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Review

# Drug resistance in liver flukes

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ABSTRACT

Liver flukes include *Fasciola hepatica*, *Fasciola gigantica*, *Clonorchis sinensis*, *Opisthorchis* spp., *Fascioloides magna*, *Gigantocotyle explanatum* and *Dicrocoelium* spp. The two main species, *F. hepatica* and *F. gigantica*, are major parasites of livestock and infections result in huge economic losses. As with *C. sinensis*, *Opisthorchis* spp. and *Dicrocoelium* spp., they affect millions of people worldwide, causing severe health problems. Collectively, the group is referred to as the Food-Borne Trematodes and their true significance is now being more widely recognised. However, reports of resistance to triclabendazole (TCBZ), the most widely used anti-*Fasciola* drug, and to other current drugs are increasing. This is a worrying scenario. In this review, progress in understanding the mechanism(s) of resistance to TCBZ is discussed, focusing on tubulin mutations, altered drug uptake and changes in drug metabolism. There is much interest in the development of new drugs and drug combinations, the repurposing of non-flukicidal drugs, and the development of new drug formulations and delivery systems; all this work will be reviewed. Sound farm management practices also need to be put in place, with effective treatment programmes, so that drugs can be used wisely and their efficacy conserved as much as is possible. This depends on reliable advice being given by veterinarians and other advisors. Accurate diagnosis and identification of drug-resistant fluke populations is central to effective control: to determine the actual extent of the problem and to determine how well or otherwise a treatment has worked; for research on establishing the mechanism of resistance (and identifying molecular markers of resistance); for informing treatment options; and for testing the efficacy of new drug candidates. Several diagnostic methods are available, but there are no recommended guidelines or standardised protocols in place and this is an issue that needs to be addressed.

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**Dedication to Joe Boray**

In Memorium

Joseph (Joe) Boray 1926 – 2018

During the preparation of this review, we were saddened to hear of Joe's passing.

"Standing on the shoulders of giants" is a wholly appropriate accolade for Joe, as he was a genuine titan in the world of fluke. Perhaps he will be best remembered for being the driving force behind the development of triclabendazole, a drug that has had such a huge impact on fluke control. But Joe's contribution was so much more than that, driven as he was by the practical application of his research, in order for farmers to benefit from it. He worked to standardise tests for the evaluation of drug efficacy and to establish and implement control programmes based on sound epidemiological knowledge. He had the vision to anticipate and monitor the almost inevitable emergence of drug resistance, and was responsible for developing new therapies to deal with it. Also, to isolate and culture drug-resistant flukes, giving over a bathroom in his own house to be a snail breeding laboratory for his library of isolates. He appreciated the value of having these isolates available for use in work to understand resistance.

So much research on fluke has gone on since Joe allegedly "retired" in 1999, though it is doubtful if he ever really did. The research has built on the foundations he laid down. Indeed, even this review could not have been written without Joe's groundwork. He leaves a great and unparalleled legacy behind him.

For a comprehensive account of Joe's life and career, do read his obituary in Vet. Parasitol. 261, 104-105, 2018.

We dedicate this review to his memory

## 1. Introduction

Liver flukes include *Fasciola hepatica*, *Fasciola gigantica*, *Clonorchis sinensis*, *Opisthorchis* spp. and *Dicrocoelium* spp., which parasitise both animals and humans, as well as *Fascioloides magna* and *Gigantocotyle explanatum*, which infect ruminants. Together with lung flukes and intestinal flukes, liver flukes form a group known as the Food-Borne Trematodes. Historically, these parasites have been overlooked as far as human infections are concerned, but they are now recognised as Neglected Tropical Diseases, causing significant health problems, and exerting a considerable economic impact, affecting as they do more than 10% of the world population (Keiser and Utzinger, 2009). For example, it has been estimated that 35 million people are infected with the Chinese liver fluke, *C. sinensis* with 601 million at risk of infection; for *Opisthorchis viverrini* and *Opisthorchis felineus*, the comparative infection figures are 10 million and 1.2 million, respectively, and combined the at-risk population is 80 million (Keiser and Utzinger, 2009). These three flukes occur predominantly in Southeast Asia and are classified as Type 1 carcinogens, as infection can lead to bile duct cancer, or cholangiocarcinoma (CCA) (Sithithaworn et al., 2012; Prueksapanich et al., 2018). In Thailand alone, opisthorchiasis and CCA have been estimated to cause economic losses of US$120 million per annum in medical care and lost wages (Andrews et al., 2008). Although this figure is from 2002, it still gives a measure of the considerable burden of this infection.

Among the "lesser" liver flukes, the giant liver fluke, *F. magna* originated in N. America and was introduced into Europe. It infects a wide range of ruminants, its principal host being the deer (Juhásová et al., 2016). *Gigantocotyle explanatum* is an amphistome parasite infecting the bile duct of water buffaloes, with a very high prevalence in the Indian sub-continent. It affects the health and productivity of the animals and this is significant because much of the rural population depends on livestock production. The precise economic impact of infection is unknown (Malik et al., 2017). The lancet flukes, *Dicrocoelium* spp. occur in a wide range of ruminant and other animals, and occasionally humans. *Dicrocoelium dendriticum* has the widest distribution of *Dicrocoelium* species, being endemic in some 30 countries. While the clinical symptoms of disease are generally mild, infection can lead to serious economic losses, in terms of milk and meat production and liver condemnation (Otranto and Traversa, 2002; Arbabi et al., 2011).

*Fasciola hepatica* has a very wide geographic distribution, possibly the widest of any helminth parasite, occurring in all continents except Antarctica. It is present in temperate regions of the world, whereas *F. gigantica* occurs in more tropical areas of Africa and Asia. It has been estimated that some 550 million animals (cattle and sheep) are infected worldwide (Boray, 1994), although that figure is old and is likely to have risen since that time (Fairweather, 2011b). In the mid-1990s, economic losses in the livestock industry due to fasciolosis were estimated at US $3 billion per annum (Boray, 1994), but again are likely to be far higher than that now. As with livestock infections, up-to-date data on human infections are hard to come by. The estimates most frequently quoted (and most often inappropriately referenced) in the literature of the number of people infected globally cover a range of 2.4–17 million, with 180 million at risk of infection (Rim et al., 1994; Hopkins, 1992; and Anon, 1995, respectively). A higher figure of 35–72 million people infected has been given by Nyindo and Lukambagire (2015), but the source of that figure was not provided; an adjusted figure (which ignores China) of 91.1 million people at risk has been estimated by Keiser and Utzinger (2005). Although the data are old, the figures emphasise the importance of the disease and it is now recognised as a major public health problem. The disease in livestock is known as fasciolosis and that in humans as fascioliasis, and that convention will be followed in this review.

There have been a number of reviews in the last few years, each covering different aspects of the disease, its control and drug resistance, in both animals and humans (eg Fairweather, 2011b; Khan et al., 2013; Mas-Coma et al., 2014; Nyindo and Lukambagire, 2015; Cwiklinski et al., 2016; Kelley et al., 2016; Carmona and Tort, 2017; Mehmood et al., 2017). In this review, which focuses on drug resistance, the topics to be covered are: the state of play with respect to resistance (in the field); what is known about resistance mechanisms; drug-related approaches to overcome resistance; farm management control strategies; and diagnosis. The review does not cover the epidemiology and forecasting of disease, nor the development of vaccines, as these topics have been well covered in recent reviews (eg Toet et al., 2014; Molina-Hernandez et al., 2015; Cwiklinski et al., 2016; Carmona and Tort, 2017; Mehmood et al., 2017; Beesley et al., 2018).

