Current Threat of Triclabendazole Resistance in *Fasciola hepatica*

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Abstract

Triclabendazole (TCBZ) is the only chemical that kills early immature and adult *Fasciola hepatica* (liver fluke) but widespread resistance to the drug greatly compromises fluke control in livestock and humans. The mode of action of TCBZ and mechanism(s) underlying parasite resistance to the drug are not known. Due to the high prevalence of TCBZ resistance (TCBZ-R), effective management of drug resistance is now critical for sustainable livestock production. Here, we discuss the current status of TCBZ-R in *F. hepatica*, the global distribution of resistance observed in livestock, the possible mechanism(s) of drug action, the proposed mechanisms and genetic basis of resistance and the prospects for future control of liver fluke infections using an integrated parasite management approach.

TCBZ-R is a current and widespread threat to livestock production systems

Fasciola species (*Fasciola hepatica*, *Fasciola gigantica*), also known as liver fluke parasites, are distributed worldwide in sheep and cattle and their **prevalence** in some regions is so great that serious clinical disease, termed **fasciolosis** (see **Glossary**), occurs [1, 2]. Globally, fasciolosis due to both fluke species is conservatively thought to cause production losses of over US\$3 billion p.a.[3]. The benzimidazole derivative triclabendazole (TCBZ), one of the major drugs used to control fasciolosis (Table 1), was first introduced in the early 1980s as a **flukicide** to treat and control acute and chronic fasciolosis in ruminants [4, 5]. It had high efficacy (>98%) against adult flukes and, more importantly,

unique efficacy against early **immature** and immature flukes. Other single flukicides only target more mature fluke ranging in age from 8-14 weeks (Table 1) [4]. As a result, TCBZ rapidly became the drug of choice for treating fluke infections, especially in sheep, as it was safe and allowed producers the relative luxury of not having to test for the stage of fluke present in their livestock [6, 7]. This over-reliance on TCBZ to treat sheep and, to a lesser extent cattle, has resulted in selection for fluke resistant to TCBZ [6, 7, 8].

The status of TCBZ-R in *F. hepatica* has been reviewed [6-13]. This review will focus on the current status of TCBZ-R worldwide, the possible mechanism(s) of action of the drug, the current knowledge of the genetics of resistance, the prospects for future control of liver fluke infections and the implementation of an **integrated parasite management (IPM)** plan to manage fasciolosis.

Current global status of TCBZ-R

Cases of TCBZ-R in livestock

Since the first appearance of TCBZ-R in *F. hepatica* [14] TCBZ-R has compromised fluke control in livestock in 11 countries or regions (Table 2; Figure 1, Key Figure). Resistance has likely appeared due to a generally poor understanding of liver fluke biology by farmers and confounding factors such as incorrect dosing, inappropriate product choice and lack of testing for **efficacy** [11, 12, 15]. The high frequency of TCBZ use, effectively TCBZ mono-therapy with no anthelmintic rotation, was a major contributing factor towards the development of TCBZ-R [14, 16]. Since TCBZ is not a persistent chemical, resistance was likely due to **head selection** in contrast to **tail selection** observed with **roundworms** [17].

The main method used to identify TCBZ-R in the field has been the **Faecal Egg Count Reduction Test (FECRT)**, with the recommended post-treatment sample collection time point at 21 days [18, 19]. Other studies using experimental infections have used 14 days for post-treatment sample collection which may not allow sufficient time for all eggs from dead parasites to be passed out of the gall bladder and be excreted [20, 21]. The use of the FECRT and the new **coproantigen ELISA (cELISA)** [22], in the form of a **Coproantigen Reduction Test (CRT)**, is now becoming common research practice [19-21, 23] but has yet to be routinely used in the field. As TCBZ kills most stages of a fluke infection in the host animal, a significant reduction (>95%) of egg count or coproantigen should occur in a susceptible fluke population using an oral TCBZ formulation. However, when adult flukicides are tested, the egg counts and coproantigen levels may not be reduced to zero at the time of re-testing (21 days post-treatment), even in a susceptible population, since young parasites not targeted by the adult flukicide will subsequently mature and release eggs or coproantigens. Several studies have shown that the signal in the cELISA is related to

