Veterinary Parasitology 309 (2022) 109766

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Flukicidal effects of abietane diterpenoid derived analogues against the food borne pathogen *Fasciola hepatica*.

Anand Chakroborty [a,b], Deiniol Pritchard [c], Marc E. Bouillon [d], Anna Cervi [c], Alan Cookson [a], Charlotte Wild [e], Caroline Fenn [e], Joseph Payne [e], Peter Holdsworth [f], Colin Capner [e], Jenna O'Neill [e], Gilda Padalino [a], Josephine Forde-Thomas [a], Sandeep Gupta [g], Brendan G. Smith [g], Maggie Fisher [e], Martina Lahmann [d,h], Mark S. Baird [c], Karl F. Hoffmann [a,*]

[a] *Institute of Biological, Environmental and Rural Sciences (IBERS), Aberystwyth University, SY23 3DA Wales, UK*
[b] *Institute of Parasitology, Biology Centre, Czech Academy of Sciences, České Budějovice 370 05, Czech Republic*
[c] *Naturiol Bangor Ltd., MSParc, Gaerwen, Anglesey LL60 6AG, UK*
[d] *School of Chemistry, Bangor University, Bangor, Wales LL57 2UW, UK*
[e] *Ridgeway Research Limited, Park Farm Buildings, Park Lane, St Briavels, Gloucestershire, GL15 6QX England, UK*
[f] *PAH Consultancy Pty Ltd, 3/27 Gaunson Crescent, Wanniassa, 2903 Canberra, ACT, Australia*
[g] *Bimeda UK, Bryn Cefni Industrial Estate, Unit 2 A, Llangefni, Anglesey, North Wales LL77 7XA, UK*
[h] *KTH Royal Institute of Technology, Biomedical Engineering and Health, Systems, Hälsovägen 11, 141 52 Huddinge, Sweden*

ARTICLE INFO

Keywords:
Fasciola hepatica
Triclabendazole
Anthelmintic drug discovery
Abietic acid
Diterpene and dehydroabietic acid

ABSTRACT

Control of liver fluke infections remains a significant challenge in the livestock sector due to widespread distribution of drug resistant parasite populations. In particular, increasing prevalence and economic losses due to infection with *Fasciola hepatica* is a direct result of drug resistance to the gold standard flukicide, triclabendazole. Sustainable control of this significant zoonotic pathogen, therefore, urgently requires the identification of new anthelmintics. Plants represent a source of molecules with potential flukicidal effects and, amongst their secondary metabolites, the diterpenoid abietic acids can be isolated in large quantities. In this study, nineteen (19) chemically modified abietic acid analogues (MC_X) were first evaluated for their anthelmintic activities against *F. hepatica* newly excysted juveniles (NEJs, from the laboratory-derived Italian strain); from this, 6 analogues were secondly evaluated for their anthelmintic activities against adult wild strain flukes. One analogue, MC010, was progressed further against 8-week immature- and 12-week mature Italian strain flukes. Here, MC010 demonstrated moderate activity against both of these intra-mammalian fluke stages (with an adult fluke EC_{50} = 12.97 μM at 72 h post culture). Overt mammalian cell toxicity of MC010 was inferred from the Madin-Darby bovine kidney (MDBK) cell line (CC_{50} = 17.52 μM at 24 h post culture) and demonstrated that medicinal chemistry improvements are necessary before abietic acid analogues could be considered as potential anthelmintics against liver fluke pathogens

1. Introduction

Fasciolosis, a food borne zoonosis caused by infection with liver flukes (e.g. *Fasciola hepatica* and *Fasciola gigantica*), remains highly prevalent across tropical and temperate regions (Keiser and Utzinger, 2009; Fox et al., 2011; Iglesias-Piñeiro et al., 2016). Worldwide, losses due to fasciolosis in agriculture and livestock sectors cost US$ 3.2 billion annually, with losses of £ 23 million per annum in the United Kingdom (UK) alone (Mazeri et al., 2017). A key problem with liver fluke management is the over-reliance on a single drug (triclabendazole, TCBZ),

* Corresponding author.
E-mail addresses: anandchakroborty@gmail.com (A. Chakroborty), deiniol.rhodri@gmail.com (D. Pritchard), marc.bouillon@endotherm.de (M.E. Bouillon), AnnaCervi@naturiol.uk (A. Cervi), akc@aber.ac.uk (A. Cookson), wild_charlie@hotmail.co.uk (C. Wild), CFenn@ridgewayresearch.co.uk (C. Fenn), JPayne@ridgewayresearch.co.uk (J. Payne), peter.paragon60@gmail.com (P. Holdsworth), colin.capner@colincapner.plus.com (C. Capner), jennaoneill3@gmail.com (J. O'Neill), gip7@aber.ac.uk (G. Padalino), jef19@aber.ac.uk (J. Forde-Thomas), sandeep.vzgupta@gmail.com (S. Gupta), BSmith@bimeda.com (B.G. Smith), Maggie@shernacre.co.uk (M. Fisher), lahmann@kth.se (M. Lahmann), markbaird@naturiol.uk (M.S. Baird), krh@aber.ac.uk (K.F. Hoffmann).

https://doi.org/10.1016/j.vetpar.2022.109766
Received 9 May 2022; Received in revised form 29 June 2022; Accepted 17 July 2022
Available online 20 July 2022
0304-4017/© 2022 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

which has led to the development of TCBZ resistant fluke populations in Australia, New Zealand, Peru, Argentina, Brazil, Bolivia, Chile, the Philippines, the United Kingdom, Ireland and other European countries (Fairweather et al., 2020; Hanna et al., 2015; Hassell and Chapman, 2012; Kelley et al., 2016; Moll et al., 2000; Olaechea et al., 2011; Ortiz et al., 2013; Romero et al., 2019; Venturina et al., 2015). In the absence of an approved vaccine, chemotherapy remains the predominant control strategy for the foreseeable future. As the World Health Organisation (WHO) also includes fascioliasis amongst the Neglected Tropical Diseases (NTDs) caused by foodborne pathogens (Casulli, 2021), the discovery of TCBZ replacement molecules is urgently needed to enable sustainable control of this economically-devastating zoonotic agent.

Some of nature's best medicines are found in plants, with methods for harvesting and modifying diverse natural products to control or reverse non-communicable or infectious diseases being rapidly developed or optimised (Rates, 2001; Faustino et al., 2019). This realisation has re-invigorated the search for plant-derived natural products with activity against helminth parasites (Liu et al., 2020). Natural products investigated to date as anthelmintics include monophenols or terpenoids (e.g. thymols and carvacrol) and flavonoids such as quercetin and cinnamic acids (Garcia-Bustos et al., 2019). Contributing to this growing body of information, we have also actively explored the anthelmintic activity of plant-derived natural products (e.g. di- and tri- terpenoids) against liver and blood fluke pathogens (Edwards et al., 2015; Crusco et al., 2018, 2019; Whiteland et al., 2018). The potential of identifying more diverse anthelmintic starting points, based on natural product scaffolds, has led to the current study exploring the bioactive properties of abietane and dehydroabietane derivatives on *F. hepatica*.

Naturally produced abietanes and dehydroabietanes are abundant in conifer resins, where they are synthesised as defense metabolites (Sofia Costa et al., 2016). Apart from being biomarkers in conifers, these naturally occurring diterpenoids have also been isolated from other terrestrial plants (González, 2015; Hadacek, 2019). Abietic acid (Fig. 1A) is the most abundant of several isomeric or closely related organic acids that constitute the solid portion of coniferous tree oleoresin (Eksi et al., 2020). This tricyclic abietane diterpenoid represents the primary irritant in pine resin and can be isolated in large quantities (Faustino et al., 2019). The first natural occurrence of abietic acid was reported from *Podocarpus ferrugineus* and *Thujopsis dolabrata* (Kitadani et al., 1970). Abietic acid has significant anti-inflammatory, anti--allergic, anti-convulsant, anti-proliferative, cell cycle arresting and pro-apoptotic, anti-microbial, anti-protozoal, osteoclastogenic, and anti-cancer effects (Aranda and Villalaín, 1997; González et al., 2015; Olmo et al., 2015; Xu et al., 2017; Faustino et al., 2019; Liu et al., 2019). Biological membranes are the predicted site of action due to the amphipathic nature of abietic acid (Aranda and Villalaín, 1997). However, abietic acid is prone to thermal or acid induced rearrangement to other isomeric dienic acids (e.g. Fig. 1B) (Takeda et al., 1968), and to dehydrogenation to the more-stable aromatic acid, dehydroabietic acid (MC003; Fig. 1C). The two conjugated double bonds in abietic acid and some of these other common 'resin acids' are also sensitive to oxidative polymerisation. This can be useful in some applications, but in others, for example as drugs, long term chemical stability is preferred (Peng and Roberts, 2000). In the search for novel anthelmintics, we have, therefore, evaluated a set of dehydroabietic acid analogues for their flukicidal properties on three different developmental stages of *F. hepatica*.