Among liver flukes, *Fasciola* spp. have been studied the most intensively, so the review will largely relate to them, but information on other flukes will be included where it is available and pertinent. In terms of drug resistance in liver flukes, the main concern is with triclabendazole (TCBZ). Its very success in the treatment of fasciolosis, underpinned by its efficacy against all the intra-mammalian lifecycle stages of fluke, inevitably has led to over-reliance on this single drug and the emergence of resistance. As it is the drug that has been most widely studied, much of the information presented on drug resistance unavoidably will apply to it, but any data on resistance to other drugs will also be included.

## 2. State of play with respect to resistance

Taking *F. hepatica* first, since the initial published report of resistance to TCBZ, in Australia in 1995 (Overend and Bowen, 1995), there have been numerous reports of resistance, in many areas of the world: in Europe, South America and Australasia. The information contained in these reports has been neatly summarised in table form in Kelley et al. (2016, Table 2) and in McMahon et al. (2016, Table 6). The Tables contain useful comparative data on the methods used to indicate the presence of resistance. Tables 1 and 2 in this review provide updated and extended data. The veracity or otherwise of some reports of resistance is a controversial topic that was raised by Fairweather (2011a, b, c) and will be returned to in Section 6, which deals with

**Table 1**
Field reports of drug resistance in *Fasciola hepatica* in sheep.

| Year | Country | Drug | Number of farms* | CET | FEC/FECRT | Sero-diagnosis | CRT | EHA | Molecular | Histology | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1995 | Australia | TCBZ | 1/1 | X | X | | | | | | 1 |
| 1998 | Scotland | TCBZ | 1/1 | | X | X | | | | | 2 |
| 2000 | The Netherlands | TCBZ | 1/1 | | X | | | | | | 3 |
| 2000 | Wales | TCBZ | 1/1 | | X | | | | | | 4 |
| 2005 | The Netherlands | TCBZ | 1/1 | | X | X | | | | | 5 |
| 2006 | Spain | ABZ, TCBZ | 1/1 | | X | | | | | | 6 |
| 2008 | Brazil | TCBZ | 1/1 | | X | | | | | | 7 |
| 2009 | Ireland | TCBZ | 1/1 | | X | X | | | | | 8 |
| 2009 | Bolivia | ABZ, TCBZ | 2/2 | X | X | X | | | | | 9 |
| 2010 | Northern Ireland | TCBZ | 1/12 | | X | | X | | | | 10 |
| 2010 | Spain | TCBZ | 1/1 | | X | X | | | | | 11 |
| 2011 | Scotland | TCBZ | 1/1 | | X | | | | | | 12 |
| 2012 | Wales, Scotland | TCBZ | 7/25 | | X | | | | | | 13 |
| 2012 | Scotland | TCBZ | n/s | X | X | X | X | | | | 14 |
| 2012 | Scotland | TCBZ | 2/2 | | X | X | X | | | | 15 |
| 2012 | New Zealand | TCBZ | 1/1 | X | X | | | | | | 16 |
| 2012 | Sweden | ABZ | 1/1 | | X | | X | | | | 17 |
| 2013 | Peru | TCBZ | 1/1 | X | | | | | | | 18 |
| 2013 | Spain | ABZ, CLORS | 2/2 | | X | | X | | X | | 19 |
| 2013 | Argentina | ABZ | 1/1 | X | | | | | | | 20 |
| 2014 | Spain | ABZ, CLORS | 1/1 | | X | | | | | | 21 |
| 2015 | Northern Ireland | TCBZ | 5/13 | | X | | X | | | X | 22 |
| 2016 | Sweden | ABZ | 2/2 | | X | X | | X | | | 23 |
| 2019 | England, Wales | TCBZ | 21/26 | | X | | | | | | 24 |
| 2019 | Uruguay | ABZ | n/s | X | | | | X | | | 25 |
| 2019 | Argentina | ABZ | 4/4 | | X | | | X | | | 25 |

CET, controlled efficacy test; FEC/FECRT, faecal egg counts/faecal egg count reduction test; CRT, coproantigen reduction test; EHA, egg hatch assay; *Number of farms on which resistance detected/total number of farms surveyed; n/s, not stated.
ABZ, albendazole; CLORS, clorsulon; TCBZ, triclabendazole.
References: 1, Overend and Bowen (1995); 2, Mitchell et al. (1998); 3, Moll et al. (2000); 4, Thomas et al. (2000); 5, Borgsteede et al. (2005); 6, Álvarez-Sánchez et al. (2006); 7, Oliveira et al. (2008); 8, Mooney et al. (2009); 9, Mamani and Condori (2009); 10, Flanagan (2010); 11, Martínez-Valladares et al. (2010); 12, Sargison and Scott (2011); 13 Daniel et al. (2012); 14, Gordon et al. (2012a); 15, Gordon et al. (2012b); 16, Hassell and Chapman (2012); 17, Novobilský et al. (2012); 18, Ortiz et al. (2013); 19, Robles-Pérez et al. (2013); 20, Sanabria et al. (2013); 21, Martínez-Valladares et al. (2014); 22, Hanna et al. (2015); 23, Novobilský et al. (2016); 24, Kamaludeen et al. (2019); 25, Ceballos et al. (2019).

diagnostic methods. It is imperative that reports provide full details of methods and results; this has not always been the case. TCBZ-resistant (TCBZ-R) fluke populations have been described in both sheep and cattle and in human infections too: for the latter, see Winkelhagen et al. (2012), Gil et al. (2014), Gülhan et al. (2015), Cabada et al. (2016), and Ramadan et al. (2019). While there is no empirical evidence for initial selection of TCBZ resistance in sheep, this is the most likely scenario, based on the amount of TCBZ that has been used historically in this host species. TCBZ resistance was first demonstrated in sheep in Australia and most likely evolved independently in other countries throughout the world. In some localities, TCBZ resistance may well have developed separately in cattle and sheep. Co-grazing and stock movements have probably contributed to the spread of resistance. Wildlife reservoir hosts such as rabbits, hares, deer and horses will also harbour flukes.

**Table 2**
Field reports of drug resistance in (a) *Fasciola hepatica* and (b) *Fasciola gigantica* in cattle.

| Year | Country | Drug | Number of farms* | CET | FEC/FECRT | Sero-diagnosis | CRT | EHA | Molecular | Histology | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) *F. hepatica* | | | | | | | | | | | |
| 2000 | The Netherlands | TCBZ | 1/1 | | X | | | | | | 1 |
| 2006 | Turkey | ABZ, RAFOX | 1/1 | | X | X | | | | | 2 |
| 2011 | Argentina | TCBZ | 1/1 | | X | X | | | | | 3 |
| 2012 | Peru | ABZ, TCBZ | n/s | | X | | | | | | 4 |
| 2012 | Peru | TCBZ | 3/5 | | X | | | | | | 5 |
| 2013 | Peru | TCBZ | 1/1 | | X | | | | | | 6 |
| 2014 | Australia | TCBZ | 5/8 | | X | | X | | | | 7 |
| 2015 | Australia | TCBZ | 1/6 | | X | | X | | | | 8 |
| 2015 | Sweden | CLOS | 2/3 | | X | | X | | | | 9 |
| 2019 | Chile | TCBZ | 1/1 | | X | | | | | | 10 |
| (b) *F. gigantica* | | | | | | | | | | | |
| 2008 | Tanzania | ABZ, OXYCLO | 1/1 | | X | | | | | | 11 |
| 2013 | Egypt | ABZ, RAFOX | n/s | | X | | | | | | 12 |
| 2015 | The Philippines | ABZ, TCBZ | n/s | | X | X | | | | | 13 |
| 2018 | Tanzania | ABZ | n/s | | X | | | | | | 14 |

CET, controlled efficacy test; FEC/FECRT, faecal egg counts/faecal egg count reduction test; CRT, coproantigen reduction test; EHA, egg hatch assay; *Number of farms on which resistance detected/total number of farms surveyed; n/s, not stated.
ABZ, albendazole; CLORS, clorsulon; CLOS, closantel; OXYCLO, oxyclozanide; RAFOX, rafoxanide; TCBZ, triclabendazole.
References: 1, Moll et al. (2000); 2, Elitok et al. (2006); 3, Olaechea et al., (2011); 4, Chávez et al. (2012); 5, Rojas (2012); 6, Ortiz et al. (2013); 7, Brockwell et al. (2014); 8, Elliott et al. (2015); 9, Novobilský and Höglund (2015); 10, Romero et al. (2019); 11, Keyyu et al. (2008); 12, Shokier et al. (2013); 13, Venturina et al. (2015); 14, Nzalawahe et al. (2018).

