear regression and the cell viability in MTS assay was statistically analyzed using the Student *t* test. The invasion efficiency and the parasites burden results were statistically analyzed using the Student *t* test. The results were expressed as mean ± SEM and values of $P < 0.05$ were considered statistically significant.

Results

Enoyl acyl carrier protein reductase is a conserved protein in *N. caninum*

To get information about ENR in *N. caninum*, we indexed the ToxoDB (ver. 9.0) database to search for ENR genes. We found a putative enoyl acyl carrier reductase (NCLIV_066970), which has a 74% similarity to the ENR in *Toxoplasma*, another apicomplexan parasite similar to *N. caninum*, at the amino acid level (Fig. 1a). We therefore named this putative enzyme NcENR. We then searched and

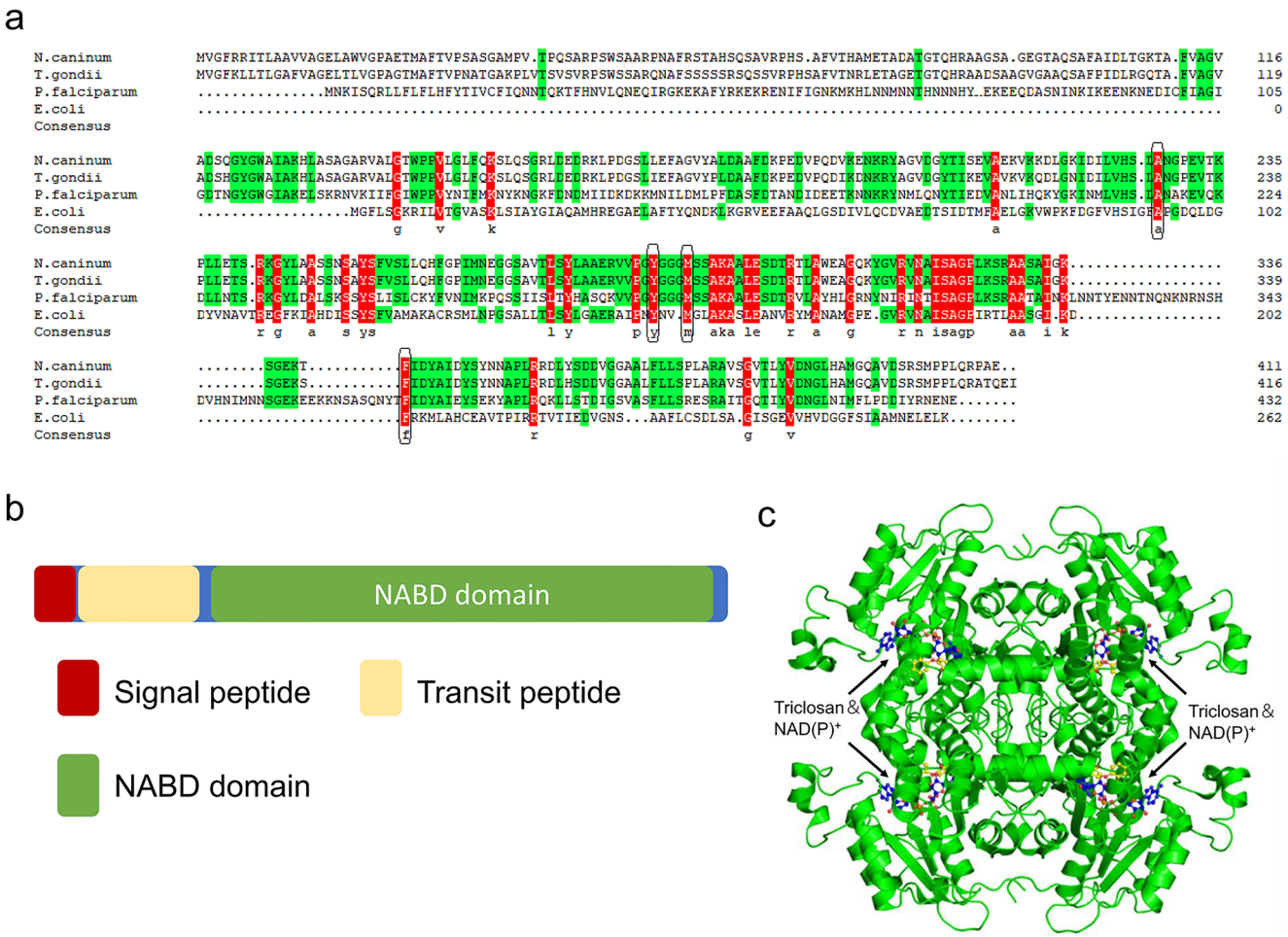

Fig. 1 ENR is a conserved enzyme among protozoa. **a** Multiple sequence alignment of ENR in *N. caninum*, *T. gondii*, *P. falciparum*, and *E. coli*. The active sites that specifically bound the triclosan are indicated by black rectangular frames. Dots indicate gaps or missing residues. The green shades stand that the amino acid residue is conserved among three-quarters of the organism and the red shades stand that the amino acid residue is conserved among the four organisms. **b** The predicted secondary structure of ENR. Red bar, signal peptide; yellow bar, transit peptide; green bar, NABD domain. **c** A structural model of ENR-NAD(P)$^+$-triclosan complex predicted by the Swiss-Model. NcENR is green and triclosan-NAD(P)$^+$ is indicated on the diagram

