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Lin-Hao Zhang： Software, Investigation, Data curation, Writing- Original draft preparation.

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Chun-Mei Jin: Resources, Supervision, Funding acquisition.

Zhi-Yu Wei: Conceptualization, Project administration, Writing - Review & Editing, Funding acquisition.

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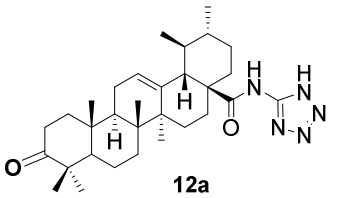

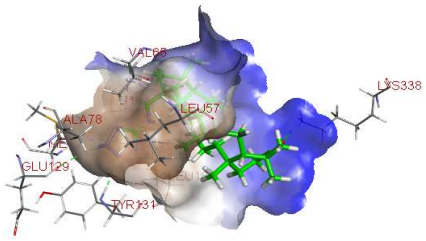

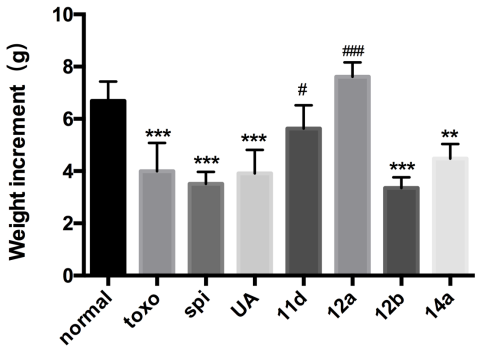

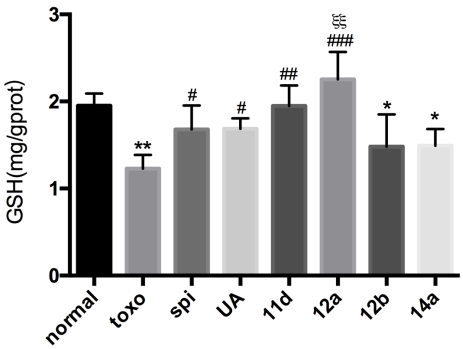

Evaluation of ursolic acid derivatives with potential anti-*Toxoplasma gondii* activity

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Abstract

Toxoplasma gondii is an important pathogen that causes serious public health problems. Currently, therapeutic drugs for toxoplasmosis cause serious side effects, and more effective and novel substances with relatively low toxicity are urgently needed. Ursolic acid (**UA**) has many properties that can be beneficial to healthcare. In this study, we synthesized eight series of **UA** derivatives bearing a tetrazole moiety and evaluated their anti-*T. gondii* activity *in vitro* using spiramycin as a positive control. Most of the synthesized derivatives exhibited better anti-*T. gondii* activity *in vitro* than **UA**, among which compound **12a** exhibited the most potent anti-*T. gondii* activity. Furthermore, the results of biochemical parameter determination indicated that **12a** effectively restored the normal body weight of mice infected with *T. gondii*, reduced hepatotoxicity, and exerted significant anti-oxidative effects compared with the findings for spiramycin. Additionally, our molecular docking study indicated that the synthesized compounds could act as potential inhibitors of *T. gondii* calcium-dependent protein kinase 1 (TgCDPK1), with **12a** possessing strong affinity for TgCDPK1 via binding to the key amino acids GLU129 and TYR131.

Keywords: ursolic acid; anti-*T. gondii*; *in vitro*; *in vivo*; docking; TgCDPK1 inhibitors

1. Introdiction

Toxoplasma gondii is a globally distributed Apicomplexa protozoan that infects a wide range of intermediate hosts, including almost all warm-blooded animals and humans (Ma et al., 2018;Hou et al., 2019). *T. gondii* can infect people through several pathways, such as eating raw or undercooked meat and drinking the milk of infected goats (Gazzonis et al., 2019). Toxoplasmosis can cause mild or no symptoms in healthy individuals; however, it may cause serious diseases and even death in immunocompromised patients, such as patients with AIDS or cancer (Grant et al., 1990). In pregnant women, *T. gondii* infection can lead to mental or psychomotor disability, chorioretinitis, microcephaly, and stillbirth in the fetus (Liesenfeld et al., 2017). In addition, because of the prevalence of AIDS and cancer, as well as increased pet ownership, *T. gondii* infection has increasingly become a serious public health problem globally, and there are few reliable anti-*T. gondii* drugs available for preventing and treating toxoplasmosis. Differing from common anti-cancer agents that directly kill cancer cells, new anti-*T. gondii* drugs must be developed in full consideration of host cell damage. Most currently used clinical anti-*T. gondii* drugs are limited by their toxicity to host cells. Therefore, the discovery of more effective and less toxic anti-*T. gondii* agents is awaited.

Ursolic acid (**UA**) is a pentacyclic triterpenoid found in most plant species (Lee et al., 2001), and it is known to possess a number of bioactive properties (Wang et al., 2017), such as anti-microbial (Zhang et al., 2013), anti-inflammatory (Nascimento et al., 2014), immunomodulatory (Ramos et al., 2010), anti-oxidant (Soica et al., 2014), and anti-cancer activities (Shanmugam et al., 2013). Recently, Choi et al. reported that **UA** improved the survival time of *T. gondii*-infected mice, illustrating its promise as a potential candidate for the development of anti-*T. gondii* drugs (Choi et a., 2018). We