**Table 3**
Details of defined drug-resistant isolates of *Fasciola hepatica*.

| Isolate name | Resistant to which drug | Country | Confirmation of resistance status | Use in studies on mechanism(s) of resistance |
|---|---|---|---|---|
| Sligo | TCBZ | Ireland | Coles et al. (2000); Coles and Stafford (2001); McCoy et al. (2005); Alvarez et al. (2009); McConville et al. (2009); Flanagan et al. (2011a); Hanna et al. (2013); Forbes et al. (2014) | Robinson et al. (2002, 2004a); Alvarez et al. (2005); Mottier et al. (2006); Ryan et al. (2008); Ceballos et al. (2010); Chemale et al. (2010); Hanna et al. (2010); Scarcella et al. (2012); Fuchs et al. (2013); Savage et al. (2013a, b, 2014); Fernández et al. (2014, 2015a, b) |
| Dutch | TCBZ | The Netherlands | Moll et al. (2000); Gaasenbeek et al. (2001); Borgsteede et al. (2005); Fairweather et al. (2012) | |
| Oberon | TCBZ | Australia | Walker et al. (2004); Keiser et al. (2007a); Flanagan et al. (2011b); Fairweather et al. (2012) | Ryan et al. (2008); Devine et al. (2009; 2010a, b, c; 2011a, b, c; 2012); Fuchs et al. (2013); Meaney et al. (2013); Fernández et al. (2014, 2015b); George et al. (2017) Radio et al. (2018) |
| Cajamarca | ABZ, TCBZ | Peru | Ortiz et al. (2013); Canevari et al. (2014); Ceballos et al. (2019) | |
| CEDIVE | ABZ | Argentina | Sanabria et al. (2013); Canevari et al. (2014) | |
| Rubino | ABZ | Uruguay | Canevari et al. (2014) | Radio et al. (2018) |
| Uru-Mon | ABZ | Uruguay | Ceballos et al. (2019) | |
| AR 1-4 | ABZ | Argentina | Ceballos et al. (2019) | |
| Santillán de la Vega (SV) | ABZ, CLORS | Spain | Robles-Pérez et al. (2013, 2014); Martínez-Valladares et al. (2014) | |
| RA | ABZ, CLORS | Spain | Robles-Pérez et al. (2014) | |
| Corrulón (CR) | ABZ, CLORS, TCBZ | Spain | Robles-Pérez et al. (2013) | |

ABZ, albendazole; CLORS, clorsulon; TCBZ, triclabendazole.

There have also been reports of resistance to non-TCBZ flukicides. For example, field isolates resistant to albendazole (ABZ), but susceptible to TCBZ, have been identified in Argentina (Sanabria et al., 2013) and in Sweden (Novobilský et al., 2012, 2016), a country where TCBZ is not registered for use. Also in Sweden, resistance to closantel (CLOS) has been reported in fluke populations in cattle, although a pour-on formulation was used, which may have influenced the outcome of the trial (Novobilský and Höglund, 2015).

Resistance to more than one flukicide in the same fluke population has been reported in Spain:

- to ABZ and TCBZ (Álvarez-Sánchez et al., 2006; Martínez-Valladares et al., 2010; and Robles-Pérez et al., 2015). However, further studies on this isolate have shown that it is TCBZ-susceptible (TCBZ-S) (this is the "Leon" isolate referred to in Fairweather, 2011a; Flanagan et al., 2011b; Fairweather et al., 2012);
- to ABZ and clorsulon (CLORS). Dual resistance has been described in the Santillán de la Vega (SV) isolate (Robles-Pérez et al., 2013); in the RA isolate from the CV flock (Robles-Pérez et al., 2014); in an unspecified isolate, possibly the SV isolate (Robles-Pérez et al., 2015); and in an unnamed isolate (Martínez-Valladares et al., 2014); and
- to ABZ, CLORS and TCBZ. The isolate, named the Corullón (CR) isolate, is TCBZ-R at the adult, not immature, stage (Robles-Pérez et al. (2013).

Fluke populations resistant to both ABZ and TCBZ have also been reported in South America (Mamani and Condori, 2009; Chávez et al., 2012).

Data on the origins and resistance status of defined drug-resistant fluke isolates is given in Table 3, together with information on their use in studies on mechanisms of resistance. More historical information relating to resistance against rafoxanide (RAFOX) in Australia, with side resistance to CLOS, but not to oxyclozanide (OXYCLO), another member of the salicylanilide group of flukicides; and cross-resistance to nitroxynil (NITROX), a halogenated phenol, is given in Fairweather and Boray (1999) (see also Boray, 1990, 1997). In Europe, reduced efficacy, indicative of resistance, has been reported to ABZ and RAFOX (but not TCBZ) (Elitok et al., 2006) and to RAFOX and NITROX (Rapic et al., 1988).

With regard to *F. gigantica*, reduced activity of ABZ has been reported in Tanzania (Nzalawahe et al., 2018). This may be due to (the widespread) use of ABZ to treat nematode infections in cattle, employing lower dosages than required to treat fluke infections. Alternatively, it may be due to the way in which Zebu cattle process the drug, compared to European breeds. Reduced activity of ABZ and OXYCLO in cattle has been reported before in the same country (Mahlau, 1970; Keyyu et al., 2008). In the latter study, reduced efficacy to ABZ did not extend to TCBZ (Keyyu et al., 2008). Reduced activity of ABZ (but not TCBZ) and RAFOX (but not OXYCLO, another salicylanilide) has been reported in an unspecified species of *Fasciola* in cattle in Egypt, the results being regarded as indicative of resistance (Shokier et al., 2013). Clearly, further work is needed to determine whether these treatment failures represent genuine resistance or not.

As far as the authors are aware, there have been no reports of drug resistance in *C. sinensis* and *Opisthorchis* spp., or in any other species of liver fluke.

## 3. Resistance mechanisms

Studies to identify the mechanism(s) of resistance to TCBZ have centred on 3 areas: tubulin binding, altered drug uptake and modified drug metabolism. These possibilities are illustrated in Fig. 1 and will be discussed separately below.