68 fluke numbers and could possibly be used to indicate the relative level of fluke infection (low, moderate,
69 high), allowing the targeted treatment of animals but further work is required to validate the cELISA under
70 field conditions [2, 22, 24, 25]. The **bulk milk tank ELISA** is also used to detect fluke infections;
71 however, this method only detects antibodies to whole fluke antigen and, since antibodies can persist
72 following treatment, this ELISA cannot be used to evaluate drug efficacy against *F. hepatica* [2, 10].
73

74 Prior to 2011, peer-reviewed reports of TCBZ-R were historically described in livestock on only six
75 properties in Australia, Scotland, Wales, the Netherlands, Spain and the Republic of Ireland [8, 10] (Table
76 2). Since then, TCBZ-R has been reliably reported in sheep or cattle on a further 24 properties in N.
77 Ireland, Scotland, Wales, Australia, New Zealand, Peru and Argentina (Table 2)[13, 19, 26-32]. Three
78 human cases of TCBZ-R are discussed below. In total, cases of TCBZ-R have been reported on at least 30
79 properties (Table 2). Several non-peer reviewed cases of TCBZ treatment failure on-farm (possible TCBZ-
80 R) are not listed in the Table. These were cited in [6, 10] as Anon.1995 (the Sligo isolate); 1998; 2006;
81 2008; 2009. Mooney et al [36] also cited two anecdotal reports for TCBZ-R in 1995 and 1998. Recent data
82 from Great Britain revealed TCBZ-R on 7/25 farms tested using a composite FECRT method [30]. Six of
83 these farms were in S. Wales and one in Scotland. In some cases resistance is absolute (no reduction in
84 FEC observed). On certain farms, egg counts actually increased post-TCBZ treatment, which is of major
85 concern. In N. Ireland, a recent FECRT and CRT survey of sheep flocks, supported by fluke histology,
86 found demonstrable TCBZ-R on 5/13 farms tested with absolute resistance on two farms [31].
87 Interestingly, a subsequent questionnaire study revealed a change in farmer behaviour over recent years
88 (2005-2011), presumably as a result of perceived TCBZ-R [33]. For example, there was a significant shift
89 away from the use of TCBZ over time in favour of Closantel, with farmers also tending to treat earlier in
90 the year.

91
92 Despite being the source of one of the earliest TCBZ-resistant fluke isolates (the Sligo isolate) [11, 34, 35]
93 there are few reliable published reports of TCBZ-R in the Republic of Ireland. Again, the general
94 perception is that resistance is widespread. In 2009, Mooney *et al.* [36] reported a lack of efficacy of TCBZ
95 in sheep on a hill farm in the west of Ireland. FECRT were in the region of 49% and 66% based on
96 arithmetic means and considered to be highly indicative of TCBZ-resistant *F. hepatica* in sheep on this
97 farm. Mooney *et al.* [36] cited two earlier reports of TCBZ-R in Ireland in 1995 and 1998 but these were
98 anecdotal with no data or evidence of TCBZ-R provided.

99 In mainland Europe, most reports of TCBZ-R have come from the lower-lying northwestern countries e.g.
100 the Netherlands [16]. There are very few, if any, reports of confirmed TCBZ-R from central or southern
101 Europe. This most likely reflects the general prevalence of fluke and the perceived need to treat. There is a

growing gradient in the prevalence of *F. hepatica* west-to-east and south-to-north in Europe, with prevailing climatic and/or underlying geological conditions probably pivotal. Fox *et al.* [37] predicted that fluke incidence will increase and spread west-to-east in the UK over the coming decades, based on modelling the **Ollerenshaw Indices** and UKCP09 climate projections. Similar trends are predicted to occur across Europe [38]. The implications of this spread of liver fluke is of serious concern in relation to TCBZ-R, since farmers in traditionally fluke-free regions will need to treat animals that may have been exposed to TCBZ-resistant flukes.

Human cases of TCBZ-resistant fluke infections

TCBZ is also the drug of choice for treating fasciolosis in humans and it is conceivable that TCBZ-resistant fluke populations, selected in livestock, could pose a zoonotic risk to human health, especially in areas such as Peru and Bolivia where there is a high incidence of human infections [39]. The first incidence of TCBZ treatment failure in humans was reported in a livestock farmer in the Netherlands, with further recent reports of 4 cases from Chile, one case from Turkey and seven cases from Peru [40, 41, 42, 43]. Clearly, TCBZ-resistant zoonotic infections are a serious emerging issue (Figure 1).