also reported **UA** derivatives bearing triazole moieties as potential anti-*T. gondii* agents, finding that compound **A** (Fig. 1A) displayed enhanced activity against *T. gondii* and reduced hepatotoxicity compared with **UA** (Luan et al., 2019). Several recent reports described various structural modifications to improve the bioactive properties of **UA**. Indeed, structural modification of the **UA** C-28 carboxylic acid group or C-3 hydroxyl group resulted in significant enhancement of its biological activities and obvious decreases in toxicity (Nedopekina et al., 2017; Bai et al., 2012; Gu et al., 2017; Yang et al., 2015; Chi et al., 2017). In addition, tetrazole is a mimetic of carboxylic acid and a five-membered aromatic heterocyclic compound containing four nitrogen atoms (Allen et al., et al., 2012). Its derivatives have diverse activities including anti-microbial (Méndez et al., 2019; Andrejević et al., 2018), anti-oxidant (Kumbar et al., 2018; Khan et al., 2018), anti-bacterial (Figueiredo et al., 2012; Chernov'yants et al., 2016), anti-HIV (Bielenica et al., 2017), anti-cancer (Aziz et al., 2018), and anti-parasitic activities (Fig. 1B and 1C) (Shaikh et al., 2017; Chen et al., 2019; Hamid et al., 2018). Several tetrazole-containing drugs have been developed for clinical use, including cefazolin (Allen et al., 2012), valsartan (Liu et al., 2019), and pentetrazol (Lu et al., 2018) (Fig. 1). Some of these drugs possess a tetrazolium group as the terminal moiety. Therefore, **UA** derivatives previously synthesized in our laboratory containing a tetrazole moiety (as presented in Scheme 1) are likely to possess anti-*T. gondii* activity and lower toxicity (Zhang et al., 2019). Thus, we report the biological evaluation of these compounds in this work.

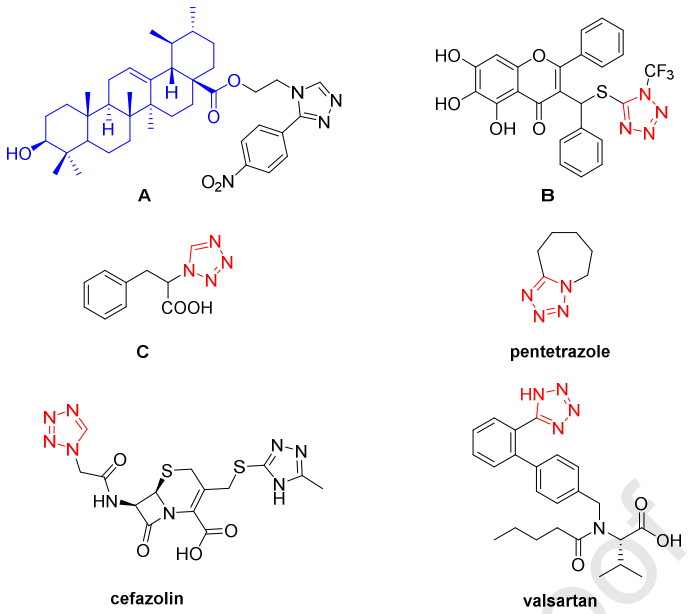
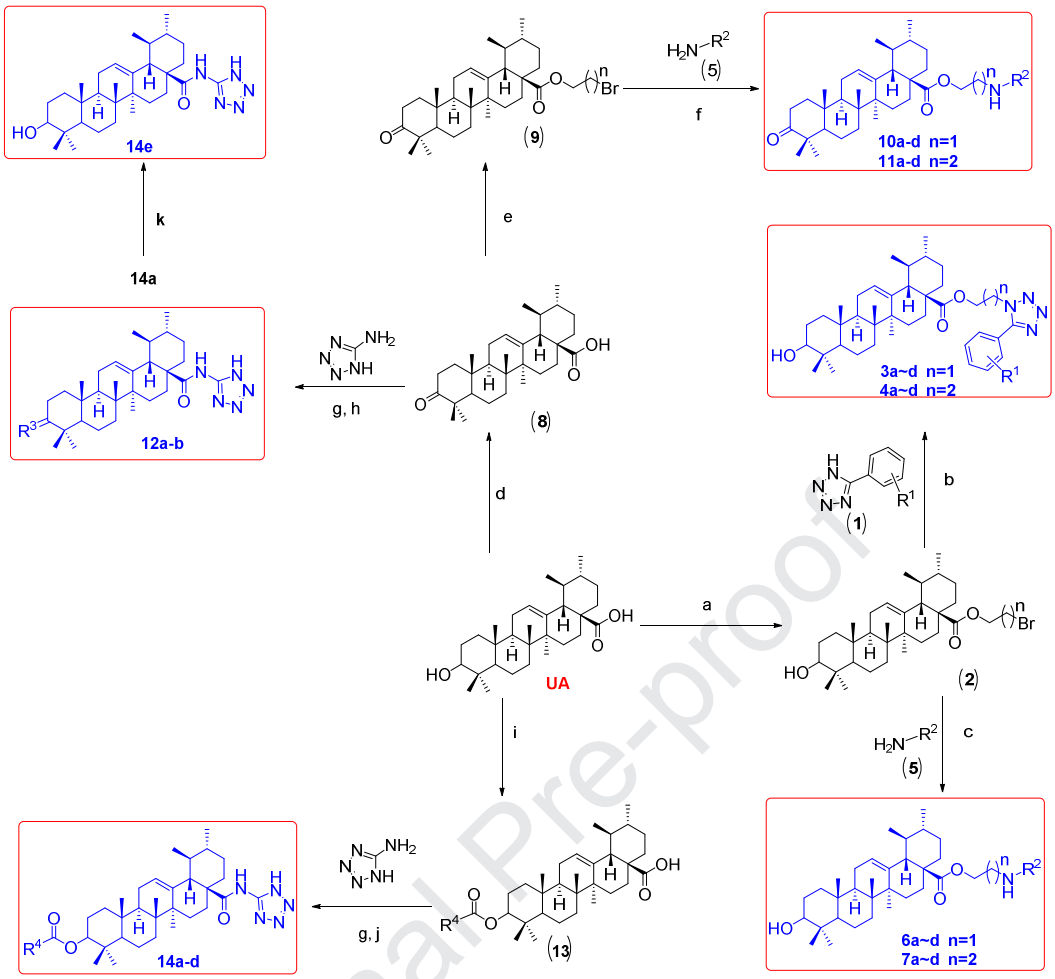


Figure 1. Structures of previously reported compounds as anti-parasitic agents and clinical medication containing tetrazole moiety.



