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In vitro and *in vivo* efficacies of novel carbazole aminoalcohols in the treatment of cystic echinococcosis

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Objectives: Cystic echinococcosis (CE), caused by the cestode *Echinococcus granulosus*, is a worldwide chronic zoonosis. Current chemotherapeutic options are limited to albendazole and mebendazole, which only exert parasitostatic effects and have to be administered at high dosages for long periods. In an effort to find alternative treatment options, the *in vitro* and *in vivo* efficacies of novel carbazole aminoalcohols were evaluated.

Methods: Carbazole aminoalcohols were tested against *E. granulosus* protoscoleces *in vitro* and metacestodes *ex vivo*. The *in vivo* chemotherapeutic effect of representative compounds was assessed in experimentally infected mice. Oral and intravenous pharmacokinetic profiles were determined in mice.

Results: The carbazole aminoalcohols exhibited potent protoscolicidal activity with LC_{50} values ranging from 18.2 to 34.3 μM. Among them, compounds **2** and **24** killed all *ex vivo* cultured metacestodes at concentrations of 34.3 and 30.6 μM. *In vivo* studies showed that oral administration of compounds **2** and **24** (25 mg/kg/day) for 30 days led to reductions of 68.4% and 54.3% in parasite weight compared with the untreated group (both groups: $P < 0.001$). Compound **2** (25 mg/kg/day) and compound **24** (50 mg/kg/day) induced significantly higher cyst mortality rates in comparison with that of the albendazole group (both groups: $P < 0.01$). Analysis of cysts collected from compound **2**- or **24**-treated mice by transmission electron microscopy revealed a drug-induced structural destruction. The structural integrity of the germinal layer was lost, and the majority of the microtriches disappeared. Pharmacokinetic profiling of compounds **2** and **24** revealed low clearance and decent oral bioavailability (>70%).

Conclusions: Our study identifies carbazole aminoalcohols as a class of novel anti-CE agents. Compounds **2** and **24** represent promising drug candidates in anti-CE chemotherapy.

Introduction

Cystic echinococcosis (CE), also known as cystic hydatid disease, is a zoonosis caused by infection with the larval stage of the tapeworm *Echinococcus granulosus*. The disease has a wide distribution, with high endemic regions including Mediterranean countries, Central Asia, North and East Africa, and South America.[1] Intermediate hosts, including humans, become infected by ingesting the eggs of parasites in the faeces of definitive hosts, with resulting release of oncospheres in the intestine and the development of hydatid cysts in the internal organs. The unilocular hydatid cysts in humans develop mainly in the liver (70%) and lungs (20%) with the remaining 10% of cases in other organs including the kidneys, spleen, brain, heart and bone.

Treatment options for CE are limited to surgery and/or chemotherapy, with surgical excision as the only way to cure the disease. However, surgical treatment requires highly qualified surgeons, an expert team and professional facilities. As a result, most patients with CE receive non-surgical treatment, including chemotherapy. Currently, benzimidazoles (albendazole and mebendazole) remain the only option for anti-CE chemotherapy. Albendazole is the only

drug recommended by WHO for human CE treatment. However, albendazole acts parasitostatically rather than parasitocidally and requires long-term application. A retrospective study showed that albendazole had a recovery rate in CE cases ranging from 11.8% to 35.2%, while 40% of the cases did not respond favourably to drug treatment.[2,3] Long-term application of albendazole resulted in severe adverse side effects.[4] Limited and low efficient chemotherapeutic options are stumbling blocks to the treatment of CE. Novel and improved chemotherapeutic drugs are urgently needed.

Since molecular mechanisms and cellular signalling pathways of *Echinococcus* largely remain unclear, using rational drug design theory to identify new chemical entities targeting a specific molecular target (or multiple targets) is a challenge for drug discovery. Considering that echinococcal cysts demonstrate some of the same characteristics as malignant tumours, such as unlimited cell proliferation, sustained angiogenesis, tissue invasion and unexpected metastasis,[5] several anticancer agents were subjected to phenotypic screening to evaluate their anti-CE potential. As expected, a few of them were identified to exhibit anti-echinococcosis activity *in vitro* and/or *in vivo*,[6–9] including doxorubicin,[10] 5-fluorouracil,[11] paclitaxel,[11] tamoxifen,[12] bortezomib[13] and imatinib.[14]

Carbazole has been regarded as a privileged core structure in antiproliferative chemotypes.[15,16] Several carbazole derivatives exhibit antitumour activity by targeting DNA topoisomerases.[17,18] In our recent studies, novel carbazole aminoalcohols were identified with antiproliferative activity.[19] Human DNA topoisomerase I (*h*topo I) was identified as one of the targets of the compounds. DNA topoisomerase I (topo I) has been a popular drug target for anticancer agents since it was discovered in 1972,[20] and is able to relax supercoiled DNA to control DNA replication.[21] Topo I inhibitors are broadly used cancer chemotherapeutic drugs in clinical practice.[22] They can block topo I-mediated cleavage of DNA, generating single-strand DNA breaks, which subsequently leads to cell cycle arrest and apoptosis. Recently, we presented data outlining the genome and transcriptome of *E. granulosus*.[23] Comparative analyses of the transcriptome sequences with other taxa provide a new idea to facilitate the development of alternative chemotherapeutic drugs.[24] In the case of topo I, it is worth noting that, according to the results of sequence alignment, *E. granulosus* topoisomerase I (*Eg*topo I) shares 55.8% identity with *h*topo I; specifically, the amino acids known to mediate the inhibitor–enzyme binding are highly conserved. Thus, it is reasonable to hypothesize that the described molecular target of carbazole aminoalcohols in humans is also a drug target for *E. granulosus*. These findings prompted us to investigate the anti-echinococcal potential of carbazole aminoalcohols.

In the present study, we evaluated the *in vitro* parasiticidal efficacy of synthetic carbazole aminoalcohols against *E. granulosus* protoscoleces (PSC) and metacestodes. The *in vivo* chemotherapeutic effect of representative compounds was also assessed by employing an experimentally infected mouse model.

Materials and methods

The synthesis of carbazole aminoalcohols is described in the Supplementary data available at *JAC* Online. Unless otherwise specified, all chemicals were purchased from Sigma–Aldrich. For *in vivo* testing, 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. The sheep livers were collected from abattoirs in Urumqi, Xinjiang Uyghur Autonomous Region, China.

In vitro culture of E. granulosus PSC and drug screening

E. granulosus PSC were aspirated from hydatid cysts of naturally infected sheep livers. The PSC were digested with 1.0% of pepsin in Hank's at pH 2.0 for 30 min. The PSC were then washed five times with PBS (pH 7.2). The parasite vitality was evaluated by 0.1% methylene blue. Only PSC batches exhibiting >98% viability were used.