Biochemistry of TCBZ action

Despite years of research, the precise mode of action of TCBZ is still unclear [reviewed in 6-10]. TCBZ is a benzimidazole (BZ) derivative and all available evidence in gastrointestinal roundworms indicates that BZ **anthelmintics** bind to α and β -tubulins within the parasite's cells, causing disruption of vital processes e.g. feeding, digestion. Several morphological studies, examining the effects of TCBZ and its active metabolites on *F. hepatica*, have been performed on the fluke's **tegument**, **vitellaria** and testis; all of these tissues showed significant signs of ultrastructural disruption, consistent with inhibition of microtubule-based processes [7]. There is also a concurrent loss of tubulin immunostaining in the tegumental syncytium, further implicating an interaction with tubulin as the primary mode of action of TCBZ [44, 45]. That said, this has not helped inform our understanding of TCBZ-R, because TCBZ-resistant flukes do not carry the F200Y/E198A or F167Y mutations in β -tubulin, implicated in BZ resistance in nematodes [46], suggesting that alterations to β -tubulin are not a key component of TCBZ-R [8].

Recently, TCBZ was reported to inhibit adenylate cyclase activity in yeast and/or inhibit the association of GTP-Ras with adenylate cyclase [47]. Interestingly, *F. hepatica* has one of the most active adenylate cyclase activities in biology and this activity is associated with the parasite's membrane fraction [48]. One of the first signs of TCBZ-induced damage in *F. hepatica* is tegumental blebbing and disruption of the tegumental ultrastructure [10, 49, 50]. If TCBZ was shown to inhibit adenylate cyclase activity the effects of TCBZ on the fluke's metabolism would likely be pleiotropic due to the second messenger function of

cAMP and its effects on protein kinases, carbohydrate metabolism and motility [48]. These results suggest that an evaluation of the sensitivity of fluke adenylate cyclase to inhibition by TCBZ may be informative. In addition, an analysis of sequence polymorphisms in adenylate cyclase gene(s) and/or GTP-Ras gene(s) in resistant and susceptible fluke populations is warranted, to determine if there is any selection on those sequences in resistant flukes.

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141 *New approaches to understanding the mode of action of TCBZ*

142 The multiplicity of studies reporting different mechanisms of resistance to TCBZ [7, 8, 10] suggests that
143 the mode of action of TCBZ and/or the effects on fluke metabolism are complicated, but the advent of new
144 technologies could allow the target of TCBZ to be unravelled in the foreseeable future. One approach is
145 **affinity purification** of the putative protein target whereby TCBZ is immobilised to a solid support and a
146 protein extract is passed over the column, followed by elution of any bound target proteins. This has
147 resulted in identification of protein targets against several types of drugs [51-53]. However, these methods
148 seem best suited for situations where a high-affinity ligand binds a relatively abundant target protein.

149

150 A new approach to understanding the mode of action of small molecules is the application of
151 **metabolomics**, a whole organism assay approach that identifies metabolic perturbations in a cell upon
152 exposure to drugs. This technique identifies the metabolomic compounds via **mass spectrometry** or
153 **nuclear magnetic resonance** and has been applied to several drug studies in various parasites [54]. One
154 such study with the protozoan parasite *Trypanosoma cruzi* and the drug benznidazole (BZN) revealed that
155 metabolised BZN generated covalent adducts of redox active thiols such as glutathione and cysteine that
156 were toxic to *T. cruzi* [55]. This is in contrast with affinity purification studies that identified the Sec23A
157 orthologue, the bacterial YjeF homologue and aldo-ketoreductase as proteins that could bind immobilised
158 BZN [53]. Thus, a combination of approaches may be required to fully characterize on-target and off-target
159 effects of TCBZ and to clearly define the mechanism(s) of TCBZ action.