2. Materials and methods

2.1. Ethics statement

Adhering to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986 as well as the European Union Animals Directive 2010/63/EU (approved by RRL Animal Welfare and Ethical Review Bodies), the *F. hepatica* (Italian strain) life cycle was maintained in sheep under project licenses PPL P6D805744 and PA09B4E45.

2.2. Synthesis and structural elucidation of abietic acid analogues

MC001 (Fig. 2) was prepared directly from commercial rosin (Fisher Scientific, Loughborough, UK) by reaction with maleic anhydride at 120 °C; the cyclohexadiene containing isomer (Fig. 1B) being trapped in a Diels-Alder reaction (Lloyd and Hendrick, 1961). For this work, dehydroabietic acid (MC003) was prepared by dehydrogenation of abietic acid using a slight modification of published procedures (Littmann, 1938; Fleck and Palkin, 1938). MC003 was converted into seventeen additional derivatives (Fig. 2), by changes mostly occurring on either position 4 or 18 (position 12 for MC013), as described in detail in the supplementary file (Appendix 1).

2.3. Study design

Following synthesis, the compounds were subjected to a sequential screening campaign on *F. hepatica* (Fig. 3). Firstly, all 19 compounds were co-incubated (10 μM final concentration) with the newly excysted juvenile (NEJ) stage of *F. hepatica* (lab-reared Italian strain; Rinaldi et al., 2015); these flukes were chosen as they were readily available, susceptible to TCBZ and have been previously characterised (isolated from Campania, Italy on August, 6, 2014 and continuously passaged since). Motility and ultrastructural effects were independently scored and compared to NEJs cultured with 0.1 % dimethyl sulfoxide (DMSO, the solvent used for all abietic acid derivatives) and 10 μM TCBZ in 0.1 % DMSO. The hit molecules (those capable of killing all NEJs within 24 h of a 72 h *ex vivo* treatment) were further screened (72 h at 40 μM final concentration) against adult *F. hepatica* (provenance unknown) obtained from a local abattoir (Randall Parker Foods, Llanidloes). This step was used as a filter for determining the hit compound(s)' activity against locally and easily sourced adult flukes (eliminating the need to produce flukes from an experimentally infected animal). In order to complete the screening programme in all intra-mammalian life stages of a single fluke strain, the most potent hit molecule's (MC010) activity was measured in lab-reared Italian strain immature and adult fluke assays (72 h at 40 μM

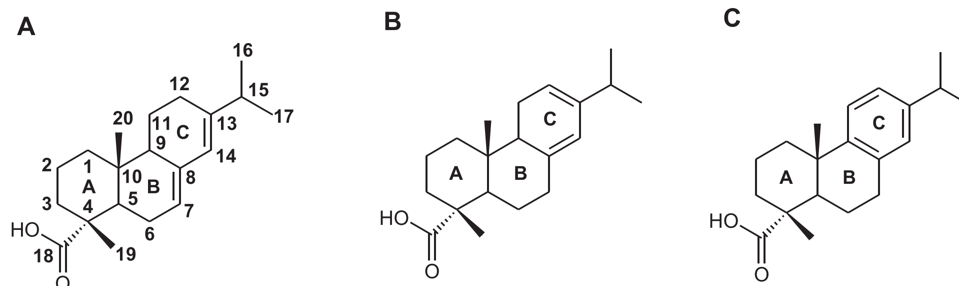

Fig. 1. Abietic acid and derivatives. (A) abietic acid; (B) abietic acid isomer; (C) dehydroabietic acid (MC003). The compounds are numbered and the rings labelled according to convention.

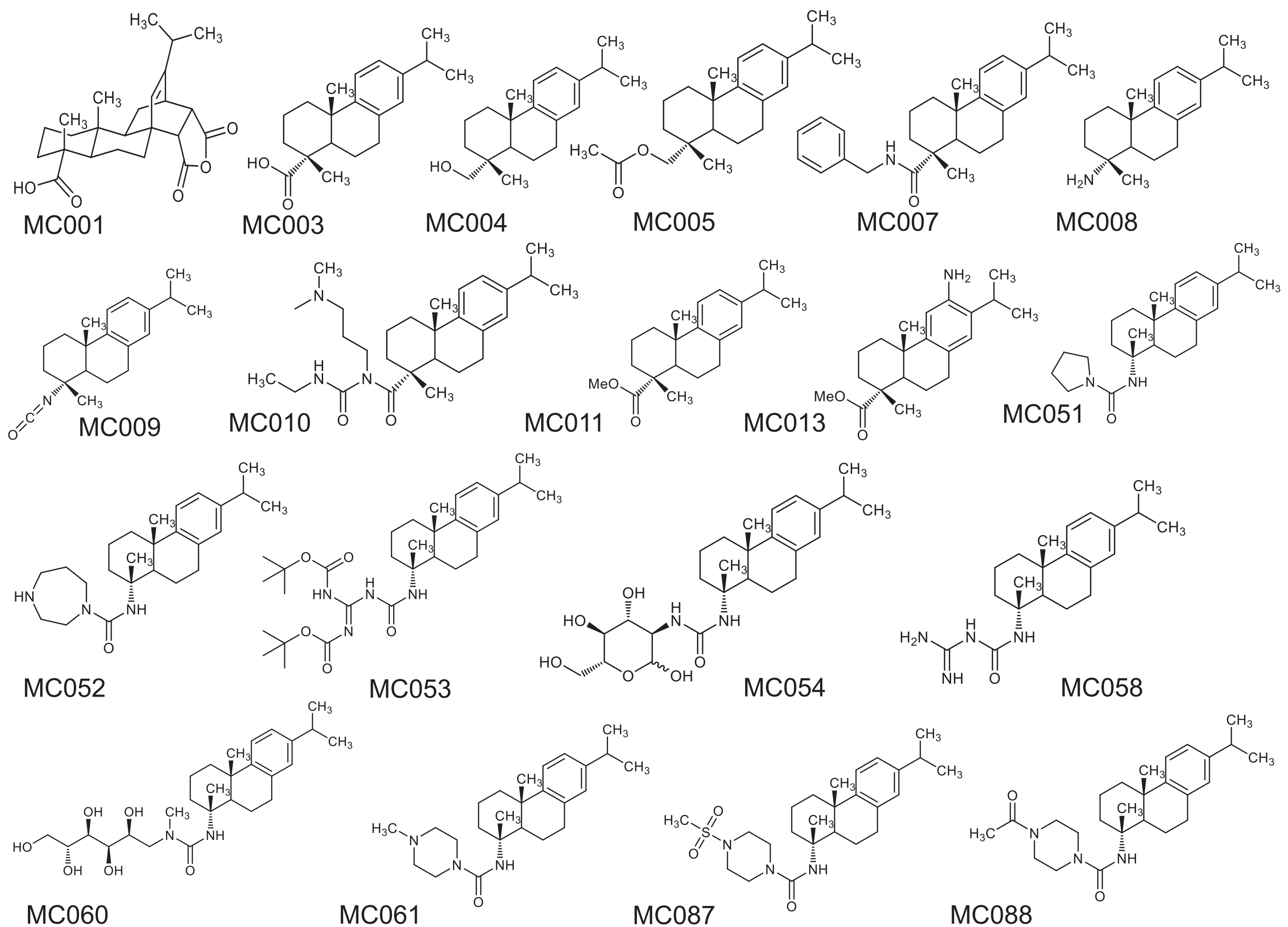

Fig. 2. Structures of abietic acid analogues evaluated in this work. Compound MC001 was prepared directly from abietic acid by reaction with maleic anhydride; the remaining compounds were prepared from MC003 by reactions described in Appendix 1.

final concentration). The most potent hit molecule (MC010) was subsequently subjected to a dose response titration on adult *F. hepatica* Italian strain flukes (72 h at 40 μM, 13.3 μM and 4.4 μM final concentrations) as well as MDBK cells (200 μM, 100 μM, 75 μM, 50 μM, 40 μM, 30 μM, 25 μM, 15 μM, 10 μM, 5 μM and 1 μM final concentrations for determining overt cytotoxicity).