analyzed the conserved domains of NcENR using the Batch CD-Search in the Conserved Domain Database (CDD). The CD-Search results revealed that NcENR has a signal peptide, after which there is a transit peptide, driving the protein to the apicoplast. Additionally, we observed an NABD domain, which can potentially bind NAD(P)H or $NAD(P)^+$ to complete the enzyme's function (Fig. 1b). By analyzing the conserved sequences, we confirmed that NcENR has all the residues that are crucial for the activity of the enzyme (Mili et al. 2004), suggesting the possibility that its activity could be inhibited by triclosan, an inhibitor of ENR in other organisms (Fig. 1a). Based on this, we modeled ENR with triclosan and $NAD(P)^+$ using the Swiss-Model. The result showed that they formed the complex of ENR-$NAD(P)^+$-triclosan, which is inactive for the synthesis of fatty acid (Fig. 1c). Taken together, we proposed that ENR is conserved in *N. caninum* and may be inhibited by triclosan.

NcENR is localized in the apicoplast

To explore the localization of NcENR in *N. caninum*, the signal peptide and transit peptide truncated NcENR protein fused with His Tags was expressed in BL21 Star (DE3) cells (Fig. 2a) and used to produce anti-rNcENR polyclonal antibody in mice. Western blot showed there are two forms of ENR in *N. caninum*, a precursor form (~ 42 kDa) and a mature form (~ 35 kDa) (Fig. 2b), which is in agreement with the previous research that the transit peptide on ENR needed to be truncated before its translocation to the apicoplast (Muench et al. 2006). IFA showed that ENR was colocalized with acyl carrier protein (ACP), an apicoplast marker (Fig. 2c). The results suggest that NcENR is localized in the apicoplast, and this is in agreement with our previous conclusion that NcENR is an enzyme participating in the type II fatty acid synthesis pathway localized in the apicoplast.

