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Hedera helix-derived α−hederin (IVL-11) demonstrates both *ex vivo* and *in vivo* flukicidal activities against *Fasciola hepatica*

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ABSTRACT

Infection with *Fasciola hepatica* (liver flukes) causes fascioliasis in humans and fasciolosis in ruminants. The current strategy for controlling fascioliasis/fasciolosis is predominantly mediated by chemotherapy with triclabendazole (TCBZ). As this flukicide targets newly excysted juveniles (NEJs), immature flukes and mature liver flukes, it's use as the frontline chemotherapy for over four decades has led to widespread drug resistance. New chemotherapeutics are, therefore, urgently required. Here, continuing our studies of flukicidal phytochemicals derived from *Hedera helix* (common ivy), we investigated the anthelmintic properties of three ivy fruit saponins, including α-hederin (**IVL-11**), against *F. hepatica*. During *ex vivo* culture, **IVL-11** was as effective as TCBZ in killing NEJs and immature flukes. However, this saponin acted more quickly than TCBZ when tested against mature flukes. Microscopic examination of **IVL-11** treated liver flukes revealed surface integrity breaches, actin disorganisation and cell membrane permeabilisation. Upon *in vivo* studies, using a novel method to isolate **IVL-11** in large quantities from *H. helix* leaf, oral delivery of **IVL-11** (up to 250 mg/kg bodyweight) to *Ovis aries* (sheep) was found to be safe, well-tolerated and detectable in the liver, peripheral blood, hepatic portal blood and bile. **IVL-11** (250 mg/kg bodyweight) treatment of *O. aries* infected with *F. hepatica* led to a significant reduction in fluke body sizes as well as a delay and decrease in fecundity during *in vivo* efficacy studies. Together,

Abbreviations: AUC, area under the curve; AWERB, Animal Welfare Ethical Review Body; BHA, butylated hydroxyanisole; BLQ, below limits of quantification; BSA, bovine serum albumin; CC50, half maximal cytotoxicity concentration; CO_2, carbon dioxide; DAPI, 4′,6-diamidino-2-phenylindole; DDU, Drug Discovery Unit; DMSO, dimethyl sulfoxide; DR, dose response; EC_{50}, half maximal effective concentration; ELSD, evaporative light scattering detector; EPG, eggs per gram; FBS, fetal bovine serum; FCS, fetal calf serum; FEC, faecal egg count; FITC, fluorescein isothiocyanate; GGT, gamma-glutamyl transferase; HDPE, high-density polyethylene; HPLC, high performance liquid chromatography; IVL-11, α-hederin;; IVL-12, hederoside B; IVL-13, δ-hederin; LC-MS, liquid chromatography-mass spectrometry; MBDK, Madin-Darby bovine kidney; MS, mass spectrometry; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; NEJ, newly-excysted juvenile; NMR, nuclear magnetic resonance; PBSTx, phosphate buffered saline containing 0.3 % v/v triton-X100; PSA, penicillin-streptomycin-amphotericin; RFC, rumen fluke conditioned; RH, relative humidity; RPMI or RPMI 1640, Roswell Park Memorial Institute medium; RRL, Ridgeway Research Limited; SEM, scanning electron microscopy; SP, single point; TAMRA, 5-carboxytetramethylrhodamine; TCBZ, triclabendazole; UPLC, ultra-performance liquid chromatography; UV, ultraviolet; WHO, World Health Organisation.

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our results confirm that **IVL-11** exhibits flukicidal characteristics suitable for progression as a renewably obtained, natural product for treating fasciolosis.

1. Introduction

Food-borne pathogens continue to pose significant challenges to global public health, contributing to considerable morbidity and mortality worldwide. Among these, *Fasciola hepatica* and *Fasciola gigantica*, the causative agents of fascioliasis, currently infect over 2.4 million people in more than 75 countries, with several million more at risk of infection in endemic regions [24,25]. These parasites are also a significant cause of disease (fasciolosis) in ruminants with current estimates indicating annual losses of US$ 3.2 billion in the livestock farming sector due to reduced yields of dairy and animal products [10]. Transmission primarily occurs through ingestion of water or aquatic vegetation contaminated with encysted metacercaria larval stages, which develop after eggs are shed by infected definitive mammalian hosts [12].

Amongst integrated strategies currently in use to control these liver fluke parasites (e.g. field and herd management, intermediate snail host control, environmental monitoring for the presence of flukes and intermediate snail hosts), infected mammals can be treated with nitroxinil, albendazole, closantel, rafoxanide and clorsulon. However, triclabendazole (TCBZ) is the most commonly employed anthelmintic due to this drug's activity against all intra-mammalian parasite lifecycle stages (i.e. newly excysted juveniles (NEJs), immature and mature flukes) [26]. Furthermore, only TCBZ is recommended by the WHO for the treatment of human fascioliasis [3]. Unfortunately, over-reliance on TCBZ as the frontline chemotherapy has led to documented, TCBZ-resistant liver fluke populations across Europe, Africa, South America, Asia and Australia [15]. While research for commercially successful vaccines targeting *F. hepatica* continues, new chemotherapies are urgently required to replace TCBZ and sustain fascioliasis/fasciolosis control in the foreseeable future.

Arguing that cost effective flukicides could originate from plants easy to cultivate throughout the British Isles, we have previously investigated the activities of phytocompounds derived from temperate conifers (*Abies procera* and *Abies grandis*), wolfberry/goji berry (*Lycium chinense*) and common ivy (*Hedera helix*) against *F. hepatica* [4,6,9,13,32]. Amongst the ivy-derived compounds studied, those with activity against NEJs, immature and adult flukes were triterpenoid saponin derivatives containing a hederagenin moiety. Here, we extended these findings and assessed the *ex vivo* anthelmintic activities of three hederagenin based monodesmosidic saponins from *H. helix* fruit (α-hederin**, IVL-11;** hederoside B**, IVL-12;** and δ-hederin, **IVL-13)** against diverse strains (TCBZ-resistant and sensitive) of *F. hepatica* and a field isolate of *Calicophoron daubneyi* (rumen fluke). We further developed a method to purify significant quantities of the most active molecule, **IVL-11**, from *H. helix* leaf, developed a stable formulation in corn oil, and evaluated this in sheep during tolerability and liver fluke efficacy studies.

2. Materials and methods

2.1. Ethics statement

The *F. hepatica* (Italian, Aberystwyth, Kilmarnock and Penrith strains) lifecycle was maintained according to project licenses PPL 40/3593, P6D805744, PA09B4E45 and PP6589959 and adhered to the United Kingdom Home Office Animals (Scientific Procedures) Act of 1986. All procedures were approved by Ridgeway Research Limited's (RRL) Animal Welfare and Ethical Review Body (AWERB).

2.2. Isolation and purification of IVL-11 (α-hederin), IVL-12 (hederoside B) and IVL-13 (δ-hederin)

The isolation of the three compounds, **IVL-11** (α-hederin), **IVL-12** (hederoside B), and **IVL-13** (δ-hederin) used for *ex vivo* assays from *Hedera helix* leaves, has been previously described [1,21,23]. In this work, these compounds were isolated from *H. helix* fruit with details provided in Supplementary Data 1–4. The **IVL-11** used for the *in vivo* studies was isolated from *H. helix* leaves by treatment with water at 40 °C for an extended time, followed by extraction of the residual solids with ethanol. Details are provided in Supplementary Data 5–7.

2.3. Parasite material and ex vivo screening

The *F. hepatica* lifecycle was propagated as previously described [6] and metacercaria were transformed into newly excysted juveniles (NEJs) as outlined by Gayo *et al.* [17] and slightly modified by Crusco *et al.* [9]. NEJs were transferred into 24-well or 96-well plates containing RPMI supplemented with 1 % v/v fetal bovine serum (FBS), 1 % v/v L-glutamine and 1 % v/v penicillin-streptomycin-amphotericin (PSA) at a density of 5–25 NEJs per well (5 individuals/200 μl for 96 well plates; 25 individuals/1000 μl for 24 well plates). Following a 30-minute recovery period in a humidified environment between 37° – 39°C and 5 % CO_2, parasites were initially dosed with RPMI (+/- 0.1 % v/v DMSO, n = 25 NEJs in 24 well plates), **IVL-11** (n = 25 NEJs in 24 well plates), **IVL-12** (n = 25 NEJs in 24 well plates), **IVL-13** (n = 25 NEJs in 24 well plates) or TCBZ (n = 25 NEJs in 24 well plates) as single point (SP) tests at 10 μM in RPMI containing 0.1 % v/v DMSO (maintaining temperature, humidity and CO_2 settings). All compounds were prepared as 10 mM stocks in DMSO with working concentrations diluted in culture medium (RPMI-1640 + 1 % v/v FBS, 1 % v/v glutamine and 1 % v/v PSA). This experiment was replicated two additional times. To quantify **IVL-11** (and TCBZ) EC_{50}s, NEJs were subsequently subjected to dose response (DR) titrations (10, 7.5, 5.0, 3.75, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, 0.02 and 0 μM in RPMI containing 0.5 % v/v DMSO) maintaining temperature, humidity and CO_2 settings. Here, DR titrations were performed across six replicates and were divided between two independent operators (three replicates per operator; n = 5 NEJs/replicate in 96 well plates). All SP and DR NEJs were scored via brightfield microscopy for phenotype or motility at 24-, 48- and 72-hr post exposure. Parasites were scored as described by Chakroborty *et al.* [5]; motility was scored as ranging from 1 to 5 and phenotype from 1 to 6, with higher values representing less viable parasites. EC_{50} values were generated from phenotypic scores by non-linear regression of $\log_{10}$ compound concentration (using 0.5 % v/v DMSO as zero value) against normalised viability scores (0 % viability = 6; 100 % = 1) in Prism (GraphPad, version 8.4.3).