2.4. Compound handling and storage

TCBZ (Sigma-Aldrich, UK) and all synthesised abietane analogues were solubilized in DMSO (Fisher Scientific, Loughborough, UK) and stored at − 20 °C at a stock concentration of 10 mM.

2.5. Ex vivo F. hepatica assays

Italian strain *F. hepatica* NEJs were produced from metacercariae as previously described (Crusco et al., 2018). Briefly, NEJs were treated with diterpenoid abietanes at a final concentration of 10 μM (in 0.1 % DMSO) in RPMI 1640 media containing 1 % Foetal Bovine Serum (FBS, Gibco, Paisley, UK), 1 % v/v antibiotics and 25 μg/ml amphotericin B (Sigma Aldrich, UK). NEJ/compound co-cultures were incubated at 37 °C in a humidified environment containing 5 % CO_2 for 72 h. Positive (10 μM TCBZ dissolved in 0.1 % DMSO) and negative (0.1 % DMSO in RPMI 1640; RPMI 1640 only) controls were also included. Independent motility and ultrastructure assessments were noted by brightfield microscopy at 24 h intervals based on previously described scoring metrics (Edwards et al., 2015). Briefly, motility was scored on a scale from 1 to 5 with 1 representing good/normal movement and 5 representing a complete absence of movement. Ultrastructure was scored on a scale from 1 to 6 with 1 representing a good/normal ultrastructure and 6 signifying a severely dissolved/granulated parasite.

Adult flukes (provenance unknown) were collected from animals (cattle breed unknown) processed at Randal Parker Foods or from experimental infection of Texel Mule X lambs (n = 6) with 200 metacercariae at 12 weeks post infection. Immature liver flukes (Italian strain) were obtained by experimental infection of Texel Mule X lambs (n = 6) with 200 metacercariae at 8 weeks post infection. Parasites were incubated in RPMI 1640 media supplemented with 10 % FBS (Gibco, Paisley, UK), 1 % v/v antibiotics and 25 μg/ml of amphotericin B (Sigma-Aldrich) for several hours before being prepared for screening. In the case of adult worms, the media was replaced 6 times (every hour for the first 3 h and then every 6 h) to remove accumulated bile prior to initiating compound screening. Immature worms (n = 4/condition) and adult flukes (n = 3/condition) were co-incubated with compounds for 72 h as previously described for NEJs; the scores were recorded by brightfield microscopy at 24, 48 and 72 hr timepoints using motility metrics as previously described (Crusco et al., 2018; Edwards et al., 2015). Positive (40 μM TCBZ dissolved in 0.4 % DMSO) and negative (RPMI1640 with 0.4 % DMSO; RPMI1640 without 0.4 % DMSO) controls were included in these screens. Dose response experiments were additionally performed for MC010 (40 μM, 13.3 μM and 4.4 μM final concentrations).

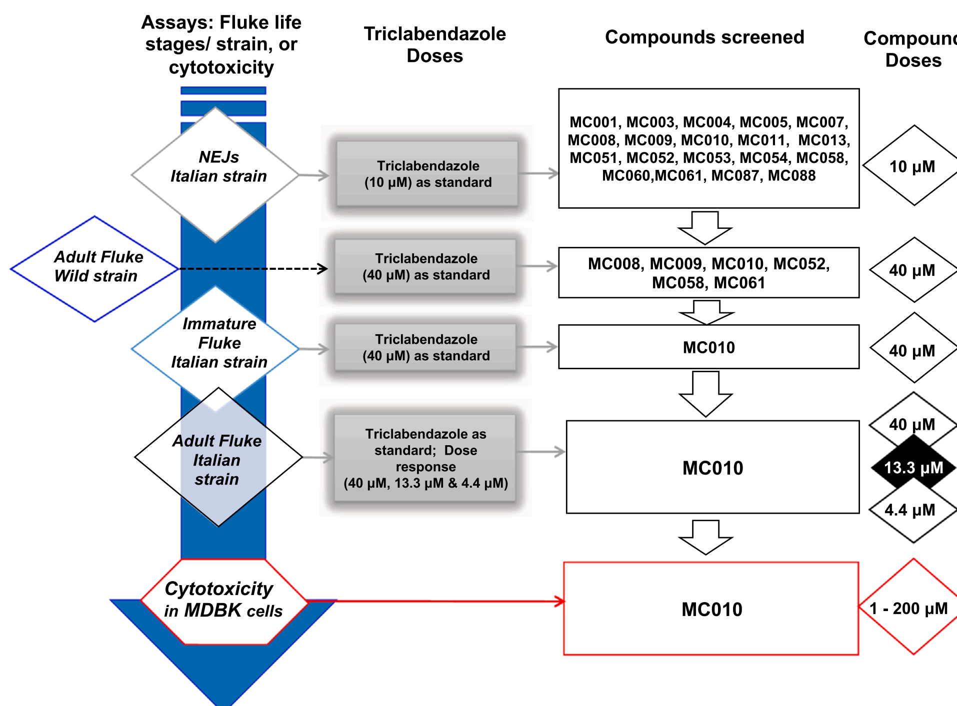

Fig. 3. Screening pipeline of abietic acid derivatives against *Fasciola hepatica*. A total of 19 freshly synthesised compounds were subjected to a sequential screening campaign on *F. hepatica*. The arrow pointing downwards indicates the study plan workflow (described in Materials and Methods).

2.6. Cytotoxicity screening on MDBK cells

Cytotoxicity of MC010 was assessed on MBDK cells (n = 2/condition; in each replicate, the conditions were tested in triplicate) using the MTT (3-(4,5-dimethylthiazol-2-yl)– 2,5-diphenyltetrazolium bromide) reagent as previously described (Crusco et al., 2018; Whiteland et al., 2018). Briefly, MDBK (NBL-1) cells (90,050,801, Sigma-Aldrich, UK) were grown to 80 % confluency in Minimum Essential Medium Eagle (Sigma Aldrich, UK) supplemented with 2 mM L-Glutamine (Sigma Aldrich, UK), 1 % v/v non-essential Amino Acids (Sigma Aldrich, UK), 1 % v/v antibiotic/antimycotic (Fisher Scientific, Loughborough, UK) and 10 % FBS (Fisher Scientific) before being seeded at a density of 1.5×10^5 cells/ml (50 µl/well) into 96-well black-sided, clear bottom tissue culture plates and incubated at 37 ^{o}C in a humidified environment containing 5 % CO_2. During each MDBK cytotoxicity assay, both negative (1 % v/v Triton X-100, Sigma-Aldrich, UK) and positive (1.25 % v/v DMSO or media only) controls were also included. After compound addition, the plates were incubated for a further 20 h before addition of the MTT reagent. After 4 h, the developed purple formazan crystals were dissolved by treatment with a (1:1) DMSO: isopropanol mixture and absorbance measured at 570 nm using a POLARstar Omega (BMG Labtech, UK) microtiter plate reader. Dose response curves were generated using the corrected average absorbance of the replicates in GraphPad Prism 8 software and the CC_{50} (the concentration of compound that reduced cell viability by 50 %) value was estimated.

2.7. Scanning electron microscopy

MC010 (40 µM) treated adult liver flukes (and DMSO controls) were removed from culture at 72 h, fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) and 1 % osmium tetroxide solution in 0.1 M sodium cacodylate at (pH 7.2), respectively. The specimens were subsequently rinsed in wash buffer (0.1 M sodium cacodylate) and ultrapure water before dehydrating in an aqueous alcohol series (30 %, 50 %, 70 %, 95 % and 100 %); each incubation lasted for at least 30 min. The dehydrated specimens underwent critical point drying in hexamethyldisilazane (TAAB Laboratories Equipment Ltd, Aldermaston, UK) for at least three hours and then were allowed to air-dry overnight on watch glasses inside a laminar airflow hood. The specimens were then attached to self-adhesive conductive carbon tabs on 1" head diameter aluminium specimen stubs (both Agar Scientific, Stansted, UK). Finally, the mounted specimens were gold-coated for five minutes in a Polaron E5000 SEM Coating Unit and subjected to imaging on a Hitachi S-4700 FESEM microscope using the Ultra High Resolution mode (Crusco et al., 2018).