160

161 **Mechanism(s) of TCBZ-R**

162 The actual biochemical mechanism underlying TCBZ-R in *F. hepatica* is not clear [reviewed in 6-10].
163 Evidence for metabolic differences between TCBZ- susceptible and resistant isolates have been reported
164 (e.g. sulfoxidation of TCBZ to TCBZ.SO and on to TCBZ.SO₂), suggesting that altered
165 uptake/efflux/metabolism of TCBZ plays a more important role in TCBZ-R than tubulin-based processes.
166 **P-glycoprotein (Pgp)** activity has also been shown to have an effect on the uptake and efficacy of TCBZ
167 *in vitro* using both resistant and susceptible isolates [56-60], with resistance to TCBZ reversed in the
168 presence of the Pgp inhibitor R(+)-verapamil. A role for mutations in one Pgp gene in TCBZ-R was
169 proposed but the few mutations that have been described [61] appear to be more related to variation

170 between strains and isolates of *F. hepatica* than a true TCBZ-R phenotype since these mutations were not
171 associated with resistance in Australian or Scottish TCBZ-resistant isolates [62, 63]. A single amino acid
172 substitution in glutathione *S*-transferase (GST) has been implicated in a TCBZ-resistant fluke isolate, but
173 this has not yet been confirmed in any other TCBZ-resistant isolates [64]. An analysis of the total soluble
174 **proteome** of TCBZ-resistant and susceptible flukes was also performed, with and without exposure to
175 TCBZ-SO, to search for key differences in the protein response to TCBZ exposure in adult flukes [65]. The
176 majority of differentially expressed proteins identified, such as molecular chaperones, fatty acid binding
177 proteins, thioredoxin peroxidase and GSTs, were thought to be related to a general stress response. It
178 appears that TCBZ treatment causes up-regulation of proteins that protect cells from damage which is not
179 surprising as several drug studies in other systems have shown similar responses [52, 66, 67].
180

181 **Genetics of TCBZ-resistant fluke populations**

182 Molecular studies on well defined TCBZ-resistant fluke populations, using both nuclear and mitochondrial
183 DNA (mtDNA), have demonstrated that such populations are genetically diverse [8, 68, 9. Intrapopulation
184 diversity (diversity within one animal) was illustrated in a calf and ewe infected with the laboratory TCBZ-
185 resistant isolates Oberon and Sligo, respectively, with 10 mtDNA **haplotypes** identified from 18 Oberon
186 fluke analysed [68]. A high level of diversity was also seen in two TCBZ-resistant fluke populations (7-9
187 haplotypes in 24-30 flukes) in Australian cattle [69] suggesting that drug selection pressure does not lead to
188 genetic bottlenecks of TCBZ-resistant fluke populations [69, 70]. The genetic analysis of 48 TCBZ-
189 resistant flukes recovered from cattle and sheep in Australia showed that mtDNA haplotypes are shared
190 between the hosts indicating that there is no host selection being applied to fluke populations [69], as seen
191 in N. Ireland [71].
192

193 Following a considerable research effort, a draft genome assembly has recently been published for *F.*
194 *hepatica* revealing that the liver fluke has a large, polymorphic genome and one of the largest pathogen
195 genomes sequenced to date at 1.3Gb [72]. As a result, *F. hepatica* is likely to be genetically predisposed to
196 developing resistance to anthelmintics. Moreover, any resistant individual *F. hepatica* which is selected in
197 the field will undergo clonal amplification when parasites develop within the snail intermediate host,
198 potentially leading to rapid dissemination of resistant parasites [10]. Hodgkinson *et al.* [8] reported a
199 strategy to employ genome-wide **single nucleotide polymorphisms (SNPs)** to map TCBZ-R in
200 recombinant *F. hepatica* progeny derived from genetic crossing of parental TCBZ-resistant and TCBZ-
201 susceptible clones. Initial results from this genome-wide approach were reported at the World Association
202 for the Advancement of Veterinary Parasitology Conference at Liverpool in August 2015 (Hodgkinson *et*
203 *al.*, Unravelling triclabendazole resistance in the liver fluke *Fasciola hepatica*;
204 http://www.waavp2015.com/bio_jane_hodgkinson.php). Pooled genotyping and subsequent analysis of

205 SNP allele frequencies on a number of replicates of TCBZ-exposed F2 parasites relative to untreated
206 controls identified a number of scaffolds within the draft *F. hepatica* genome sequence associated with the
207 TCBZ-R phenotype. Early indications suggest that, currently, there is no evidence that the obvious
208 candidate TCBZ-R genes lie in the regions of the genome under selection. The precise loci, and therefore
209 genes, involved are still to be defined but the approach being taken is a powerful one and is likely to
210 provide novel insights into the molecular basis of TCBZ-R [8].