Immature (4-wk old) and adult (8-wk old) Italian strain liver flukes used for *ex vivo* assays were produced by infection of 6-month-old Texel Mule X lambs. Adult flukes of provenance unknown (i.e. wild strain), used in *ex vivo* assays, were obtained from Randall Parker Foods, Llanidloes (Wales, UK) and washed as previously described [9]. Both immature and adult flukes were screened with compounds (**IVL-11**, **IVL-12**, **IVL-13** and TCBZ single point tests; 40 μM in RPMI containing 0.4 % v/v DMSO) at 37 °C in a humidified environment containing 5 % CO_2 as previously described [9]. For adult worms (scored at 24 and 72 hr), motility was scored from 1 to 6 where 1 equals good movement (curled, sticking on surface, movement on petri plate or conical flask), 2 equates to moderate movement (less vigour but more than 10 sec pulses or peristaltic waves), 3 equates to resting (less than 10 sec pulses in head and body), 4 equates to lethargy (less than 2 s pulses in head and body),

5 equates to faint movement of suckers (movement of oral or ventral suckers only, whole body paralysed) and 6 equates to no movement at all (or dead). The movement of immature parasites was scored (at 24 hr) from 1 to 5 (1 = normal, 2 = moderate, 3 = low, 4 = negligible and 5 = none).

Adult rumen flukes (*Calicophoron daubneyi*) of provenance unknown were obtained from Randall Parker Foods, Llanidloes (Wales, UK). After washing the parasites as previously described [9], the worms were screened with compounds (**IVL-11**, **IVL-12**, **IVL-13** and oxyclozanide single point tests; 10 μM; 5 ml assays within 15 ml falcon tubes) in rumen fluke conditioned (RFC) media containing 0.1 % DMSO; RFC media consists of 1:1 Coleman-simplex buffer solution [8] and RPMI 1640 supplemented with 1 % v/v fetal calf serum (FCS) and 1 % v/v antibiotics/antimycotics. Two replicate (each containing five parasites) experiments were performed (total n = 10) for each compound (**IVL-11**, **IVL-12**, **IVL-13** and oxyclozanide). All assays were conducted for 24 hr at 37 °C in a humidified environment containing 5 % CO_2. Afterwards, the rumen flukes were observed under brightfield microscopy and their survival assessed as previously described [20]. Briefly, motility of parasites was scored as follows: 1 = undulating movement or sticking to bottom of flask, 2 = slow movement, 3 = slight movement of mouthparts and 4 = no movement at all or dead.

2.4. Fluorescence and brightfield imaging of F. hepatica NEJs

NEJs, co-cultured in DMSO, **IVL-11** or TCBZ (as described above) were removed from their media and were stained according to methodologies described by Wendt and Collins [31], with minor modifications. Briefly, to assess the integrity of gut and tegument surfaces, NEJs were stained via incubation with 5 mg/ml dextran-TAMRA and biotin (mini-Ruby, Thermofisher D3312) in RPMI for 15 min, followed by fixing in 4 % (v/v) formaldehyde in PBSTx (phosphate buffered saline containing 0.3 % v/v Triton X-100) for 4 hr. Fixed parasites were then counterstained overnight with phalloidin conjugated to Alexa Fluor 488 (diluted 1:40 in 1 % v/v BSA in PBSTx) to label actin-enriched muscle structures and 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/ml w/v in double distilled water) to stain nuclei. Triply stained parasites were mounted on slides in 80 % v/v glycerol (in 1 X PBS) and imaged at 40 x magnification using a Cytation 7 multi-modal imager with green (469/525; phallodin), red (531/593; dextran) and blue (377/447; DAPI) fluorescence filter cubes. Brightfield and fluorescent images were visualized using Biotek Gen5 (Agilent Technologies, version 13.2) software.

2.5. Scanning electron microscopy of adult F. hepatica and C. daubneyi

Immediately after 24 hr of *ex vivo* culture, adult *F. hepatica* and *C. daubneyi* worms were separated from the media and fixed with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) and 1 % v/v osmium tetroxide solution in 0.1 M sodium cacodylate (pH 7.2), respectively. All specimens were subsequently washed, dehydrated, dried at critical point, mounted on stubs, gold-coated and imaged on a Hitachi S-4700 FESEM microscope using the Ultra High-Resolution mode as previously described [5].

2.6. Mammalian cell cytotoxicity

Mammalian cell cytotoxicity of **IVL-11**, **IVL-12** and **IVL-13** was assessed at 24 hr via a viability assay using the MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Madin Darby Bovine Kidney (MDBK) epithelial cells as previously described [4, 32] (Supplementary Data 8). Briefly, to derive CC_{50} values (concentration in which the compound exhibited 50 % cytotoxicity), each viability assay was performed on three separate occasions (n = 3) with each saponin concentration (100 μM, 10 μM, 1 μM, 0.1 μM and 0.01 μM) tested in triplicate.

2.7. Formulation and stability of IVL-11 for oral delivery

Corn oil (830 g) was added to a 3 L glass beaker and warmed to 70–80 °C with mixing at 340–360 rpm; butylated hydroxyanisole (BHA, 0.1 g) was added slowly maintaining the temperature and mixing continued for 20 min give a clear solution. At this time, Aerosil 200 (10 g) was added slowly maintaining the temperature at 70–80 °C and mixing continued for 20 min to give a clear solution free from any particles. The mixture was transferred to Silverson mixer and mixing continued at 4000 rpm maintaining the temperature at 70–80°C for 30 min followed by cooling to less than 30 °C. Mixing was increased to 6000–7000 rpm and **IVL-11** (100 g) was added over 15 min followed by mixing for a further 60 min. The mixture was made up to 1000 ml final volume with corn oil and the stirring continued at 6000–7000 rpm for 60 min. The particle size distribution was assessed (target < 80 μM) and the final mixture was transferred into High Density Polyethylene (HDPE) containers for storage.

Stability of the **IVL-11** (100 mg/ml) oral suspension was demonstrated through a series of accelerated and long-term studies conducted under controlled conditions. Samples stored at 40 °C/75 % relative humidity (RH) for up to 6 months and at 25 °C/60 % RH for up to 10 months were evaluated for critical quality attributes including appearance (slightly yellow, smooth oily appearance), **IVL-11** integrity (by high performance liquid chromatography, HPLC) and **IVL-11** related degradant (by HPLC). HPLC analyses were performed on a Waters HPLC system equipped with a photodiode array detector and a UV-Vis detector. A Zorbax Eclipse XDB C-18 (250 mm×4.6 mm) column (5 μm) was used. A volume of 20 μL was injected with a flow rate of 1.0 ml/min applied; the mobile phase consisted of (50:50) 0.1 % phosphoric acid 85 %: acetonitrile, the run lasted 15 min and **IVL-11** was detected using a wavelength of 207 nm. Throughout the study period, **IVL-11** content remained consistent (90–110 mg/ml), with no meaningful decline in the HPLC retention time (**IVL-11** standard = 5.961 min; formulated **IVL-11** = 5.954 min) observed. Total degradants remaining within established limits and without any significant **IVL-11** degradation. Collectively, these results confirm that the formulation maintains its chemical and physical integrity under both accelerated and ambient storage conditions, supporting the conclusion that the product can be stored for extended periods.

2.8. Dose escalation tolerability studies of orally-delivered IVL-11 in O. aries

Five 4–7 month-old, mixed-sex *O. aries* (Ile de France X Aberfield breed) were orally-dosed with the corn-oil based formulation of **IVL-11** (50 mg/kg bodyweight group, n = 2; 75 mg/kg bodyweight group, n = 1; 250 mg/kg bodyweight group, n = 2). Sheep were indoor housed, fed *ad libitum* using hay (or haylage) and concentrates twice daily, with unrestricted access to water throughout the study. Peripheral blood was collected from the jugular vein at 24 hr prior to dosing (day −1) and 48 hr after dosing (day +2). Sera (1.0 ml) collected from each individual at both timepoints were used to detect a variety of biochemical parameters (e.g. total protein, albumin, urea, creatine, glutamate dehydrogenase, gamma-glutamyl transferase, beta-hydroxybutyrate and non-esterified fatty acids). Whole blood (0.5 ml) was used to quantify haematological parameters including total red blood cell numbers, haemoglobin, packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, platelets and white blood cell numbers. All **IVL-11** associated values were compared to normal ranges reported for these parameters (Axiom Veterinary Laboratories Ltd, https://axiomvetlab.com/).

2.9. Pharmacokinetics sampling of orally-delivered IVL-11 in O. aries

In a primary experiment, corn-oil formulated **IVL-11** was orally-delivered to mixed-sex groups of 4–7 month-old *O. aries* (n = 15; Ile

de France X Aberfield breed) at 75 mg/kg bodyweight. Sheep were housed, fed and provided unrestricted access to water as described above. Peripheral blood was collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hr post-dosing. At 4, 12, 24, 48 and 96 hr post-dosing, postmortem collection of liver tissue (200 g per animal), bile (20 ml per animal) and hepatoportal blood from randomly selected individuals (n = 3) was also performed. Euthanasia was performed using captive bolt followed by pithing and exsanguination, except for when hepatoportal blood samples were required, in which case animals were not exsanguinated.

In a secondary experiment, **IVL-11** was orally-delivered to a mixed-sex group of 4–7 month-old *O. aries* (n = 2; Ile de France X Aberfield breed) at 250 mg/kg bodyweight. Peripheral blood was collected at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hr post-dosing.

2.10. Test sample preparation/extraction

Calibration samples (100 μl) with concentrations ranging from 0.1 to 1000 ng/ml were prepared by "spiking" a volume (5 μL) of solutions of the test compound prepared in 50:50 acetonitrile:water (v/v) into aliquots (95 μL) of control diluted sheep blood (1:2 v/v with water). Quality control samples were prepared in the same way as the calibration samples but from a separately weighed and diluted sample of the test compound. Blank samples were prepared by "spiking" a volume (95 μL) of diluted control blood with a volume (5 μL) of 50:50 acetonitrile:water (v/v).

Diluted study samples in Micronics tubes were received and stored frozen until analysis. To extract the test compound, acetonitrile (300 μL) was added to all calibration samples, QC samples and blanks. The samples were then vortexed (5–10 s) to mix and precipitate proteins. For study samples, an aliquot (50 μL) from each thawed sample was removed and acetonitrile (150 μL) added. Samples were then vortexed (5–10 s).