2.8. Statistics and software

GraphPad Prism 8 software (https://www.graphpad.com/scientific-software/ prism/) was used for all statistical analyses to determine significant differences amongst population means. As the data was not normally distributed, a non-parametric test (Kruskal-Wallis) was used followed by Dunn's multiple comparison test. A p value < 0.05 was considered significant.

3. Results

In an initial screen, the flukicidal effects of 19 synthetic diterpenoid abietanes at 10 µM were quantified on *F. hepatica* NEJs (Italian strain). The molecules tested included MC001 (prepared directly from commercial rosin), the dehydroabietic acid MC003 and 17 derived from MC003 (Fig. 2) as described in Appendix 1. Applying previously published numerical metrics (Crusco et al., 2018; Edwards et al., 2015), the ultrastructure and motility of NEJs were recorded after 24, 48 and 72 h of incubation (Supplementary Table 1 and Supplementary Fig. 1). Similar to the deleterious *ex vivo* activity of TCBZ (10 µM; green dots in Supplementary Fig. 1), six dehydroabietic acid analogues (MC008, MC009, MC010, MC052, MC058 and MC061; blue dots in Supplementary Fig. 1; coloured rows in Supplementary Table 1) all led to significantly reduced NEJ motility and abnormal ultrastructural alterations ($p < 0.05$). While some other derivatives (MC004, MC005, MC007, MC011, MC013, MC087 and MC088) also affected NEJ ultrastructure and motility, they were not as uniformly active on all parasites during co-incubation (Supplementary Table 1 and Supplementary Fig. 1).

The six most active molecules (MC008, MC009, MC010, MC052, MC058 and MC061) were next screened against adult *F. hepatica* (wild strain; provenance unknown) for 72 h at 40 μM; motility was compared to parasites co-incubated with TCBZ (40 μM in 0.4 % DMSO) and DMSO (0.4 %) for 72 h (Fig. 4). Abattoir-derived wild strains of *F. hepatica* were used for this stage of the study due to the availability of mature flukes from this source. Reassuringly, all TCBZ-treated parasites were dead after 72 h. The motility scores of MC009 and MC010 were statistically different to DMSO (0.4 %) treated parasites (**, $p < 0.05$). While two parasites were completely immobile and two demonstrated reduced movement due to MC009 treatment, all MC010 treated flukes suffered from partial paralysis and irregular contraction of their oral suckers.

Based on its highly reproducible activity on NEJs (Supplementary Table 1, Supplementary Figs. S1 and S2) and adult flukes, MC010 was further tested (40 μM) on 4-week immature flukes (laboratory-derived Italian strain); motility scores were recorded at 24, 48 and 72 h post incubation and compared to TCBZ (40 μM in 0.4 % DMSO) and DMSO (0.4 %) treated flukes (Fig. 5). Similar to the kinetics of TCBZ, MC010 was lethal to immature worms as early as 24 h post treatment. Damage to the posterior region of immature flukes cultivated with MC010 was sometimes observed (Supplementary Fig. S2).

Having established that MC010 was reproducibly active against Italian strain NEJs and immature flukes as well as field obtained adult flukes in single point assays (10 μM for NEJs; 40 μM for immature and adult flukes), a three-point dose response titration (40 μM, 13.3 μM and 4.4. μM in 0.4 % DMSO) was next conducted for Italian strain adult flukes (Fig. 6). Adult fluke motility was measured at 24, 48 and 72 h post MC010 treatment and compared to flukes co-cultivated with TCBZ (40 μM, 13.3 μM and 4.4. μM in 0.4 % DMSO), RPMI 1640 only and RPMI 1640 + DMSO (0.4 %) during the same interval. In RPMI 1640 and RPMI 1640 + DMSO (0.4 %) controls, the motility of worms consistently remained at score 1 (normal movement) throughout the assay. After 72 h of treatment, the TCBZ (40 μM) treated parasites were dead, as expected; marginal but significant variation in motility was observed at a sub-lethal dosage (13.3 μM). MC010 treated adults at the maximal dosage (40 μM) suffered from paralysis by the end of 72 h. Upon removing the parasites from MC010, one of the flukes treated with 40 μM MC010 partially recovered whereas the two other parasites continued to demonstrate immobility (score 5). The recovered worm exhibited movement (twitching) only on the right side of the ventral body surface, while the left side remained in paralysis with irregular contraction of the oral suckers. The submaximal dosage (13.3 μM) altered motility at 72 h post treatment and, under the same conditions, the lowest dosage (4.4 μM) had no demonstrable effect on worm movement at any time point. Although not as potent as TCBZ (40 μM), MC010 also induced a time and concentration dependent decrease in adult fluke motility (at 72 h, $EC_{50} = 12.97$ μM).

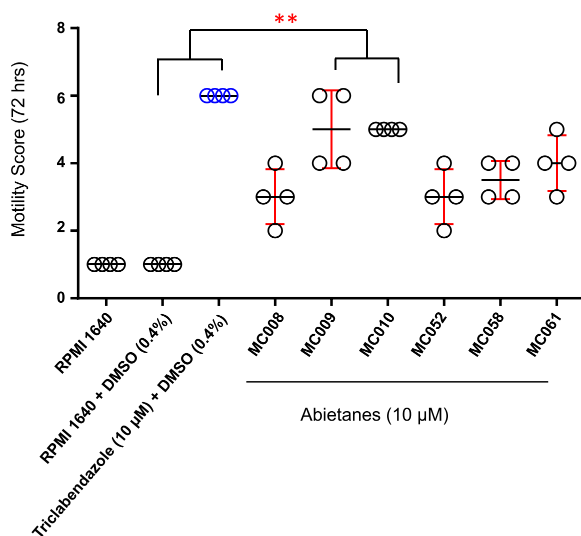

Fig. 4. Adult *Fasciola hepatica* (wild strain) motility is affected by dehydroabietic acid derivatives. Adult *F. hepatica* (wild strain; provenance unknown; n = 4) were assayed with TCBZ and the 6 modified dehydroabietic acid derived compounds capable of killing NEJs. Each compound/parasite co-culture was performed for 72 h at 40 μM final concentration. Parasites co-cultivated in the presence or absence of 0.4 % DMSO served as controls. Motility of adult worms was scored from 1 to 6 where 1 equates to good movement (curled, sticking on wall, movement on petri plate or conical flask), 2 equates to moderate movement (less vigour but more than 10 s pulses or peristaltic waves), 3 equates to resting (less than 10 s pulses in head and body), 4 equates to lethargy (less than 2 s pulses in head and body), 5 equates to body paralysis to faint movement of suckers and 6 equates to no movement or death. Motility scores obtained from flukes cultured with MC009 and MC010 were statistically significant from those obtained from the control flukes (RPMI 1640 + 0.4 % DMSO). * * = $p < 0.05$.

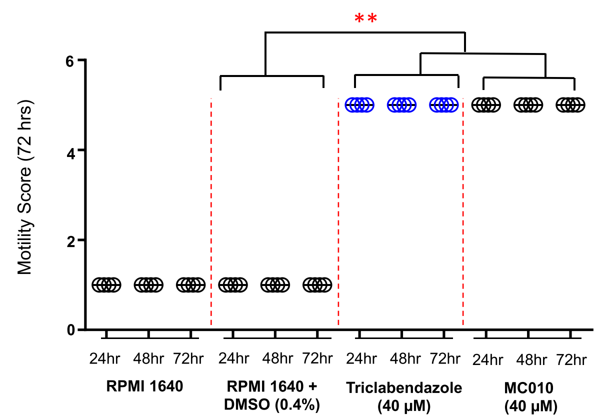

Fig. 5. MC010 is lethal to immature-stage *Fasciola hepatica* (Italian strain). Motility (1 for normal movement, 2 moderate, 3 low, 4 very little and 5 for no movement or death) of immature flukes (n = 4) was quantified at 24, 48 and 72 h after co-incubation with TCBZ (40 μM) and MC010 (40 μM). Parasites (n = 4) co-cultivated in the presence or absence of DMSO (0.4 %) served as controls. All TCBZ and MC010 treated parasites were dead by 24 h of incubation (**, $p < 0.05$; compared to control flukes cultured with RPMI 1640 + 0.4 % DMSO).