211

212 **Anthelmintic control of TCBZ-resistant flukes: role for dual-active flukicide formulations**

213 The only chemical options for the control of TCBZ-resistant fluke are, depending on the host species,
214 treatment with clorsulon, nitroxylin, closantel, albendazole or oxclozanide [4, 10] (Table 1). The fact that
215 these chemicals are available to control TCBZ-resistant fluke is of benefit but none of these chemicals is
216 administered as a single active dose that is able to kill the early immature stage of the parasite i.e. <5-
217 weeks of age. This raises the issue of what to do, practically, in the face of an acute fluke outbreak,
218 especially in sheep, when the flukes are likely to be, or confirmed to be, TCBZ-resistant. This scenario was
219 realised recently in N. Ireland during the winter of 2012-2013; the advice given by veterinary practitioners
220 and advisors was to use TCBZ as an emergency treatment, followed up by (but not mixed with!) a different
221 flukicide (e.g. closantel or nitroxylin) to kill any fluke that survived the initial TCBZ treatment [73; Edgar,
222 H. *et al* (2012) Liver fluke forecast for Northern Ireland, 2012-2013, <http://tinyurl.com/o73chqt>]. Closantel
223 is a viable alternative for TCBZ in sheep but it is only effective against >8 week old fluke [74]. However,
224 the first case of fluke resistant to closantel was recently reported in cattle in Sweden [75] and resistance to
225 albendazole, clorsulon and nitroxylin has been reported (Table 1). These results highlight the importance of
226 protecting existing adult flukicides by effective on-farm fluke management.

227

228 The use of dual-active flukicides has been recommended to control a *F. hepatica* isolate which was
229 resistant to albendazole and clorsulon when these drugs were administered individually; this isolate was
230 susceptible to these drugs when given as a dual-active formulation [76]. When such formulations have a
231 synergistic effect (i.e. have greater efficacy than the sum of the actives) this may increase the lifespan of
232 the respective actives. Synergy has been seen with several dual-active flukicides (e.g. TCBZ+ Clorsulon,
233 TCBZ+ Luxabendazole) against TCBZ-resistant fluke in sheep [reviewed in 4, 10].

234 **What can be done to manage the impact of TCBZ-R on livestock production?**

235

236 *Vaccines as an alternative control strategy for fluke control*

237 An alternative approach to control TCBZ-R would be the development of a livestock vaccine for *F.*
238 *hepatica* which would reduce fluke burdens irrespective of the drug resistance status of the flukes and not

239 compromise fluke control during lactation. The need for a vaccine is becoming urgent, particularly to
240 protect sheep from acute fluke infections in N. Ireland, Republic of Ireland, NW Europe and Australia, as
241 exemplified by the 2012-2013 fluke season in N Ireland [73; Edgar, H. *et al* (2012) Liver fluke forecast for
242 Northern Ireland, 2012-2013. <http://tinyurl.com/o73chqt>]. This need will be exacerbated by the widespread
243 prevalence of TCBZ-R (Figure 1). However, no commercial liver fluke vaccine exists although several
244 experimental vaccines for livestock are under development [77]. No vaccine has shown reproducibly high
245 efficacy (>60%) in cattle to warrant commercial production although the leucine aminopeptidase (LAP)
246 vaccine has shown very high efficacy (up to 89%) in sheep [77]. It is important to note that a vaccine with
247 only partial efficacy (50-60%) may still provide economic benefits to producers experiencing infection
248 with TCBZ-resistant fluke, depending on the intensity of fluke infection in a herd, since production losses
249 are associated with fluke burden: the threshold for economic losses in cattle is about 30-40 flukes [10, 78,
250 79] but may be as low as 10 flukes [80]. Thus, until a new anthelmintic is developed which kills all
251 developmental stages, including the early immature fluke, a vaccine is the only alternative treatment that
252 could provide ongoing control of fluke infections in livestock in regions where TCBZ-R is endemic.

253

254 *Integrated parasite management (IPM) plan for farms*

255 Farms are not closed systems and there are many factors that influence the prevalence of *F. hepatica*, such
256 as soil type, irrigation, temperature, rainfall, pasture management and livestock management practices
257 [2,73, 81, 82](Figure 2). The management practices on farms generally rely solely upon anthelmintics and
258 appear to have contributed to the development of resistance [13, 83]. Management practices must change to
259 preserve the longevity of existing flukicides, as the likelihood of any new flukicides coming to market in
260 the near future is low [73].

