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Research paper

Old drug repurposing for neglected disease: Pyronaridine as a promising candidate for the treatment of *Echinococcus granulosus* infections

Jun Li[a,1], Weisi Wang[b,1], Junmin Yao[b], Tian Wang[a], Shizhu Li[b], Wenjing Qi[a], Shuai Han[b], Yuan Ren[a], Zhisheng Dang[b], Xiumin Han[c], Gang Guo[a], Baoping Guo[a], Liqin Wang[d], Liping Duan[b,c,*], Wenbao Zhang[a,**]

[a] State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medical Research Institute, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China
[b] National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, WHO Collaborating Centre for Tropical Diseases, Key Laboratory of Parasitology and Vector Biology of the Chinese Ministry of Health, Shanghai 200025, China
[c] Qinghai Provincial People's Hospital, Xining 810007, China
[d] Biotechnology Research Institute, Xinjiang Academy of Animal Science, Urumqi 830000, China

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ABSTRACT

Background: Cystic echinococcosis (CE), a condition caused by the larval stage of the dog tapeworm *Echinococcus granulosus sensu stricto*, is a globally distributed zoonotic disease. Current treatment options for CE are limited, and an effective and safe anti-echinococcal drug is urgently required.
Methods: Drug repurposing strategy was employed to identify new therapeutic agents against echinococcal cysts. An *in vitro* protoscolicidal assay along with *in vivo* murine models was applied in the drug screening. A microinjection procedure was employed to mimic the clinical PAIR (puncture, aspiration, injection and reaspiration) technique to evaluate the potential application of the candidate drug in clinical practice.
Findings: We repurposed pyronaridine, an approved antimalarial drug, for the treatment of CE. Following a three-dose intraperitoneal regimen (57 mg/kg, q.d. for 3 days), pyronaridine caused 100% cyst mortality. Oral administration of pyronaridine at 57 mg/kg, q.d. for 30 days significantly reduced the parasitic burden in the pre-infected mice compared with albendazole group ($p < 0.001$). Using a microinjection of drug into cysts, pyronaridine (200 μM) showed highly effective in term of inhibition of cyst growth ($p < 0.05$, compared with saline group). Pharmacokinetic analysis revealed that pyronaridine was highly distributed in the liver and lungs, the most affected organs of CE. Function analysis showed that pyronaridine inhibited the activity of topoisomerase I (IC_{50} = 209.7 ± 1.1 μM). In addition, classical apoptotic hallmarks, including DNA fragmentation and caspase activation, were triggered.
Interpretation: Given its approved clinical safety, the repurposing of pyronaridine offers a rapidly translational option for treating CE including PAIR.
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1. Introduction

Cystic echinococcosis (CE) is a globally distributed zoonosis caused by the larval cyst stage of the dog tapeworm *Echinococcus granulosus sensu stricto* (*E. granulosus ss*) [1]. The prevalence of the disease in parts of South America, Northern Africa and Central Asia (especially western China) has reached as high as 5–10% [2,3]. Although as a chronic disease, CE may cause death due to the presence of large echinococcal cysts in the liver and lungs. The disease impacts population health and causes economic loss to farmers. The global burden of CE is estimated over 1 million DALYs (disability adjusted life years) per annum [4]. Annual costs incurred by CE on case treatment and the livestock industry are considerable with up to US$ 3 billion [3,5].

Currently available treatment options for CE include: i) surgery, ii) percutaneous treatment including the puncture, aspiration, injection,

* Corresponding author.
** Corresponding author at: State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia, Clinical Medical Research Institute, The First Affiliated Hospital of Xinjiang Medical University, Urumqi 830054, China.
E-mail addresses: wenbaozhang2013@163.com (W. Zhang), duanlp@nipd.chinacdc.cn (L. Duan).
[1] Both authors contributed equally to this work.

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Research in context

Evidence before this study

Human cystic echinococcosis (CE) is a neglected disease caused by the larval stage (cyst) of the tapeworm *Echinococcus granulosus sensu stricto* (*E. granulosus ss*). The disease is globally distributed, affecting mainly low-income and rural populations. CE may result in death if inadequate care management is provided. Current treatment options are limited. Drug repurposing represents a cost-effective strategy for identifying new drug candidates. Previous studies have reported that a couple of clinically approved drugs showing anti-echinococcal activity. However, none of these drugs have proceeded to potential clinical application.

Added value of this study

Through a phenotypic screening of characterized compounds, we identified pyronaridine (a China Food and Drug Administration (CFDA) approved drug) as a potent anti-echinococcal agent. We showed that oral administration of pyronaridine significantly reduced the parasitic burden in mice and exhibited more capability of killing echinococcal cyst than that of albendazole, the only drug recommended by WHO. Using a microinjection procedure (a mimic of the clinical PAIR technique), pyronaridine effectively inhibited the parasite growth, which demonstrated its potential in clinical applications. Pyronaridine predominantly distributes in the liver and lungs, which are the most affected organs of echinococcosis. Furthermore, pyronaridine inhibited topoisomerase I and induced apoptosis in *E. granulosus ss*.

Implications of all the available evidence

Given its approved clinical safety and established drug profiles, pyronaridine could be a drug candidate for the treatment of cystic echinococcosis, even for the clinical trials.

re-aspiration (PAIR) technique, and iii) anti-parasitic treatment with albendazole (ABZ) [6]. Although surgical removal of echinococcal cysts represents a curative treatment, ABZ anti-parasitic treatment is a widely used therapeutic modality accounting for most of CE cases (80–90%) in China (Prof. Hao Wen, The First Affiliated Hospital of Xinjiang Medical University, personal communication). However, ABZ is a parasitostatical drug with very limited parasitocidal effects against *E. granulosus ss* cysts. A previous clinical study revealed that approximately 40% of CE cases did not respond favorably to ABZ treatment [5]. Furthermore, this drug requires long-term and high-dose regimens, which usually result in side-effects and poor patient compliance [7].

The molecular mechanisms underlying the development and growth of *E. granulosus ss* remain to be elucidated; the lack of knowledge in this area is currently affecting the development of anti-echinococcal drugs. However, drug repurposing represents a new means of identifying anti-echinococcal drug candidates. Indeed, a range of existing clinical anti-infective and anticancer drugs have been subjected for anti-CE studies. Some of these drugs exhibited anti-echinococcosis activity *in vitro* and/or *in vivo*, but few demonstrated greater efficacy than ABZ [8–13].