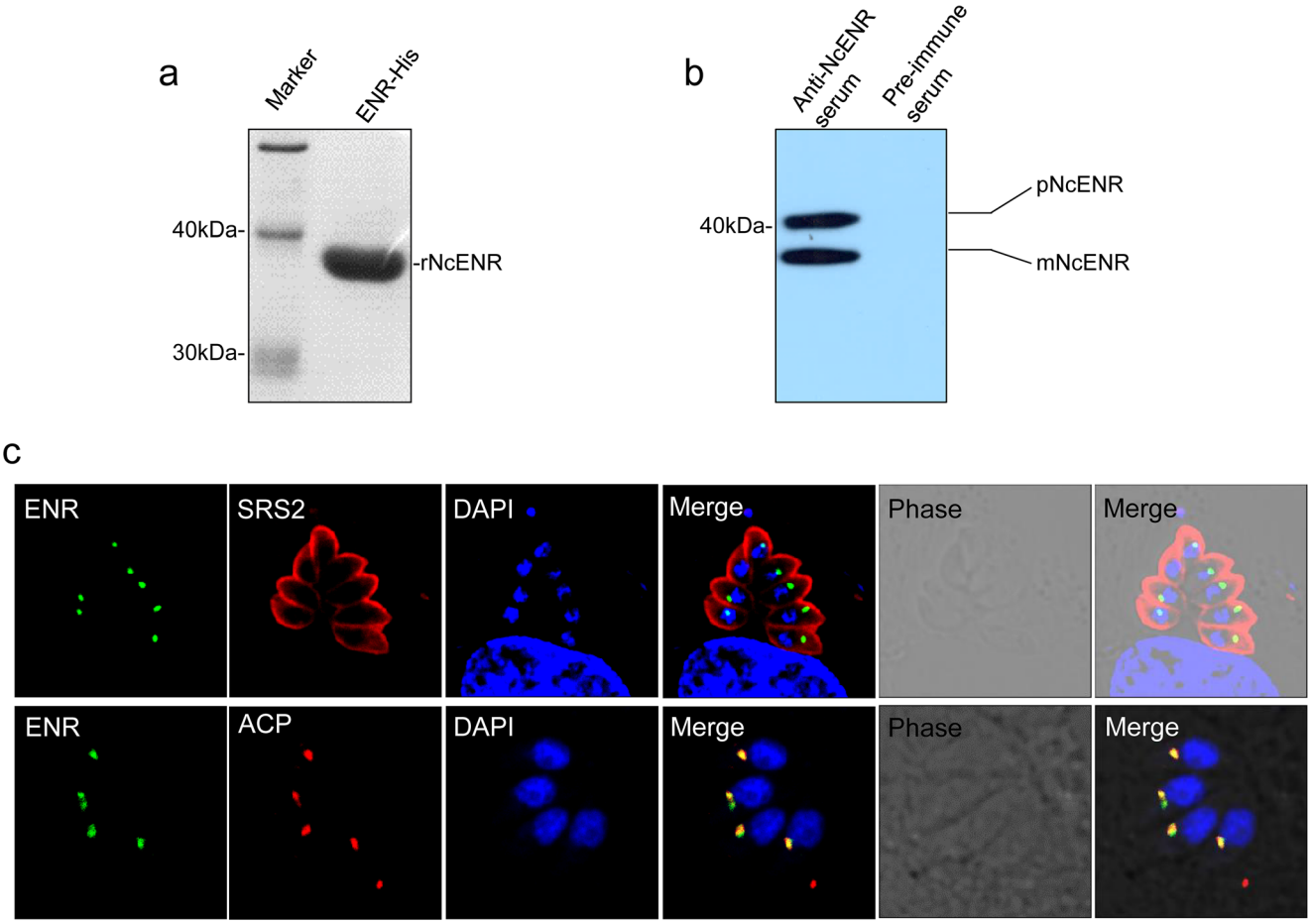

Fig. 2 ENR is expressed in *N. caninum* and localized in the apicoplast. **a** Expression and purification of the recombinant truncated NcENR without the signal and transit peptides, then analysis by SDS-PAGE. **b** Western blot analysis using ENR antibodies showed that ENR is expressed in the tachyzoite stage of *N. caninum* and NcENR exists in precursor (~ 42 kDa) and mature forms (~ 35 kDa). p, precursor form; m, mature form. **c** IFA analysis of NcENR localization. NcENR is localized in the apicoplast. In the upper panel, tachyzoites were stained with mouse anti-rNcENR polyclonal antibody (green) and rabbit anti-rNcSRS2 polyclonal antibody (red, stains the shape of the parasites); tachyzoites in the lower panel were stained with rabbit anti-rNcENR polyclonal antibody (green) and mouse anti-rNcACP polyclonal antibody (red, marker of the apicoplast), and the nucleus DNA was stained with Hoechst (blue)