In vitro treatment of PSC was performed in a 96-well plate with each well containing ~200 PSC. The pepsin-treated PSC were incubated in RPMI 1640 (Invitrogen, San Diego, CA, USA) culture solution supplemented with 10% FCS (HyClone, Logan, UT, USA) and antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin; HyClone) at 37°C in 5% CO_2. The drugs were dissolved in DMSO and added to each well (containing 200 μL of medium) resulting in corresponding final concentrations (carbazole aminoalcohols concentration range: 2.4–71.4 μM; albendazole concentration range: 18.8–150.8 μM). For drug tests, 5 μL of drug (in DMSO) was added to each well; 5 μL of DMSO was added to each control well. The plates were incubated at 37°C in a 5% CO_2 incubator for 24 h. The viability of PSC was assessed by 0.1% methylene blue staining test and motile behaviour observation. The corresponding numbers of viable/non-viable PSC were determined in 10 randomly chosen fields by using a phase contrast microscope. The LC_{50} of each compound was calculated by Bliss methods in the SPSS 20.0 statistical software.

Ex vivo culture of E. granulosus metacestodes and drug screening

BALB/c mice were infected with cultured microcysts according to our previous publication.[25] After 10 weeks post-infection, the mice were euthanized and the cysts were collected from the peritoneal cavity. The collected cysts were divided according to the size (small-sized cysts: diameter ≈5 mm, containing no PSC; middle-sized cysts: diameter ≈10 mm, some containing PSC; large-sized cysts: diameter ≥15 mm, containing PSC). The cysts were cultured in bottles and maintained in RPMI 1640 culture solution supplemented with 10% FCS and antibiotics. Compounds **2** and **24** were dissolved in DMSO and added to each culture bottle resulting in corresponding final concentrations. Control cultures were also supplemented with equal volumes of DMSO. The culture bottles were incubated in a 5% CO_2 incubator at 37°C for 5 h. The viability of metacestodes was assessed by visual check using an inverted optical microscope. The evaluation criteria for dead cysts include the loss of turgidity and the collapse of the germinal layers (clearly detached from the laminated layer).

Experimental infection of mice and in vivo treatment with compounds 2 and 24

To obtain successfully infected mice, *E. granulosus* PSC were pre-cultured *in vitro* to generate small cysts (microcysts, 200–300 μm in diameter).[25] Each mouse was intraperitoneally transplanted with 50 microcysts, suspended in 0.4 mL of RPMI 1640 medium. Compounds were formulated in 0.5% carboxymethyl cellulose (CMC) at corresponding concentrations. After 10 weeks post-infection, all BALB/c mice were randomly divided into eight groups with 10 mice in each group. The mice were treated by gavage for a period of 30 days with 200 μL of 0.5% CMC (control group), 200 μL of albendazole (50 mg/kg in 0.5% CMC, albendazole group) and 200 μL of compound **2** or **24** at corresponding dosages (from 25 up to 100 mg/kg in 0.5% CMC, drug groups), respectively. During treatment, animals were carefully observed and checked for clinical signs of impaired health including weight loss, ruffled coat, hunched back and changes in behaviour. At the

end of treatment, all mice were euthanized. After necropsy, cysts were collected and their numbers, sizes and weight were measured. The viability of cysts was assessed as described above.

Transmission electron microscopy (TEM)

For probing tissue changes after drug treatments, cysts were processed for TEM analysis as described by Hemphill and Croft.[26] Briefly, cysts were fixed for 2 h in 2.5% glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2) at room temperature, then post-fixed for 2 h in 2% OsO_4 in 100 mM sodium cacodylate buffer (pH 7.2). Samples were washed with distilled water and treated with 1% uranyl acetate for 30 min. Then samples were washed again with distilled water, dehydrated in a graded alcohol series and embedded in Epon 812 resin. Polymerization lasted overnight at 65°C. Sections were cut on a Reichert and Jung ultramicrotome and stained with uranyl acetate and lead citrate. Micrographs were taken on a JEOL JEM-1230 transmission electron microscope.

Pharmacokinetic analysis

Pharmacokinetic studies on selected compounds were performed in mice, since this is the host species for the *E. granulosus in vivo* efficacy model. Compounds **2** and **24** were dosed in male ICR mice (20–22 g, 3 animals per timepoint) via intravenous and oral routes. Compounds were dissolved in 10% DMSO, 10% Solutol HS 15 and 80% PBS for the intravenous dose and 0.5% CMC for the oral dose. Blood samples (150 μL) were collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, 24, 32 and 48 h post-intravenous dose and 0.25, 0.5, 1, 2, 4, 6, 8, 10, 24, 32 and 48 h post-oral dose into centrifuge tubes containing sodium heparin and then plasma was isolated by centrifugation at 4°C. Plasma samples were extracted by the addition of acetonitrile containing a generic internal standard (propranolol). Samples were centrifuged at 2600 **g** for 15 min at 4°C, and the resulting supernatants were collected and analysed. The concentration of the compounds in plasma was determined by LC-MS/MS, and the data were analysed by non-compartmental methods using WinNonlin software.

Cytotoxicity assay

Human embryonic lung fibroblasts (WI38) were grown and harvested at log phase. Cells were plated in a 96-well plate at 10000 cells per well in 180 μL of MEM supplemented with 10% FBS and 1% penicillin/streptomycin. After incubation for 12 h at 37°C in 5% CO_2 to allow the cells to adhere, 20 μL aliquots of 2-fold serial dilutions of compounds **2** and **24** were added to wells in triplicate. The final concentrations of compounds were 50.0, 25.0, 12.5, 6.3, 3.2, 1.6 and 0.8 μM. Plates were incubated for another 2 days at 37°C in 5% CO_2. Then supernatants were removed, and 90 μL of fresh medium and 10 μL of MTT solution (Amresco, USA) were added. After incubation for another 4 h, the supernatants were removed and 110 μL of DMSO was added to each well. The plates were swirled gently for 10 min, and then the absorbance was read at 490 nm. Inhibition rate was calculated by the following formula:

$$\text{Inhibition rate} = \left(1 - \frac{OD_{treated} - OD_{blank}}{OD_{control} - OD_{blank}}\right) \times 100\%$$

CC_{50} values were then calculated.

Sequence analyses

The amino acid sequences of *Eg*topo I (GenBank: EUB61517.1), *h*topo I (NCBI Reference Sequence: NP_003277.1) and murine topo I (*m*topo I) (GenBank: EDL06289.1) were obtained from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) databank. The sequence alignment of *Eg*topo I, *h*topo I and *m*topo I was performed using the NCBI protein BLAST program.