Scheme 1. Synthetic scheme for the synthesis of compounds **3a-d**, **4a-d**, **6a-d, 7a-d, 10a-d**, **11a-d**, **12a-b** and **14a-e**. Reagents and conditions: (a) 1,2-dibromoethane or 1,3-dibromopropane, K_2CO_3, KI, DMF, 50 °C, 6h; (b) K_2CO_3, KI, acetone, reflux, 8h; (c) K_2CO_3, KI, acetone, reflux, 10h; (d) Jones' reagent acetone, 0 °C, 5 h, 90%; (e) 1,2-dibromoethane or 1,3-dibromopropane, K_2CO_3, KI, DMF, 50 °C, 6h; (f) K_2CO_3, KI, acetone, reflux, 10h; (g) $SOCl_2$, DMF/DCM (1:1), trimethylamine; (h) Phenyl hydrazine hydrochloride, acetic acid, ethanol, reflux,8h; (j) anhydride, DMAP, DCM, trimethylamine, r.t., overnight; (k) 10%NaOH, 4NHCl, 50% method in water.

2. Materials and methods

2.1. Drugs and reagents

Spiramycin(spi) were purchased from Sigma Chemical Company (St. Louis, MO,

USA). UA derivatives were prepared according to the procedures we have been reported. (Zhang et al., 2019). All sera,antibiotics and RPMI 1640 for cell culture were obtained from Invitrogen (Biological Industry, Israel). All other chemicals were of reagent grade.

2.2. Cells, parasites and animals

GES-1 cells were cultured in DMEM, supplemented with 0.01% Penicillin-Streptomycin and 10% heat-inactivated FBS and maintained at 37 □ and 5% CO_2. Cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Tachyzoites used in our study were from the virulent RH strain of *T. gondii* (Yanbian University School of Medicine, China) and maintained by serial intraperitoneal passage in KM female mice, which were purchased from Experiment Center, Yanbian University. All experimental procedures were approved by animal experimental center (Number of license: SCXK 2017-0003), Yanbian University and complied with the relevant international animal ethics regulations for the care and use of experimental animals. All mice were kept in a central animal care facility with free access to water and rodent food during the experiment.

2.3. In vitro studies

The anti-*T. gondii* activity and cytotoxicity of compounds to host cells (GES-1) *in vitro* were evaluated by the published method of (Jin et al., 2009). The cells were plated in 96-well plates at an appropriate density to ensure exponential growth throughout the experimental period and then allowed to adhere for 24 h at 37□. The cells were infected with *T. gondii*, followed by incubation for 24 h. All compounds were prepared in dimethyl sulfoxide (DMSO) at a stock concentration of 100 μM. Serial dilutions (1–1000 μM) of each compound were tested. The aim of our

modification of structure was to obtain compounds with low toxicity and high efficiency, therefore, spi with low toxicity was selected as a positive control. After 24h of incubation, 10 μL of 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide solution were added to each well and cells were incubated for a further 4 h. The optical density (OD) was read on a microplate reader at a wavelength of 492 nm. The IC_{50} in GES-1 cells, IC_{50} in *T. gondii* and selectivity index were calculated using Microsoft Excel.

2.4. In vivo studies

2.4.1. *Inhibition rate of tachyzoites and body weight in vivo*

Forty-two female KM mice (weighing 18–22g) were used to establish an animal model of acute *T. gondii* infection. These were randomly divided into seven groups: infected untreated, normal, infected with spi treatment, infected with **UA** treatment, infected with **11d** treatment, infected with **12a** treatment, infected with **12b** treatment and infected with **14a** treatment. Each group consisted of six mice. To unify the various compounds to maintain the same effect comparison, and the main consideration was the limited amount of drugs and the total usage of the mouse, we almost always choose the concentration of 100 mg/kg. Four hours after infection, 100 mg/kg of the compounds was administered to the mice by gavage, once a day for 4 consecutive days, whereas the untreated group was administered the same dose of physiological saline. On the fifth day, the body weight of mice infected with *T. gondii* from different groups was collect. Furthermore, blood from the eyes of mice was collected and they were sacrificed by cervical dislocation. Their abdominal cavity was rinsed with sterile physiological saline to collect the parasites/tachyzoites. These were counted under the light microscope, and the inhibition rate of parasites was calculated.

2.4.2. Measurement of visceral weights and liver biochemical parameters

The liver and spleen were dissected and liver and spleen indexes, serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and liver homogenate glutathione (GSH) and malonaldehyde (MDA) were determined (Luan et al., 2019; Guo et al., 2019; Zhang et al., 2016).

Serum levels of AST and ALT were measured by the method of (Zhang et al., 2016). The quintuple substrate reaction of ALT or AST and serum was carried out under incubation at 37□ for 30 min, then added 1mmol/L 2, 4-DNPH and held for 20 min. Finally, 0.4 mol/L NaOH was added and allowed to react for 5 min. The absorbance at 505 nm was measured.

The GSH was measured according to the method of (Zhang et al., 2016; Beutler et al., 1963).The double volume liver homogenate was mixed with trichloroacetic acid (20%, w/v) and centrifuged at 4000 rpm for 10 min. Then, 0.3 mol/L phosphate buffer and DTNB (0.04%, w/v) were added to the separated supernatant and mixed thoroughly. After 5 min at room temperature, the absorbance was measured at 412 nm. MDA was measured by the standard method (Zhang et al., 2016; Ohkawa et al.,1979) with minor modifications. The liver homogenate supernatant was mixed with thiobarbituric acid (0.5%, w/v) and heated in the bath of boiling water for 60 min, then cooled quickly and centrifuged at 3000 rpm for 10 min, the absorbance of pink colored supernatant was measured at 532 nm. Tetraethoxypropane replaced the liver homogenate in the standard sample.