Triclosan inhibits *N. caninum* in vitro

To characterize the function of NcENR, a specific inhibitor of ENR, triclosan, was used. First, we observed the growth of *N. caninum* in HFF cell treated with triclosan. Through Giemsa stain, we observed the parasites exhibited normal growth in the PVs in the control group treated with DMSO after 24 h (Fig. 3a), 48 h, or 72 h; the tachyzoites were released from the cells and the cells were disrupted severely (Fig. 3b, c). However, in the group treated with triclosan, the parasites stop proliferating in the PVs after 24-h treatment (Fig. 3d), and after 48 h or 72 h, the cells were not be disrupted; we speculated the tachyzoites have died (Fig. 3e, f). The results suggest that triclosan inhibits the proliferation of *N. caninum* in the host cell.

To determine the EC50 of triclosan on *N. caninum* intracellularly, the MTS assay was used (Pan et al. 2016). Vero cells grew normally with low triclosan concentrations; however, when the concentrations of triclosan were greater than 10 μM, the cell viability was decreased, suggesting that the triclosan was cytotoxic to the cells at high concentrations, with a CC50 of 9 μM (Fig. 3g). The viability of the cells inoculated with *N. caninum* was increased with the increasing concentrations of triclosan, while the viability was decreased at concentrations toxic to the cell, suggesting that triclosan is capable of inhibiting *N. caninum*, with an EC50 of 6 μM, confirming our above results (Fig. 3h).

Triclosan inhibits the invasion and proliferation of *N. caninum*

To further investigate that triclosan inhibits *N. caninum* in vitro, the invasion and proliferation abilities of *N. caninum* were investigated using triclosan treatment. The invasion efficiency of freshly released tachyzoites exposed to 6 μM triclosan for 1 h was detected and compared with that of the DMSO control. We observed that the invasion ability of triclosan-treated tachyzoites was attenuated, and invasion efficiency decreased from 30 to 15% (Fig. 4a). In addition, proliferation assay was performed to detect the effect of triclosan on *N. caninum* intracellular growth. After incubation in HFF for 24 h, significantly more PVs of the triclosan-treated group contained 1 or 2 tachyzoites. In the DMSO control group, however, approximately 60% PVs developed to 4 tachyzoites and 10% PVs developed to 8 tachyzoites (Fig. 4b). These results suggest that triclosan inhibits *N. caninum* in vitro through weakening both its invasion ability and its intracellular proliferation ability.

Triclosan inhibits *N. caninum* growth in vivo

Then we assessed the anti–*N. caninum* activity of triclosan in vivo. We first determined the optimal dose of triclosan in vivo through a pilot assay. The minimal effective and clinically safe dose of triclosan (10 mg/kg) was chosen for the subsequent experimental design (data not shown).

Three groups of BALB/c mice (five mice per group) were given different treatments. The experimental group was intraperitoneally injected with 5×10^6 tachyzoites and then treated with 10 mg/kg triclosan every other day (Nc-1+triclosan group). The control group was only intraperitoneally injected with 5×10^6 tachyzoites (Nc-1 group). The triclosan control group was treated with only triclosan (triclosan group). Over a 30-day period, both groups infected with tachyzoites exhibited a 40% mortality rate. The Nc-1+triclosan group exhibited a delayed death by a few days and a slower rate of weight loss, while the difference was not significant between the two groups. Expectedly, the group treated with only triclosan gained weight and presented a good physical condition (Fig. 5a, b).