Ethics

This study was conducted in accordance with the Chinese Laboratory Animal Administration Act (1988). The *in vivo* studies obtained the approval of the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of Xinjiang Medical University (approval number: IACUC-20140424008). The protocols for using samples from mice were approved by the Animal Ethic Committee of the First Affiliated Hospital of Xinjiang Medical University.

Results

Carbazole aminoalcohols exhibit potent parasiticidal activity against E. granulosus PSC and metacestodes in vitro

A total of 26 carbazole aminoalcohols were initially screened for their protoscolicidal potential using a concentration of 20.0 mg/L. As expected, the five compounds with confirmed *h*topo I inhibitory ability killed 100% of the *E. granulosus* PSC. The efficacy of the five compounds on PSC was further confirmed by testing graded concentrations of the drugs (Figure 1). The strength of the effect of the compounds on PSC proved dose-dependent. Their parasiticidal effects were shown in both invaginated and evaginated PSC. The five carbazole aminoalcohols demonstrated potent protoscolicidal activity with LC_{50} values ranging from 18.2 to 34.3 μM (Figure 1), which were all superior to that of albendazole ($LC_{50} = 79.2$ μM). Specifically, compounds **2** and **24** exhibited more potent parasiticidal activity against *E. granulosus* PSC than others did with LC_{50} values of 18.2 and 20.0 μM, respectively, which were 4-fold more effective than albendazole. Control PSC (DMSO treated) showed little change in viability and morphology throughout the experimental period.

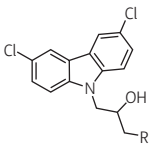
Based on the effectiveness of the carbazole aminoalcohols on PSC, we further employed compounds **2** and **24** to culture with *E. granulosus* cysts (collected from mice infected with microcysts) at concentrations ranging from 12.8 to 34.3 μM for 5 h. Three different sizes of *E. granulosus* cysts were used for evaluation. As shown in Figure 2, a significant metacestodicidal effect was observed after treatment with either **2** or **24**. Both compounds showed dose-dependent parasiticidal activity against the *E. granulosus* metacestodes. Treatment with 34.3 μM of compound **2** resulted in the killing of all cysts regardless of cyst size, and ∼50% of the cysts were killed at the concentration of 14.3 μM. Compound **24** produced similar results in the killing of cysts. When evaluating effects based on cyst size, we found that compound **2** was more effective against the large cysts, while compound **24** was more effective against the small cysts.

Incubation of *E. granulosus* metacestodes in the presence of compounds **2** and **24** resulted in dramatic morphological change. After a 4 h incubation period, the control cysts were remaining in the normal shape of cysts, with a smooth surface and intact germinal and laminated layers (Figure 3a and c). In contrast, cysts treated with compound **2** showed a complete disruption with reduced turgidity, thin translucent membranes and irregular and fissured surfaces (Figure 3b). The parasite germinal layer was collapsed, detached from the laminated layer and formed a densely

packed aggregate inside of the cyst. Similar results were observed in the cysts treated with compound **24** (Figure 3d).

Oral application of compounds 2 and 24 reveals significant in vivo activity in secondarily infected mice

The *in vivo* efficacy of compounds **2** and **24** against *E. granulosus* cysts was investigated in BALB/c mice infected with cultured microcysts. The average parasite weights in each group are shown in Figure 4. Daily oral application of albendazole yielded the expected results, primarily, significantly reduced parasite weights ($P < 0.001$, compared with the control group). All dosage groups of compounds **2** and **24** also showed a highly significant reduction in parasite burden in comparison with mice treated with CMC (all groups: $P < 0.001$). The mice treated with 25 mg/kg/day and 100 mg/kg/day of compound **2** exhibited 68.4% and 67.9% reduction of parasite weight, respectively, which were both higher than that of albendazole-treated mice (56.5%). However, despite the improved mean efficacy of compound **2**, there was no statistical difference in terms of parasite weight between the albendazole- and compound **2**-treated groups. During the entire treatment period, the body parameters of the treated mice, including body weight, body temperature and physical activity, were recorded and compared with those of control mice, and there was no



Compound	R	LC_{50} (μM)	CC_{50} (μM)[a]
2	HN	18.2	7.3
11	HN	30.8	NT
24	HN	20.0	5.9
25	HN	31.3	NT
26	HN	34.3	NT
Albendazole	–	79.2	NT

NT, not tested.
[a]Mean value of two independent experiments.

Figure 1. *In vitro* protoscolicidal activity (LC_{50}) and cytotoxicity (CC_{50}) of carbazole aminoalcohols.

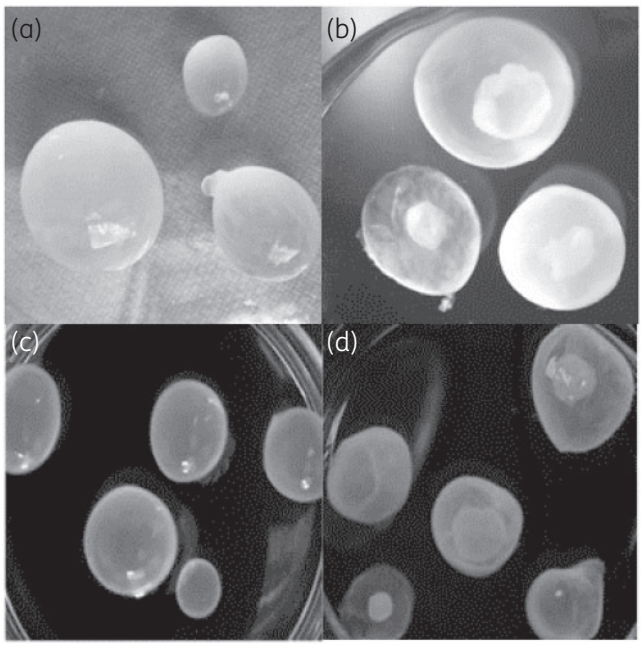


Figure 3. Morphological analysis showing the effect of compounds **2** and **24** on *E. granulosus* cysts. Control cysts with diameter of 8–15 mm (a) and 5–10 mm (c) cultured with DMSO. (b) Cysts with diameter of 8–15 mm cultured with compound **2** at a concentration of 28.6 μM for 4 h. (d) Cysts with diameter of 5–10 mm cultured with compound **24** at a concentration of 25.5 μM for 4 h.