261 Throughout the year there are periods in which the risk of fluke infection is higher and these periods
262 fluctuate depending upon location and prevailing climatic conditions, but do provide a set of guidelines to
263 determine when treatment may be required [37, 38, 84, 85]. If farmers combine strategic treatments with
264 FECs and the cELISA during high risk periods this approach could be used to determine when to drench,
265 which drench to use, or whether treatment is required at all, based on the known thresholds for economic
266 loss [2, 79, 80]. Well executed strategic treatments will minimize the need for further treatments
267 throughout the year and therefore help to preserve the efficacy of existing flukicides [73]. Regular drug
268 efficacy testing, using FECRT and/or CRT, to preserve the efficacy of existing flukicides or TCBZ is
269 essential to allow producers to avoid using products with reduced efficacy and prevent economic losses
270 resulting from unidentified resistance [73]. Movement of livestock can also contribute to the spread of
271 resistant *F. hepatica*. All newly purchased livestock or livestock housed over winter (eg in Europe) should
272 be quarantine treated upon arrival and cleaned out with a dual-active formulation [10, 13, 73, 83].

273 Flukicides should always be administered according to the product specifications and best practice methods
274 which include: weighing individual animals or the heaviest in the mob to determine dose, calibrating
275 drench equipment prior to use and during treatments, selecting the most potent formulations of product and,
276 where possible, regularly rotate effective products [73].

277 In addition, we must also look at how pastures, drinking water and irrigation can be better managed to
278 decrease the likelihood of *F. hepatica* infection (Figure 2). Pasture management can allocate low risk
279 pastures (such as newly sown paddocks, hay or silage paddocks) to young animals during the high risk
280 periods, to limit the chances of parasite transmission [73]. Often these practices are difficult to implement
281 within an existing system due to the timing of harvest and high risk periods. Old studies suggest that
282 metacercariae in herbage can be infective for 270-340 days whereas, in silage and hay, metacercariae will
283 be killed if ensiled for a period between 35-57 days and well cured hay with low moisture content poses
284 less risk [86, 87]. There is very limited recent literature investigating the viability of metacercariae on
285 pasture or in hay or silage and this requires further investigation. Through major overhauls of irrigation
286 infrastructure it may be possible to better target the liver fluke's intermediate host, the mud snail *Galba* spp.
287 in Europe and the UK, or *Austropeplea* spp. (formerly *Lymnaea* spp.) and *Pseudosuccinea* spp., and thus
288 break the life cycle [84]. However, such intervention is very costly and thus unappealing to farmers.
289 However, there have been successes: cementing of ditches near rice fields was used to assist the
290 elimination of schistosomiasis in Japan in 1950-70 [88]. Alternative cheaper options such as fencing off
291 high risk areas and regular maintenance of irrigation channels/drains will help to reduce the likelihood of
292 liver fluke infection in livestock [73, 84] but are set against a trend towards introduction of agri-
293 environment wetland schemes, at least in Europe, aimed at increasing biodiversity and reducing
294 greenhouse gas emissions [e.g. 10, 89]. The successful implementation of IPM strategies is inherently
295 difficult as the program will undergo continual change over the first few years while the strategy is fine-
296 tuned to suit the requirements of farms and the unique local issues affecting each property. This requires
297 the time, skill and knowledge of expert extension staff to continually assist the farmer throughout the
298 transitional period [73].

299 300 **Concluding Remarks and Future Perspectives**

301 The widespread incidence of TCBZ-R in livestock is a major threat to global livestock production and
302 producers need alternative treatments such as new flukicides or vaccines to control infections. Some key
303 outstanding questions need to be addressed if we are to fully understand the basis of TCBZ-R and provide
304 producers with better management options to control fasciolosis and reduce economic losses (see
305 **Outstanding Questions Box**). Affinity purification of target proteins using TCBZ as ligand, or
306 metabolomic analysis of the parasite response to TCBZ, may allow the identification of the protein target