All samples were then centrifuged to pellet any precipitated proteins. Study samples in Micronics tubes were centrifuged using a Beckman Coutler Allegra C-12R for 10 min, 3750 rpm. All other samples were centrifuged in Eppendorf tubes using a SIGMA SciQuip 1–14 for 10 min, 14800 rpm/16163 g. Supernatants from study samples (100 μL) and calibration samples, QCs, blanks, and double blanks (200 μL) were then transferred to wells of a 96-well block containing Milli-Q water (50 μL or 100 μL respectively). Samples (ng/ml for liquid samples or ng/g for tissue samples) were then analysed using LC-MS/MS

The Drug Discovery Unit (DDU) at the University of Dundee performed the LC-MS/MS analyses of both peripheral and hepatoportal samples, while Sygnature Discovery performed the LC-MS/MS analyses of bile and liver samples. LC-MS/MS analysis was conducted on a Waters Acquity LC system equipped with a Waters Xevo TQ-XS triple quadrupole MS (Dundee) or on a HPH-C18 column equipped with a Poroshell Sciex 5500 Qtrap MS (Sygnature Discovery). Chromatography of the test compound was achieved using a Waters Acquity BEH C18 UPLC column, (2.1 ×50 mm, 1.7 μm particle size) and an acetonitrile/water gradient elution method (with 0.1 % ammonia in both phases as the modifier) at a flow rate of 0.6 ml/min. The gradient elution used was as follows: initial conditions, 95 % water/5 % acetonitrile, held for 0.5 min; then linear increase to 95 % acetonitrile over 1.5 min, hold at 95 % acetonitrile for 0.5 min. Column re-equilibration time was 0.5 min (note the first 0.5 min of the gradient were run to waste and not into the mass spectrometer).

Mass spectrometric analysis was conducted in negative MRM mode. The following mass spectrometer conditions were used: Capillary voltage 0.8 kV; desolvation temperature 600°C; Source temperature 150°C, desolvation gas flow 1000 L/h; Cone gas flow 150 L/h. The analyte was optimised using the Waters QuanOptimize software within MassLynx (v4.2) and the following conditions were selected: cone voltage 12 V; collision energy 40 V; precursor ion *m/z* 749.5 Da; fragment ion *m/z* 471.3 Da. The lower limit of quantification (BLQ) was 2 ng/ml.

2.11. Pharmacokinetics analyses of LC-MS/MS data

Pharmacokinetics were subsequently calculated from the LC-MS/MS-derived **IVL-11** concentrations in each of the four matrices (bile, liver, peripheral blood and hepatoportal blood). Data analysis was conducted using Waters TargetLynx Software (v4.2). Peak concentration (C_{max}) and corresponding time (t_{max}), compound half-life ($t_{1/2}$) and area under plasma concentration–time curve ($AUC_{0\text{-}Inf}$) values were derived by the non-compartmental method using PKsolver [33].

2.12. Efficacy of orally-delivered IVL-11 in F. hepatica infected O. aries

In two successive efficacy studies, mixed-sex groups of 4–7-month-old *O. aries* (Ile de France X Aberfield breed) were given 200 metacercaria/individual derived from a *F. hepatica* TCBZ-resistant Kilmarnock strain by oral gavage. In the first efficacy study, sheep were orally-dosed with either the excipient control (corn-oil based formulation; n = 3), closantel (10 mg/kg bodyweight; n = 5) or **IVL-11** (250 mg/kg bodyweight; n = 6) at day 85 post-infection. After an additional 17 days, all animals were euthanised and adult flukes were recovered from the livers. In the second efficacy experiment, infected sheep were orally-dosed with the excipient control (n = 9) or **IVL-11** (250 mg/kg bodyweight; n = 10) at day 73 post-infection. To facilitate the collection of egg per gram faces (EPG) data, rectal grabs were performed at days 70 (pre-treatment), 73 (day of treatment), 76, 80 and 83 post-treatment. Flukes were recovered from the liver at post-mortem as in the previous study. After counting flukes at the end of the studies, parasites were washed three times in 1 X PBS and fixed with 2.5 % v/v glutaraldehyde in 0.1 M sodium cacodylate buffer. Fixed flukes were stored at 4°C until they could be weighed. In a fume cabinet, wet weights of individual flukes were collected using an Ohaus PR224 analytical balance.

2.13. Faecal sampling and faecal egg counts

For rectal sampling of restrained sheep, one or two fingers were gently inserted into the rectum and directed dorsally toward the tail head to stimulate defecation by gentle movement along the rectal roof. Faecal material was retrieved manually, yielding a minimum of 3 g, which was sufficient for determining *F. hepatica* faecal egg counts (FECs). Where animals defecated naturally, freshly voided faeces were collected.

A maximum 10 g of faeces/sample was weighed and then transferred to a 300 ml beaker containing three glass marbles and approximately 100–200 ml of tap water. The sample was mixed using a plastic spatula for approximately 5 min until all pelleted material was broken up and in suspension. The mixed suspension was poured onto a stack of three stainless steel sieves (top – 300 μm; middle – 150 μm; bottom – 38 μm) and washed through them using a shower head on high pressure to force the eggs/filtered liquid to collect on the bottom sieve. Washing was complete when the flow of water from the 38 μm sieve ran clear.

The sediment concentrated in the 38 μm sieve was then transferred to a 500 ml glass and topped up to 500 ml with tap water. The contents were then left to settle for 4 min. Using an aspirator, the suspension was siphoned down to 200 ml, refilled to 500 ml with tap water and left to settle for an additional 4 min. This was repeated until the supernatant was clear, which took approximately 100 cycles. Once clear, the suspension was then siphoned down to 100 ml and decanted into a 150 ml beaker. After leaving to settle for another 4 min, the sample was siphoned down to 50 ml and transferred into a 50 ml tube. After leaving to settle for another 4 min, the sample was carefully drawn down until the bottom of the meniscus was in line with the 10 ml mark. Prior to examining the sample, it was agitated to ensure the sediment was randomly mixed and evenly suspended in the liquid. A 1 ml sample was pipetted into a petri dish and eggs counted using a dissecting microscope

at x12 magnification. These counts were used to calculate eggs per gram (EPG) faeces values.

2.14. Statistical analyses

Given that all datasets were not normally distributed (or represented ranked, categorical data in the case of *ex vivo* fluke experiments), non-parametric comparisons were performed between groups using either Kruskal-Wallis with Bonferroni correction for multiple comparisons (*in vivo* experiments with ≥ 3 groups and all *ex vivo* assays including *F. hepatica* NEJs, adult fluke and rumen fluke phenotype and motility scores) or Mann-Whitney U (*in vivo* experiments with 2 groups) distributional analysis. For the final **IVL-11** efficacy study, average daily egg production was correlated with fluke sizes using a Spearman's rank order correlation. All statistical analyses were performed in SPSS version 29.

3. Results

To assess the flukicidal properties of **IVL-11** (α-hederin), as well as two related molecules (**IVL-12**; hederoside B and **IVL-13**; δ-hederin), the purified *H. helix*-derived saponins (Supplementary Data 1) were separately co-cultured with two *F. hepatica* intra-mammalian lifecycle stages (Fig. 1). Under *ex vivo* conditions, all three structurally related saponins (Fig. 1**A**) induced motility defects in Italian strain NEJs (Fig. 1**B**; 10 μM assays) and immature flukes (Fig. 1**C**; 40 μM assays) similar to TCBZ after 24 hr of co-culture (NEJ assays were repeated and led to identical results; Supplementary Figure 1). Since the saponin-treated NEJs and immature flukes showed no response to mechanical stimulation and appeared granular (e.g. inset images of NEJs, Fig. 1**B**), they were considered dead at 24 hr. These saponins also killed adult flukes (both Italian and Wild strains) more quickly than TCBZ (40 μM assays; compare 24 hr vs 72 hr timepoints; Fig. 2**A vs 2B**). Upon scanning electron microscopy (SEM) of saponin-treated adults (40 μM for 24 hr), all three compounds induced similar phenotypes including damage to the surface (i.e. spine erosion, sloughing) and protrusion of the cirrus