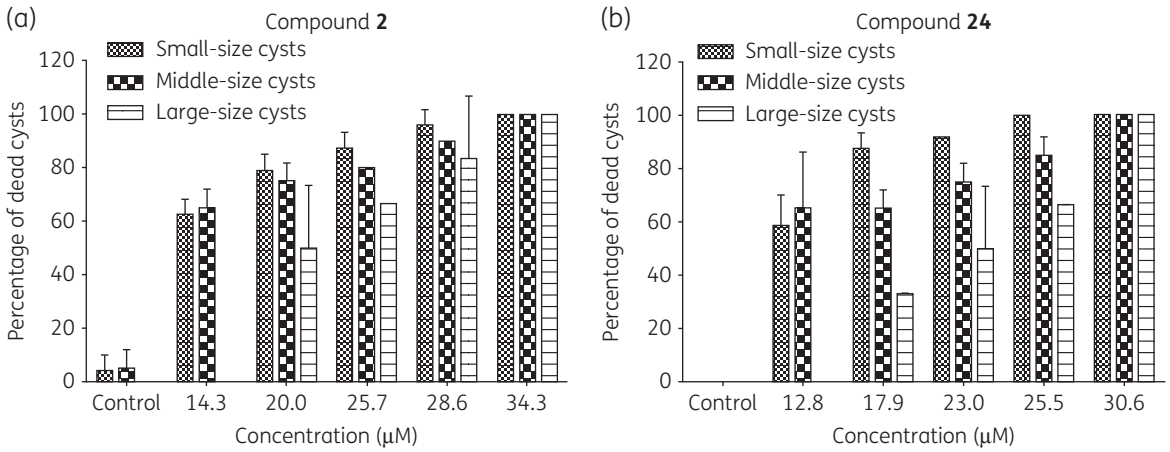


Figure 2. *Ex vivo* effect of compounds **2** and **24** on killing *E. granulosus* cysts. Three sizes of *E. granulosus* cysts were incubated in the presence of different concentrations of compound **2** (a) or compound **24** (b) for 5 h (small-sized cysts: diameter ≈5 mm, containing no PSC; middle-sized cysts: diameter ≈10 mm, some containing PSC; large-sized cysts: diameter ≥15 mm, containing PSC).

difference between them. After necropsy, the liver, spleen, heart and kidneys of each mouse were weighed and carefully examined. There was also no difference between control and treated mice (data not shown). All these data suggest that the mice well-tolerated compounds **2** and **24** at the experimental dosages.

In addition, for each group, the cysts were collected and their mortality was determined (Figure 4). The mortality of the cysts demonstrated that treatments of oral compounds **2** and **24** exhibited a significantly greater cysticidal effect than albendazole treatment. The groups treated with compound **2** at 25 mg/kg/day (31.5%, $P < 0.01$), 50 mg/kg/day (35.7%, $P < 0.001$) and 100 mg/kg/day (51.2%, $P < 0.001$) or with **24** at 50 mg/kg/day (30.7%, $P < 0.01$) and 100 mg/kg/day (43.1%, $P < 0.001$) revealed a significantly higher mortality of cysts than those treated with albendazole (22.2%).

Compounds 2 and 24 induce severe ultrastructural change on E. granulosus metacestodes

The *in vivo* anti-echinococcosis ability of compounds **2** and **24** was further confirmed by the ultrastructural images of TEM. Untreated metacestodes showed no ultrastructural alteration in parasite tissue. The entire parasite tissue was surrounded by an acellular laminated layer. Microtriches protruded from the tegument to the laminated layer. The metacestode tissue consisted of relatively densely packed tissue containing the germinal layer, nucleoli, muscle cells, connective tissue, undifferentiated cells and numerous mitochondria (Figure 5a). TEM micrographs taken after 30 days of administration of albendazole, compound **2** and compound **24** (50 mg/kg/day, oral) are shown in Figure 5(b–d), respectively. The albendazole-treated group revealed a less dense germinal layer, but microtriches, nucleoli, undifferentiated cells and mitochondria were still present (Figure 5b). After treatment with compound **24**, structurally intact parasite tissue could no longer be observed. Most residues of the germinal layer remained attached to the tegument, which was covered with sparse and elongate microtriches (Figure 5d). Treatment with compound **2** showed an even more dramatic effect, and resulted in complete destruction of the metacestode tissue (Figure 5c). The structural integrity of the germinal layer was totally lost and the majority of the microtriches were absent. A very large part of the germinal layer collapsed and detached from the tegument. Only a few residues of tegumental tissue were left.

In vivo pharmacokinetic profiles

Pharmacokinetic parameters of compounds **2** and **24** were subsequently determined in mice after a single intravenous (5 mg/kg) and oral (10 mg/kg) dose. The results are presented in Table 1. Compounds **2** and **24** both had excellent oral bioavailability (77.2% and 70.5%), reasonably low plasma clearance (7.8 and 8.9 mL/kg/min) and acceptable half-lives. Peak plasma concentration ($C_{max} = 2.2$ μM) of compound **2** was achieved 1.0 h after the oral dose. Administration of compound **24** resulted in a C_{max} value of 1.6 μM with a T_{max} of 7.3 h. The overall exposure for compound **2** appeared to be somewhat greater than compound **24** as reflected by a higher value for the area under the curve.

Sequence analysis of Egtopo I

In our previous studies, *h*topo I was identified as one of the drug targets of carbazole aminoalcohols.[19] Combining with their potent parasiticidal ability against *E. granulosus in vitro* and *in vivo*, we wondered whether the described molecular target of carbazole aminoalcohols in humans was also present in *E. granulosus*. Accordingly, the amino acid sequence alignment of *h*topo I, *Eg*topo I and *m*topo I was performed. As shown in Figure 6, *Eg*topo I shares 54.4% identity and 66.1% similarity with *h*topo I, and 53.6% identity and 65.7% similarity with *m*topo I. In particular, the amino acids involved in the binding of inhibitor and *h*topo I-DNA