In this study, we identified that pyronaridine (PND), an antimalarial drug approved by the China Food and Drug Administration (CFDA) [14], significantly reduced the parasitic burden of *E. granulosus ss* in mice following both intraperitoneal and oral treatment regimens. In addition, a mimic of PAIR procedure with cysts microinjected with PND showed highly effective in preventing cyst growth. Furthermore, we revealed that PND inhibited *E. granulosus ss* topoisomerase I and induced DNA fragmentation and caspase activation, which may result in cyst death. Thus, we report here that the repurposing of PND offers a safely and rapidly translatable option for CE treatment.

2. Results

2.1. *Pyronaridine kills* E. granulosus ss *protoscoleces* in vitro

A phenotypic screening of our in-house anti-parasitic compound library (Table S1) revealed that PND showed potent killing activity against *E. granulosus ss* protoscoleces (PSCs). The parasitocidal strength of PND was in a dose-dependent manner. PND exhibited protoscolicidal activity with an LC_{50} value of 49.0 ± 0.2 μM, which was better than that of ABZ (LC_{50} = 79.2 ± 3.9 μM).

2.2. *Intraperitoneally administered pyronaridine significantly reduces the parasitic burden in secondarily infected mice*

For malaria treatment, PND is administered either orally (taking tablets) or by intramuscular and intravenous injections. These routes of administration were well tolerated. In this study, we initially adopted an intraperitoneal administration approach to ensure drug absorption. To investigate whether PND kills the larval cysts in a short time, a three-dose regimen was applied to the mice previously infected with CE. The *in vivo* efficacy was determined by measuring the wet weight and mortality of the cysts collected from the infected animals. All three dosages of PND successfully suppressed the growth of cysts. Compared with the unmedicated mice, the wet cyst weight in the mice administered with 57 mg/kg of PND was significantly reduced (38.7%, $p < 0.05$, one-way ANOVA, Fig. 1a), and PND killed all cysts (100% of cyst mortality) compared with vehicle controls showing most of the cysts survived ($p < 0.001$, nonparametric Kruskal-Wallis test, Fig. 1b). In addition, the PND regimen resulted in dramatic morphological changes in the cysts. The control cysts were round in shape with smooth surfaces and intact germinal and laminated layers (Fig. 1d). In contrast, cysts collected from the mice treated with PND at the dosage of 57 mg/kg, exhibited completely structural disruption with dramatically reduced turgidity and loss of cyst fluid as well as irregular and fissured surfaces (Fig. 1g). The parasite germinal layer was teared off from the laminated layer and formed densely packed aggregates. Calcification of cysts was often observed. Similar results were also observed in the other two low-dosage groups (Fig. 1e and f) with some cysts being round in shape.

All the mice in the drug experimental groups exhibited normal behavioral activities including eating, drinking and movement without obvious adverse effects. Compared to the control group, no significant pathological changes in the liver, kidneys, thymus and body weight were observed in the treated mice, except for a minor increase in liver weight in the high-dosage group (Fig. 1c). The hematological and biochemical indexes of the mice in the three dosage groups were almost all within normal limits (Table S2).

2.3. *Oral administration of pyronaridine kills* E. granulosus ss *cysts in mice*

Oral administration has drawn much attention for treating chronic diseases. To investigate whether PND is orally available, secondarily infected mice were treated with PND through the oral route. Treatment of PND at 57 mg/kg reduced 42.4% of parasite wet weight, which showed a significant reduction compared with that of the vehicle control mice ($p < 0.05$, nonparametric Kruskal-Wallis test, Fig. 2a). Comparably, 50 mg/kg of ABZ p.o. reduced 48.1% of cyst weight, no significant difference between PND and ABZ groups ($p > 0.05$, nonparametric Kruskal-Wallis test). However, PND treatment killed 90.7% of cysts, significantly higher than the mortality rate observed in the ABZ-treated group (22.2%, $p < 0.001$, one-way ANOVA, Fig. 2b). During the entire treatment, no explicit toxicity was observed. A minor loss of body weight was observed in the