2.5. Docking simulations

T. gondii calcium-dependent protein kinase 1 (TgCDPK1) plays a crucial role in

the motility and gliding of *T. gondii*, and the enzyme, together with adenosine kinase and purine nucleoside phosphorylase, serves as a key purine metabolic enzyme for *T. gondii* (Vidadala et al., 2016; Schumacher et al., 2000). Luan et al reported that UA derivative was potential TgCDPK1 inhibitor (Luan et al., 2019). Therefore, the development of molecular docking was determined. Molecular computation studies were carried out by using Discovery Studio 2017 (DS, Accelrys, San Diego,CA, USA). The X-ray crystal structure of Calcium-Dependent Protein Kinase 1 from *T. gondii* (TgCDPK1) in complex with inhibitor **UW1561** was obtained from protein data bank (PDB: 4TZR). The water molecules, heavy atom and **UW1561** in protein were removed and the protein was prepared by adding hydrogen and correcting incomplete residues using Clean Protein tool of DS. ChemBioDraw 14.0 was used to draw the structure of the docking compounds and save them in the molar format. The structures of **UA**, compound **12a** and **UW1561** were sketched in 2D and converted into 3D using the DS molecule editor. LibDock was used to investigate whether the synthesized compounds had potential TgCDPK1 inhibitory effects. CDDOCK studies were carried out to investigate the binding mode of **UA**, compound **12a** and **UW1561** in the crystal structure of 4TZR from 2D images. The 3D image was chosen for analyzing the binding features of **UA**, compounds **12a** and **UW1561** with TgCDPK1 (Zhao et al., 2018).

3. Results and discussion

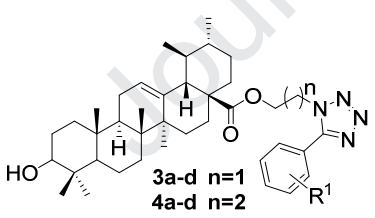
3.1. In vitro results and discussion

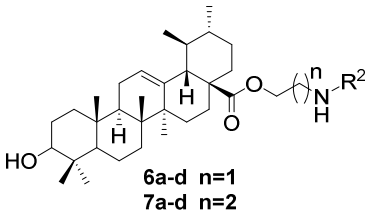
The *in vitro* anti-*T. gondii* activities of the **UA** derivatives are summarized in Tables 1–2. The selectivity index (SI) is usually applied to evaluate anti-*T. gondii*

agents. It is a measure of specific resistance to *T. gondii*, and it is generally believed that larger SI values indicate stronger inhibitory activity against *T. gondii* and lower cytotoxicity (Jin et al., 2009). As shown in Table 1, the SI of **UA** (0.6) was lower than that of the positive control spi (0.8), indicating the poorer anti-*T. gondii* effects of **UA**. Almost all of the **UA** derivatives exhibited stronger anti-*T. gondii* activity than the parent compound, and 27 compounds exhibited similar or slightly greater activity than spi. In addition, with the exception of compound **14b**, the IC_{50} values of all other compounds in GES-1 cells were higher than that of **UA**, indicating that these compounds are less cytotoxic than **UA**. Regarding compounds **3a–3d** and **4a–4d**, a 3,4-(OCH_2O) substituent on the benzene ring attached to the tetrazole moiety was found to be more beneficial, and compounds **4c** (SI = 0.9) and **4d** (SI = 0.9) exhibited potent growth-inhibiting effects on *T. gondii* in *vitro*. Compounds **6a–6d** and **7a–7d**, which feature a shorter linkage (n = 1) between **UA** and the tetrazole moiety, exhibited stronger inhibitory activities than compounds bearing longer linkages (n = 2). In these two series, compounds containing *ortho*-tetrazole moieties displayed relatively good activity. Interestingly, compound **6d**, which lacks a benzene ring between the shorter linkage and tetrazole moiety, displayed enhanced potency compared with the effects of compounds containing the benzene ring. However, among compounds **7a–7d** with a longer linkage, **7d** exhibited lower inhibitory activity than the other compounds (**7b–7d**). Compounds in series **10** and **11** exhibited slightly more potent activity than those in series **6** and **7**, in which the structures only differed at position 3. The former series featured a ketone at this position, whereas compounds in the latter series carried a hydroxyl group. Furthermore, in series **10** and **11**, the compounds featuring *meta*-tetrazole (**10b**, **11b**) or those lacking a benzene group (**10d**, **11d**) exhibited relatively good potencies, with compound **11d** displaying the strongest

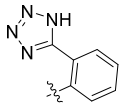
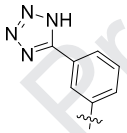
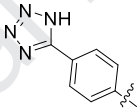
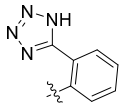
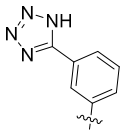
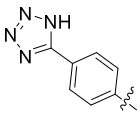
activity with an SI of 1.0. Compounds **12a** and **12b** also exhibited higher inhibitory activities than other compounds with an SI of 1.0. Almost all of the compounds in series **14** displayed good activities (SI = 1.0, 0.8, 0.8, and 0.9, respectively), excluding **14b**. From the biological results for the compounds in series **12** and **14**, in which the tetrazole group was directly linked to a nitrogen atom of the C-28 amide group and the C-3 hydroxyl group was changed to ketone, ester, or hydrazine, we can observed that such a modification at the C-3 position results in enhanced activity of *T.gondii* inhibition. It was found that the number of *T. gondii* invading host cells was significantly reduced after administration, and it was difficult to observe *T. gondii* extracellularly, and the growth status of the cells was no different from that of normal cells.

Table 1. In *vitro T. gondii* growth inhibition and cytotoxicity of the series **3**, **4**, **6** and **7**.





Compound	R^1	R^2	IC_{50} (μM)		SI [c]
			GES-1cells [a]	*T.gondii* [b]	
UA	–	–	44.8	72.2	0.6
3a	H	–	198.2	256.3	0.8
3b	4-Cl	–	189.6	246.6	0.8

3c	4-CH_3O	–	165.5	234.5	0.7
3d	3,4-(OCH_2O)	–	222.0	275.7	0.8
4a	H	–	212.3	256.7	0.8
4b	4-Cl	–	181.8	228.3	0.8
4c	4-CH_3O	–	207.0	243.1	0.9
4d	3,4-(OCH_2O)	–	213.5	237.9	0.9
6a	–		181.7	193.2	0.9
6b	–		178.0	224.2	0.8
6c	–		169.6	199.9	0.8
6d	–		205.0	227.5	0.9
7a	–		147.2	186.9	0.8
7b	–		125.0	191.8	0.7
7c	–		155.9	175.0	0.9
7d	–		54.6	84.9	0.6
spi	–	–	207.8	250.5	0.8

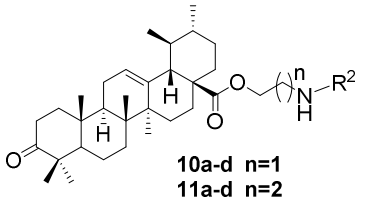
[a] IC_{50} in GES-1cells: Median toxicity dose, a measure of cytotoxicity against host cells. IC_{50} of UA in HeLa cells. Values were shown as mean, n=3.