Having established MC010's flukicidal activity, we next addressed this compound's overt mammalian cell cytotoxicity on the bovine MBDK cell line (Supplementary Fig. S3). Here, based on a dose response titration, MC010 demonstrated a $CC_{50} = 17.52$ μM at 24 h post culture.

To generate further information regarding potential mechanism of action and building upon the brightfield microscopic analyses of MC010 treated NEJs and immature flukes (Supplementary Fig. S2), we next explored MC010-associated adult fluke surface alterations by SEM (Fig. 7). Here, MC010 treatment (40 μM; parasites were alive, but motility was severely affected, Fig. 6) caused surface damage to 50 % of the adult flukes examined (2/4) whereas no damage was observed in the DMSO treated individuals (0/4). This damage was restricted to the dorsal region of the fluke and broadly consisted of membrane blebbing/sloughing and spine alterations.

4. Discussion

Abietic acids and their derivatives (also referred as abietanes) are amphipathic diterpenes currently being considered for veterinary (Buommino et al., 2021) and biomedical uses (Xu et al., 2017; Liu et al., 2019). Diene sub-groups in these molecules are rather unstable to acid and heat, but can be readily converted into aromatic dehydroabietanes The increased stability of these aromatic compounds facilitates their use in biomedical and veterinary applications (Fig. 1C) (Fleck and Palkin, 1938; Littmann, 1938). As the properties of chemically modified dehydroabietanes have not been widely examined as anthelmintics, we have quantified the activity of 19 related molecules on the food-borne liver fluke pathogen, *Fasciola hepatica*. A single dehydroabietane (1 *R*,

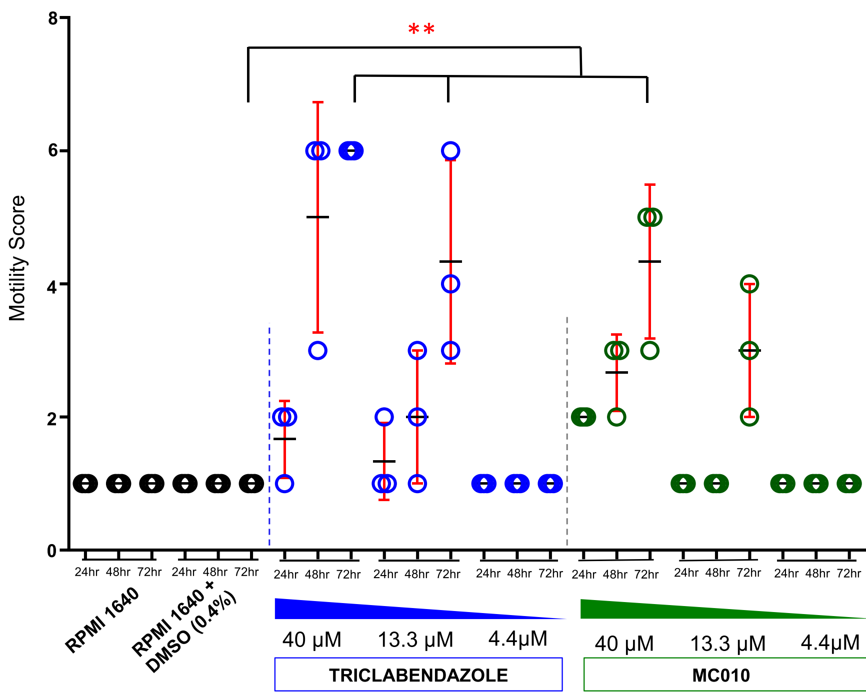

Fig. 6. MC010 affects adult *Fasciola hepatica* (Italian strain) motility in a dose and time-dependent manner. *F. hepatica* (n = 3) were co-cultured with MC010 at 40 µM, 13.3 µM and 4.4 µM; motility was scored at 24, 48 and 72 h. Parasites (n = 3) co-cultured with TCBZ at identical concentrations were used as positive controls and RPMI 1640 cultivated parasites (n = 3) incubated in the presence or absence of 0.4 % DMSO served as negative controls. Motility of adult worms was scored from 1 to 6 where 1 equates to good movement (curled, sticking on wall, movement on petri plate or conical flask), 2 equates to moderate movement (less vigour but more than 10 s pulses or peristaltic waves), 3 equates to resting (less than 10 s pulses in head and body), 4 equates to lethargy (less than 2 s pulses in head and body), 5 equates to body paralysis to faint movement of suckers and 6 equates to no movement or dead. At the end of the assay, the scores of TCBZ (40 µM and 13.3 µM) and MC010 (40 µM) were statistically significant (*** $= p < 0.05$) from those obtained from the control flukes (RPMI 1640 + 0.4 % DMSO) at a similar timepoint (72 h post treatment).

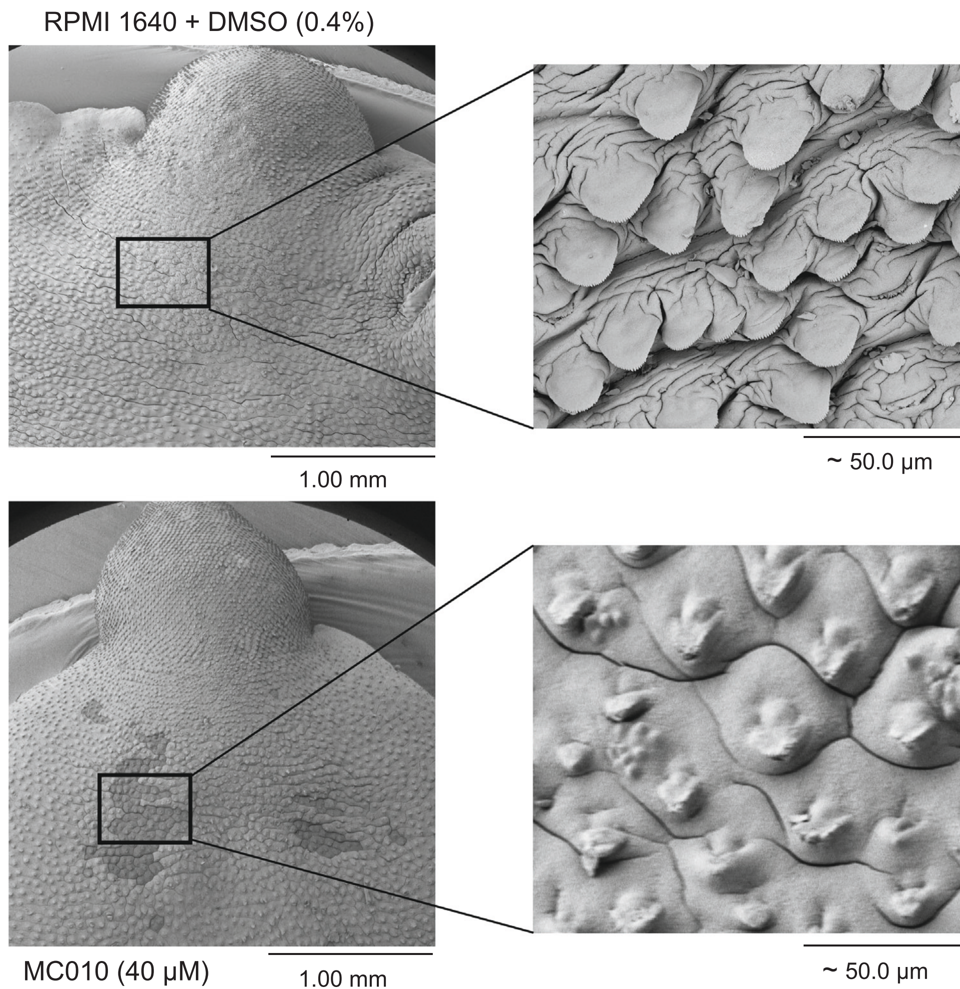

Fig. 7. MC010 induces surface damage on paralysed adult *F. hepatica*. Post *ex vivo* assays, all adult *F. hepatica* of provenance unknown (harvested from infected cattle liver at Abattoir) were fixed with glutaraldehyde and prepared for viewing under a scanning electron microscope (SEM) according to the Materials and Methods. The control liver flukes (RPMI 1640 + 0.4% DMSO) showed no significant damage on the dorsal side surface. MC010 (40 µM) led to surface damage on the dorsal side. Images magnified from areas outlined by a black box support the differential damage of the two representative specimens. MC010 led to this type of surface damage in 50 % of the parasites examined.