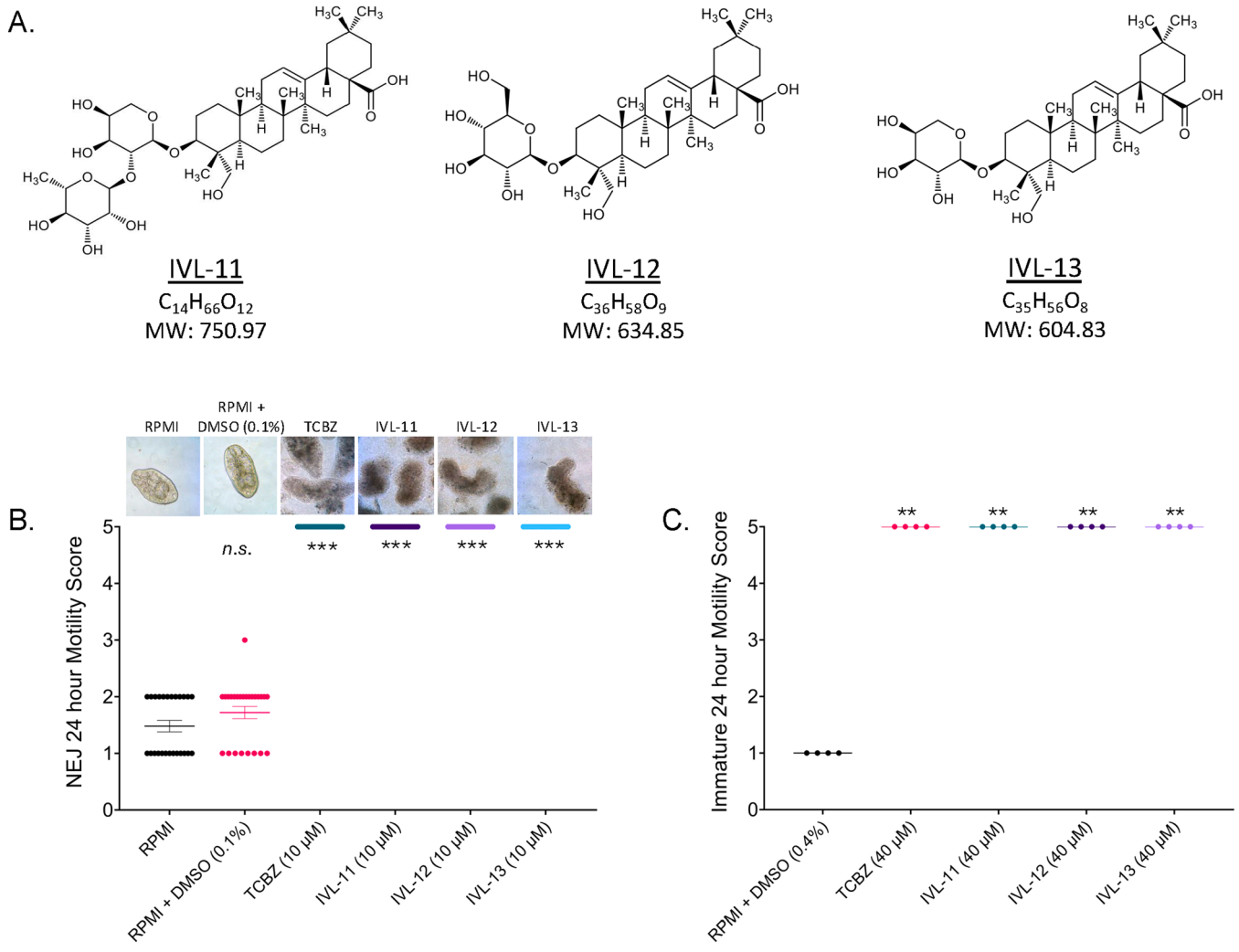

Fig. 1. *Hedera helix* derived saponins (IVL-11, IVL-12 and IVL-13) are lethal to *ex vivo* cultured *Fasciola hepatica* (Italian strain) newly excysted juveniles and immature flukes. A) Chemical structures, molecular formula and molecular weights of **IVL-11** (α-hederin), **IVL-12** (hederoside B) and **IVL-13** (δ-hederin). **B)** Motility scores for newly excysted juveniles (NEJs) cultured for 24 hr in RPMI media only or RPMI containing 0.1 % v/v DMSO (negative controls, n = 25) and 10 μM triclabendazole (TCBZ, n = 25), **IVL-11**, **IVL-12** or **IVL-13** (all in RPMI containing 0.1 % v/v DMSO, n = 25 for each treatment). All three IVLs were able to induce a significant reduction in motility (***; $p < 0.001$) in comparison to negative control parasites, equivalent to TCBZ. Examination of the phenotypes of parasites treated with TCBZ, IVL-11, IVL-12 and IVL-13 indicated that 10 μM treatments were fatal given their dissolved appearance (example images provided). C) Motility scores for immature flukes (n = 4 parasites/assay) cultured for 24 hr in RPMI + 0.4 % v/v DMSO or 40 μM triclabendazole (TCBZ), IVL-11, IVL-12 or IVL-13 (in RPMI containing 0.4 % v/v DMSO). All treatments induced a significant (**; $p < 0.01$) reduction in parasite motility.

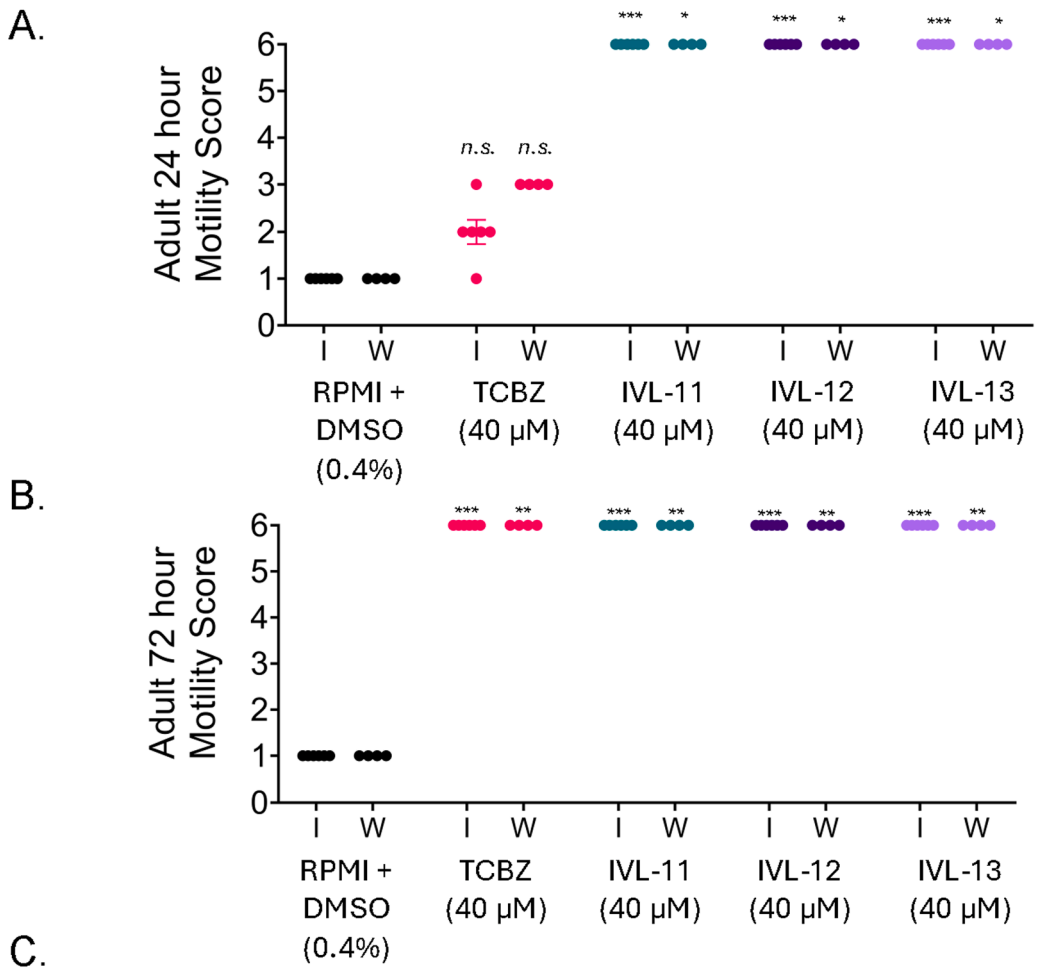

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Fig. 2. The flukicidal properties of IVL-11, IVL-12 and IVL-13 extend to *ex vivo* cultivated adult *F. hepatica* (wild type and Italian strains). A) Motility scores for wild-type (W; n = 4 parasites/assay) or Italian (I; n = 6 parasites/assay) strain adults cultured for 24 hr in RPMI containing 0.4 % v/v DMSO (negative control) or 40 μM triclabendazole (TCBZ), **IVL-11**, **IVL-12** or **IVL-13** (all in RPMI containing 0.4 % v/v DMSO). In both strains, TCBZ failed to significantly impact motility, whereas all IVL series compounds each induced significant reductions in motility (***; $p < 0.001$ for Italian strain and *; $p < 0.05$ for wild-type). B) After 72 hr of culture, all treatments induced significant reductions in parasite motility (***; $p < 0.001$ for Italian strain and **; $p < 0.01$ for wild-type). C) The IVL-mediated reductions in adult fluke (Italian strain; n = 3 parasites/replicate; 2 replicates per assay) motility at 24 hr were correlated with surface damage (spine erosion and sloughing) and protrusion of the cirrus as observed by scanning electron microscopy. As these phenotypes were not observed in the RPMI (containing 0.4 % v/v DMSO) or TCBZ controls, the IVL-mediated paralysis of adult flukes led to death (represented by bright field micrographs) in these individuals during the 24 hr culture.

(Fig. 2**C**). These abnormalities were not found in controls (adults cultured in either 40 μM TCBZ or RPMI + 0.4 % DMSO).

To determine if these saponins were also active on a related fluke species commonly found to parasitise UK cattle and sheep [16], *ex vivo* assays were conducted on the rumen fluke *Calicophoron daubneyi* (Supplementary Figure 2). Here, **IVL-11**, but not **IVL-12** or **IVL-13**, induced paralysis in adults (after 24 hr co-culture) equivalent to the effect seen in oxyclozanide-treated parasites (Supplementary Figure 2A). As these **IVL-11** treated rumen flukes were unresponsive to external stimulation and displayed ultra-structural damage (e.g distortions to tegumental surface, oral sucker, posterior acetabulum and genital pore) similar to oxyclozanide-killed rumen flukes (Supplementary Figure 2B), they were considered dead. When taken together, **IVL-11** (α-hederin), exhibited the best flukicidal activities amongst the tested saponins (comparable to TCBZ against liver flukes and oxyclozanide against rumen flukes).

To assess the generic cytotoxicity of these saponins, preliminary viability assays on MDBK epithelial cells were subsequently performed (Supplementary Data 8). Here, all three saponins displayed moderate MDBK cytotoxicity with **IVL-11** (CC_{50} ~ 0.75 μM) exhibiting greater activity when compared to either **IVL-12** (CC_{50} ~ 3.5 μM) or **IVL-13** (CC_{50} ~ 2.9 μM). General mammalian cell cytotoxicity of saponins, including α-hederin/**IVL-11**, has been previously described [11,29,30]. However, as earlier investigations clearly demonstrated that high dose administration (500 mg/kg, 800 mg/kg and 800 mg/kg: 10 day interval between treatments) of α-hederin/**IVL-11** to *Dicrocoelium*-infected ewes had no long-term adverse effects even after 50 days post-administration [21], further investigations of this saponin were performed.