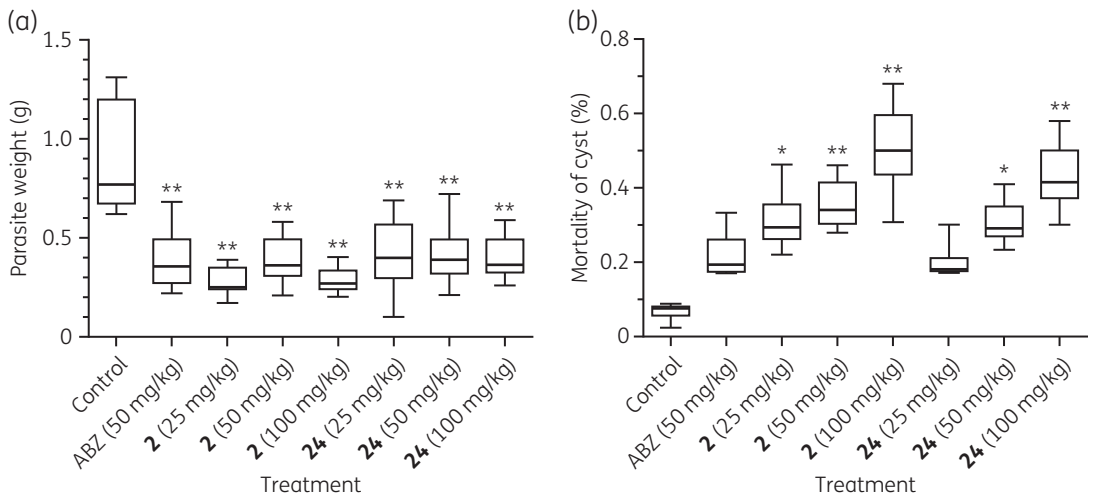


Figure 4. *In vivo* treatment of secondary *E. granulosus*-infected BALB/c mice with compounds **2** and **24**. Treatment was initiated 10 weeks post-infection. Each treatment group comprised 10 animals. The control group received CMC by gavage. Treatment groups were given daily albendazole (50 mg/kg), compound **2** (25, 50 and 100 mg/kg) and compound **24** (25, 50 and 100 mg/kg) in CMC by gavage for 30 days. After euthanasia, the cysts were resected and weighed. Parasite weight (a) and mortality of cysts (b) visualized as box plots. For statistical analysis, the results were tested with one-way ANOVA and pairwise *t*-test, and compared with the control group (a) and the albendazole-treated group (b). $^{*}P < 0.01$ and $^{**}P < 0.001$. ABZ, albendazole.

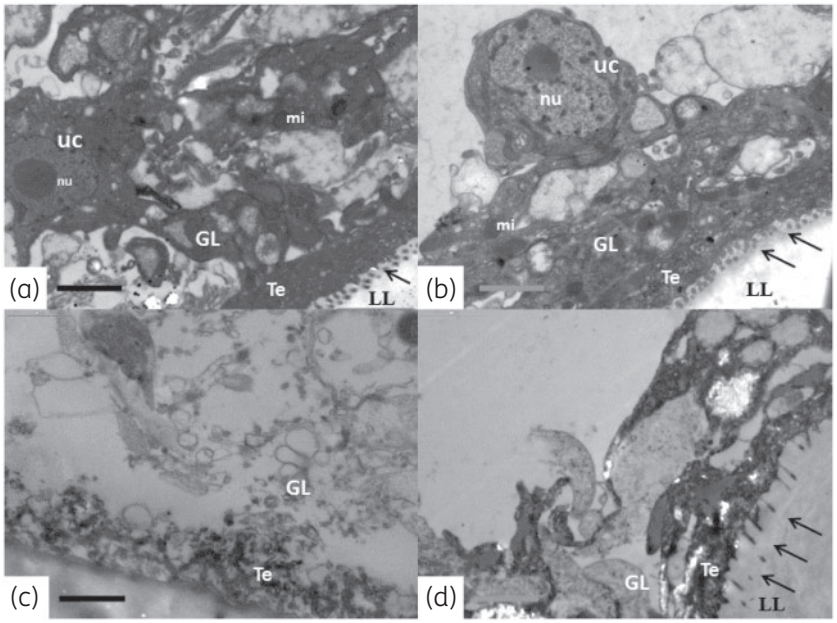


Figure 5. TEM analyses of *E. granulosus* metacestodes after *in vivo* treatment with albendazole, compound **2** and compound **24**. (a) A non-treated cyst of *E. granulosus*, showing the laminated layer (LL), tegument (Te) and the germinal layer (GL) of an echinococcal cyst. Clearly visible cells are undifferentiated stem cells (uc) and the microtriches protrude from the tegument wall to LL (arrows). mi, mitochondrion; nu, nucleolus. (b–d) Representative micrographs of the metacestode collected from the secondarily infected BALB/c mice after the daily gavage of albendazole (50 mg/kg, b), compound **2** (50 mg/kg, c) and compound **24** (50 mg/kg, d) for 30 days. All bars = 2 μm.

Table 1. *In vivo* pharmacokinetic parameters of compounds **2** and **24** in ICR mice

Compound	Dose (mg/kg)	Route	Oral bioavailability (%)	C_{max} (μM)	T_{max} (h)	AUC_{0-t} (μM·h)	$t_{1/2}$ (h)	Clearance (mL/kg/min)
2	5	intravenous	–	–	–	29.8	8.3	7.8
	10	oral	77.2	2.2	1.0	44.9	10.1	–
24	5	intravenous	–	–	–	23.4	9.3	8.9
	10	oral	70.5	1.6	7.3	32.6	9.6	–

cleavage complex are all exactly conserved in *Eg*topo I (Figure 6). In addition, the homology modelling structure of *Eg*topo I showed a similar structure of the inhibitor-binding site with *h*topo I (Figure S1, available as Supplementary data at *JAC* Online). As a result, we believe that the parasiticidal effect of carbazole aminoalcohols on CE was probably mediated by inhibiting *Eg*topo I.

Discussion

Benzimidazoles show limited treatment efficacy against CE, and patients usually have severe side effects from long-term exposure. Developing new chemotherapeutic options is needed urgently given that this disease has caused significant health problems globally. In the present study, the *in vitro* and *in vivo* efficacies of novel carbazole aminoalcohols in the treatment of CE were confirmed. In addition, topo I was investigated as a potential drug target of *E. granulosus*.

We recently showed that carbazole aminoalcohols have antitumour efficacy,[19] which allowed us to consider their anti-CE potential given that the target molecules (topo I) are similar in *E. granulosus* and humans. In addition, *E. granulosus* metacestodes and cancer cells show similar growth behaviour. As expected, several carbazole aminoalcohols, specifically compounds **2** and **24**, with confirmed *h*topo I inhibitory activity, exhibited potent parasiticidal activity against *E. granulosus* PSC *in vitro* and metacestodes *ex vivo*. Molecular docking analyses were carried out to predict the possible binding modes of compound **2** (*R* and *S* isomers) with the *Eg*topo I–DNA complex (Figure S2). Like camptothecin and topotecan, (*R*)- and (*S*)-**2** also intercalated into DNA at the DNA cleavage site and formed base-stacking interactions with downstream (–1) T-A and upstream (+1) G-C base pairs. (*R*)-**2** formed one H-bond with Asp332, and (*S*)-**2** formed one with Arg164. Both residues are near the active site and known to be necessary for *h*topo I sensitivity to camptothecin.[27] Given the significant sequence identity of *Eg*topo I and *h*topo I together with the homology building and molecular docking results, it is likely that the anti-CE effect of carbazole aminoalcohols was mediated by targeting *Eg*topo I.