4a*S*)-*N*-((3-(dimethylamino)-propyl)carbamoyl)-*N*-ethyl-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene-1-carboxamide (MC010), displaying broad anti-flukicidal activities, was highlighted as a candidate for progressing further in the search for novel anthelmintics.

4.1. The modified diterpenoid abietanes

All of the diterpenoid abietanes screened against *F. hepatica* are derived from dehydroabietic acid (MC003), except the pentacyclic Diels-Alder adduct MC001. The latter was obtained by thermal isomerisation of abietic acid into its cyclohexadiene isomer followed by trapping this with maleic anhydride. Reaction of the carboxylic group produced the benzyl amide MC007 and the key amide MC010. The methyl ester (MC011) was further derivatised by the introduction of an amino group in position 12 (MC013). Reduction of the carboxylic acid to the corresponding alcohol gave MC004 and subsequent acetylation produced the acetate MC005. Conversion of the carboxyl group into an isocyanate intermediate MC009 followed by treatment with a suitable amine resulted in a 9-member library of urea derivatives (MC051, MC052, MC053, MC054, MC058, MC060, MC061, MC087, MC088). Hydrolysis of isocyanate MC009 gave the 4-amino derivative MC008.

4.2. Structure activity relationships

While the available data is insufficient for an in-depth structure activity relationship discussion, some trends can be identified. Six of the 19 compounds (Fig. 2) were found to perform particularly poorly in both the NEJ motility and ultrastructure assays (Supplementary Fig. S1, Supplementary Table S1). Many natural compounds, in particular terpenoid saponins produced by plants and consisting of an aglycone unit (sapogenin) linked to a carbohydrate moiety, gain their pharmaceutical properties due to their amphiphilicity (Chen et al., 2015; Li et al., 2018). As membrane disruptors, loss of the hydrophilic sugar part has been reported to be responsible for loss of activity. Due to the presence of a carboxylic acid group, abietic acids are amphiphilic by nature. Interestingly, the saponin-like structures displaying an N-linked D-glucosyl residue (MC054) in its common cyclic or in its open-chain (MC060) form, did not show activity in the assays; nor did the 2 free acids, dehydroabietic acid MC003 itself and the Diels-Alder adduct MC001. The latter also displays an increased steric demand. In addition, the pyrrolidine urea derivative MC051, with diminished amphiphilicity, performed poorly as did the Boc protected version (MC053) of the active guanidyl urea derivative (MC058). An additional seven compounds (MC004, MC005, MC007, MC011, MC013, MC087, MC088) either showed inconclusive results or did not pass the threshold (i.e. all NEJs dead within 24 h of *ex vivo* treatment) to be taken into the secondary assays (i.e. adult liver flukes). None of these has a pronounced amphiphilic nature; MC004 carries a primary hydroxy group at C18 (reduced abietic acid) and MC013 displays a weakly basic amino group on the aromatic system (aniline group). On the other hand, except for the isocyanate MC009, all compounds that were included in the best performing group carry a nitrogen derived basic function on the A side of the abietic acid derivative.

While compound MC058 carries the most basic guanidyl group, there is little difference in the basicity of the remaining amines. Thus, as long as the A side is not too basic (e.g. a guanidinium group as opposed to simple amine, when ionic interactions will dominate), distance and flexibility seem to be other factors of importance. A possibility is that the dimethylpropylamino group of MC010 is able to interact with the carboxyl group of an amino acid residue (within a currently unknown *F. hepatic* target (s)) and, in this way, contributes to MC010's greater anti-flukicidal activity (amongst the other derivatives).

4.3. Anthelmintic effects of (1R,4aS)-N-((3-(dimethylamino)propyl)-carbamoyl)-N-ethyl-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene-1-carboxamide (MC010)

The mechanism(s) by which MC010 negatively impacts *F. hepatica* motility, ultrastructure and viability is currently unknown. However, due to its amphiphilic nature and potential to act on biological membranes, MC010 may be exerting its effects on all three *F. hepatica* lifecycle stages through disruption of surface membranes covering the liver fluke's syncytial tegument (Halton, 2004). It is a well-known phenomenon that amphiphilic compounds affect the function and integrity of cell membranes (Hägerstrand and Isomaa, 1989). Dehydroabietic acid, specifically, also affects the physical state of eukaryotic cytoskeletal proteins and phospholipid bilayers in biological membranes (Aranda and Villalaín, 1997; Bushnell et al., 1985; Butterfield et al., 1994). In addition, several prokaryotic studies have also confirmed that dehydroabietic acid derivatives lead to the destruction of the cell wall by disturbing fatty acid branching (Burčová et al., 2018; Jagalski et al., 2016; Popova et al., 2021). As shown in the dipalmitoylphosphatidylcholine (DPPC) membrane model, the cytotoxicities of dichlorodehydroabietic acid and dehydroabietic acid are likely due to these molecules co-localising within the lipid tail region of cellular membranes leading to alterations in fluidity, curvature and shape (Jagalski et al., 2016). Therefore, based on the damage observed to the surface of NEJs (some parasites exhibited a dissolved surface, Supplementary Fig. S2), immature flukes (most noticeable at the posterior end, Supplementary Fig. S2) and adults (sloughing/abrasions/spine alterations), we propose that MC010's mechanism of activity operates by disrupting surface heptalaminate membrane integrity. This structure is essential to survival of the parasite in both *ex vivo* and in vivo environments (Halton, 2004). We speculate that the consequences of disrupting surface membranes could lead to the dysregulation of tegumental/sub-tegumental cytoskeletal proteins that participate in locomotion or ion channels that participate in muscle contractions (Graham et al., 1999; Jang et al., 2012, 2004). MC010-mediated surface damage could also lead to *F. hepatica* membrane depolarisation and neurological impairment as previously shown for related abietanes in mammalian models (Nicholson, 1994a, 1994b; Villeneuve et al., 1977). Preliminary support for these contentions is found in the dose response experiments where MC010 treatment of adults at 40 μM led to partial paralysis at 72 h, but no effect on motility at 4.4 μM (Fig. 6). Maximal dosage of MC010 also resulted in damage to the dorsal side of adults, which included sloughing/blebbing of membranes and phenotypic alterations of tegumental spines (Fig. 7). Tegumental alterations similar to those reported here have been previously observed in liver (and blood) flukes treated with 7-keto-sempervirol, a structurally similar diterpenoid isolated from *Lycium chinense* (Edwards et al., 2015).

While MC010 displays activity against all intra-mammalian *F. hepatica* lifecycle stages, it also demonstrates general cytotoxicity against a representative bovine cell line (Supplementary Fig. S3). Therefore, in depth medicinal chemistry investigations are required to identify MC010 analogues with an acceptable selectivity index.

5. Conclusions

In the search for novel flukicides, we investigated the anthelmintic qualities of 19 diterpenoid abietanes from rosin. The most active compound, (1*R*,4a*S*)-*N*-((3-(dimethylamino)propyl)carbamoyl)-*N*-ethyl-7-isopropyl-1,4a-dimethyl-1,2,3,4,4a,9,10,10a-octahydrophen-anthrene-1-carboxamide (MC010), demonstrated promising effects on NEJs, immature flukes and mature flukes. However, significant optimisation of the abietic acid molecules to reduce their cytotoxicity is required before they could be considered as useful anthelmintics.