(s) of TCBZ. The current search for SNPs associated with TCBZ-R in the fluke genome, using carefully selected parasite lines, will likely provide completely new insights into the possible mechanism of TCBZ-R as well as tools to monitor the appearance of TCBZ-R in herds [8]. The cELISA could possibly be used to estimate the approximate intensity of fluke infections, allowing targeted treatment of livestock with moderate-high fluke burdens, especially in cattle [2, 22, 24, 25]. Farmers must be more proactive and use an IPM approach to optimise their practices, monitor flukicide efficacy and test regularly for liver fluke prevalence to preserve the longevity of existing flukicides [10, 73, 83]. Given the increasing level of awareness of the impact of fasciolosis on production and the prevalence of TCBZ-R [2, 73], we anticipate that future control of fasciolosis will occur in three stages: (i) in the short term, better delivery of advice to producers from veterinarians and government or private extension advisors that will lead to more effective IPMs tailored to address the issue of fasciolosis and TCBZ-R on each farm and local region; (ii) in the medium term, the development of a new drench that has a similar efficacy spectrum to TCBZ; (iii) in the longer term, the application of a livestock vaccine would provide a more sustainable control strategy [77, 90]. The world market for a liver fluke vaccine is estimated at about US\$182m per annum(<https://www.ucd.ie/nova/ucdlicensingopportunities/novel%20vaccine%20for%20protection%20of%20liver%20fluke.pdf>) suggesting that a major commercial opportunity exists for this niche vaccine which would likely overcome the futile cycle of flukicide development and implementation followed by the inevitable selection for drug resistance.

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Figure 1. Global distribution of reports of triclabendazole resistance (TCBZ-R) in livestock and humans 1995-2015. Grey: 1 reported case of TCBZ-R; purple: 2-4 reported cases; yellow: ≥ 5 reported cases of TCBZ-R. Hashed lines indicate reported human cases of TCBZ-R. Image created in SmartDraw 2015.

Figure 2. Integrated parasite management. A schematic of the factors that influence *Fasciola hepatica* prevalence on a farm. The key areas are colour coded: climate, water bodies and irrigation in dark blue; pasture management in yellow; anthelmintic practices in green; concurrent disease in grey; and livestock in purple. Image created in SmartDraw 2015.

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571 **Table 1. List of currently available TCBZ products and other drugs to control *F. hepatica* in cattle and sheep worldwide¹**

Flukicides: Active Compound(s)	Method of administration available	Age of <i>F. hepatica</i> killed	Reports of resistance on-farm	Refs
TCBZ and TCBZ based combinations	Oral, pour-on	From early immature	30 cases	Table 2
Albendazole	Oral, Intraruminal	From adult	2 cases	[76, 91]
Clorsulon	Injectable, Oral	From adult; from late immature for oral	3 cases	[35, 76, 95]
Closantel	Pour-on, Injectable, Oral	From late immature	1 case	[75]
Nitroxynil	Injectable	From adult	1 case	[4]
Nitroxynil + Clorsulon	Injectable	From early immature	No reports to date	
Oxyclozanide	Oral	From adult	No reports to date	

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573 ¹ Label claims for efficacy of products containing each compound can vary as a result of differences in formulation and regulatory systems in different
574 countries.

575
576 ² Age of parasites: **early immature**: 1-4 weeks, parenchyma migration stage; **late immature**: 6-8 weeks, prepatent stage in bile ducts; **adult**: 12-14
577 weeks, bile duct stage [18].
578

Table 2. Published peer-reviewed reports of TCBZ-R in livestock on-farm or in humans and methods of assessment of resistance.