Furthermore, after the 30-day experimental period, the surviving mice were sacrificed, and the parasite burden in the brain was determined by qRT-PCR. As expected, the Nc-1+triclosan group had a significantly reduced parasite burden, while the parasite burden in the Nc-1 group is quadrupled that is significantly different from the Nc-1+triclosan group ($P < 0.05$, Student's *t* test) (Fig. 5c). The result suggested triclosan inhibits the parasite proliferation in the mice. Taken together, triclosan is capable of reducing the parasite burden in the brain significantly and of attenuating the virulence in mice moderately, although it cannot protect the mice from death.

Discussion

Neosporosis is one of the greatest threats to the livestock industry, so there is an urgent need to develop anti-neospora agents. Apicoplast is an optimum target, as the type II fatty acid pathway is localized in this organelle (Vaishnava and Striepen 2010). The ENR is an enzyme of the type II fatty acid pathway and is not found in mammals (Jolly et al. 2006; Ramakrishnan et al. 2012; White et al. 2005). Being an ENR inhibitor, triclosan is extensively used in daily necessities, such as detergents, and was effective against several apicomplexa, such as *Plasmodium falciparum*, *Toxoplasma gondii* (Mcleod et al. 2001), *Babesia* (Sabine et al. 2003), *Eimeria tenella* (Lu et al. 2007), and *Trypanosoma brucei* (Paul et al. 2004).

In the present study, we first identified the existence of ENR in *N. caninum*. As expected, NcENR was found to be localized in the apicoplast and was inhibited by triclosan. Further experiments showed that triclosan inhibited the invasion and proliferation of *N. caninum*. The results from in vivo studies in the mouse showed that triclosan reduced the parasite burden in the brain significantly and attenuate the virulence in mice mildly, although it cannot protect the mice from death. It suggests that triclosan inhibits *N. caninum* in vivo mildly.