CRediT authorship contribution statement

Anand Chakroborty: Writing – original draft, review, Methodology & Investigation. **Deiniol Pritchard:** Investigation. **Marc E. Bouillon:** Investigation. **Anna Cervi:** Investigation and Project administration. **Alan Cookson:** Investigation. **Charlotte Wild:** Resources. **Caroline Fenn:** Resources. **Joseph Payne:** Project administration. **Peter Holdsworth:** Project administration. **Colin Capner:** Investigation. **Jenna O'Neill:** Investigation. **Gilda Padalino:** Investigation. **Josephine Forde-Thomas:** Investigation. **Sandeep Gupta:** Project administration. **Brendan G. Smith:** Project administration. **Maggie Fisher:** Resources and Supervision. **Martina Lahmann:** Supervision, review, Methodology and editing. **Mark S. Baird:** Supervision, Methodology, review, editing, Funding acquisition and Project administration. **Karl F. Hoffmann:** Supervision, Methodology, editing, Funding acquisition and Project administration.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Brendan G Smith, Sandeep Gupta reports a relationship with Biimeda UK that includes: employment. Charlotte Wild, Caroline Fenn, Joseph Payne, Colin Capner, Jenna O'Neill, Maggie Fisher reports a relationship with Ridgeway Research that includes: employment. Peter Holdsworth reports a relationship with PAH Consultancy that includes: employment. Deiniol Pritchard, Anna Cervi, Mark S. Baird reports a relationship with Naturiol Bangor Ltd that includes: employment.

Acknowledgements

This work was supported and funded by UK Research and Innovation: Innovate UK grant number 102101.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2022.109766.

References

Aranda, F.J., Villalaín, J., 1997. The interaction of abietic acid with phospholipid membranes. Biochim. Biophys. Acta Biomembr. 1327, 171–180. https://doi.org/10.1016/S0005-2736(97)00054-0.

Buommino, E., Vollaro, A., Nocera, F.P., Lembo, F., Dellagreca, M., De Martino, L., Catania, M.R., 2021. Synergistic effect of abietic acid with oxacillin against methicillin-resistant *Staphylococcus pseudintermedius*. Antibiotics 10, 1–12. https://doi.org/10.3390/antibiotics10010080.

Burčová, Z., Kreps, F., Greifová, M., Jablonský, M., Ház, A., Schmidt, Š., Šurina, I., 2018. Antibacterial and antifungal activity of phytosterols and methyl dehydroabietate of Norway spruce bark extracts. J. Biotechnol. 282, 18–24. https://doi.org/10.1016/j.jbiotec.2018.06.340.

Bushnell, P.G., Nikinmaa, M., Oikari, A., 1985. Metabolic effects of dehydroabietic acid on rainbow trout erythrocytes. Comp. Biochem Physiol. C Comp. Pharmacol. Toxicol. 81, 391–394.

Butterfield, D.A., Trad, C.H., Hall, N.C., 1994. Effects of dehydroabietic acid on the physical state of cytoskeletal proteins and the lipid bilayer of erythrocyte membranes. Biochim. Biophys. Acta Biomembr. 1192, 185–189. https://doi.org/10.1016/0005-2736(94)90117-1.

Casulli, A., 2021. New global targets for NTDS in the WHO roadmap 2021–2030. PLoS Negl. Trop. Dis. 15, 1–10. https://doi.org/10.1371/journal.pntd.0009373.

Chen, Y., Dong, Z., Huang, D., Chen, W., Yao, F., Xue, D., Sun, L., 2015. Brevistylumsides A and B, diterpene glycosides from *Illicium brevistylum*. Phytochem. Lett. 14, 27–30. https://doi.org/10.1016/j.phytol.2015.08.014.

Crusco, A., Bordoni, C., Chakroborty, A., Whatley, K.C.L., Whiteland, H., Westwell, A.D., Hoffmann, K.F., 2018. Design, synthesis and anthelmintic activity of 7-keto-sempervirol analogues. Eur. J. Med. Chem. https://doi.org/10.1016/j.ejmech.2018.04.032.

Crusco, A., Whiteland, H., Baptista, R., Forde-Thomas, J.E., Beckmann, M., Mur, L.A.J., Nash, R.J., Westwell, A.D., Hoffmann, K.F., 2019. Antischistosomal properties of sclareol and its heck-coupled derivatives: design, synthesis, biological evaluation, and untargeted metabolomics. ACS Infect. Dis. 5, 1188–1199. https://doi.org/10.1021/acsinfecdis.9b00034.

Edwards, J., Brown, M., Peak, E., Bartholomew, B., Nash, R.J., Hoffmann, K.F., 2015. The diterpenoid 7-keto-sempervirol, derived from *Lycium chinense*, displays anthelmintic activity against both *Schistosoma mansoni* and *Fasciola hepatica*. PLoS Negl. Trop. Dis. 9, e0003604 https://doi.org/10.1371/journal.pntd.0003604.

Eksi, G., Kurbanoglu, S., Erdem, S.A., 2020. Analysis of diterpenes and diterpenoids. In: In: Recent Advances in Natural Products Analysis. Elsevier, pp. 313–345. https://doi.org/10.1016/B978-0-12-816455-6.00009-3.

Fairweather, I., Brennan, G.P., Hanna, R.E.B., Robinson, M.W., Skuce, P.J., 2020. Drug resistance in liver flukes. Int. J. Parasitol. Drugs Drug Resist. 12, 39–59. https://doi.org/10.1016/j.ijpddr.2019.11.003.

Fleck, E.E., Palkin, S., 1938. On the nature of pyroabietic acids. J. Am. Chem. Soc. 60, 921–925. https://doi.org/10.1021/ja01271a047.

Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts of climate change on *Fasciola hepatica* risk. PLoS One 6, e16126. https://doi.org/10.1371/journal.pone.0016126.

Faustino, C., Neto, Í., Fonte, P., Macedo, A., 2019. Cytotoxicity and chemotherapeutic potential of natural rosin abietane diterpenoids and their synthetic derivatives. Curr. Pharm. Des. 24, 4362–4375. https://doi.org/10.2174/1381612825666190112162817.

Garcia-Bustos, J.F., Sleebs, B.E., Gasser, R.B., 2019. An appraisal of natural products active against parasitic nematodes of animals. Parasit. Vectors 12, 306. https://doi.org/10.1186/s13071-019-3537-1.

González, M.A., 2015. Aromatic abietane diterpenoids: their biological activity and synthesis. Nat. Prod. Rep. 32, 684–704. https://doi.org/10.1039/c4np00110a.

Graham, M.K., McGeown, J.G., Fairweather, I., 1999. Ionic mechanisms underlying spontaneous muscle contractions in the liver fluke, *Fasciola hepatica*. Am. J. Physiol. Integr. Comp. Physiol. 277, R374–R383. https://doi.org/10.1152/ajpregu.1999.277.2.R374.

Hadacek, F., 2019. Encyclopedia of lipidomics. Encycl. Lipidom. 1–4. https://doi.org/10.1007/978-94-007-7864-1.

Hägerstrand, H., Isomaa, B., 1989. Vesiculation induced by amphiphiles in erythrocytes. Biochim. Biophys. Acta Biomembr. 982, 179–186. https://doi.org/10.1016/0005-2736(89)90053-9.

Halton, D.W., 2004. Microscopy and the helminth parasite. Micron 35, 361–390. https://doi.org/10.1016/j.micron.2003.12.001.

Hanna, R.E.B., McMahon, C., Ellison, S., Edgar, H.W., Kajugu, P.-E., Gordon, A., Irwin, D., Barley, J.P., Malone, F.E., Brennan, G.P., Fairweather, I., 2015. *Fasciola hepatica*: a comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. Vet. Parasitol. 207, 34–43. https://doi.org/10.1016/j.vetpar.2014.11.016.

Hassell, C., Chapman, V., 2012. Case report: suspect lack of triclabendazole efficacy in liver fluke in sheep in the Taranaki. Proc. Soc. Sheep Beef Cattle Vet. N. Z. Vet. Assoc. 2012, 4.14.1-144.14.2.

Iglesias-Piñeiro, J., González-Warleta, M., Castro-Hermida, J.A., Córdoba, M., González-Lanza, C., Manga-González, Y., Mezo, M., 2016. Transmission of *Calicophoron daubneyi* and *Fasciola hepatica* in Galicia (Spain): Temporal follow-up in the intermediate and definitive hosts. Parasit. Vectors 9, 610. https://doi.org/10.1186/s13071-016-1892-8.

Jagalski, V., Barker, R., Topgaard, D., Günther-Pomorski, T., Hamberger, B., Cárdenas, M., 2016. Biophysical study of resin acid effects on phospholipid membrane structure and properties. Biochim. Biophys. Acta Biomembr. 1858, 2827–2838. https://doi.org/10.1016/j.bbamem.2016.08.008.