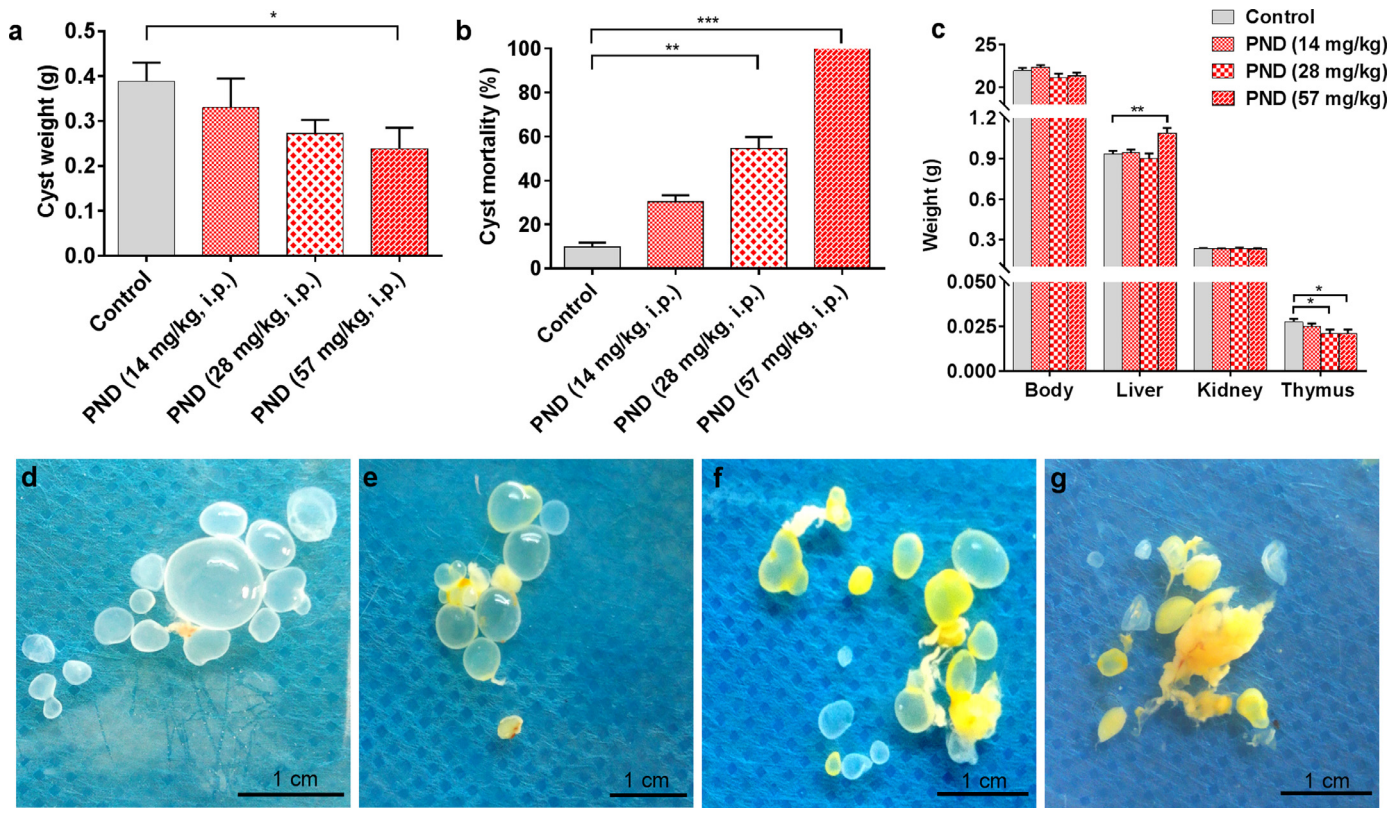


Fig. 1. *In vivo* efficacy of pyronaridine (PND) against secondary infection (cysts) of *E. granulosus ss*. The treatment groups (n = 10 for each) were intraperitoneally injected with PND at a dosage of 14, 28 or 57 mg/kg once daily for three days. The control group (n = 12) received saline. After euthanasia, cyst weight (a) and mortality (b) were measured and visualized by column plots. The liver, kidney, thymus and body weights of mice were measured (c). The cysts collected from each group were presented (d: controls; e: 14 mg/kg PND; f: 28 mg/kg PND; g: 57 mg/kg PND). Data are represented as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA or nonparametric Kruskal-Wallis test).

PND-treated mice as well as in the ABZ group; this reduction in body weight was deemed acceptable following long-term administration (Fig. 2c). Hematological and biochemical indexes did not show any statistical difference among the groups (Table S3).

2.4. *Ultrastructual analysis of* E. granulosus ss *cysts following pyronaridine treatment*

Transmission electron microscopy (TEM) was performed to evaluate the efficacy of PND based on the ultrastructure of cysts. Cysts from the ABZ-treated group (50 mg/kg, p.o., Fig. 3e) did not exhibit obvious ultrastructural changes in the germinal and laminated layers compared with that of the cysts from the control mice (Fig. 3a). Nucleoli and undifferentiated cells were intact. Microtriches were arranged in a regular fashion. Treatment with PND (both i.p. and p.o.) resulted in the devastating damage of the cyst tissues, and no structurally intact cysts were observed (Fig. 3b and c). Indeed, all the cysts were collapsed and the germinal layers were teared off from the laminated layers, and the structural integrity was completely lost. The cytoplasm of parenchyma cells became concentrated and apoptotic bodies were formed (Fig. 3d). Normal nuclei, nucleoli and undifferentiated cells could not be found, suggesting that the daughter cyst

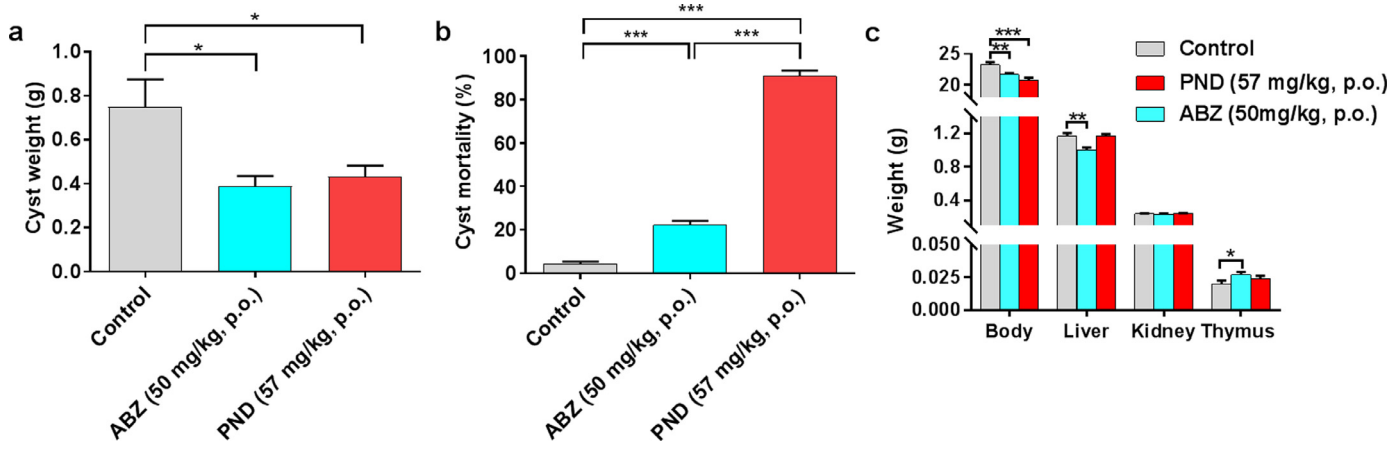


Fig. 2. The efficacy of pyronaridine (PND) taken orally against *E. granulosus* cyst infection in mice**.** Drugs were orally given for 30 days to the mice preciously infected with microcysts: the control group was unmedicated; the treatment groups were administered with albendazole (ABZ, 50 mg/kg) in 0.5% carboxymethyl cellulose or PND (57 mg/kg) in double distilled water on a daily basis by gavage (n = 10 for each treatment). After euthanasia, cyst weight (a) and mortality (b) were measured. The liver, kidney, thymus and body weights of mice were measured (c). Data are represented as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way ANOVA or nonparametric Kruskal-Wallis test).