Year	Country or Region	Host			Diagnostic method										Number of Farms	Refs
		Sheep	Cattle	Human	FECRT or FEC ¹	CRT ¹	PM ¹ and TFC ¹	GLDH ¹	GGT ¹	PCV ¹	GOT ¹	Histo ¹	ELISA - Serum	<i>F. hepatica</i> isolated		
1995 ²	Australia	✓			✓		✓							✓	1	[14]
1998	Scotland	✓			✓		✓		✓						1	[92]
2000	Netherlands	✓	✓		✓									✓	1	[16, 93]
2000	Wales	✓			✓		✓								1	[94]
2006	Spain	✓			✓										1	[95]
2009	Republic of Ireland	✓			✓			✓	✓	✓					1	[36]
2011	Scotland	✓			✓		✓								1	[13]
2011	Argentina		✓		✓				✓		✓			✓	1	[26]
2012	Wales and Scotland	✓			✓		✓					✓			7	[30]
2012	Scotland	✓			✓	✓	✓	✓	✓				✓		2	[29]
2012	Netherlands ²			✓	✓				✓				✓		N/A	[40]
2012	New Zealand	✓			✓		✓								1	[32]
2013	Peru		✓		✓									✓	1	[27]
2014	Chile ^{2, 3}			✓	✓								✓		N/A	[41]
2014	Australia ⁴		✓		✓	✓									5	[19]
2015	Australia		✓		✓	✓									1	[28]
2015	Northern Ireland	✓			✓	✓						✓			5	[31]
2015	Turkey ²			✓					✓			✓	✓		N/A	[42]
2016	Peru ²				✓								✓		N/A	[43]

582 ¹ FECRT, faecal egg count reduction test; CRT, coproantigen reduction test; PM, postmortem; TFC, total fluke counts; GLDH, glutamate
583 dehydrogenase; GGT, gamma-glutamyl transferase; PCV, packed cell volume; GOT, glutamic oxaloacetic transaminase; Histo, Histology .

584 ² N/A: not applicable, these are human cases of TCBZ-R

585 ³ Only the abstract was available in English.

586 ⁴ Five farms were found to have resistance (<90%) by CRT; only 4 farms were confirmed with resistance by FECRT.

587 **Glossary**

- 588 **Affinity purification:** the purification of a protein based on its ability to bind to a substrate or drug
- 589 **Anthelmintic:** a chemical drug used to treat a worm infection
- 590 **Bulk milk tank ELISA:** the level of specific worm antibody detected by an ELISA assay in the combined
591 milk samples from several dairy cattle
- 592 **Coproantigen:** an antigen found in faeces
- 593 **Coproantigen Reduction Test (CRT):** the evaluation of the level of a worm antigen in faeces before and
594 after chemical treatment of the host
- 595 **Efficacy:** the effectiveness of a drug to kill worms in an infected animal
- 596 **ELISA:** a technique used to measure the level of an antigen or antibody in an animal
- 597 **Faecal Egg Count (FEC):** the number of worm eggs found in the faeces of an infected animal
- 598 **Fasciolosis:** the disease caused by infection with *F. hepatica* or *F. gigantica*
- 599 **Faecal Egg Count Reduction Test (FECRT):** the evaluation of the level of worm eggs in faeces before
600 and after chemical treatment of an animal
- 601 **Flukicide:** a chemical that kills *F. hepatica*
- 602 **Haplotype:** set of DNA sequence variations in a chromosome that are inherited together
- 603 **Head selection:** selection for anthelmintic resistant roundworms at the time of treatment
- 604 **Immature fluke:** a liver fluke aged up to 5 weeks old
- 605 **Integrated Parasite Management (IPM):** the use of a range of methodologies to manage the incidence of
606 parasites in a herd
- 607 **Mass Spectrometry:** the analysis of the mass to charge ratio of a molecule
- 608 **Metabolomics:** the analysis of metabolic perturbations in a cell upon exposure to drugs
- 609 **Nuclear Magnetic Resonance:** an analytical chemistry technique to determine the content and purity of a
610 sample and its molecular structure
- 611 **Ollerenshaw Indices:** an estimate of the risk of liver fluke disease, based on the calculated
612 evapotranspiration rate and its likely impact on intermediate host snails and shedding of metacercarial
613 cysts.
- 614 **P-glycoprotein:** a protein involved in pumping a chemical from a cell
- 615 **Pour-on:** a chemical delivered externally on the back of an animal
- 616 **Prevalence:** the percentage of animals in a herd infected by a parasite
- 617 **Proteome:** the full protein content of a cell or parasite
- 618 **Resistance:** the ability of a worm to resist the effects of a chemical normally lethal to the worm
- 619 **Roundworm:** a nematode parasite
- 620 **Single nucleotide polymorphisms (SNPs):** DNA sequence variations at one position in a chromosome

- 621 **Tail selection:** selection for anthelmintic resistant infective roundworm larvae at the time of treatment
- 622 **Tegument:** the surface layer of tissue on the liver fluke
- 623 **Vitellaria:** tissues in a fluke that produce egg shell material

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