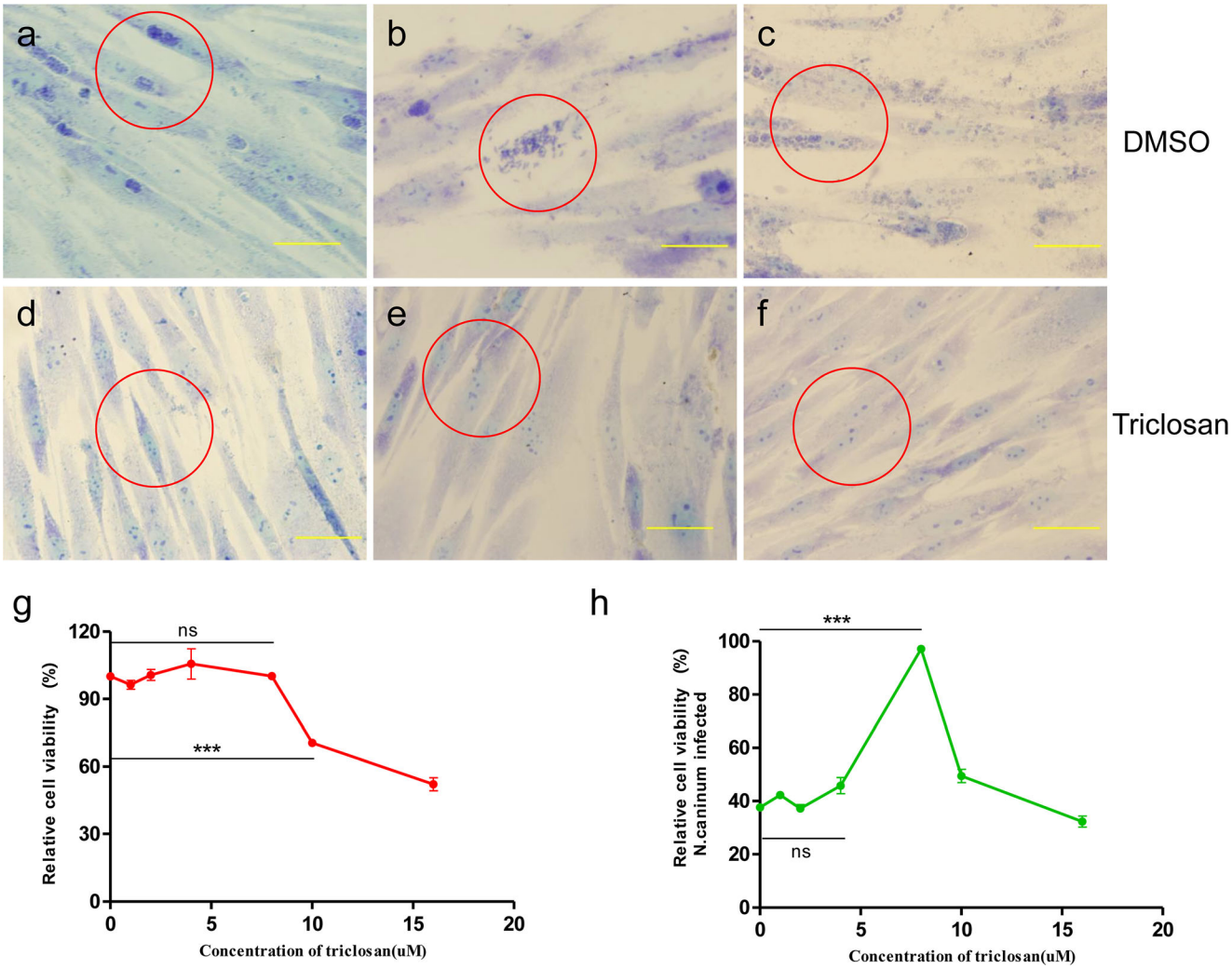

Fig. 3 Triclosan inhibits the proliferation of *N. caninum* tachyzoites at low concentration. **a**–**f** Microscopic images following Giemsa stain showing the proliferation of *N. caninum* treated with DMSO or 10 μM triclosan at different times. The red circles indicate the representative PVs. **g** MTS assay showed triclosan was cytotoxic to the cells at high concentrations (*t* test, ***$P < 0.0001$; ns, not significant), with a CC50 of 9 μM (non-linear regression). **h** MTS assay showed triclosan inhibited *N. caninum* growth expressed by increasing viability of Vero cells infected with *N. caninum* (*t* test, ***$P < 0.0001$; ns, not significant), with an EC50 of 6 μM (non-linear regression)

Some studies about ENR and triclosan in other apicomplexa protozoa have been published (Lu et al. 2007; Mcleod et al. 2001; Paul et al. 2004; Sabine et al. 2003); some of the research showed triclosan inhibits the parasite by inhibiting ENR specifically. We must point out that we did not assess the activity of NcENR as an enzyme that can be specifically inhibited by triclosan in vitro through biochemical methods as other studies have done (Surolia and Surolia 2001). This is because ENR is a conserved enzyme in apicomplexa as well as in *E. coli*, and the active site of ENR is conserved too (Fig. 1a). Through the Swiss-Model tool, we observed that triclosan binds the NcENR perfectly (Fig. 1b). So, it suggests that triclosan is a specific inhibitor of NcENR. Actually, the combination mode of triclosan and ENR is a two-state process, where the triclosan interacts with the $NAD(P)^+$ cofactor first and then is packed by an α-helix of ENR (Muench et al. 2013).

However, in our study, we used the MTS assay to determine the EC50 of triclosan on *N. caninum* for the first time and we demonstrated triclosan inhibits *N. caninum* in vitro through attenuating both its invasion ability and its intracellular propagation proliferation ability, which is more detailed and may provide a reference for future research.

Triclosan is hydrophobic, so the bioavailability is low if we administrated the mice orally (Vandhana et al. 2010). Therefore, in this study, we dissolved the triclosan with soybean oil and used subcutaneous injection to treat the mice. Although the MTS assay indicated triclosan is toxic to the cell at high concentration, the concentration used in mice in our study did not affect the health of the mice.