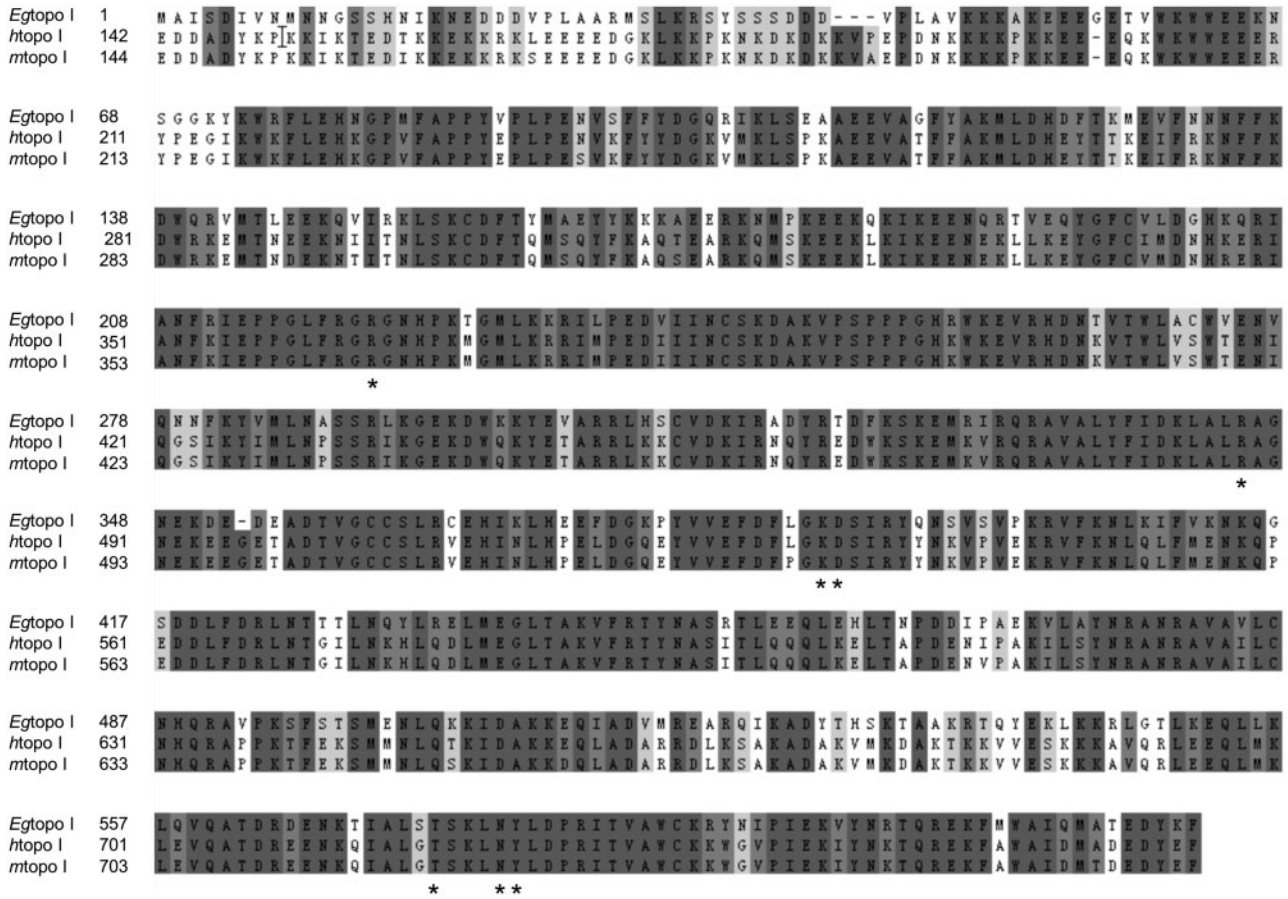


Figure 6. Alignment of the sequences of *Eg*topo I (GenBank: EUB61517.1), *h*topo I (NCBI Reference Sequence: NP_003277.1) and *m*topo I (GenBank: EDL06289.1). Identical residues are highlighted in dark grey. Residues sharing strong and weak similarity are highlighted in middle and light grey, respectively. Amino acids involved in the inhibitor–enzyme binding are indicated by asterisks.

Topo I is a valid and credible antitumour drug target. Topo I inhibitors, such as irinotecan and topotecan, have been used as first-line anticancer drugs for decades. They are widely used for treating small cell lung cancer and advanced cervical cancer.[28] In parasites, such as *Plasmodium* spp.,[29,30] *Leishmania* spp.,[31,32] *Cryptosporidium parvum*,[33] *Toxoplasma gondii*,[34] *Trypanosoma* spp.[35,36] and *Brugia malayi*,[37] DNA topoisomerases have been long investigated as potential drug targets. However, to date, DNA topoisomerases have not been considered as drug targets of *E. granulosus*. In addition to the introduction of carbazole amino-alcohols as a novel anti-CE compound class, this study also encourages the use of topo I inhibitors to treat CE. By establishing an assay system for *Eg*topo I, it should be possible to screen the anti-echinococcosis potential of currently available topo I inhibitors.

Murine bioassay results confirmed the *in vitro* findings and demonstrated that oral administration of compounds **2** and **24** led to significantly reduced parasite weight and remarkably increased mortality of cysts. Besides the parasite weight, the mortality of cysts was also used as an evaluation criterion, as it can reliably reveal the viability and infectivity of cysts. Although there was no statistical difference in terms of reduction in parasite weight between the albendazole- and compound **2**-treated groups, the mortality of cysts showed the treatments of compounds **2** (25, 50 and 100 mg/kg/day) and **24** (50 and 100 mg/kg/day) exhibited significantly improved cysticidal effects compared with albendazole treatment (all groups: $P < 0.01$). *In vivo* pharmacokinetic studies demonstrated that compounds **2** and **24** were orally absorbed well, readily taken up into plasma and slowly cleared. Since the combination treatment of clinical anti-CE drugs (i.e. albendazole) and novel agents usually yields a synergistic effect,[13,38,39] both *in vitro* and *in vivo* studies focused on a combination of albendazole and compound **2** or **24** should be further performed.