IVL-11 dose response titrations were firstly employed to quantify the potency of this saponin on *ex vivo* cultured NEJs derived from both TCBZ sensitive (Aberystwyth) and insensitive (Penrith) liver fluke strains (Fig. 3). Here, **IVL-11** was found to be similarly active on both Aberystwyth and Penrith strain NEJs (e.g. EC_{50} for Aberystwyth strain at 72 hr post-treatment = 4.34 μM; EC_{50} for Penrith strain at 72 hr post-treatment = 4.31 μM). Furthermore, this saponin's potency approximated the level measured for TCBZ against Aberystwyth NEJs (e.g. EC_{50} at 72 hr post-treatment = 3.97 μM; Fig. 3**A**). Tri-colour fluorescence staining of parasites (Aberystwyth strain) to visualize actin (FITC-phalloidin), nuclei (DAPI) and intact tegumental membranes (Dextran-TAMRA) revealed phenotypic abnormalities in **IVL-11** treated NEJs when compared to RPMI/0.5 % v/v DMSO controls (Fig. 3**B** and Supplementary Figure 3). At 10 μM, **IVL-11** (as well as TCBZ) led to a complete dissolution of actin structures (including dissociation of the actin rich ventral and oral suckers), breakdown of heptalaminate membrane/tegumental integrity (at surface and the gut) and disruption of cell membranes (coalescence of DAPI signal). While cellular/organ/tissue architecture was unaffected in parasites treated with 0.625 μM **IVL-11** (similar to RPMI/0.5 % v/v DMSO controls), many defects (e.g. disruption of heptalaminate membrane/tegumental integrity, breakdown of longitudinal and transverse actin-containing muscle structure) remained in some NEJs (13 % of individuals) treated with concentrations of **IVL-11** (3.75 μM) approaching the EC_{50}.

Having established that **IVL-11** was: 1) active against key intra-mammalian *F. hepatica* lifecycle stages (NEJs, immature flukes and mature flukes), 2) equally capable of killing *F. hepatica* strains differing in TCBZ sensitivities (Italian, Aberystwyth and Penrith strains), 3) lethal to both liver and rumen fluke species (*F. hepatica* and *C. daubneyi*) and 4) similarly potent to TCBZ during NEJ dose response titrations, this single saponin was next subjected to *in vivo* tolerability/safety studies in *O. aries*. These studies required significant quantities of **IVL-11**; this was achieved by isolating the active saponin from ivy leaf in a process that incorporated a treatment with water at 40 °C, which converted hederacoside C in the leaf into **IVL-11** (Supplementary Data 5). Corn-oil formulated **IVL-11** was orally administered (once) to individuals as part of a dose escalation investigation (50 mg/kg, 75 mg/kg and 250 mg/kg bodyweights). In most haematological and biochemical parameters assessed (pre and 48-hr post **IVL-11** treatment at all dosages; Supplementary Table 1), the values recorded were within the normal ranges for healthy adult sheep based on laboratory standards (Axiom Veterinary Laboratories, https://axiomvetlab.com/). Where some values were elevated above normal ranges, they were either already high in pre-treated individuals and remained high after **IVL-11** administration (comparing all GGT pre-treatment to 48 hr post-treatment values) or were lowered to normal ranges upon **IVL-11** gavage (e.g. haemoglobin in one out of two sheep in the 250 mg/kg treatment group). In only three examples did **IVL-11** administration impact upon normal haematological and biochemical parameters; these examples included lowering platelet numbers in one of two individuals in the 50 mg/kg group, elevating mean corpuscular haemoglobin concentration (MCHC) in one out of two sheep in the 250 mg/kg group and elevating urea in one out of two sheep in the 50 mg/kg group (Supplementary Table 1). As these abnormal readings were not reproducibly observed in every animal under study, it is unlikely that the effects recorded are due to **IVL-11**.

Based on the observations that **IVL-11** was well-tolerated in sheep up to 250 mg/kg bodyweight, pharmacokinetic (PK) investigations were next pursued to determine tissue associated C_{max}, t_{max}, $t_{1/2}$ and AUC values (Fig. 4). Firstly, a single oral administration of corn-oil formulated **IVL-11** (75 mg/kg bodyweight) was given to a group (n = 15) of healthy sheep. At different time points (see Materials and Methods) post-dosing, liver tissue, bile fluid, hepatoportal- and peripheral- blood samples were obtained from individuals and subjected to LC/MS-MS analysis to quantify **IVL-11** concentrations (Fig. 4**A**). At this dosing level, **IVL-11** reached highest concentrations in these tissues between 4 and 12 hr, with C_{max} values ranging between 38.2 ng/ml (bile) to 261.80 ng/ml (peripheral blood). Clearly, **IVL-11** can reach compartments in which liver flukes transiently migrate through (i.e. liver) or permanently reside (bile ducts). A further experiment was conducted to quantify how peripheral blood PK values shifted when sheep (n = 2) were given a single oral dose of **IVL-11** at the highest tolerable dose tested in this investigation (250 mg/kg bodyweight; Fig. 4**B**). Here, at several timepoints (see Materials and Methods) post administration, peripheral blood C_{max}, T_{max}, $T_{1/2}$ and AUC values were calculated for **IVL-11** in these two animals. A 3-fold increase in administered **IVL-11** (from 75 mg/kg bodyweight to 250 mg/kg bodyweight) led to an approximate 5-fold increase in peripheral blood exposure to **IVL-11** ($AUC_{0\text{-}inf}$ 75 mg/kg bodyweight = 4, 088 ng/hr*ml; $AUC_{0\text{-}inf}$ 250 mg/kg bodyweight = 20, 312 ng/hr*ml; Fig. 4**B**).

The ability to safely administer **IVL-11** to *O. aries* and to detect it in various tissues upon PK investigations led to the initiation of *in vivo* efficacy studies in *F. hepatica* infected sheep (Fig. 5). Initially, a small pilot study compared the efficacy of a single oral dose of **IVL-11**

A.

Time after dosing (hours)	TCBZ (Aberystwyth strain)			IVL-11 (Aberystwyth strain)			IVL-11 (Penrith strain)		
	EC_{50} (μM)	95% C.I. (μM)	EC_{50} (ng/ml)	EC_{50} (μM)	95% C.I. (μM)	EC_{50} (ng/ml)	EC_{50} (μM)	95% C.I. (μM)	EC_{50} (ng/ml)
24	4.59	4.41 - 4.77	3446.95	5.36	5.08 - 5.66	4025.20	5.05	4.38 - 5.86	3792.40
48	4.11	3.86 - 4.37	3086.49	4.68	4.36 - 5.01	3514.54	4.36	3.83 - 4.98	3274.23
72	3.97	3.70 - 4.24	2981.35	4.34	4.03 - 4.69	3259.21	4.31	3.80 - 4.93	3236.68

B.