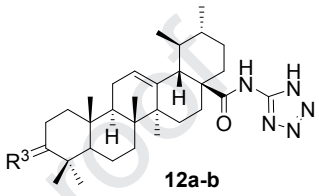
[b] IC_{50} in *T. gondii*: Median inhibitory concentration, a measure of tachyzoite inhibition. Values

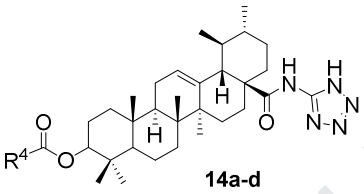
were shown as mean, n=3.

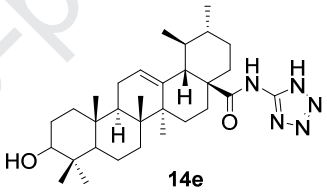
[c] SI: Selectivity index, a measure of efficacy, calculated by IC_{50} in GES-1 cells/IC_{50} in *T. gondii*. Values were shown as mean, n=3.

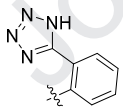
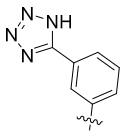
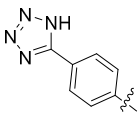
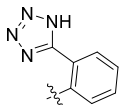
Table 2. In *vitro T. gondii* growth inhibition and cytotoxicity of the series **10**, **11**, **12** and **14**.

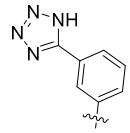
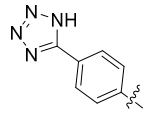
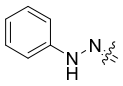
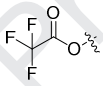
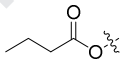
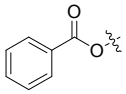








Compound	R^2	R^3	R^4	IC_{50} (μM)		SI [c]
				GES-1cells [a]	*T.gondii* [b]	
10a		–	–	192.6	242.2	0.8
10b		–	–	270.1	310.1	0.9
10c		–	–	264.3	315.3	0.8
10d		–	–	161.1	179.7	0.9
11a		–	–	152.8	187.6	0.8

11b		–	–	271.4	316.4	0.9
11c		–	–	246.2	306.2	0.8
11d		–	–	188.2	192.8	1.0
12a	–		–	213.2	218.6	1.0
12b	–		–	245.0	248.5	1.0
14a	–	–		130.3	136.8	1.0
14b	–	–		–	–	–
14c	–	–		92.8	114.1	0.8
14d	–	–		74.3	96.2	0.8
14e	–	–	–	202.7	220.9	0.9

[a] IC_{50} in GES-1cells: Median toxicity dose, a measure of cytotoxicity against host cells. Values were shown as mean, n=3.

[b] IC_{50} in *T. gondii*: Median inhibitory concentration, a measure of tachyzoite inhibition. Values were shown as mean, n=3.

[c] SI: Selectivity index, a measure of efficacy, calculated by IC_{50} in GES-1 cells/IC_{50} in *T. gondii*. Values were shown as mean, n=3.

3.2. In vivo results and discussion

We performed an in-depth study of the anti-*T. gondii* activities of compounds **11d**, **12a**, **12b**, and **14a** in mice due to their good anti-*T. gondii* activity *in vitro* and their different structural characteristics.

3.2.1. Inhibition rate of tachyzoites and body weight in vivo

In order to study the degree of inhibition of *tachyzoites* by compounds, the inhibition rate experiment was carried out. The results of inhibition rate of *tachyzoites* are summarized in Table 3. After treatment with the compounds at a dosage of 100 mg/kg, the **UA** derivatives had different inhibitory effects on the number of *T. gondii* specimens in the abdominal cavity of mice, among which **12a** exerted the strongest inhibitory effect (55.3%), exceeding that of spi (51.8%) in the maternal body. However, the inhibitory activity of these four compounds showed different results *in vivo* and *in vitro*. We speculate that the substituents at C-3 position of compounds **12b** (29.4%), **14a** (31.8%) and **12a** are different, and the substituents at C-28 position of compounds **11d** (16.5%) and **12a** are different, which leads to their different absorption, distribution, metabolism and excretion *in vivo*, thus leading to the difference of activities.

Table 3. Effect of compounds on the inhibition rate of tachyzoites in mice.

	spi	**11d**	**12a**	**12b**	**14a**
Inhibition rate (%)	51.8%$^{###}$	16.5%	55.3%$^{###}$	29.4%$^{#}$	31.8%$^{#}$

Inhibition rate(%) of tachyzoites in mice peritoneal cavity treated with compounds, compared to toxo group; n = 6, # $P< 0.05$ compared with toxo group and ### $P< 0.001$ compared with toxo group.

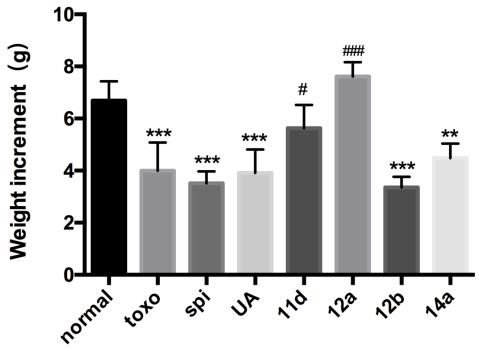


Figure 2. Effect of compounds on body weights in *T.gondii*-infected KM mice, $^{**}P$<0.01compared with normal group; $^{***}P$<0.001compared with normal group; $^{\#}P$<0.05 compared with toxo group; $^{\#\#\#}P$<0.001 compared with toxo group; $^{\S\S}P$<0.01compared with spi group; $^{\S\S\S}P$<0.001compared with spi group.