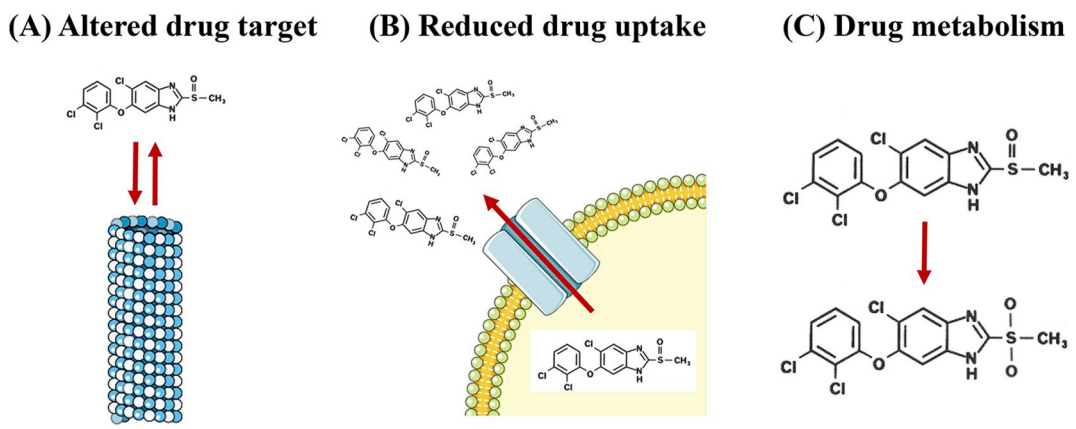


**Fig. 1.** Proposed mechanisms of TCBZ resistance in *Fasciola hepatica*. (A) Initial studies focused on the putative target of TCBZ, namely β-tubulin, although no mutations conferring resistance have been identified. (B) Several studies suggest that TBCZ is a substrate for membrane transporters such as P-glycoprotein. Their activity is increased in TCBZ-resistant flukes which may reduce the intracellular concentration of the drug at its site of action. (C) Metabolism of active forms of TCBZ to comparatively inert metabolites (e.g. TCBZ sulphoxide to TCBZ sulphone as shown here) is increased in TCBZ-resistant flukes. Medical art provided by Les Laboratories Servier, https://smart.servier.com/.

### 3.1. Altered tubulin binding

Initial attempts to understand the mechanism of resistance to TCBZ understandably focused on the presumed target of the drug, β-tubulin, because benzimidazole (BZ) compounds are known to disrupt microtubule-based processes in other helminth parasites (Fennell et al., 2008). As a consequence of BZ binding, microtubule assembly is inhibited and microtubule-based processes affected, including tissue penetration, feeding and reproduction. The results of a large number of morphological studies carried out by the group at the Queen's University of Belfast have shown that treatment with TCBZ and its metabolites stops the movement of secretory vesicles, inhibits the mitotic division of germ and somatic cells and the meiotic division of germ cells; it also leads to a loss of tubulin immunostaining (for references, see previous reviews by Fairweather, 2005, 2009, 2011b). These changes are typical of microtubule inhibition and are not seen in TCBZ-R flukes. Subsequent studies were carried out to sequence β-tubulin isotypes and attempt to identify the binding site in the β-tubulin molecule. The binding site is presumed to be different from that for other BZs, in order to account for the narrow spectrum of activity of TCBZ and the refractoriness of *F. hepatica* to BZs in general. While no computational models of fluke tubulin have been made, some insights have been achieved with models from other organisms, including nematodes. Although the process is complicated, essentially (and insofar as it might apply to *F. hepatica*), the binding of BZs is accepted as being associated with the colchicine binding site. However, this site is buried deep within the β-tubulin molecule and is inaccessible to ligands. It has been suggested that an inter-domain movement in β-tubulin (as occurs during dimer dissociation) would expose the amino acids involved in binding (Ravelli et al., 2004; Robinson et al., 2004b). Of the residues concerned, $Glu^{198}$ is essential for binding, forming H-bonds with the carbamate and benzimidazole moieties of the ligand. Residue 165 (Asn) also forms an H-bond with the BZ (Aguayo-Ortiz et al., 2013a, b, 2017; Guzmán-Ocampo et al., 2018). A Phe-Tyr mutation affects binding, (with) the formation of an H-bond between $Asn^{165}$ and $Tyr^{200}$ closing off the binding pocket; $Tyr^{200}$ also forms an H-bond with $Glu^{198}$, which stops $Glu^{198}$ forming bonds with the ligand (Aguayo-Ortiz et al., 2013a, b; Guzmán-Ocampo et al., 2018). $Phe^{200}Tyr$ is the predominant mutation that has been linked to BZ resistance in nematodes (Aguayo-Ortiz et al., 2013a, b, 2017). $Tyr^{200}$ is present in β-tubulin from drug-susceptible *F. hepatica* (Robinson et al., 2001, 2004b; Ryan et al., 2008; Chambers et al., 2010; Fuchs et al., 2013) and this may help to explain why the fluke is refractory to BZ compounds. TCBZ will not fit in the binding site for albendazole sulphoxide (ABZ.SO) in the *H. contortus* β-tubulin model (Robinson et al., 2004b). The idea of a colchicine binding "site" has been extended to become a "domain", encompassing 3 zones (Dorléans et al., 2009; Massarotti et al., 2012). Using the *H. contortus* β-tubulin model originally constructed by Robinson et al. (2004b), it has been predicted that TCBZ binds to zone 2, while other BZs, such as mebendazole (MBZ) and ABZ bind to the region of overlap between zones 1 and 2 and to zone 3, respectively (Ranjan et al., 2017). Interestingly, other flukicidal compounds can be docked into this model as well: for example, CLORS, RAFOX and OXYCLO to the region overlapping zones 1 and 2; NITROX to zone 3; while Coriban (diamphenethide) binds exclusively to zone 2, along with TCBZ (Ranjan et al., 2017). So, the results suggest that TCBZ binds at a different site (on β-tubulin) than colchicine or other BZs. Sequence comparisons between TCBZ-S and TCBZ-R flukes showed that there were no sequence differences in β-tubulin isotypes and no differences in expression levels between isolates (Robinson et al., 2002; Fuchs et al., 2013). Moreover, the expression levels did not change after exposure to the therapeutically active sulphoxide metabolite of TCBZ (TCBZ.SO) (Chemale et al., 2010).

In conclusion, then, we still do not fully understand how TCBZ interacts with the β-tubulin of *F. hepatica* or why other BZs have a low affinity for *F hepatica*. To date, there is no evidence of any changes to β–tubulin in TCBZ-R flukes. The focus of attention has shifted to exploring other potential resistance mechanisms, most notably in relation to altered drug uptake and drug metabolism.

### 3.2. Altered drug uptake

The uptake of TCBZ and TBCZ.SO by TCBZ-R fluke isolates is significantly less than that by TCBZ-S flukes (Alvarez et al., 2005; Mottier et al., 2006). This suggests that P-glycoprotein (Pgp)-linked drug efflux pumps may be involved in resistance. Resistance can be reversed by co-incubation *in vitro* with ivermectin (IVM) (Mottier et al., 2006). A number of morphological experiments involving the P-glycoprotein (Pgp) inhibitor, R(+)-verapamil have shown that it can potentiate TCBZ action *in vitro* in TCBZ-R, but not TCBZ-S, flukes (Meaney et al., 2013; Savage et al., 2013a, b, 2014). A single nucleotide polymorphism (SNP) $T^{687}G$ in the Pgp gene of *F. hepatica*, which results in a serine to arginine change at residue 1144, has been described by Wilkinson et al. (2012). It is located in a region of the Pgp molecule that is linked to its transporter function. However, the SNP was not found in TCBZ-R and TCBZ-S isolates from Australia (Elliott and Spithill, 2014), or in isolates

from Latin America (Solana et al., 2018) or Scotland (P. Skuce, unpublished observations), so it may be related more to inherent haplotype variation between fluke populations than to TCBZ resistance *per se*, particularly as the isolates studied by Wilkinson et al. (2012) were not properly defined and the number of individual fluke samples analysed was low. So, this SNP could not be used as a marker for TCBZ resistance. The Latin American isolates showed more variable SNPs than those in the earlier studies, but none were associated with TCBZ resistance (Solana et al., 2018). Clearly, more work is required to identify any resistance markers. Docking studies with a molecular model of *C. elegans* Pgp-1 (Cel-Pgp-1) demonstrated that macrocyclic lactones (MLs) may bind to a site within the inner chamber delineated by the transmembrane domains in the transporter protein (David et al., 2016). Interestingly, TCBZ could be docked into a site(s) in the inner chamber that partially overlapped the ML binding site, suggesting that it could be transported by the Pgp, that is, TCBZ is a substrate of Cel-Pgp-1 (David et al., 2016). That being so, Pgp transporters in *F. hepatica* may have a role to play in the development of resistance to TCBZ. The results of the docking studies re-enforce the pharmacology data. The flukicide CLOS could also be docked into the inner chamber in the *C. elegans* Pgp-1 model. This model could potentially be used for the screening of novel drugs.