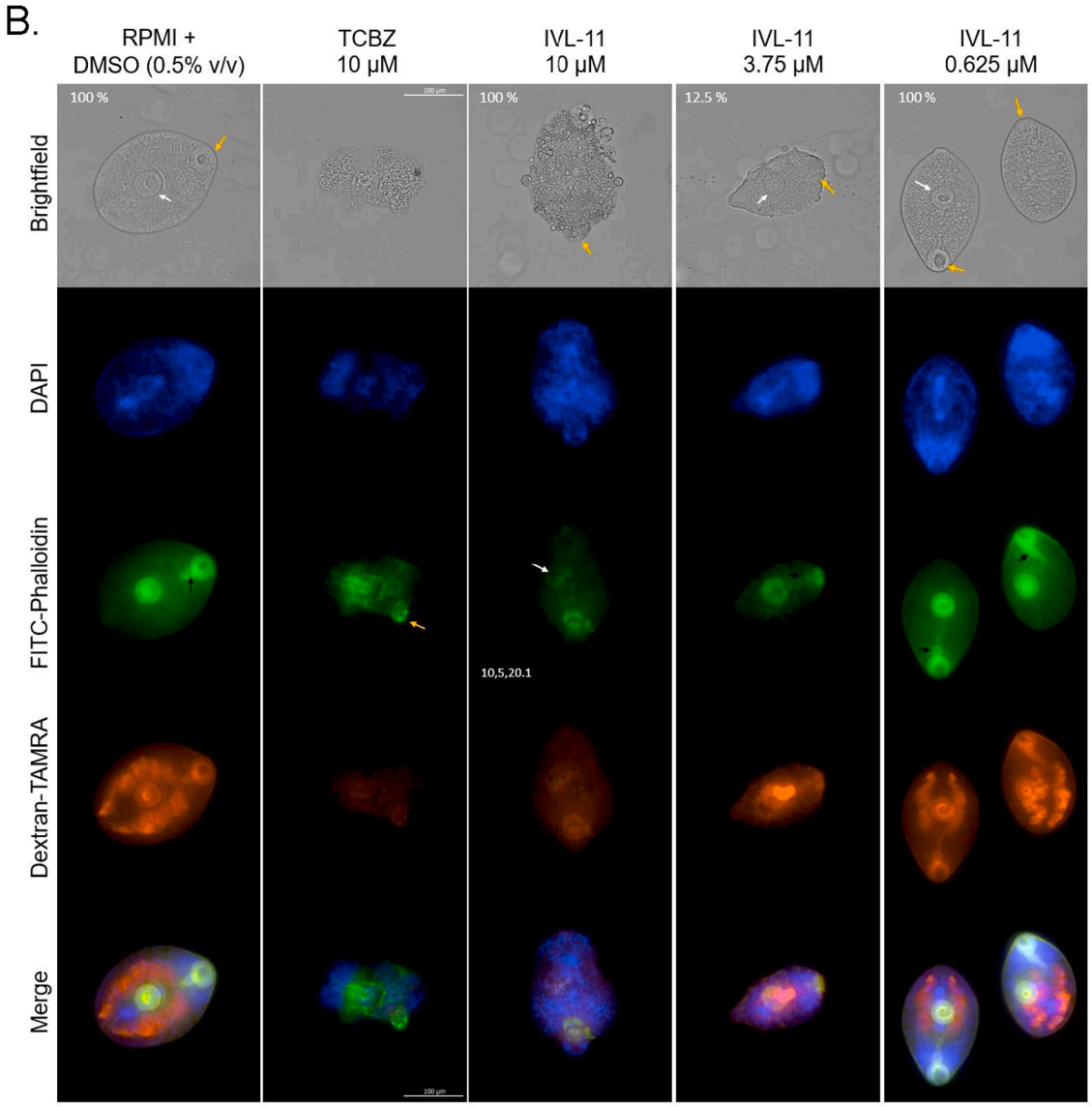

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Fig. 3. IVL-11 has similar flukicidal potencies as TCBZ against *ex vivo* cultured NEJs derived from both TCBZ - sensitive (Aberystwyth) and insensitive (Penrith) strains. A) EC_{50} values calculated from *ex vivo* exposure of sensitive (Aberystwyth) and insensitive (Penrith) NEJs to **IVL-11** and triclabendazole (TCBZ). Data was produced from six replicates (5–12 NEJs/replicate) and two independent operators. Phenotype scores from a categorical ranking system (from score 1 = normal phenotype to 6 = fully dissolved) were converted into percentage viability (score 6 = 0 % viability, score 1 = 100 % viability) for the purposes of calculation using a continuous scale. **B)** Microscopic visualisation of NEJs at 72-hours post-culture with RPMI + 0.5 % v/v/ DMSO, IVL-11 (10 μM, 3.75 μM and 0.625 μM in 0.5 % v/v DMSO) or 10 μM TCBZ (in 0.5 % v/v/ DMSO). Parasites were stained to highlight structures across three wavelengths: blue (ex. 377, em. 447) = DAPI, cell nuclei; green (ex. 469, em. 525) = phallodin, muscle fibres and red (ex. 531, em. 593) = dextran-TAMRA, surface integrity, including gut. Images were captured at 40 x magnification on a Cytation 7 using consistent exposure settings for each wavelength, except for the green channel for 10 μM **IVL-11** and 10 μM TCBZ, on which the gain was adjusted to account for low signal (altered settings marked on figure). Arrows marking the images represent structures of interest, including oral and ventral suckers (orange and white arrows, respectively) and pharynx musculature (black arrows). Images were captured at a consistent scale for all wavelengths and scale bars (top and bottom right) apply to all images. Percentages represent the total number of sampled individuals displaying the phenotype described (n ≥ 10 per replicate; all images available in supplementary data (Supplementary Figure 2).

(250 mg/kg bodyweight; n = 6) to a single oral dose of closantel (10 mg/kg bodyweight; n = 5) in reducing liver fluke (TCBZ-insensitive Kilmarnock strain) burdens (Fig. 5**A**). At the termination of the experiment (Day 17 post **IVL-11** or closantel administration), a significant reduction in worm burdens was only observed in the closantel treated group when compared to the excipient controls (n = 3). However, both closantel and **IVL-11** treatment groups led to significant reductions in liver fluke body sizes as quantified by wet weight of individuals (Fig. 5**B**). When the experimental group sizes were increased in a second experiment (excipient control, n = 9; 250 mg/kg bodyweight **IVL-11**, n = 10), **IVL-11** again failed to reduce fluke numbers (Fig. 5**C**), but significantly suppressed liver fluke sizes (Fig. 5**D**).

As adult fluke body sizes were impacted by **IVL-11** treatment in both efficacy experiments, we next assessed if egg production was also negatively affected (Fig. 6). While egg counts were reduced in **IVL-11** treated animals compared to excipient controls (i.e. post-treatment data; days 76, 80 and 83 post-infection, Fig. 6**A**), these reductions did not reach significance according to a Mann-Whitney *U* test. At day 80 post-infection (7 days post-treatment), this reduced egg production difference neared significance (23.50 ± 21.35 EPG in excipient control and 6.45 ± 17.23 EPG in **IVL-11** treated animals, p = 0.065). In addition to these reductions, the peak in egg production was delayed by **IVL-11** treatment (i.e. day 7 post-dosing in excipient control and day 10 post-dosing in **IVL-11** treatment group). Finally, when average fluke weights were compared to average daily EPG faeces (in both excipient and **IVL-11** treatment groups), a monotonic relationship between measurements was found demonstrating that smaller flukes (in both groups) produced fewer eggs (Fig. 6**B**).

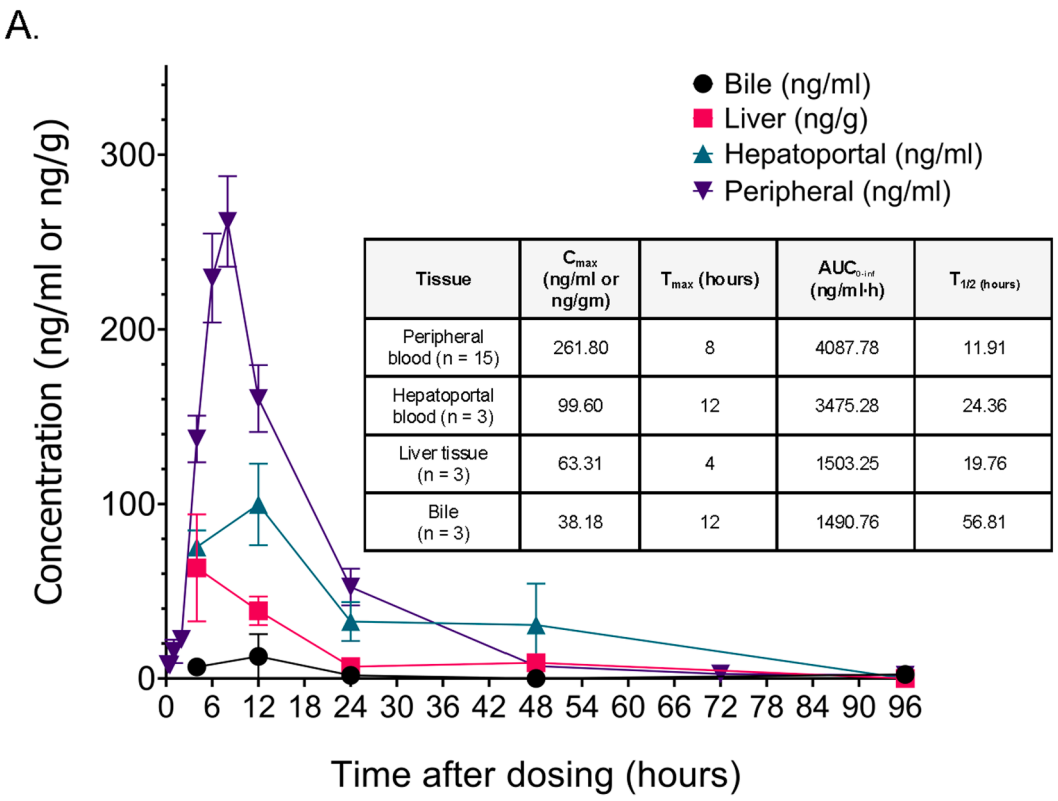
4. Discussion

Until a liver fluke vaccine is developed and deployed, the cornerstone intervention for controlling fasciolosis/fascioliasis is chemotherapy with TCBZ. Because of its optimal properties (i.e. effective against NEJ-, immature fluke- and mature fluke- lifecycle stages), TCBZ usage across the globe has expanded and led to a rise in drug-resistant *F. hepatica* populations (e.g. Kelley et al., [2,22]). In the absence of a TCBZ-replacement drug with multi- lifecycle stage efficacy, the spread of liver fluke infection is a real threat to livestock and human health. Here, we provide both *ex vivo* and *in vivo* evidence that *H. helix* derived α-hederin (**IVL-11**) contains promising properties suitable for further development as an urgently-needed, next-generation anthelmintic for fasciolosis/fascioliasis.

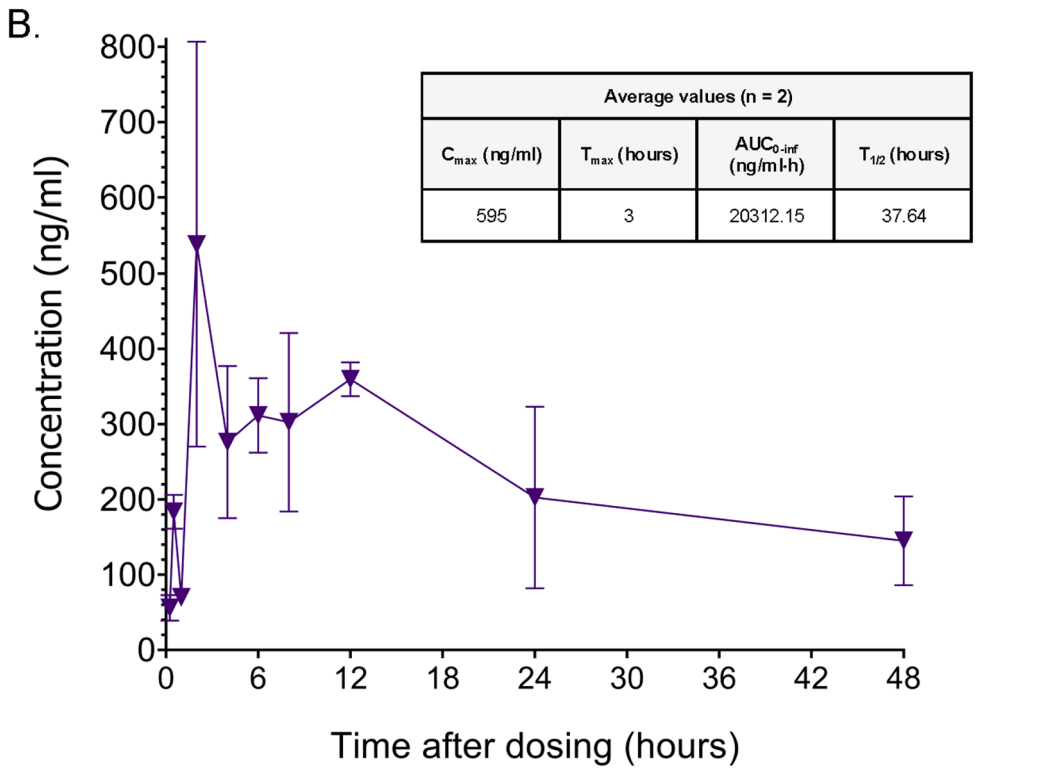
During *ex vivo* testing, we found that **IVL-11** performed as well as TCBZ against *F. hepatica* NEJs, immature flukes and mature flukes (Figs. 1–3**)**. In fact, both compounds were similarly potent against NEJs when tested side by side in dose response assays (Fig. 3**A**). However, **IVL-11** acted more quickly than TCBZ in exerting its immobilizing/flukicidal activity against adult flukes (Fig. 2**A**). Furthermore, all *F. hepatica* strains (Italian, Aberystwyth, Penrith) tested, regardless of their TCBZ-sensitivities, were similarly impacted by **IVL-11**. This flukicidal activity was also observed for an exemplar wild fluke population obtained from a local abattoir and against *C. daubneyi* rumen flukes (Supplementary Figure 2). Collectively, our results broadly support a previous study in which the *ex vivo* anthelmintic activities of α-hederin/**IVL-11** were clearly demonstrated against abattoir-derived adult *F. hepatica* and *Dicrocoelium dentriticum* (lancet liver fluke) derived from France [21]. Specifically, the flukicidal concentration of **IVL-11** used against immature and adult *F. hepatica* in our study (40 μM; 30 μg/ml) was well within the range of lethal concentrations (5 μg/ml – 5000 μg/ml) tested by Julien *et al.* [21].