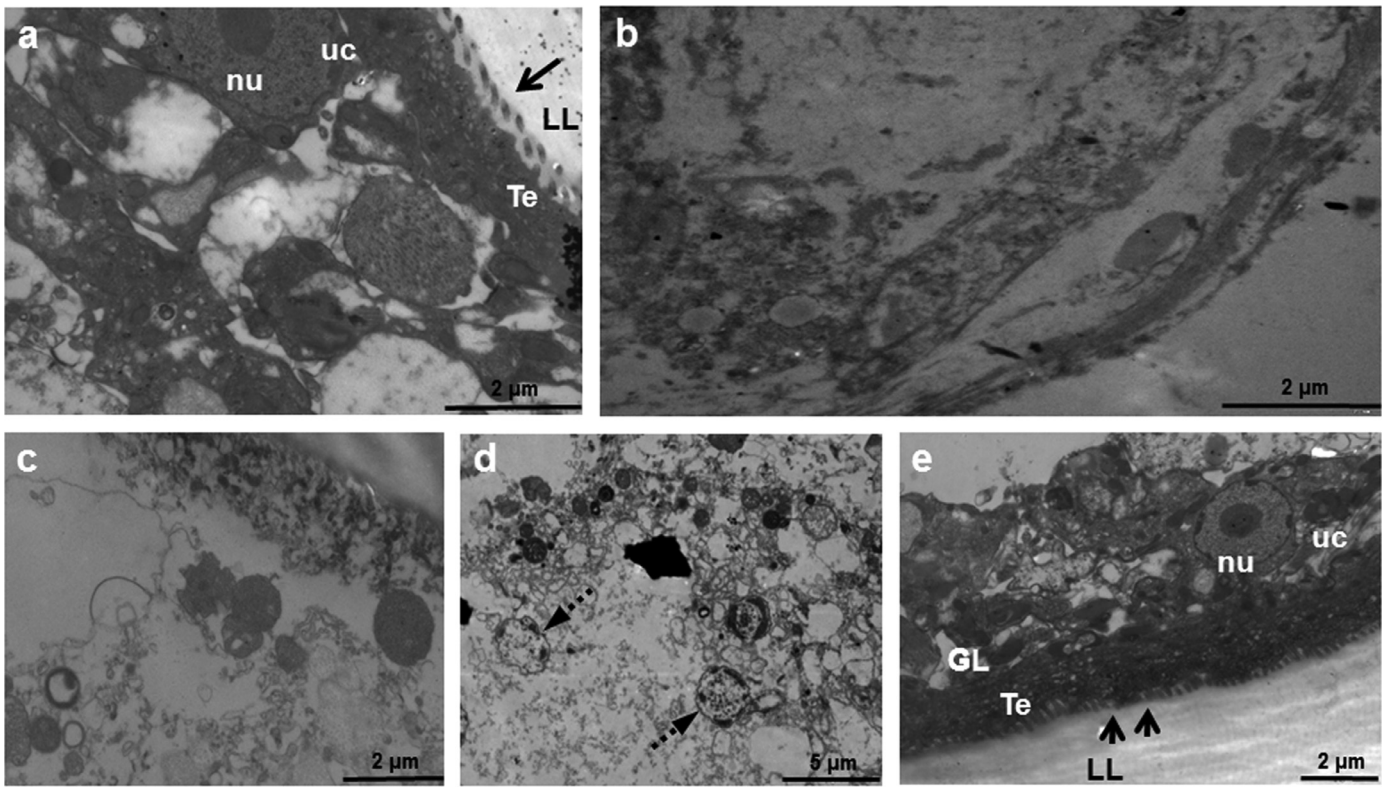


Fig. 3. TEM analysis shows the ultrastructure of *E. granulosus ss* cysts from mice treated with pyronaridine (PND). (a) Structure of intracellular organelles of a cyst from an untreated mouse. (B-E) Representative micrographs of cysts collected from the mice treated with PND (b, 57 mg/kg, i.p.; c and d, 57 mg/kg, p.o.) and ABZ (e, 50 mg/kg, p.o.). LL: laminated layer; Te: tegument; GL: germinal layer; uc: undifferentiated cells; nu, nucleolus. Microtriches: solid arrows; apoptotic bodies: dotted arrows.

generation system was destroyed. PND treatment caused a devastating impact on the tegument layer with complete shedding of microtriches, and only a few residues of tegumental tissue were left. Gross ultrastructural alterations were also observed in the laminated layer. Normal regular and densely packed laminated layer was replaced by a distorted and vacuolated structure.

2.5. Microinjection of pyronaridine kills cysts in mice

PAIR is a highly recommended option for treating echinococcosis. In this study, we developed an *in vitro* microinjection procedure to inject PND into echinococcal cysts using a micromanipulator. The *in vitro*-treated cysts were then transferred into mice and allowed to develop without intervention. After 30 days, as expected, the transferred cysts showed effective responses. The wet weight and counts of cysts were reduced in both PND groups, and the most therapeutic effect was observed in the high concentration group (Fig. 4). The parasite wet weight of the PND group at a concentration of 200 μM was significantly reduced by 52.3% compared with that of the saline group ($p < 0.05$, one-way ANOVA, Fig. 4a). There was a significant difference in the cyst counts between the PND group (6.4 $\pm$ 1.2, 200 μM) and the saline group (14.4 $\pm$ 2.1, $p < 0.05$, one-way ANOVA, Fig. 4b). After injection with PND, most of the cysts were devitalized and failed to grow and develop in mice. The dead cysts were transformed into calcification foci, and were observed in the abdominal cavity during the autopsy.

2.6. Pyronaridine predominantly distributes in the liver and lungs

In order to better understand the distribution of PND in organs, the pharmacokinetic properties and tissue distribution of PND were determined in mice following administration of a single intraperitoneal dose (Fig. 5). PND exhibited a significantly higher exposure in the liver than in the plasma at all time points; we also observed a difference in drug distribution between the lungs and plasma. The average concentrations of the drug in the liver and lungs from 0 to 48 h were 435.7 and 122.5 μmol/kg, whereas the average plasma concentration was 1.5 μM; this resulted in a ratio of liver/plasma of 290.5 and a ratio of lungs/plasma of 81.7 (assuming the density of the liver = 1.0 g/ml). We also found that PND sustained high concentrations in the livers without an obvious decline in 48 h after taking the drug, which indicated that PND may be administered every two days (or longer).

The liver is an important metabolic organ, as well as a major target organ of CE. Hepatic metabolic properties of PND were of great interest to us. We revealed that, in human hepatocytes, PND exhibited great metabolic stability with a reasonably long half-life ($T_{1/2}$ = 7.8 h) and slow clearance rate (Cl_{int} = 3.8 ml/min/kg). Cytochrome P-450 (CYPs) are the major enzymes involved in drug metabolism. Few studies have attempted to analyze the relationships between CYPs and PND. The capacity of PND to inhibit the catalytic activity of five CYP isoforms was evaluated in this study. No significant inhibition of other CYP isoforms ($IC_{50} > 10$ μM for CYP1A2, CYP2C9, CYP2C19 and CYP3A4) was observed except for moderate inhibition of CYP2D6 (IC_{50} = 1.53 μM), suggesting that potential alterations in pharmacokinetic properties and drug interactions would need to be taken into consideration in future studies.