A number of flukicides have been tested for their ability to inhibit Pgp-mediated rhodamine 123 transport in a recombinant cell line (LLC-PK1 cells) over-expressing Pgp. Of the drugs tested, TCBZ, TCBZ.SO, CLOS and RAFOX inhibited transport, whereas TCBZ sulphone (TCBZ.$SO_2$), ABZ, mebendazole (MBZ), NITROX and CLORS were without effect (Dupuy et al., 2010). This is an important approach to understanding potential interactions between drugs and Pgp transporters, and could be used to predict any such interactions in the design of combinations to alter parasite Pgp activity and enhance drug bioavailability.

Many ATP binding cassette transporters (ABC transporters) have been identified in *O. felineus* and Pgp activity was inhibited by verapamil (Mordvinov et al., 2017a), although the involvement of these in drug resistance in this species has yet to be determined.

### 3.3. Altered drug metabolism

The metabolism of TCBZ to TCBZ.SO and TCBZ.SO to TCBZ.$SO_2$ is greater in TCBZ-R than TCBZ-S isolates (Robinson et al., 2004a; Alvarez et al., 2005), suggesting that drug metabolism is upregulated in TCBZ-R flukes. In a series of morphological studies, co-incubation of TCBZ and TCBZ.SO with flavin monooxygenase (FMO) and cytochrome P450 (CYP450) inhibitors led to a potentiation of drug action in TCBZ-R flukes that was not seen in TCBZ-S isolates (Devine et al., 2009, 2010b; 2011b, 2012). A CYP450 enzyme has been isolated from *O. felineus* and, while its activity has been shown to be sensitive to ketoconazole (KTZ), an established CYP450 inhibitor, it is not yet known if anthelmintic compounds are substrates of the enzyme (Pakharukova et al., 2012, 2015).

Glutathione S-transferase (GST) is another enzyme involved in drug metabolism. Its activity is greater in TCBZ-R than TCBZ-S flukes (Scarcella et al., 2012; Fernández et al., 2014, 2015a). Among the various isoenzymes of the GST family, it is the mu type that is differentially expressed at elevated levels in TCBZ-resistant flukes (Fernández et al., 2014). A comparison of the GST mu gene in a TCBZ-S and in a TCBZ-R isolate of *F. hepatica* revealed an amino acid change in the TCBZ-R fluke, where threonine is replaced by serine at position 143 (Fernández et al., 2015b). The mutation is in the C-terminal alpha helical domain of the protein, in the area of the active site; it is located on the outside of the protein, where it can bind ligands and so modify the enzyme's activity. This suggests that this substitution may be involved in the resistance mechanism (Fernández et al., 2015b). A GST has been cloned, expressed as a recombinant protein and shown to bind TCBZ.SO, indicating that it might have a role to play in resistance to TCBZ (Chemale et al., 2010). Elevated levels of GST activity have also been linked to salicylanilide resistance in *F. hepatica* (Miller et al., 1994).

A transcriptomics approach to understanding TCBZ resistance has been adopted by Radio et al. (2018). Comparisons were made between 3 isolates with varying susceptibilities to TCBZ and ABZ: one resistant to TCBZ and ABZ; one resistant to ABZ, but susceptible to TCBZ; and the third susceptible to both drugs. The levels of expression of cytoskeleton-related proteins were lower in the TCBZ-R and ABZ-R isolate than in the other two: the proteins were α- and β-tubulin, kinesins and dyneins (Radio et al., 2018). Moreover, there was a downregulation of a number of drug metabolism enzymes in this isolate, although the GST mu protein was upregulated (Radio et al., 2018). Upregulation of an ABC transporter-like protein was also observed in the double-resistant isolate. Interestingly, there was a downregulation of adenylate cyclase (AC); recently, inhibition of AC by TCBZ has been described in yeast (Lee et al., 2013). The AC enzyme in *F. hepatica* is one of the most active in eukaryotes and is likely to play significant roles in fluke biology through its established roles in cell signalling mechanisms (Fairweather, 2004; Kelley et al., 2016). The impact of TCBZ on fluke AC activity warrants further study. The results of the investigation by Radio et al. (2018) are compatible with those of previous studies (morphological, biochemical and proteomic) on drug actions and targets and on the response of drug-resistant fluke isolates to drug action; they emphasise that drug resistance is likely to be polygenic in nature, as is typical with anthelmintics (Kotze et al., 2014). Indeed, the mode of action of the drug itself may have a similar multifactorial basis. To complicate matters further for TCBZ, it is metabolised to different forms: TCBZ.SO, TCBZ.$SO_2$ and hydroxy forms of TCBZ, TCBZ.SO and TCBZ.$SO_2$. There has been a long-held view that TCBZ.SO is the only active form of TCBZ and that TCBZ.$SO_2$ is inert. That is not so: TCBZ.$SO_2$ has activity *in vivo* against juvenile fluke in sheep (Büscher et al., 1999) and the various metabolites cause morphological disruption to the fluke *in vitro*. Consequently, overall TCBZ action may be due to the combined and sequential effects of different metabolites, not to one active form of the drug; for greater discussion of this point, see Fairweather (2009, 2011b).

### 3.4. Summary of section

Some progress has been made in understanding the binding of TCBZ to β-tubulin, but the picture is far from complete. With respect to alternative resistance mechanisms, altered drug uptake and metabolism seem likely to be involved, but the molecular basis for each of these possibilities has yet to be identified. So, it is likely that drug resistance in *F. hepatica* is polygenic in nature.

When carrying out experimental studies with specific fluke isolates, it is important to know the drug sensitivity of the isolate used and that information should be documented, otherwise the results are open to misinterpretation (Fairweather, 2011c). Unfortunately, the provenance data are not always provided.

## 4. Overcoming resistance: drug-related approaches

### 4.1. Alternative flukicides and the re-purposing of existing antiparasitic drugs

A number of existing flukicides are active against adult TCBZ-R flukes: they are ABZ, CLORS, CLOS, NITROX and OXYCLO (Coles et al., 2000; Moll et al., 2000; Coles and Stafford, 2001; McKinstry et al., 2009; Martínez-Valladares et al., 2010; Hanna et al., 2015). The susceptibility to NITROX does not extend to the juvenile (4-week) stage (Forbes et al., 2014). Combinations of flukicides, anthelmintics and other drugs have been shown to be effective: they will be discussed below (Section 4.2.).