Body weight is significantly decreased in mice following *T. gondii* infection (Spano et al., 2002). As shown in Figure 2, the weight of mice treated with the test compounds (spi, **14a**, and **12b**) was significantly lower than that of the normal group, which weight was similar to toxo group, indicating that mouse weight was not regained by treatment with these compounds to *T. gondii*-infected mice. However, compared with the findings in *T. gondii*-infected mice, **12a** and **11d** significantly increased body weight to the same levels observed in normal mice. Thus, compounds **12a** and **11d** could alleviate the weight reduction caused by acute *Toxoplasma* infection, leading to the speculation that these two compounds can affect the gastrointestinal digestive system and restore the normal feeding status of mice or that they can reduce the metabolic level and energy consumption of mice, thereby restoring weight.

From the effect of these four compounds on body weights in *T.gondii*-infected

KM mice, we deduce that the carbonyl substitution at C-3 position of compounds **12b** and **11d** are beneficial to weight recovery to mice. On the contrary, the hydrazone and hydroxyl substituents at C-3 position of compounds **14a** and **12b** are not conducive to weight recovery.

3.2.2. Liver and spleen indexes

The liver is the main part of histopathology, and the spleen plays an important role in coordinating adaptation and innate immune responses (Khan et al., 2017; Znalesniak et al., 2017). Therefore, liver and spleen indices are used to evaluate the protective effects of drugs on viscera. As shown in Figure 3, liver and spleen enlargement can occur after acute infection by *T. gondii* in mice, but the liver index was unchanged compared with that in normal mice. It was noteworthy that the spleen index was increased significantly by all **UA** derivatives compared with the normal group findings, especially by **11d** and **12a**, which also significantly increased the spleen index versus that observed in the infected group. Because splenomegaly is an early sign of portal hypertension in cirrhosis, attention should be paid to splenomegaly in mice infected by *T. gondii* following treatment.

From the effect of compounds on spleen and liver weights in *T.gondii*-infected KM mice, we infer that natural product **UA** binding to tetrazolium moiety could increase the spleen index evidently, especially the C-3 position was modified by carbonyl group as well.

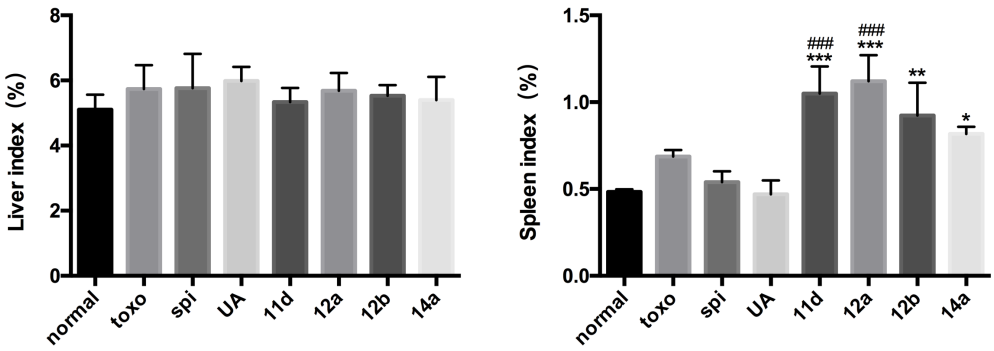


Figure 3. Effect of compounds on spleen and liver weights in *T.gondii*-infected KM mice, $^{*}P<0.05$compared with normal group; $^{**}P<0.01$compared with normal group; $^{***}P<0.001$compared with normal group; $^{\#\#\#}P<0.001$ compared with toxo group; $^{\S}P<0.05$compared with spi group; $^{\S\S\S}P<0.001$compared with spi group.

3.2.3. ALT and AST

The serum levels of ALT and AST are extremely sensitive indicators of liver injury, and their elevation roughly reflects the degree of liver injury (Liu et al., 2014). To further study the toxicity of these compounds, serum ALT and AST levels were measured in mice infected with *T. gondii*. As shown in Figure 4, compared with the normal group levels, acute infection by *T. gondii* resulted in significant increases of serum ALT and AST levels. Serum ALT and AST levels were significantly higher in mice treated with **11d**, **12b**, or **14a** than in those in the normal and toxo groups, indicating that these three derivatives caused liver damage without similar protective effects to spi. However, compound **12a** was linked to significantly decreased ALT and AST levels in *T. gondii*-infected mice with similar protective effects to spi. The results suggest that **12a** can provide resistance to *Toxoplasma*-mediated hepatotoxicity.

From the effect of compounds on ALT and AST levels in *T.gondii*-infected KM

mice, we conjecture that the C-28 position of **UA** is linked to tetrazole by esterification and alkylation, and hydrazone formation or retention of hydroxyl group at C-3 position has no regulation effect on AST and ALT indexes. Conversely, oxidation at C-3 position of **UA**, and C-28 linked to tetrazole by amide bond can be beneficial to protective effect on liver.