2.7. Pyronaridine inhibits E. granulosus ss topoisomerase I and induces apoptosis

Although the antimalarial mechanism of PND remains unclear, it has previously been reported that PND can inhibit *Plasmodium* topoisomerases [15,16]. We subsequently speculated as to whether PND targets homologues in *E. granulosus ss.* In order to investigate this potentiality, we cloned and expressed *E. granulosus ss* topoisomerase I (*Eg*topo I). The recombinant protein demonstrated topoisomerase activity by relaxing supercoiled DNA (Fig. S3). Furthermore, PND

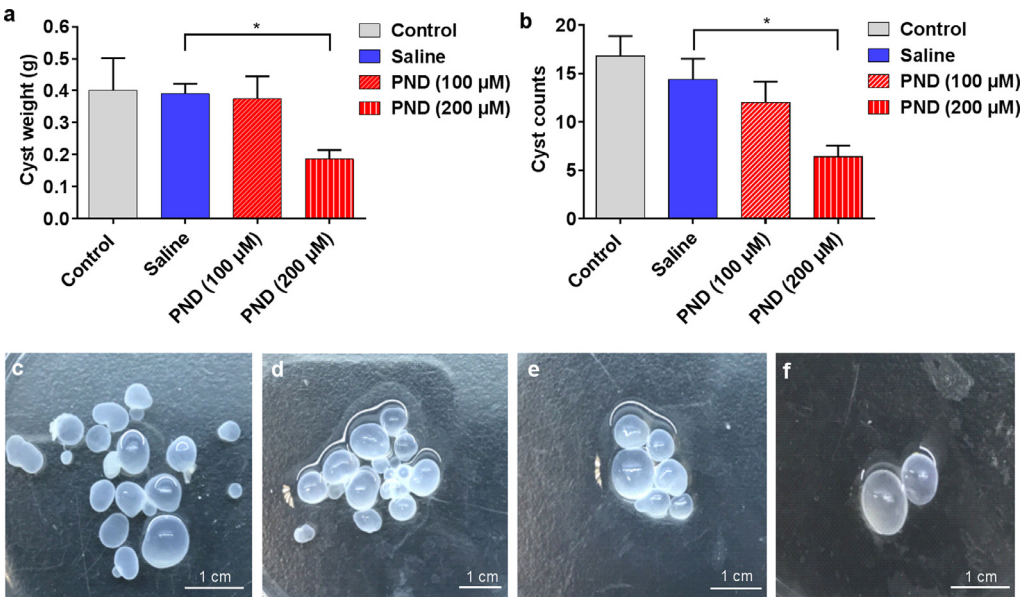
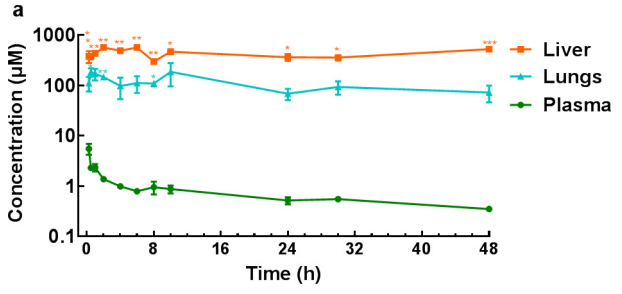


Fig. 4. *In vivo* anti-echinococcal efficacy of pyronaridine (PND) through a microinjection technique. After *in vitro* microinjection of PND or saline, cysts were intraperitoneally injected into mice (35 cysts/mice, $n = 5$ for each treatment). At 30 days post-treatment, cyst weight (a) and counts (b) were recorded. The cysts collected from each group were presented (c: controls; d: saline; e: 100 μM PND; f: 200 μM PND). Data are represented as means ± SEM. * $p < 0.05$ (one-way ANOVA).

inhibited *Eg*topo I activity in a dose-dependent manner with a moderate IC_{50} value of 209.7 ± 1.1 μM (Fig. 6).

Topo I inhibition usually triggers apoptosis. Typical apoptosis-induced morphological changes were observed in the cysts collected from PND-treated mice using the TEM. These morphological changes included the formation of apoptotic bodies (Fig. 3d) and the presence of condensed cytoplasm. In this study, we also characterized apoptotic phenotypes of PSCs (including DNA fragmentation and caspase activation) treated with PND. Typically, upon induction of classical apoptosis, DNases are activated, leading to DNA fragmentation. Indeed, in PND-treated PSCs, abundant TdT dUTP nick-end labeling (TUNEL)-positive cells were observed, a phenomenon that is indicative of DNA fragmentation (Fig. 7a). In addition, when DNA extracted from PND-treated PSCs was subjected to agarose gel electrophoresis, a clear pattern of DNA fragmentation (DNA ladder) was observed, which was absent in DNA obtained from control PSCs (Fig. 7b). Caspase-3 is a cysteine-aspartic acid protease that plays a core role in the execution phase of apoptosis. PND treatment resulted in a 20-fold increase in caspase-3 activity compared with the vehicle controls, suggesting that the apoptosis induced by PND is most likely associated with caspase-3 activation (Fig. 7c).



b

Parameters	PND (57 mg/kg, i.p., $n = 3$)		
	Plasma	Liver	Lungs
T_{max} (h)	0.3	6.0	10.0
C_{max} (μM)	5.5	565.1	186.1
$T_{1/2}$ (h)	28.3	ND*	81.5

*ND, not determined. Since the drug concentration did not obviously decline within 48 h, the $T_{1/2}$ cannot be calculated.

Fig. 5. *In vivo* pharmacokinetics of pyronaridine (PND). (a) Concentrations of PND in the plasma, liver and lungs were respectively measured over time after a single intraperitoneal dose (57 mg/kg). The mean PND concentration of three replicates at each time point is shown ($n = 3$). Data are represented as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (one-way repeated measures ANOVA). Pharmacokinetic parameters: $T_{1/2}$, C_{max} and T_{max} are provided in (b).