Given the high costs of drug discovery and development (which can

run into billions of dollars: Geary and Thompson, 2003; Geary et al., 2015), and the length of time it takes to bring drugs to market, the idea of drug Re-purposing has gained traction in recent years. Re-purposing entails the testing of old or existing drugs for potential new applications and so provides a way round the problems associated with the development of novel actives. For helminth infections, the topic has been well covered in the review by Panic et al. (2014). A few examples will be picked out here for liver flukes, to illustrate the idea: they are tribendimidine, nitazoxanide and oxfendazole (OXF). Other examples are the artemisinins and Mirazid: they will be covered in Section 4.4.

**Tribendimidine** was originally developed to treat infections of humans with soil-transmitted helminths such as *Ascaris lumbricoides* and hookworms (Xiao et al., 2013). It has also been examined for potential activity against trematode parasites, especially in situations where control is dependent on a single drug such as praziquantel (PZ) against schistosomiasis. In these situations, the development of resistance would have serious consequences for control, and so it is important to identify alternative drugs. Tribendimidine has been shown to be active against *C. sinensis* and *O. viverrini* in a rodent model, but not against *F. hepatica* or *Schistosoma mansoni* (Keiser et al., 2007b). The results of clinical trials with patients infected with *C. sinensis* and *O. viverrini* have shown that tribendimidine has an efficacy comparable to that of PZ, which is the current drug of choice, but with a higher safety profile (Soukhathammavong et al., 2011; Qian et al., 2013; Xiao et al., 2013; Sayasone et al., 2016, 2018). In addition, it has been shown to be as effective as PZ against *O. felineus* in a rodent study (Pakharukova et al., 2019).

**Nitazoxanide** has broad-spectrum activity against intestinal protozoal and helminth infections in humans (Gilles and Hoffman, 2002; Hemphill et al., 2006). It has been tested as an alternative to TCBZ in human fascioliasis, again to reduce dependence on a single drug, TCBZ, and to avoid the problems stemming from the development of resistance. A viable alternative is also important, because TCBZ is not widely registered and available for human use (Keiser et al., 2011; Cwiklinski et al., 2016). Nitazoxanide has been shown to be effective in the treatment of human fascioliasis (Rossignol et al., 1998; Kabil et al., 2000; Favennec et al., 2003; Zumquero-Ríos et al., 2013). However, it should be noted that nitazoxanide was ineffective in the treatment of a human case of apparent TCBZ resistance in the Netherlands (Winkelhagen et al., 2012). More recently, nitazoxanide has been shown to be partially successful in the treatment of human cases of TCBZ failure in Egypt (Ramadan et al., 2019).

**Oxfendazole (OXF)** is normally used to treat nematode and cestode infections and is not indicated for fasciolosis. Nevertheless, at increased dose rates, it has been shown to be effective against *F. hepatica* infections in sheep and pigs (Furmaga et al., 1982; Gomez-Puerta et al., 2012; Ortiz et al., 2014). It exerts ovicidal activity against the eggs of TCBZ-S flukes (Alvarez et al., 2009). OXF is also present in selected combination products (see below: Section 4.2).

### 4.2. Drug combinations: use and rationale

Drug combinations are used routinely for the control of parasitic infections in livestock. As multi-actives, they can be used to treat mixed infections (of nematodes and fluke, for example), so broadening the spectrum of activity of the individual drugs, or infections of parasites from the same phylum. Combinations are also used to slow down the development of drug resistance, thereby potentially serving to extend the useful lifespan of the individual drugs (in the combination) (Bartram et al., 2012; Geary et al., 2012, 2015). TCBZ is marketed in combination products alongside levamisole (LEV), OXF, IVM, abamectin or moxidectin (MOX). If the drugs in the combination are from different chemical groupings and have different mechanisms of action, this raises the possibility of the combination having additive or synergistic effects, and would permit the use of lower drug concentrations, with consequent environmental benefits and reduced selection pressure for resistance (Bartram et al., 2012; Geary et al., 2012). Synergism between TCBZ + CLORS and TCBZ + luxabendazole (LUX) at considerably lower dose rates than normal has been demonstrated for 6-week-old infections of TCBZ-R *F. hepatica* in sheep. Combinations of CLOS + TCBZ, CLORS, LUX or OXF, and of NITROX + CLOS or CLORS have been shown to be synergistic against salicylanilide-resistant isolates of *F. hepatica* in sheep, at different ages and at reduced dose rates (for details, see Fairweather and Boray, 1999). Synergism between TCBZ and either artemether or artesunate (two artemisinin derivatives), was demonstrated in a rodent model, but the effect was limited to the adult fluke. In the same study, there was no synergy with a more fully synthetic artemisinin derivative, OZ78 (Duthaler et al., 2010). A fluke population in a sheep flock in Spain deemed to be resistant to ABZ and CLORS, but susceptible to TCBZ, was treated successfully with a combination of ABZ + CLORS at their normal concentrations, but not with a combination at half their therapeutic concentrations (Martínez-Valladares et al., 2014). No synergy was demonstrated between TCBZ and NITROX (at normal dose rates) against juvenile TCBZ-R flukes in sheep (Forbes et al., 2014). A pour-on formulation of TCBZ + MOX was shown to be effective against flukes from 4 weeks post-infection (pi) to the adult stage in cattle, a greater efficacy range than that for IVM + CLOS (late immature and adult) or IVM + CLORS (adult only) (Geurden et al., 2012). A combination of NITROX + CLORS + IVM has been shown to be highly active against early juvenile flukes (2 and 4 weeks old) of the Sunny Corner isolate, with a level of efficacy either equal to that of a TCBZ + OXF combination, or greater than combinations of TCBZ + IVM or TCBZ + abamectin (Hutchinson et al., 2009). This is an interesting result, as flukes of the Sunny Corner isolate are less susceptible to TCBZ at these early juvenile stages than are older flukes, which are fully susceptible (Fairweather, 2011a). Combinations of OXF + OXYCLO and TCBZ + LEV have been shown to be more effective than the individual drugs (on their own) in treating a natural *Fasciola* infection in sheep (Khan et al., 2017). The impact of a combination of TCBZ + CLORS at half-normal dose rates on the morphology of adult flukes has been assessed in a rodent model. The combination induced greater disruption than either drug on its own (at reduced and normal levels), supporting the idea of at least additive, if not synergistic, effects (Meaney et al., 2006, 2007).

Drug combinations can be used in another way, by manipulating drug pharmacokinetics (PKs) and pharmacodynamics (PDs), applying the considerable advances made in our understanding of the pharmacology of anthelmintic drugs (see reviews by Lanusse et al., 2015, 2018). The objective of this approach is to enhance the bioavailability of the active drug, as measured by parameters such as the peak plasma concentration, time to peak concentration, mean residence time and elimination half-life. This will serve to increase the exposure of the parasite to the drug, thereby potentially improving drug efficacy. This can be achieved by combining anthelmintics with inhibitors of drug uptake or metabolism. Again, the aim is to optimise their activity and extend their active life, by delaying the emergence and spread of resistance. With regard to the manipulation of drug transport, the model described by Dupuy et al. (2010), which could be used to test the potential of drug combinations, has already been discussed in Section 3.2.

For *F. hepatica*, data on the PKs of TCBZ has been covered by Fairweather (2009) and Moreno et al. (2014). Co-administration of TCBZ with IVM has been shown to enhance the PKs of both compounds in sheep, but the impact of this increase on drug efficacy was not tested in the study (Lifschitz et al., 2009). In a similar manner, co-administration of TCBZ + KTZ led to a greater bioavailability of TCBZ in sheep, but again no efficacy experiments were carried out (Virkel et al., 2009). However, in a separate study by Ceballos et al. (2010), in which TCBZ was administered with both IVM and methimazole (MTZ) (an FMO inhibitor), the combination did not increase the PKs of TCBZ, nor did it improve the efficacy of TCBZ against a TCBZ-R fluke isolate. Part of the explanation for this, and the discrepancy between the studies by Lifschitz et al. (2009) and Virkel et al. (2009), may lie in the different

routes of drug administration used in the three studies: intra-ruminal (Ceballos et al., 2010), intravenous (Lifschitz et al., 2009) and oral (Virkel et al., 2009).