Moreover, TEM micrographs of the cysts collected from mice demonstrated the drug-induced ultrastructural alterations. The treatment with compounds **2** and **24** resulted in the breakdown of the structural integrity of the germinal layer and the parasite tissue. The germinal layer collapsed and detached from the tegument. Most of the parasite tissues were compressed to residues of the germinal layer. In particular, compound **2** had a pronounced effect on the microtriches, resulting in their almost complete disappearance. Microtriches are microvilli-like protrusions presented over the tegument of metacestodes. They are analogous in structure and function to the brush border of gut epithelia[40] and thought to be strongly involved in the acquisition of nutrients and the expulsion of waste materials.[41]

Since *Eg*topo I, *m*topo I and *h*topo I share high identity in protein sequences, there is a possibility that the effect of carbazole aminoalcohols may not be parasite-specific, which might consequently result in unexpected toxic side effects on human and murine hosts during killing of the parasites. In addition, CE treatment usually requires long-term and high-dose administration, which may increase the probability of the occurrence of adverse effects. Thus, the toxic side effects of carbazole aminoalcohols must be an area of focus. During the entire *in vivo* treatment period, all dosage regimens of compounds **2** and **24** were well-tolerated by the secondarily infected mice, and none of them exhibited adverse effects or a toxic reaction. The haematological parameters and biochemical indices of the mice were tested including blood routine indices, electrolytes, and liver and renal function (Table S1). Almost all indices of the treated groups were normal when compared with the healthy mice. The most influenced indices were mean corpuscular haemoglobin concentration and blood platelets. However, in comparison with the control mice, the mean corpuscular haemoglobin concentration and platelets level were within the acceptable range. In addition, the cytotoxicity of compounds **2** and **24** against human embryonic lung fibroblast WI38 was evaluated (Figure 1; CC_{50}). Both compounds showed cytotoxicity in the micromolar range. Negative selectivity indices of cytotoxicity and *in vitro* protoscolicidal activity were found. However, *in vivo*, drugs have to undergo extremely complex pharmacokinetic processes. To obtain a more comprehensive understanding of the toxicity profiles of compounds **2** and **24**, further *in vivo* toxicity analyses were performed in normal BALB/c mice (see the Supplementary data available at *JAC* Online). The mice were intragastrically administered compound **2** or **24** twice in a day at an interval of 6 h at dosages of 4000 (as maximum tolerated dose), 1000, 500 and 100 mg/kg, respectively. After administration, the mice were inspected for 7–14 days. No death was found in any group during the entire period. All the mice showed normal behaviour in terms of taking in water and food and physical activity. The acute toxicity test indicated that the mice could tolerate 4000 mg/kg of compound **2** or **24** in a day and showed no significant change in body weight compared with that of control mice ($P > 0.05$; Tables S2 and S3). After autopsy, in all groups, no visible damage was observed in the main organs of mice, and the weight of the liver, spleen and kidneys did not exhibit any dose-dependent changes (Tables S4 and S5). According to the results of haematological analysis, blood biochemistry test and electrolyte analysis (Tables S6 and S7), there were no significant changes observed in the haematological parameters, blood biochemical indices and electrolyte parameters of all dosage groups compared with the control mice. In summary, at a maximum tolerated dose of 4000 mg/kg, there were no toxic effects and clinical signs of impaired health observed in the mice administered compounds **2** and **24**, which was 160-fold of their *in vivo* effective dosage (25 mg/kg). It is worth noting that, for CE, a much longer treatment regimen (several months to years) has to be envisaged. Long-term toxicity of compounds **2** and **24** in rats and dogs should be carefully investigated in further studies.

In conclusion, to the best of our knowledge, this study demonstrated the use of carbazole aminoalcohols as a class of anti-CE agents for the first time. The potent *in vitro* and *in vivo* parasiticidal activity in conjunction with the good pharmacokinetic properties warrants further development of compounds **2** and **24** as promising drug candidates for CE treatment. More studies should be pursued to optimize the therapeutic effect of compounds **2** and **24**. *Eg*topo I as an interesting potential drug target of *E. granulosus* also requires further validation. Investigations to understand the detailed mechanism of action of their parasite-killing effects on *E. granulosus* are in progress.

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Transparency declarations

None to declare.

Supplementary data

Supplementary data, including Figures S1 and S2 and Tables S1 to S7, are available at *JAC* Online.

References

1 McManus DP, Gray DJ, Zhang W *et al*. Diagnosis, treatment, and management of echinococcosis. *BMJ* 2012; **344**: e3866.

2 Horton RJ. Albendazole in treatment of human cystic echinococcosis: 12 years of experience. *Acta Trop* 1997; **64**: 79–93.

3 Horton RJ. Chemotherapy of *Echinococcus* infection in man with albendazole. *Trans R Soc Trop Med Hyg* 1989; **83**: 97–102.

4 Kern P. Clinical features and treatment of alveolar echinococcosis. *Curr Opin Infect Dis* 2010; **23**: 505–12.

5 Hemphill A, Stadelmann B, Rufener R *et al*. Treatment of echinococcosis: albendazole and mebendazole—what else? *Parasite* 2014; **21**: 70.

6 Küster T, Lense N, Barna F *et al*. A new promising application for highly cytotoxic metal compounds: η^6-areneruthenium(II) phosphite complexes for the treatment of alveolar echinococcosis. *J Med Chem* 2012; **55**: 4178–88.

7 Spicher M, Naguleswaran A, Ortega-Mora LM *et al*. *In vitro* and *in vivo* effects of 2-methoxyestradiol, either alone or combined with albendazole, against *Echinococcus* metacestodes. *Exp Parasitol* 2008; **119**: 475–82.

8 Gelmedin V, Caballero-Gamiz R, Brehm K. Characterization and inhibition of a p38-like mitogen-activated protein kinase (MAPK) from *Echinococcus multilocularis*: antiparasitic activities of p38 MAPK inhibitors. *Biochem Pharmacol* 2008; **76**: 1068–81.

9 Naguleswaran A, Spicher M, Vonlaufen N *et al*. *In vitro* metacestodicidal activities of genistein and other isoflavones against *Echinococcus multilocularis* and *Echinococcus granulosus*. *Antimicrob Agents Chemother* 2006; **50**: 3770–8.

10 Liance M, Nemati F, Bories C *et al*. Experience with doxorubicin-bound polyisohexylcyanoacrylate nanoparticles on murine alveolar echinococcosis of the liver. *Int J Parasitol* 1993; **23**: 427–9.

11 Pensel PE, Albani C, Gamboa GU *et al*. *In vitro* effect of 5-fluorouracil and paclitaxel on *Echinococcus granulosus* larvae and cells. *Acta Trop* 2014; **140**: 1–9.