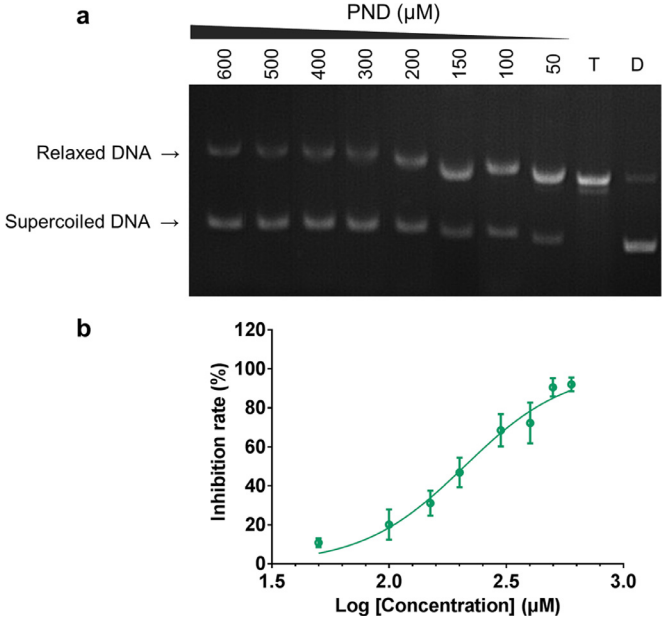


Fig. 6. Inhibition of *Eg*topo I-mediated DNA relaxation by pyronaridine (PND). Supercoiled pBR322 plasmid DNA and *Eg*topo I were incubated with PND at indicated concentrations. DNA relaxation was visualized by electrophoresis using a 1% agarose gel (a) and the IC_{50} value of PND against *Eg*topo I was measured (b, $n = 3$). Data are represented as means ± SEM. *D* = pBR322, *T* = pBR322 + *Eg*topo I.

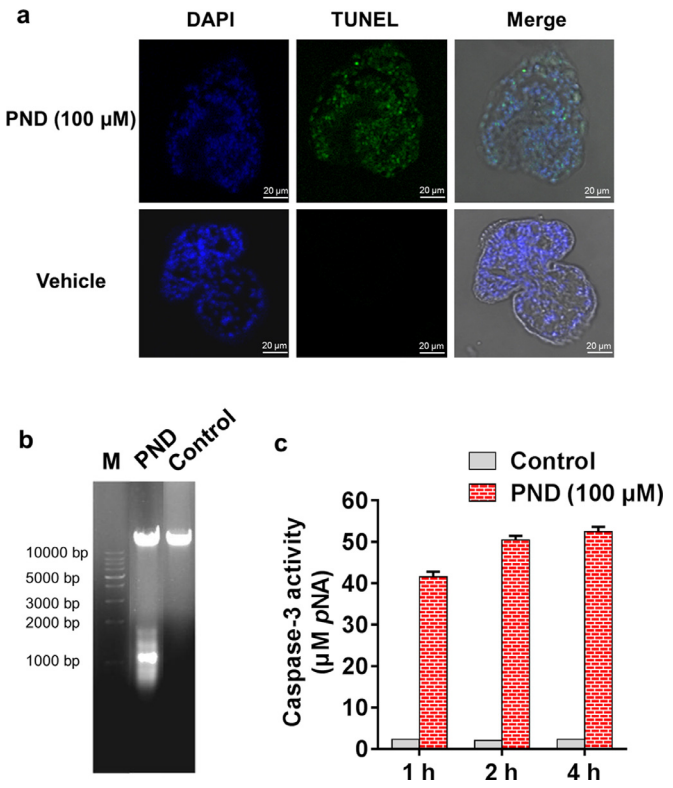


Fig. 7. Apoptosis induced by pyronaridine (PND) in *E. granulosus ss* PSCs. (a) PND-treated PSCs and vehicle controls were stained using the TUNEL assay system. Apoptotic cells were observed using TUNEL (green) and nuclei were stained with DAPI (blue). (b) DNA fragmentation in PSCs was analyzed using agarose gel electrophoresis after incubated with 50 μM of PND for 24 h. (c) The caspase-3 activity in PSCs treated with or without PND was analyzed (n = 2). Data are represented as means ± SEM.

3. Discussion

Repurposing old drugs for treating different diseases has accelerated drug R&D process in recent years and saved 40% of the overall cost [17]. Several studies have reported that some clinically available drugs showing anti-CE activity, such as artemisinins [8], mefloquine [9], nitazoxanide [9], 5-fluorouracil [11], imatinib [13] and metformin [18]. However, none of these drugs has proceeded to clinical application. In this study, we revealed that PND can be used as an anti-CE agent following either intraperitoneal or oral administration. Both of the regimens facilitate an increased distribution in the CE target organs, rendering PND as a promising candidate for the treatment of *E. granulosus ss* larval infections.

The *in vitro* assay showed that the efficacy of PND against PSCs was better than that of ABZ. We subsequently evaluated the anti-CE efficacy of PND in mice. A three-dose intraperitoneal regimen of PND in three days resulted in great therapeutic effects against CE in the murine model. This was a short-course regimen which obtained 100% cyst mortality. As far as we know, there are currently no drugs showing such high killing effect against the larval stage of the parasite. In addition, the cysts from the treated mice were in yellow color, the color of the drug, indicating that PND (when administered orally or intraperitoneally) can penetrate through the laminated and germinal layers of cysts and kill the cysts.

Regarding the oral regimen, although there was no statistical difference in terms of the reduction in parasite wet weight between the ABZ- and PND-treated groups (likely due to the short-term of treatment), the treatment of PND exhibited significantly greater cysticidal effects compared with that of ABZ. The results of viability tests were confirmed at the ultrastructural level of cellular organelles by TEM. The damage induced by PND was more severe than that by ABZ. The ultrastructural changes include the completely damaged germinal layer, the distortion of internal tissue with the presence of vacuolated areas, and the presence of apoptotic bodies.

In the study, PND was orally administered to mice using a dose of 57 mg/kg/day for 30 days. Based on the human equivalent dose (HED) formula [19], this dose is equivalent in humans to 4.6 mg/kg of PND. Previous sub-acute toxicity studies showed that oral administration of PND at a dosage of 24 mg/kg/day (HED = 13.0 mg/kg/day) for 30 days was well tolerated by dogs without gross alterations in clinical laboratory parameters or electrocardiogram results [20]. In some recent studies, the no observed adverse effect levels (NOAEL) of PND following 28 days oral treatment in the rat and dog were 23 mg/kg/day (HED = 3.7 mg/kg/day) and 5 mg/kg/day (HED = 2.7 mg/kg/day), respectively [16], which were both very close to the oral dosage used in our study. Throughout the treatments, the behavior and appearance of the animals were normal and no obvious adverse effects or toxic reactions were observed, indicating all regimens were well-tolerated by the mice. There were no significant changes of the hematological parameters and biochemical indices between experimental and control mice.