The impact of a combination of TCBZ with inhibitors of drug metabolism is discussed below, in Section 4.3.

### 4.3. New drugs against specific targets

Although the empirical screening of compounds has underpinned the discovery of anthelmintics in the past and this approach will no doubt continue, a more rational, mechanism-based approach, directed against specific targets, may play a greater role in the future. This will depend on the identification of suitable target molecules, perhaps based on a greater understanding of the basic biology of the parasite and benefitting from "-omics" studies, of drug actions or of the mechanism of drug resistance. Initially, this work may well be carried out in academic research laboratories but then needs to be picked up by the Animal Health industry for further validation and development (Geary and Thompson, 2003; Woods et al., 2007; Woods and Knauer, 2010; Geary et al., 2015; Vercruysse et al., 2018). For the liver fluke, a number of studies have been carried out to explore the possibility of designing drugs to target specific molecules, for example, cathepsins and CYP450. Cathepsins (which are also vaccine candidates) play vital roles in fluke biology, with different cathepsin family members being expressed at different times in the life cycle (Cancela et al., 2008; Beckham et al., 2009; Robinson et al., 2008, b; Smooker et al., 2010). Inhibitors have been shown to evoke an anti-fecundity effect, with a retardation of development, reduced egg output and decreased egg viability (Alcalá-Canto et al., 2006, 2007). In addition, the cathepsin-specific inhibitor Z-Phe-Ala-$CHn_2$ reduced excystment of *F. hepatica* metacercariae *in vitro* by 99% (Robinson et al., 2009). A more recent study screened a library of 40 synthetic flavonoids against recombinant cathepsin L1 and cathepsin L3 enzymes (*Fh*CL1 and *Fh*CL3, respectively). *Fh*CL3 is the only cathepsin L expressed by the newly-excysted juvenile fluke (NEJ), while *Fh*CL1 is the main cathepsin produced by the adult fluke (Robinson et al., 2008). One compound, C34, showed particular activity: it also decreased the ability of NEJs to migrate through the gut wall and reduced NEJ motility, leading to their death (Ferraro et al., 2016). Molecular docking studies identified one binding site for C34 in both *Fh*CL1 and *Fh*CL3; the site occupied a similar position within the active site of each enzyme. A second site was observed in *Fh*CL3; it had a different orientation and is not located as deeply within the binding site as the other binding site (Ferraro et al., 2016). The data suggest that C34 is a suitable candidate for further drug development.

CYP450 is an important enzyme, involved in drug metabolism and the synthesis of secondary metabolites and other important molecules within eukaryotes. Liver flukes possess a single CYP450 gene (Pakharukova et al., 2012, 2015) and this enzyme may represent a potential target for drug action. A range of CYP450 inhibitors has been tested for anthelmintic activity against the human liver fluke, *O. felineus* using motility and mortality assays with newly-excysted metacercariae and adult flukes. Miconazole, clotrimazole and KTZ were the most effective inhibitors (Mordvinov et al., 2017b). A large number of natural compounds have been screened against *O. felineus* CYP450 (OfCYP450), using a series of computational studies which included modelling, structure-based virtual screening, docking, molecular simulation and binding energy analyses. The initial 165,869 compounds were whittled down to 3 which had excellent inhibitor activity and could act as lead compounds for drug discovery (Shukla et al., 2018). KTZ in combination with TCBZ has been shown to potentiate the action of TCBZ against TCBZ-R *F. hepatica* (Devine et al., 2011b, 2012). Co-treatment of TCBZ + KTZ led to an increase in the bioavailablity of TCBZ in sheep (Virkel et al., 2009) but, as indicated in the previous Section (Section 4.2), the impact of this on drug efficacy was not tested.

For *O. felineus*, synergism between the current drug of choice, PZ, and the inhibitors clotrimazole and miconazole has been demonstrated *in vitro*, but was not confirmed *in vivo*, so the combination offered no benefit over praziquantel monotherapy (Pakharukova et al., 2018).

### 4.4. Natural products

Traditional medicines, based on natural plant products, have long been used in developing countries to treat parasitic diseases in both humans and livestock (Tagboto and Townson, 2001; Iqbal et al., 2003; Kayser et al., 2003; Ndjonka et al., 2013). They remain attractive propositions because of their easy availability and affordability. Identification of the active component in the extract could lead to the design of synthetic analogues with more defined properties for further evaluation. Several natural products have been shown to possess activity against *F. hepatica* (for references, see Fairweather, 2009). In a more recent study, 15 tropical plant extracts used in traditional Mexican medicine were screened for activity against NEJs: five of the fifteen, including *Artemisia mexicana* (see the section on artemisinins below) showed promising activity (Alvarez-Mercado et al., 2015). Plumbagin, a naphthoquinone derived from the roots of *Plumbago indica*, the Indian leadwort, has been tested against NEJs and 4-week-old immature flukes of *F. gigantica*. On the basis of the results of several assays, plumbagin was shown to be more effective than TCBZ at similar concentrations (Lorsuwannarat et al., 2014). A diterpenoid, 7-keto-sempervirol, isolated from the wolfberry plant, *Lycium chinense* affects NEJ movement and viability, also adult morphology (Edwards et al., 2015). Synthesis of 30 structural analogues of the compound revealed one, designated 7d, that displayed greater activity than 7-keto-sempervirol (Crusco et al., 2018). Extending this work on structurally-related phytochemicals, ten triterpenoids isolated from the bark of the fir tree, *Abies procera* have been tested for activity (in terms of their effects on motility and changes to surface morphology) against 3 life cycle stages of both *F. hepatica* and *S. mansoni*; for *F. hepatica*, this involved NEJs, 4-week-old immature flukes and 8-week-old adult flukes, and for *S. mansoni* it involved schistosomula, 3-week-old juveniles and 7-week-old adult worms. Compound 700015 showed the most potent anthelmintic activity against both species and further work to develop analogues of this triterpenoid is warranted (Whiteland et al., 2018). A number of natural products have displayed activity against the eggs and miracidia of *F. hepatica* (Pereira et al., 2016; Hegazi et al., 2018; Nwofor et al., 2018). Another possibility is to use anthelmintically active phytochemicals in combination with existing anthelmintics: this has been discussed as an option for nematode control by Lanusse et al. (2018). While the idea of using natural products is interesting, far more rigorous identification of active components, together with their subsequent evaluation and testing, is necessary before their true value to future parasite control can be properly judged. It is comparatively easy to find compounds that are active *in vitro*, but less so to translate that activity to efficacy *in vivo* and to commercial viability. Added to which, the costs associated with the discovery and development of new drugs are extremely high. Having said that, a number of plant-derived compounds have undergone further development, including the artemisinins and Mirazid.