While hederoside B (**IVL-12**) and δ-hederin (**IVL-13**) demonstrated similar *ex vivo* activities to **IVL-11** against liver flukes, these two related saponins were inactive against rumen flukes. It is currently unclear why this structural activity relationship exists for related compounds (Fig. 1**A**), especially since these two fluke species are also closely related [7]. If a *bona fide* liver or rumen fluke target does not exist for **IVL-11** and other saponins (including **IVL-12**, **IVL-13**), then perhaps activity against rumen flukes is linked to greater hydrophilicity due to the presence of more than one glycosidic moiety (**IVL-11** has two; both **IVL-12** and **IVL-13** only have one) or the type of glycosidic moiety (**IVL-11** contains an α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl moiety/side chain, **IVL-12** contains a β-D-glucopyranosyl moiety/side chain and **IVL-13** contains an α-L-arabinopyranosyl moiety/side chain). Alternatively, the expansion of saposin-like proteins (SAPLIPs) in the *C. daubneyi* genome may also contribute to the more efficient degradation of lipophilic molecules and underpin an intrinsic insensitivity to some saponins (e.g. **IVL-12** and **IVL-13**) [7]. Until these outstanding queries can be resolved, microscopic (brightfield, tri-colour fluorescence- and SEM imaging) observations of **IVL-11** treated liver and rumen flukes have provided some clues as to the mechanism(s) of this saponin's differential activities. While all three saponins damaged liver fluke surface membranes (NEJs and adults) similar to *F. hepatica* NEJs treated with chemically-modified hederagenin derivatives derived from hydrolysed ivy saponins [6], this effect was only observed for **IVL-11,** and not **IVL-12** nor **IVL-13,** on rumen fluke adults (compare Figs. 1–3 with Supplementary Figure 2). In addition to surface damage, **IVL-11** also affected gut integrity, actin fiber organisation (most noticeable in musculature tissues) and cell membrane permeability in *F. hepatica* NEJs (Fig. 3**B** and Supplementary Figure 3). These data collectively suggest that liver flukes (to a greater extent than rumen flukes) are susceptible to the membrane-damaging/permeabilising effects of ivy saponins (here **IVL-11**, **IVL-12** and **IVL-13**), which, in turn, contributes to downstream disruption of deeper tissue (actin disorganization) and cellular (membrane instability) structures within individuals.

Despite moderate *in vitro* MBDK cytotoxicity observed in our studies (Supplementary Data 8), we have confirmed previous observations [21] demonstrating that oral administration of **IVL-11** (here, formulated in corn oil) in sheep was well-tolerated and safe, even at concentrations as high as 250 mg/kg bodyweight (Supplementary Table 1). Our PK investigations further revealed that **IVL-11** was subjected to absorption, distribution, metabolism and elimination (Fig. 4). When compared to PK studies of TCBZ (10 mg/kg bodyweight) where very little parent compound was found in plasma after post-TCBZ oral or intraruminal dosing [19,28], metabolism/elimination of orally-administered **IVL-11** was similarly affected by first-pass hepatic metabolism ($t_{1/2}$ = 12 hr at



Tissue	C_{max} (ng/ml or ng/gm)	T_{max} (hours)	$AUC_{0\text{-}inf}$ (ng/ml·h)	$T_{1/2}$ (hours)
Peripheral blood (n = 15)	261.80	8	4087.78	11.91
Hepatoportal blood (n = 3)	99.60	12	3475.28	24.36
Liver tissue (n = 3)	63.31	4	1503.25	19.76
Bile (n = 3)	38.18	12	1490.76	56.81



Average values (n = 2)			
C_{max} (ng/ml)	T_{max} (hours)	$AUC_{0\text{-}inf}$ (ng/ml·h)	$T_{1/2}$ (hours)
595	3	20312.15	37.64

Fig. 4. IVL-11 is detectable in bile, liver, peripheral blood and hepatoportal tissues upon oral dosing of *O. aries*. A) Fifteen, 4–7 month old, mixed-sex sheep (Ile de France X Aberfield breed) were given a single oral dose (75 mg/kg bodyweight) of corn-oil formulated **IVL-11**. LC-MS/MS was used to quantify **IVL-11** in peripheral blood at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 hr post-dosing. **IVL-11** was also quantified in liver tissue (200 g per animal), bile (20 ml per animal) and hepatoportal blood from randomly selected individuals (n = 3) at 4, 12, 24, 48 and 96 hr post-dosing. Fluid measures are given in ng/ml (bloods, bile) and tissue measures (liver) are given in ng/g. **B)** Two sheep (Ile de France X Aberfield breed) were given a single oral dose (250 mg/kg bodyweight) of corn-oil formulated **IVL-11**. LC-MS/MS was used to quantify **IVL-11** in peripheral blood at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 hr post-dosing. C_{max}, T_{max}, $AUC_{0\text{-}Inf}$ and $T_{1/2}$ metrics were calculated according to the Materials and Methods.