PAIR is highly recommended as the first treatment option for CE patients who are asymptomatic [21]. Currently, 20% hypertonic saline, 95% alcohol and 3% hydrogen peroxide are commonly used as protoscolecides in the PAIR procedure [6]. However, the application of these agents increases the morbidity of sclerosing cholangitis, a complication that can occur during PAIR. In the current study, we show that more than 90% of cysts were killed after microinjection with PND, which indicated that, in addition to oral administration, PND can also be injected intracystically and might represent an alternative parasiticide that can be applied during the PAIR procedure. In addition, when used as a topically administered drug, only a very small amount of PND is released to the tissues surrounding the cysts or the circulatory system of the host; this limited amount of drug is probably tolerated as it has previously been shown that dogs and rodents can tolerate very high doses of the drug following oral and intramuscular administration [16].

The liver and lungs act as the first two natural mechanical filters for the parasite. The hydatid cysts in humans develop mainly in the liver (70%) and lungs (20%) [22]. The histological structure of *E. granulosus* cysts determines that passive diffusion is the major route of entry for chemotherapeutic drugs into parasite tissues (endocyst) [23,24]. Thus, increased distribution into the two afore-mentioned organs would be very beneficial for anti-CE compounds. Indeed, PND was highly distributed in the liver and lungs of mice after i.p. administration. For oral administration, according to previously published data, the liver also ranks as the first site of tissue distribution in mice [25] and rats [26]. In addition, we found that PND exhibited a sustained high-level distribution with a very slow clearance rate in the liver. The hepatic cysts were contained in a high drug concentration after treatment, which is undoubtedly beneficial for the drug to penetrate the laminated layer and germinal layer of echinococcal cyst and concentrate in the cyst fluid. Bearing in mind the high distribution of PND in the liver and lungs, the drug most likely exhibits greater therapeutic effects in the cases of hepatic and pulmonary echinococcosis. This needs to be further investigated using liver or lung echinococcosis models.

So far, there is no standard method for cyst viability assessment. We previously tried to use methylene blue method to stain the cysts treated or no treated, which showed that the germinal layers were not clearly stained and it was difficult to identify whether the cysts alive or dead. In this study, we proposed a classification to identify the CE cysts collected from experimentally infected mice (Table S4). To verify the reliability of the classification, for each cyst type, we transferred some of those cysts into the abdominal cavity of mice. After three weeks, we necropsied the mice and examined the cysts.

Type 1 cysts survived and developed. Type 2 cysts could not survive and develop, and almost all of them were ruptured. Type 3 cysts were still ruptured and collapsed, and cyst residues became more consolidated and calcified. In addition, for Type 2 cysts, we also stained the cyst section with H&E, which showed that the germinal layers were broken and teared off from the laminated layers.

We showed that PND inhibited the activity of *E. granulosus ss* topoisomerase I (*Eg*topo I). Topo I is a drug target that has been validated in clinical practice. Irinotecan and topotecan, two classical Topo I inhibitors, have been used for the treatment of tumors for decades [27,28]. Some studies have reported the potential of topo I inhibitors against parasites including *Plasmodium* spp. [29,30], *Leishmania* spp. [31,32], *Cryptosporidium parvum* [33], and *Trypanosoma* spp. [34,35]. PND has been reported to kill plasmodia by targeting topoisomerases. Since the core structures of topoisomerases are highly conserved in organisms [36], we speculated that PND has a similar effect on *E. granulosus ss* topoisomerases. Indeed, according to our results, PND inhibited *Eg*topo I-mediated cleavage of DNA; the inhibitory ability was not strong, and other mechanisms (*e.g.* apoptosis) may be involved in killing *E. granulosus ss* PSCs and cysts. A recent study indicated that PND induces apoptosis and cell cycle arrest in cancer cell lines [37]. Our results also revealed that DNA fragmentation and caspase-3 activation, two defined apoptotic hallmarks, were observed in PND-treated PSCs. However, targeting topoisomerases may result in genotoxicity in associated cells. Fortunately, earlier studies have addressed this problem. Following a micronucleus test in mice, there was no increase in micronucleus frequency in the bone marrow, and no structural damage to chromosomes or the spindle apparatus after PND treatment [38,39]. Following the Ames mutagenicity test, PND did not induce mutations in four *Salmonella typhimurium* strains (TA100, TA98, TA1535 and TA1538), but there was evidence of bacterial mutagenicity for strain TA1537 [40].

In conclusion, this study demonstrated that PND is a potent and orally active anti-echinococcal agent, which can be used as a promising drug candidate for treating CE. PND could be released into the market in a relatively short time frame since the toxicity profile is already available. Importantly, PND was highly concentrated in the liver and lungs also indicating that the drug might exhibit greater efficacy and cause less adverse effects than currently available alternatives. Furthermore, given the growing popularity of minimally invasive treatments, the utilization of PND as a protoscolecide and cyst-killer in PAIR management or as an interventional anti-parasitic treatment before/after PAIR procedure may render it as a beneficial addition to current treatment options.

4. Materials and methods

4.1. E. granulosus ss *PSC killing assay*

E. granulosus ss PSCs were aspirated from echinococcal cysts of naturally infected sheep livers collected from an abattoir in Urumqi, Xinjiang Uyghur Autonomous Region, China. The *in vitro* culture of PSCs was performed following a previously described method [41]. A drug activity assay was performed in 96-well plates. PND was synthesized in-house (Fig. S1 and S2). The drug was made up to a 10 × stock solution with deionized water and the concentrations ranged from 340 μM to 1140 μM. Viable PSCs were aliquoted in each well (200 PSCs/well) containing 190 μl of culture medium. Then, 10 μl of stock PND solution was added into each of the wells with final drug concentrations ranging from 34 to 114 μM. PSCs in the medium only were used as the control. The plates were placed in an incubator at 37 °C in a 5% CO_2 atmosphere for 24 h. The viability of PSC was assessed using methylene blue staining test. Briefly, 0.1% methylene blue solution was added to each well. After 5 min, the PSC were observed under an inverted microscope. Dead PSC were stained in blue color, while the living ones remained colorless. The experiments were carried out in triplicate. The LC_{50} value was calculated with GraphPad Prism software using a nonlinear regression equation.