12 Nicolao MC, Elissondo MC, Denegri GM *et al*. *In vitro* and *in vivo* effects of tamoxifen against larval stage *Echinococcus granulosus*. *Antimicrob Agents Chemother* 2014; **58**: 5146–54.

13 Stadelmann B, Aeschbacher D, Huber C *et al*. Profound activity of the anti-cancer drug bortezomib against *Echinococcus multilocularis* metacestodes identifies the proteasome as a novel drug target for cestodes. *PLoS Negl Trop Dis* 2014; **8**: e3352.

14 Hemer S, Brehm K. *In vitro* efficacy of the anticancer drug imatinib on *Echinococcus multilocularis* larvae. *Int J Antimicrob Agents* 2012; **40**: 458–62.

15 Schmidt AW, Reddy KR, Knölker HJ. Occurrence, biogenesis, and synthesis of biologically active carbazole alkaloids. *Chem Rev* 2012; **112**: 3193–328.

16 Głuszyńska A. Biological potential of carbazole derivatives. *Eur J Med Chem* 2015; **94**: 405–26.

17 Saulnier MG, Langley DR, Frennesson DB *et al*. Novel 3′,6′-anhydro and N12, N13-bridged glycosylated fluoroindolo[2,3-*a*]carbazoles as topoisomerase I inhibitors. Fluorine as a leaving group from sp3 carbon. *Org Lett* 2005; **7**: 1271–4.

18 Nakamura K, Uenaka T, Nagasu T *et al*. A novel carbazole topoisomerase II poison, ER-37328: potent tumoricidal activity against human solid tumors *in vitro* and *in vivo*. *Cancer Sci* 2003; **94**: 119–24.

19 Wang W, Sun X, Sun D *et al*. Carbazole aminoalcohols induce antiproliferation and apoptosis of human tumor cells by inhibiting topoisomerase I. *ChemMedChem* 2016; **11**: 2675–81.

20 Champoux JJ, Dulbecco R. An activity from mammalian cells that untwists superhelical DNA—a possible swivel for DNA replication. *Proc Natl Acad Sci USA* 1972; **69**: 143–6.

21 Chen SH, Chan NL, Hsieh TS. New mechanistic and functional insights into DNA topoisomerases. *Annu Rev Biochem* 2013; **82**: 139–70.

22 Pommier Y. Drugging topoisomerases: lessons and challenges. *ACS Chem Biol* 2013; **8**: 82–95.

23 Zheng H, Zhang W, Zhang L *et al*. The genome of the hydatid tapeworm *Echinococcus granulosus*. *Nat Genet* 2013; **45**: 1168–75.

24 Zhang W, Wang S, McManus DP. *Echinococcus granulosus* genomics: a new dawn for improved diagnosis, treatment, and control of echinococcosis. *Parasite* 2014; **21**: 66.

25 Zhang WB, Jones MK, Li J *et al*. *Echinococcus granulosus*: pre-culture of protoscoleces *in vitro* significantly increases development and viability of secondary hydatid cysts in mice. *Exp Parasitol* 2005; **110**: 88–90.

26 Hemphill A, Croft SL. Electron microscopy in parasitology. In: M Rogan, ed. *Analytical Parasitology*. Heidelberg: Springer-Verlag, 1997; 227–68.

27 Staker BL, Hjerrild K, Feese MD *et al*. The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci USA* 2002; **99**: 15387–92.

28 Pommier Y. Topoisomerase I inhibitors: camptothecins and beyond. *Nat Rev Cancer* 2006; **6**: 789–802.

29 Cortopassi WA, Penna-Coutinho J, Aguiar AC *et al*. Theoretical and experimental studies of new modified isoflavonoids as potential inhibitors of topoisomerase I from *Plasmodium falciparum*. *PLoS One* 2014; **9**: e91191.

30 Mudeppa DG, Kumar S, Kokkonda S *et al*. Topoisomerase II from human malaria parasites: expression, purification, and selective inhibition. *J Biol Chem* 2015; **290**: 20313–24.

31 Saha S, Mukherjee T, Chowdhury S *et al*. The lignan glycosides lyoniside and saracoside poison the unusual type IB topoisomerase of *Leishmania donovani* and kill the parasite both *in vitro* and *in vivo*. *Biochem Pharmacol* 2013; **86**: 1673–87.

32 Prada CF, Alvarez-Velilla R, Balaña-Fouce R *et al*. Gimatecan and other camptothecin derivatives poison *Leishmania* DNA-topoisomerase IB leading to a strong leishmanicidal effect. *Biochem Pharmacol* 2013; **85**: 1433–40.

33 Ordóñez C, Alfonso J, Balaña-Fouce R *et al*. Functional expression of a DNA-topoisomerase IB from *Cryptosporidium parvum*. *J Biomed Biotechnol* 2009; **2009**: 837608.

34 Dzitko K, Paneth A, Plech T *et al*. 1,4-Disubstituted thiosemicarbazide derivatives are potent inhibitors of *Toxoplasma gondii* proliferation. *Molecules* 2014; **19**: 9926–43.

35 Tang SC, Shapiro TA. Newly identified antibacterial compounds are topoisomerase poisons in African trypanosomes. *Antimicrob Agents Chemother* 2010; **54**: 620–6.

36 Steverding D, Wang X. Evaluation of anti-sleeping-sickness drugs and topoisomerase inhibitors in combination on *Trypanosoma brucei*. *J Antimicrob Chemother* 2009; **63**: 1293–5.

37 Ali M, Afzal M, Abdul NS *et al*. Nanocurcumin: a novel antifilarial agent with DNA topoisomerase II inhibitory activity. *J Drug Target* 2014; **22**: 395–407.

38 Albani CM, Pensel PE, Elissondo N *et al*. *In vivo* activity of albendazole in combination with thymol against *Echinococcus multilocularis*. *Vet Parasitol* 2015; **212**: 193–9.

39 Stettler M, Rossignol JF, Fink R *et al*. Secondary and primary murine alveolar echinococcosis: combined albendazole/nitazoxanide chemotherapy exhibits profound anti-parasitic activity. *Int J Parasitol* 2004; **34**: 615–24.

40 Jha RK, Smyth JD. *Echinococcus granulosus*: ultrastructure of microtriches. *Exp Parasitol* 1969; **25**: 232–44.

41 Küster T, Stadelmann B, Aeschbacher D *et al*. Activities of fenbendazole in comparison with albendazole against *Echinococcus multilocularis* metacestodes *in vitro* and in a murine infection model. *Int J Antimicrob Agents* 2014; **43**: 335–42.