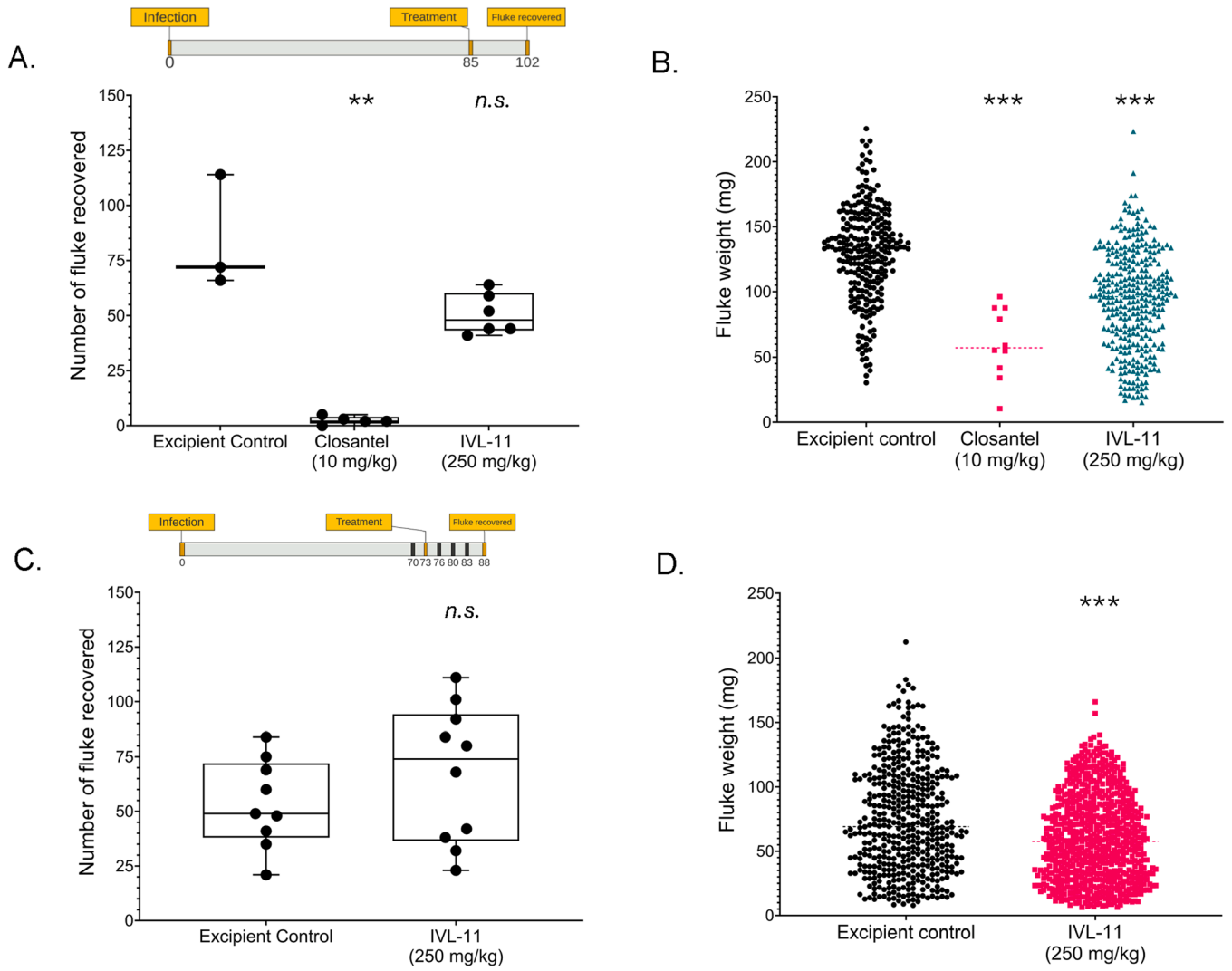


Fig. 5. Oral administration of IVL-11 affects *F. hepatica* body size in experimentally-infected *O. aries*. Groups of mixed-sex, 4–7-month-old sheep (Ile de France X Aberfield breed) were infected with 200 metacercaria/individual derived from a *F. hepatica* TCBZ-resistant Kilmarnock strain. **(A)** At day 85 post-infection, sheep were orally dosed with an excipient control (n = 3; 1.0 % aerosol 200, 0.01 % BHA, to volume with corn oil), closantel (n = 5; 10 mg/kg in the excipient formulation) or **IVL-11** (n = 5; 250 mg/kg in the excipient formulation). At day 17 post-dosing, sheep were culled and liver fluke burdens calculated. Ns = not significant. ** = statistically significant ($p < 0.01$). **B)** Weight distributions of recovered fluke from sheep treated with either the excipient control (1.0 % aerosol 200, 0.01 % BHA, to volume with corn oil), closantel (10 mg/kg in the excipient formulation) or **IVL-11** (250 mg/kg in the excipient formulation). Both closantel and **IVL-11** induced a significant (***; $p < 0.001$) reduction in fluke size as measured by weight; the two treatments were also not significantly different from each other ($p = 1.000$). **C)** At day 73 post-infection, sheep were orally dosed with the excipient control (n = 9; 1.0 % aerosol 200, 0.01 % BHA, to volume with corn oil) or **IVL-11** (n = 10; 250 mg/kg in the excipient formulation). At day 15 post-dosing, sheep were culled and liver fluke burdens calculated. Ns = not significant. **D)** Weight distributions of recovered fluke from sheep treated with either the excipient control (1.0 % aerosol 200, 0.01 % BHA, to volume with corn oil) or **IVL-11** (250 mg/kg in the excipient formulation). Treatment with **IVL-11** resulted in a highly significant reduction in fluke weight in comparison to the excipient controls (***; $p < 0.001$).

75 mg/kg bodyweight; $t_{1/2}$ = 38 hr at 250 mg/kg bodyweight), [28] While **IVL-11**'s AUC values are linked to administered dose (Fig. 4), it is likely that the sheep rumen does not offer a slow-release system for **IVL-11** movement to the posterior digestive track (and elsewhere throughout the body) as has been previously postulated for TCBZ [27]. Nevertheless, the formulation used to orally-deliver **IVL-11** (corn oil) clearly enabled the detection of this saponin in a variety of parasite associated tissues (bile and liver) upon dosing.

Although **IVL-11** treatment (a single 250 mg/kg bodyweight dose) of *F. hepatica*-infected sheep did not significantly reduce fluke burdens, it did significantly impact worm size (wet weight of individuals), reduced egg production and delayed the fecundity peak (as measured by EPG faeces) (Figs. 5–6). From *ex vivo* dose response titrations, **IVL-11** EC_{50} values against NEJs ranged from 2981 ng/ml (at 72 hr post treatment) to 3447 ng/ml (at 24 hr post treatment) (Fig. 3**A**). PK analyses of peripheral blood derived from **IVL-11** (250 mg/kg bodyweight) dosed uninfected sheep indicated an average C_{max} of 595 ng/ml (Fig. 4**B**). This 5–6-fold deficiency (*in vivo* peripheral blood C_{max} vs *ex vivo* EC_{50}) in **IVL-11** concentrations required to kill flukes likely explains why worm burdens were unaffected in treated animals (vs excipient controls); **IVL-11** concentrations never reached flukicidal concentrations in 250 mg/kg bodyweight treated animals. While we did not measure how much **IVL-11** was free or bound to plasma proteins, this process (protein binding) could also lead to substantial decreases in this saponin's availability/activity as a flukicidal agent. Nevertheless, our *in vivo* results clearly demonstrate that a single oral dose of **IVL-11** (250 mg/kg bodyweight) significantly impacts the body size of *F. hepatica* adults. Based on a previous study that identified a positive correlation between testis

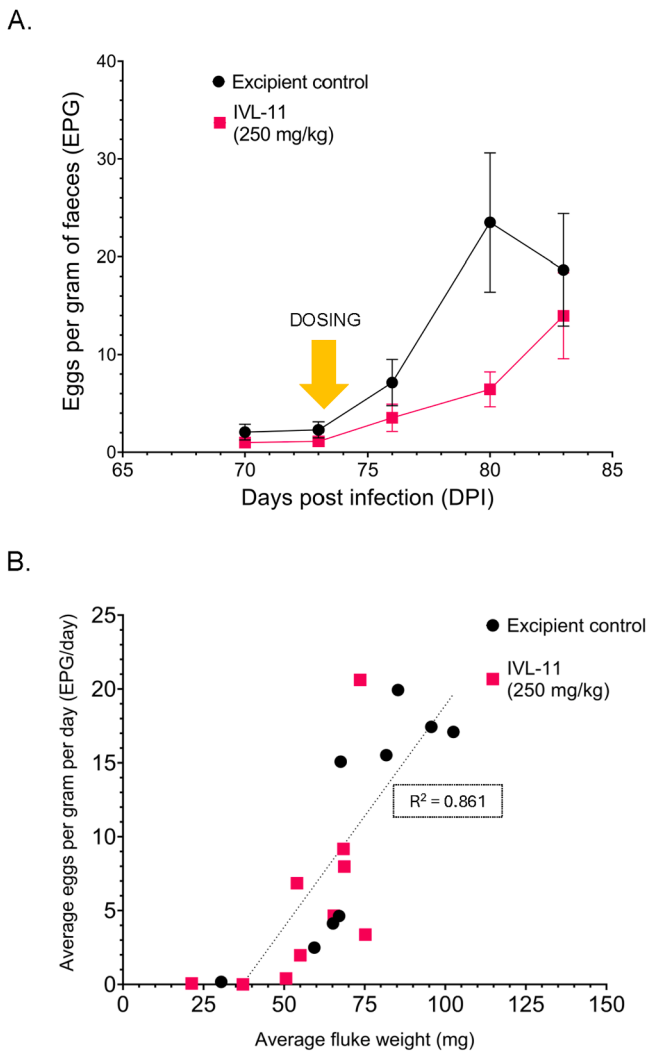


Fig. 6. IVL-11 treatment reduces *F. hepatica* fecundity and delays peak egg output in experimentally-infected *O. aries*. Groups of mixed-sex, 4–7-month-old sheep (Ile de France X Aberfield breed) were infected with 200 metacercaria/individual derived from a *F. hepatica* TCBZ-resistant Kilmarnock strain. **A)** Eggs per gram of faeces (EPG) measured from direct rectal sampling of sheep treated with either an excipient control (1.0 % aerosol 200, 0.01 % BHA, to volume with corn oil) or **IVL-11** (250 mg/kg in the excipient formulation). Yellow arrow indicates day of dosing. **B)** Correlation of average fluke weights (mg) with average daily EPG produced in each individual animal from the study. Fluke size and egg production were significantly ($p < 0.001$) positively correlated.

development and *F. hepatica* body size [18], **IVL-11** is likely to be affecting the normal development of at least gonadal tissue in adults. The fact that egg production also was negatively impacted by **IVL-11** treatment supports this contention. Our observations echo the results of a previous study in which three doses of α-hederin/**IVL-11** (500 mg/kg, 800 mg/kg and 800 mg/kg bodyweight; 10 day interval between treatments), administered to *D. dendriticum* infected ewes, substantially affected fluke fecundity as measured by EPG faeces [21]. Worm burdens were not assessed in this study. Similarly, when aqueous extracts of *H. helix* were administered to *Haemonchus contortus* (barber pole worm) infected sheep (1.13 g/kg or 2.25 g/kg bodyweight), reductions in both worm numbers and EPG faeces were also reported [14]. The authors of the *H. contortus* study did not assess the anthelmintic activities of purified α-hederin/**IVL-11**, either *ex vivo* or *in vivo*.

5. Conclusions

From these collective investigations, we conclude that altering formulation (e.g. different excipient), increasing amount (e.g. greater than 250 mg/kg bodyweight) or frequency of dosing (e.g. greater than one) with **IVL-11**, without negatively impacting tolerability/safety, are optimisations likely required to raise the therapeutic concentrations of this saponin during *in vivo* efficacy studies in sheep. Given that *H. helix* is commonly found in geographical areas sympatric with *F. hepatica*, that a scheme for purifying large amounts of **IVL-11** from *H. helix* leaf is uncomplicated and that **IVL-11** is equally active against TCBZ-sensitive/insensitive strains of *F. hepatica*, further research into the anthelmintic properties of this renewably obtained saponin is warranted.

CRediT authorship contribution statement

Joseph Payne: Writing – review & editing, Methodology, Investigation. **Peter Holdsworth:** Writing – review & editing, Investigation. **Anand Chakroborty:** Writing – review & editing, Visualization, Methodology, Investigation. **Caroline Fenn:** Writing – review & editing, Project administration. **Davey Sarah:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Laura Frame:** Methodology, Formal analysis. **Ayman Al-Najjar:** Writing – review & editing, Resources, Methodology, Formal analysis. **Gilda Padalino:** Writing – review & editing, Formal analysis. **Hoffmann Karl:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Anna Cervi:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis. **Baird Mark:** Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Thomas Dafydd:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Formal analysis. **Maggie Fisher:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Bouillon Marc:** Writing – review & editing, Resources, Methodology. **Alan Cookson:** Resources, Methodology. **Hulme Benjamin:** Writing – review & editing, Methodology, Investigation. **McNally Vincent:** Writing – review & editing, Resources, Project administration. **Burgess Adam:** Validation, Methodology, Investigation. **Martina Lahmann:** Writing – review & editing, Resources, Methodology.

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Declaration of Competing Interest

This work represents a collaboration between Aberystwyth and Swansea Universities with Ridgeway Research Ltd., Naturiol Bangor Ltd. and Bimeda Innovation Centre. All three private organisations may have a direct financial interest in the subject matter reported in the manuscript.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2026.119123.

Data Availability

Data will be made available on request.

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