4.2. In vivo *efficacy study in experimentally infected mice*

Pathogen-free female BALB/c mice, aged 6–8 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Company Limited, and raised in the animal facility of the First Affiliated Hospital of Xinjiang Medical University. Mice were infected by intraperitoneally injecting microcysts using previously described procedures [41]. At three months post-infection, the animals were randomly allocated into the following groups.

Intraperitoneal administration study: (1) saline control group (n = 12); (2) 14 mg/kg PND group (n = 10); (3) 28 mg/kg (n = 10) and (4) 57 mg/kg PND group (n = 10). PND tetraphosphate was dissolved in saline. Animals were dosed with 1.0 ml of corresponding drug solution or saline by intraperitoneal injection once a day for three days.

Oral administration study: (1) unmedicated control group; (2) 50 mg/kg ABZ group; (2) 57 mg/kg PND group. Each group has 10 mice. PND tetraphosphate was dissolved in deionized water. ABZ was formulated in 0.5% carboxymethyl cellulose. The mice in medicated groups were treated with 0.2 ml of corresponding drug solution by gavage once a day for a period of 30 days. Unmedicated control mice received an equal volume of deionized water.

At two weeks post-treatment, all mice were euthanized, and necropsy was carried out. Cysts collected from each mouse were weighed and examined. The cyst viability was assessed following the classification for *E. granulosus* cysts (CE) collected from experimentally infected mice (Table S4). According to the structural morphology, the murine CE cysts are classified into three types, the features and images (by eyes or under microscope) of each cyst type are represented in Table S4. In our previous work, for each type, we transferred the cysts to the abdominal cavity of normal BALB/c mice by surgical operation, and after three weeks, we necropsied the mice and examined the cysts. Type 1 cysts could survive and develop, thus we define it alive. Type 2 and 3 cysts could not survive and develop, so they are defined as dead cysts. During the treatment, animals were carefully observed for signs of morbidity, including emaciation, hunched back, ruffled coat, and change in appetite, as well as behavioral alterations. After euthanasia, blood samples were collected immediately, and plasma was separated by centrifugation at 2000 *g* for 15 min at 4 °C; samples were subsequently kept frozen at −20 °C until further analysis. The liver, kidneys and thymus of each mouse were subsequently weighed.

4.3. *Microinjection treatment*

Microcysts were cultured from PSCs according to a previously described method; the cysts were cultured to 300 μm in diameter [42]. Before microinjection, we selected each batch of cysts under a stereomicroscope; these semi-transparent cysts with round in shape were qualified for microinjection. Microneedles (inner diameter: 5 μM) and holding pipettes (outer diameter: 100–150 μM, inner diameter: 50–80 μM) were pulled by a P97 horizontal puller (Sutter). In a culture dish (diameter of 60 mm), 30 μl of culture medium was aliquoted as a central droplet onto the middle of the dish. In addition, a 10-μl droplet of culture medium used for the storage of microinjected cysts and one 2-μl droplet of PND (at a concentration of 14 or 28 mM) in saline were dropped close to the central droplet. Mineral oil (6 ml) was subsequently transferred to cover the droplets in the dish. Approximately 50 microcysts were transferred into the central droplet. The dish was placed on the micromanipulator and microcysts were positioned by holding pipettes. Prior to microinjection, the PND stock solution (14 or 28 mM in saline) was drawn into a microneedle. The microneedle was inserted into the cavity of microcysts and approximately 100 pl of PND solution was injected (final

concentrations: 100 and 200 μM). Microinjection was performed under an IX71 inverted microscope (Olympus) using a FemtoJet microinjector and a TransferMan NK2 micromanipulator (Eppendorf). Injection pressure was set at 800 Pa with a pulse length of 200 ms and compensation pressure of 200 Pa. An identical amount of saline was injected into cysts as a control. For each group, 35 microinjected cysts were washed three times with culture medium and intraperitoneally injected into mice (n = 5) with a syringe. At 30 days post-treatment, all mice were euthanized and therapeutic effects were examined as described for the *in vivo* efficacy study.

4.4. In vivo *pharmacokinetics*

The *in vivo* pharmacokinetic properties of PND were measured in the plasma, liver and lungs of male ICR mice (20−22 g, 3 animals/time point) after a single intraperitoneal injection (57 mg/kg). Plasma samples (40 μl) were mixed with acetonitrile (400 μl) containing internal standards (200 ng/ml of tolbutamide, 50 ng/ml of propranolol and 500 ng/ml of diclofenac sodium) in tubes. The tubes were subsequently centrifuged at 2600 g for 10 min at 4 °C, and the supernatants were collected and analyzed. Liver and lung samples were homogenized with 4 times the volume of phospherate buffer (100 mM, pH 7.4). The resultant homogenates were processed as mentioned above. The plasma and tissue concentrations at 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 30 and 48 h after administration were determined by LC-MS/MS, and the data were analyzed by non-compartmental methods using WinNonlin software.

4.5. E*gtopo I-mediated DNA relaxation assay*

The inhibitory activity of PND toward *Eg*topo I was analyzed using a DNA topoisomerase I assay kit (TaKaRa). Briefly, DNA (pBR322, 200 ng), *Eg*topo I (300 ng), 0.1% bovine serum albumin (BSA, Sigma-Aldrich) and PND (final concentrations: 50, 100, 150, 200, 300, 400 500 and 600 μM) were incubated in assay buffer for 30 min at 37 °C. The reaction was terminated by adding 10% SDS solution and DNA loading buffer. Samples were electrophoresed on 1% agarose gels, stained with ethidium bromide (5 μg/ml) and photographed at 254 nm. The curve was fitted using a nonlinear regression model by GraphPad Prism software.

4.6. *Statistical analysis*

Statistical significance was assessed using one-way ANOVA or nonparametric test (Kruskal-Wallis test). P values less than 0.05 were considered statistically significant. Unless otherwise specified, data are represented as means ± SEM ($n \geq 3$).

Ethics statement

Animal studies were conducted in accordance with the Chinese Laboratory Animal Administration Act 2017. All experiments involving animals and protocols for use of samples from mice were approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval number: IACUC-20140424008).

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Declaration Competing of Interest

The authors declare that they have no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2020.102711.

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