Jang, J.H., Kim, S.D., Park, J.B., Hong, S.J., Ryu, P.D., 2004. Ion channels of *Fasciola hepatica* incorporated into planar lipid bilayers. Parasitology 128, 83–89. https://doi.org/10.1017/S0031182003004232.

Jang, J.H., Park, J.B., Kim, S.D., Lee, S.Y., Hong, S.-J., Ryu, P.D., 2012. Property of large conductance Ca2+-activated K+ channels from Fasciola hepatica incorporated into planar lipid bilayer. Vet. Parasitol. 186, 281–288. https://doi.org/10.1016/j.vetpar.2011.11.038.

Keiser, J., Utzinger, J., 2009. Food-borne trematodiases. Clin. Microbiol. Rev. 22, 466–483. https://doi.org/10.1128/CMR.00012-09.

Kelley, J.M., Elliott, T.P., Beddoe, T., Anderson, G., Skuce, P., Spithill, T.W., 2016. Current threat of triclabendazole resistance in *Fasciola hepatica*. Trends Parasitol. 1–12. https://doi.org/10.1016/j.pt.2016.03.002.

Kitadani, M., Yoshikoshi, A., Kitahara, Y., Campello, J., McChesney, J., Watts, D., Wendert, E., 1970. Natural ar-Abietatriene. Chem. Pharm. Bull. 18, 402–405. https://doi.org/10.1248/cpb.18.402.

Li, Yuan, Lin, H.X., Wang, J., Yang, J., Lai, C.J.S., Wang, X., Ma, B.W., Tang, J.F., Li, Yong X.L., Li, Guo, J., Gao, W., Huang, L.Q., 2018. Glucosyltransferase capable of catalyzing the last step in neoandrographolide biosynthesis. Org. Lett. 20, 5999–6002. https://doi.org/10.1021/acs.orglett.8b02146.

Littmann, E.R., 1938. The resin acids. The action of palladium on abietic acid. J. Am. Chem. Soc. 60, 1419–1421.

Liu, M., Panda, S.K., Luyten, W., 2020. Plant-based natural products for the discovery and development of novel anthelmintics against nematodes. Biomolecules 10. https://doi.org/10.3390/biom10030426.

Liu, X., Chen, W., Liu, Q., Dai, J., 2019. Abietic acid suppresses non-small-cell lung cancer cell growth via blocking IKKβ/NF-κB signaling. Onco Targets Ther. 12, 4825–4837. https://doi.org/10.2147/OTT.S199161.

Lloyd, W.D., Hendrick, G.W., 1961. The Diels-alder reaction of levopimaric acid and its use in quantitative determinations 2. J. Org. Chem. 26, 2029–2032. https://doi.org/10.1021/jo01065a085.

Mazeri, S., Rydevik, G., Handel, I., Bronsvoort, B.M.D., Sargison, N., 2017. Estimation of the impact of *Fasciola hepatica* infection on time taken for UK beef cattle to reach slaughter weight. Sci. Rep. 7. https://doi.org/10.1038/s41598-017-07396-1.

Moll, L., Gaasenbeek, C.P., Vellema, P., Borgsteede, F.H., 2000. Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. Vet. Parasitol. 91, 153–158. https://doi.org/10.1016/s0304-4017(00)00267-3.

Nicholson, Russell A., 1994a. The actions of abietic acid in mammalian synaptosomal preparations. In: In: Biochemical Society Transactions. Portland Press Ltd. https://doi.org/10.1042/bst022226s.

Nicholson, Russell A., 1994b. Excitatory actions of dehydroabietic acid on mammalian synaptosomes. Pharmacol. Toxicol. 75, 274–279. https://doi.org/10.1111/j.1600-0773.1994.tb00360.x.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366. https://doi.org/10.1016/j.vetpar.2010.12.047.

Olmo, F., Guardia, J.J., Marin, C., Messouri, I., Rosales, M.J., Urbanová, K., Chayboun, I., Chahboun, R., Alvarez-Manzaneda, E.J., Sánchez-Moreno, M., 2015. Prospects of an alternative treatment against *Trypanosoma cruzi* based on abietic acid derivatives show promising results in Balb/c mouse model. Eur. J. Med. Chem. 89, 863–890. https://doi.org/10.1016/j.ejmech.2014.11.004.

Ortiz, P., Scarcella, S., Cerna, C., Rosales, C., Cabrera, M., Guzmán, M., Lamenza, P., Solana, H., 2013. Resistance of *Fasciola hepatica* against Triclabendazole in cattle in Cajamarca (Peru): a clinical trial and an in vivo efficacy test in sheep. Vet. Parasitol. 195, 118–121. https://doi.org/10.1016/j.vetpar.2013.01.001.

Peng, G., Roberts, J.C., 2000. Solubility and toxicity of resin acids. Water Res. 34, 2779–2785. https://doi.org/10.1016/S0043-1354(99)00406-6.

Popova, L., Ivanchenko, O., Pochkaeva, E., Klotchenko, S., Plotnikova, M., Tsyrulnikova, A., Aronova, E., 2021. Rosin derivatives as a platform for the antiviral drug design. Molecules 26, 3836. https://doi.org/10.3390/molecules26133836.

Rates, S.M.K., 2001. Plants as source of drugs. Toxicon 39, 603–613. https://doi.org/10.1016/S0041-0101(00)00154-9.

Romero, J., Villaguala, C., Quiroz, F., Landaeta-Aqueveque, C., Alfaro, G., Pérez, R., 2019. Flukicide efficacy against Fasciola hepatica of Triclabendazole and Nitroxynil in cattle of the central valley of Chile. Rev. Bras. De. Parasitol. Vet. 28, 164–167. https://doi.org/10.1590/S1984-296120180089.

Rinaldi, L., Biggeri, A., Musella, V., De Waal, T., Hertzberg, H., Mavrot, F., Torgerson, P. R., Selemetas, N., Coll, T., Bosco, A., Grisotto, L., Cringoli, G., Catelan, D., 2015. Sheep and *Fasciola hepatica* in Europe: the GLOWORM experience. Geospat. Health 9, 309. https://doi.org/10.4081/gh.2015.353.

Sofia Costa, M., Rego, A., Ramos, V., Afonso, T.B., Freitas, S., Preto, M., Lopes, V., Vasconcelos, V., Magalhães, C., Leão, P.N., 2016. The Conifer Biomarkers Dehydroabietic and Abietic Acids are Widespread in Cyanobacteria OPEN. https://doi.org/10.1038/srep23436.

Takeda, H., Kanno, H., Schuller, W.H., Laurence, R.V., 1968. Effect of temperature on various rosins and pine gum. IEC Prod. Res. Dev. 7, 186–189. https://doi.org/10.1021/i360027a006.

Venturina, V.M., Alejandro, M.A.F., Baltazar, C.P., Abes, N.S., Mingala, C.N., 2015. Evidence of *Fasciola spp.* resistance to albendazole, triclabendazole and bromofenofos in water buffaloes (*Bubalus bubalis*). Ann. Parasitol. 61, 283–289. https://doi.org/10.17420/ap6104.20.

Villeneuve, D.C., Yagminas, A.P., Marino, I.A., Becking, G.C., 1977. Toxicity studies on dehydroabietic acid. Bull. Environ. Contam. Toxicol. 18, 42–47. https://doi.org/10.1007/BF01686303.

Whiteland, H.L., Chakroborty, A., Forde-Thomas, J.E., Crusco, A., Cookson, A., Hollinshead, J., Fenn, C.A., Bartholomew, B., Holdsworth, P.A., Fisher, M., Nash, R. J., Hoffmann, K.F., 2018. An *Abies procera*-derived tetracyclic triterpene containing a steroid-like nucleus core and a lactone side chain attenuates in vitro survival of both *Fasciola hepatica* and *Schistosoma mansoni*. Int. J. Parasitol. Drugs Drug Resist. https://doi.org/10.1016/j.ijpddr.2018.10.009.

Xu, H., Liu, L., Fan, X., Zhang, G., Li, Y., Jiang, B., 2017. Identification of a diverse synthetic abietane diterpenoid library for anticancer activity. Bioorg. Med. Chem. Lett. 27, 505–510. https://doi.org/10.1016/j.bmcl.2016.12.032.