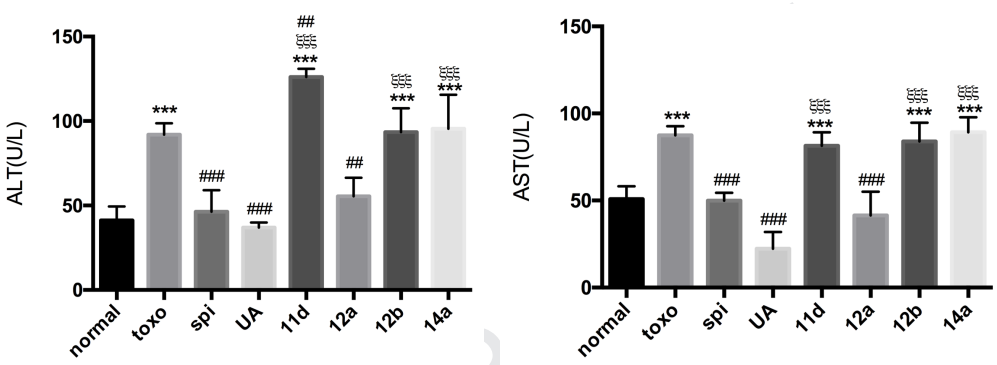


Figure 4. Effect of compounds on ALT and AST levels in *T.gondii*-infected KM mice, $^{**}P$<0.01compared with normal group; $^{***}P$<0.001compared with normal group; $^{\#\#}P$<0.01 compared with toxo group; $^{\#\#\#}P$<0.001 compared with toxo group; $^{\S}P$<0.05compared with spi group; $^{\S\S\S}P$<0.001compared with spi group.

3.2.4. GSH and MDA

GSH is an important anti-oxidant and free radical scavenger in the human anti-oxidant system that converts harmful free radicals and heavy metals into harmless substances and facilitates their excretion from the body (Shaun et al., 2008). MDA is a lipid peroxidation product formed by reactive oxygen radicals oxidizing biological membranes after enhanced oxygen stress, which is an important marker of lipid peroxidation (Gaweł et al., 2004). The levels of MDA and GSH in

liver can indirectly reflect the degree of cells damage with the degree of liver lipid peroxidation in organism (Cederbaum et al., 2009). As shown in Figure 5, compared with the normal group findings, GSH levels were significantly lower in *T. gondii*-infected mice. Nevertheless, compared with the *T. gondii*-infected group results, derivatives **12a** and **11d** significantly increased GSH content to levels exceeding that in the toxo group, supporting their liver restored after exposure to compounds **12a** and **11d**. Additionally, compared with the level in the normal group, the MDA level was significantly elevated in *T. gondii*-infected mice, whereas MDA content significantly decreased by treatment with compounds **12a**, **12b**, and **14a**, indicating that these compounds could reduce lipid peroxidation caused by acute *T. gondii* infection.

From the effect of compounds on GSH and MDA levels in *T.gondii*-infected KM mice, we believe that oxidation at C-3 position of **UA** benefits to enhance regulation effect on GSH, the C-28 position linked to tetrazole via amide bond is to prejudice of adjusting MDA index.

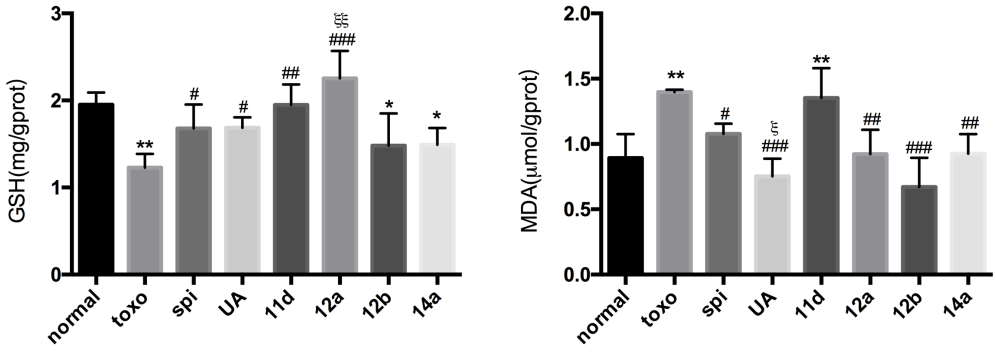


Figure 5. Effect of compounds on GSH and MDA levels in *T.gondii*-infected KM mice, $^{*}P<0.05$compared with normal group; $^{**}P<0.01$compared with normal group; $^{\#}P<0.05$ compared with toxo group; $^{\#\#}P<0.01$ compared with toxo group; $^{\#\#\#}P<0.001$ compared with toxo group; $^{\S}P<0.05$compared with spi group; $^{\S\S}P<0.01$compared with spi group.

4. Molecular docking analysis

To investigate whether the synthesized compounds inhibit TgCDPK1, we used the LibDock tool of DS software to investigate docking with TgCDPK1 protein (4TZR). The results illustrated that all synthesized compounds could dock with 4TZR, and the docking scores were higher than that of **UA** (see Supplementary material), inferring that these compounds might be potential TgCDPK1 inhibitors, although further validation is needed. Due to compound **12a** with good *T. gondii* inhibitory activity and the maximal increase in weight, splenomegaly, and alterations in the enzymatic data, we used **UA** and inhibitor **UW1561** as control to further analyzing the docking results of it with 4TZR. Table 4 presents the docking results for **12a**, **UA** and **UW1561** with 4TZR using the CDDOCK tools of DS. Compound **12a** had the most promising -CDDOCK Interaction Energy (98.89 k.cal/mol) among **UA** (54.77 k.cal/mol) and inhibitor **UW1561** (57.74 k.cal/mol). That indicated that compound **12a** may have better TgCDPK1 inhibitory activity than inhibitor **UW1561**, the TgCDPK1 inhibitory activity of **UA** may lower than that of inhibitor, which of course needs further validation. The preferred coordination modes of **12a**, **UA** and **UW1561** with the TgCDPK1 protein are presented in Fig. 6. It was observed that the tetrazole fragment of compound **12a** fit into a 4TZR cavity that was formed by GLU129, ALA78, MET112, VAL65, LEU181, and TYR131 (Fig. 6A and 6B), which the ligand of compound **12a** displayed a similar orientation in the active site of TgCDPK1, compared with inhibitor **UW1561**. The formed complex was stabilized by the formation of hydrogen bonds (H-bonds) (Zhao et al., 2018). Furthermore, H-bonds not only were formed with the key amino acids GLU129 and TYR131 (Gaweł et al.,

2004), but also with LYS338, which may be the reason that -CDDOCK Interaction Energy of compound **12a** is better than inhibitor **UW1561**. From the 3D image of **12a** bound to TgCDPK1, **12a** was wrapped in the active pocket, and the tetrazole part was buried deep in the pocket. **UA** formed H-bonds with TgCDPK1 amino acids GLU135, GLU138, LYS338, and LYS185 (Fig. 6C and 6D), however, compared to inhibitor **UW1561**, there is no H-bond formed with the key amino acid, which is the difference observed from the binding of **UA** with the protein.