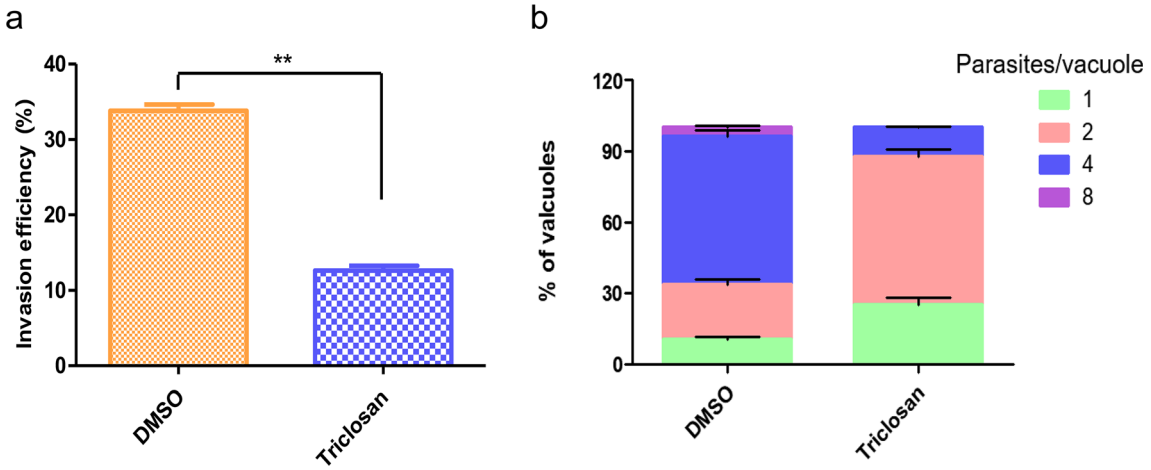

Fig. 4 Triclosan inhibits *N. caninum* in vitro through inhibiting the invasion and proliferation of the parasites. **a** An invasion assay was performed after treating the freshly released extracellular tachyzoites with DMSO or triclosan for 1 h. The ratio was based on the number of cells infected with parasites divided by the number of total cells in one field of view. Asterisks indicate statistically significant results ($P < 0.05$ as determined by the Student *t* test). Data is represented as the mean ± SEM of three independent experiments. **b** An intracellular proliferation assay was carried out through counting the number of parasites per vacuole. Freshly released tachyzoites were allowed to invade HFF cell and proliferate for 24 h with DMSO or triclosan, and then the numbers of parasites per vacuole were counted. Data were compiled from three independent assays, and in each assay, 100 total PVs of each strain were counted