Table 4. The result of docking with TgCDPK1 (PDB ID: 4TZR).

Compound	-CDDOCK Interaction Energy (k.cal/mol)
12a	98.89
UA	54.77
UW1561	57.74

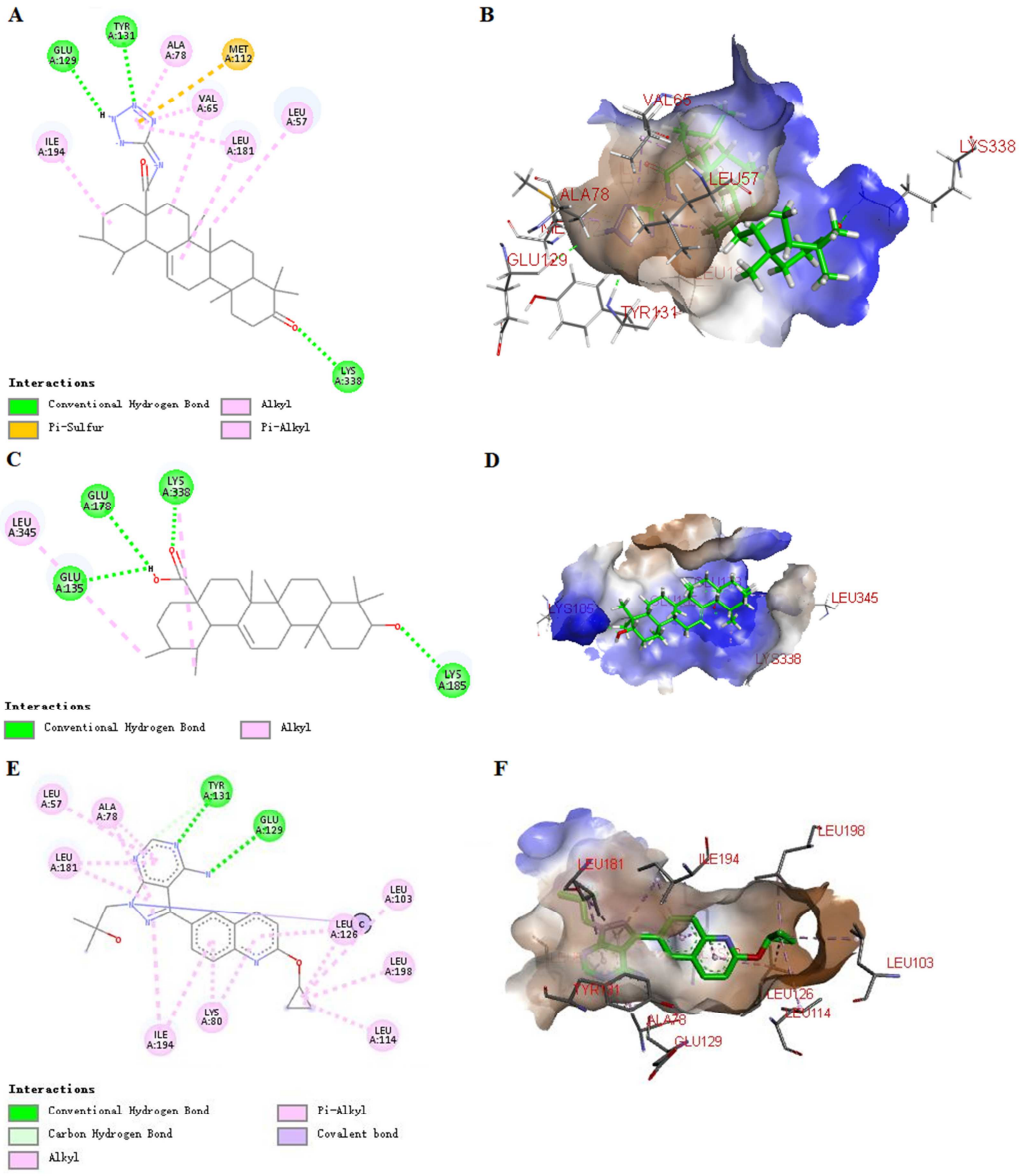


Figure 6. Docking result of compound **12a**, **UA** and **UW1561** with TgCDPK1 (PDB ID: 4TZR). (A) 2D molecular docking modeling of compound **12a** with 4TZR. (B) Key residues in binding site surrounding **12a**. (C) 2D molecular docking modeling of compound **UA** with 4TZR. (D) Key residues in binding site surrounding **UA**. (E) 2D molecular docking modeling of inhibitor **UW1516** with 4TZR. (F) Key residues in binding site surrounding **UW1516**.

5. Conclusions

Eight series of synthesized **UA** derivatives were evaluated for their anti-*T. gondii* activities. Compared with spi, several compounds exhibited potent abilities to restore the normal body weight of infected mice, better effects on liver and blood indices, and stronger effects on GSH. Of the compounds tested, **12a** displayed the promising anti-*T. gondii* activity *in vitro* with an SI of 1.0, as well as the strong effect against *T. gondii in vivo*, inhibiting tachyzoite growth by 55.3% in mice, which exceeded the inhibitory effects of the traditional drug spi on the proliferation of *T. gondii.* Molecular docking results showed that the ligand of compound **12a** displayed a similar orientation in the active site of TgCDPK1 binding to key amino acids GLU129 and TYR131, compared to inhibitor **UW1561**. Thus, compound **12a** can be used as a hit for the development of more effective anti-*T. gondii* drugs.

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Highlights:

- Eight series of synthesized ursolic acid derivatives bearing tetrazole moieties were evaluated for their anti-*T. gondii* activities.
- Compound **12a** is a promising hit for the development of new anti-*T. gondii* agents**.**
- The investigation of the mechanism *in vivo* and binding mode of action of **12a** to TgCDPK1 was performed.