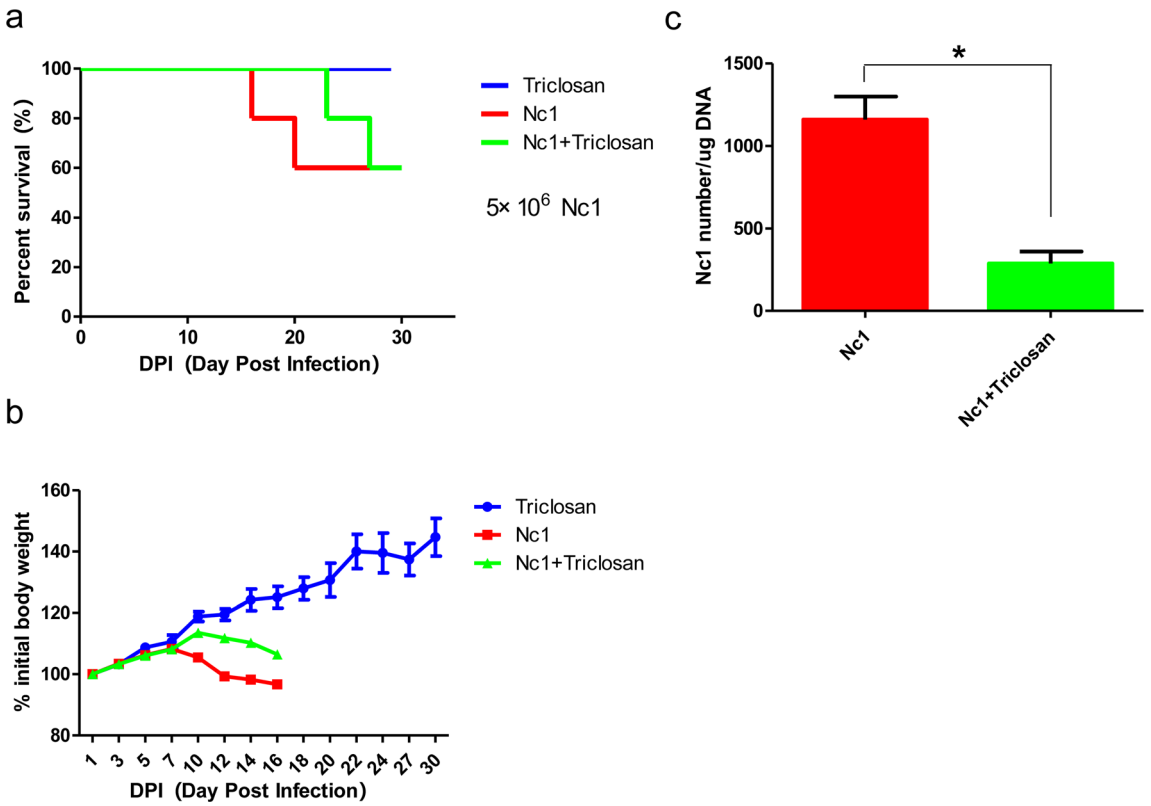

Fig. 5 Triclosan delayed the death of *N. caninum*–infected mice and decreased the parasite burden of surviving mice. **a**, **b** Survival curves and bodyweight of mice after infection with 5×10^6 Nc-1 tachyzoites and treated with triclosan or not over a 30-day period. The weight data collection was stopped on the day when the first mouse died. There are five mice in each group. The assay has been performed three times and represented results are shown here. **c** Parasite burden in the brain was detected by qRT-PCR among the alive mice after the 30-day infection. The parasite burden was represented as the number of tachyzoites per 1 mg of brain tissue DNA. There are three mice in each group. Asterisks indicate statistically significant results ($P < 0.05$ as determined by the Student *t* test). Data is represented as the mean ± SEM of three independent experiments

A gene-editing system based on CRISPR/Cas9 has been proficiently used in *N. caninum* in our laboratory (Yang et al. 2018). Before we used the inhibitor to study the function of ENR, we tried to knockout ENR in *N. caninum*; however, we were unsuccessful after many attempts, suggesting that ENR maybe essential for *N. caninum* survival (Mcleod et al. 2001), and our subsequent experiments that triclosan inhibit *N. caninum* through inhibit ENR demonstrated this speculation. Meanwhile, there are more and more strategy used to knockout or knockdown the expression of an essential gene, such as Cre-Loxp-based approach (Heaslip et al. 2010) and auxin-inducible degron (AID) fusions system (Long et al. 2017). Hence, we expect more research about the function of NcENR using the new genetic modification tools in the future.

Conclusions

The identification and characterization of *N. caninum* ENR and identification of triclosan as an inhibitor of *N. caninum* provide more choice to rationally develop novel inhibitory agents for the treatment of neosporosis.

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Availability of data and material Data supporting the conclusions of this article are included within the article.

Authors' contributions HZ and JL designed the study and analyzed the data. HZ, YF, and CSY carried out the experiments. JHX provided help for instrument operation. QL and JL contributed reagents and materials and offered advice during the research. All authors read and approved the final manuscript.

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Compliance with ethical standards

Ethics approval and consent to participate The experimental procedures were performed strictly according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and approved by the Institutional Animal Care and Use Committee of China Agricultural University (under the certificate of Beijing Laboratory Animal employee ID: CAU20161210-2).

Consent for publication Informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare that they have no conflict of interest.

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