The **artemisinins**, originally isolated from the wormwood plant, *Artemisia*, are a major group of therapeutic compounds, well-established as antimalarials and used in the treatment of human schistosomiasis (Utzinger et al., 2007; Ding et al., 2011; Keiser and Utzinger, 2012; Chaturvedi et al., 2010). Much effort has gone into assessing their potential value as fasciolicides, and this represents another example of drug re-purposing. A number of semi- and fully-synthetic artemisinin derivatives have been tested against immature and mature *F. hepatica* and activity demonstrated under *in vitro* conditions and in rodent (rat) infections, even against a TCBZ-R isolate (Keiser et al., 2006a, b, 2007a; Duthaler et al., 2010; Zhao et al., 2010; Kirchofer et al., 2011; Wang et al., 2011). Unfortunately, this activity has not been fully or consistently translated to infections in larger ruminants, such as sheep (Keiser et al., 2008, 2010a, b; Meister et al., 2013). Two studies have

been carried out to determine the efficacy of artemisinins in treating fascioliasis in humans. The first, in Vietnam, compared the response of patients with fascioliasis to treatment with artesunate to that with TCBZ. Initially, at 10 days post-treatment (pt), symptom control was better with artesunate but, by the end of the study (at 3 months pt), it was lower than that resulting from TCBZ treatment (Hien et al., 2008). In the second study, artemether was seen to exert little or no activity against fascioliasis in phase-2 trials in Egypt (Keiser et al., 2011).

Artemisinin compounds have also been tested against *C. sinensis* and *O. viverrini*. Again, activity in small animal studies was not reproduced in human trials (see review of literature by Lam et al., 2018).

**Mirazid** is a commercial preparation of myrrh, derived from the plant, *Commiphora molmol*. Primarily developed as a fasciolicide, for both human and animal use, it has activity against a range of protozoan and helminth parasites (references cited in Abdelaal et al., 2017a, b). The use of Mirazid as an antischistosomal drug has been questioned (e.g. Botros et al., 2004) and its activity against *Fasciola* spp. has also been challenged, although this may be due (at least in part) to the variable treatment protocols employed in previous studies (a point discussed by Abdelaal et al., 2017a). Recent studies *in vitro* and *in vivo* (in a rodent model), using a TCBZ-R isolate of *F. hepatica* have shown that Mirazid causes severe disruption of fluke tissues and suppression of egg production (Abdelaal et al., 2017a, b). The results warrant further investigation, to determine the most effective concentration of drug and standardise the dosing regime for commercial use.

### 4.5. New formulations and delivery systems

Traditionally, flukicides have been delivered as oral or injectable formulations. Relatively recently, pour-on products have been introduced primarily for cattle, with the flukicide in combination with a nematocide (eg TCBZ + MOX, CLOS + IVM, CLORS + IVM). The ease of administration and minimisation of animal handling and stress make them attractive products to use. However, pour-ons appear to rely, at least in part, on oral ingestion of the drug and absorption from the gut, as a result of licking behaviour by the individual or between animals. This results in variable bioavailability, with no guarantee of the recommended dose reaching the parasite. In turn, this may lead to reduced efficacy and to conditions that favour selection of resistance. Moreover, licking of pour-ons containing CLOS may lead to CNS toxicity issues (see Section 5.2). Tissue levels of drug residues are likely to be variable and this may have an impact on food safety (Bousquet-Mélou et al., 2004; Hutchinson et al., 2009; Martin et al., 2009; Toutain et al., 2012). In the case of TCBZ + MOX, the product has an inferior range of efficacy to that of oral TCBZ, only being active against late immature and adult flukes. As mentioned previously (Section 2), resistance to CLOS has been linked to the use of a pour-on product (Novobilský and Hoglund, 2015).

As with other benzimidazoles, TCBZ has low solubility in water and biological fluids, which renders its bioavailability low and limits the options available for its administration. A TCBZ prodrug, designated MFR-5, has been synthesised and shown to display an 88,000-fold increase in aqueous solubility compared to TCBZ (Flores-Ramos et al., 2017). Intramuscular injection of an aqueous solution of MFR-5 to sheep experimentally infected with *F. hepatica* led to a very high efficacy, comparable to that of TCBZ itself, together with total suppression of egg production (Flores-Ramos et al., 2017). In a separate study in cattle, intramuscular injection of the same pro-drug (named Fosfatriclaben) resulted in a similar level of efficacy to that following oral administration of TCBZ and the sub-cutaneous injection of CLOS, but at a lower concentration (Rojas-Campos et al., 2019). This opens up the possibility of developing new formulations of TCBZ, to improve the delivery of the drug.

Compound alpha is a TCBZ derivative, with a spectrum of activity similar to that of TCBZ, and it may have a similar action, targeting fluke tubulin (see reviews by Fairweather, 2005, 2009, 2011a, b). It displays high efficacy against TCBZ-S flukes *in vivo* (in sheep) but, despite showing activity *in vitro* against juvenile and adult TCBZ-R flukes, this activity was not replicated *in vivo* in a sheep trial, possibly due to the formulation of the drug or the route of delivery used (Fairweather, 2011b). A compound alpha prodrug has been synthesised with a 50,000-fold greater aqueous solubility than the parent compound (Flores-Ramos et al., 2014). When tested *in vitro* against NEJs, the prodrug had an efficacy similar to that of the TCBZ control. Its efficacy was also evaluated *in vivo* in a sheep trial, using 3 different routes of administration (oral, intramuscular, subcutaneous). The highest efficacy for the prodrug (comparable to that of oral compound alpha) was achieved following intramuscular injection and at a much reduced dose rate than that normally used for compound alpha; egg production was greatly reduced as well (Flores-Ramos et al., 2014). A subsequent trial in sheep confirmed the activity of the intramuscular injection of compound alpha prodrug against adult fluke, at a dose less than half that normally used for oral compound alpha. Activity against juvenile flukes was lower, but the flukes recovered were significantly smaller than the controls (Ibarra-Velarde et al., 2018). Again, this illustrates the value of using prodrugs for more effective routes of drug delivery.

Two alternative approaches to improving the solubility of TCBZ for the development of novel formulations have been adopted by Real et al. (2018a, b). The first involved the preparation of chitosan-based nanocapsules loaded with TCBZ (Real et al., 2018a). The second approach involved the complexing of TCBZ with the cyclodextrins, 2-hydroxylpropyl-β-cyclodextrin and methyl-β-cyclodextrin: solubility increased 256- and 341-fold, respectively, and the complexes retained their stability after prolonged storage (Real et al., 2018b). In a separate study, a combination of TCBZ and silver nanoparticles was shown to have a more significant inhibitory effect on egg hatching than TCBZ alone (Gherbawy et al., 2013).

ABZ nanocrystal formulations have been shown to improve the solubility, bioavailability and (antiparasitic) efficacy of ABZ (Paredes et al., 2018a, b; Pensel et al., 2018). It has also been demonstrated that solid dispersions of ABZ using Poloxamer 407 as carrier increase the solubility of ABZ, and this could lead to greater bioavailability (Simonazzi et al., 2018).

### 4.6. Summary of section

A number of older flukicides are available as alternatives to TCBZ, although they act against adult, rather than juvenile, *F. hepatica*. Drug combinations could be used to treat fluke infections, using existing flukicides and anthelmintics, and some combination products are already on the market. Combinations of flukicide + nematocide need to be used judiciously, particularly in relation to the decision regarding when to treat. The optimum time to treat the potential target parasites is likely to be different, so a compromise may be needed. Moreover, it is important to identify which parasites are actually present before deciding to treat. Combinations of drugs could be used in a different way, to alter their PKs and PDs in order to overcome resistance. There seems to be much interest in finding new uses for, and new ways of delivering, existing drugs – and the design of new drugs to act against specific targets.

The issue here is whether any of these studies will actually lead to commercial exploitation. The artemisinins are a case in point: despite much interest and the demonstration of promising activity, as described above, the development of these compounds seems to have stalled, as pointed out by Whitehead et al. (2018). While efficacy can be demonstrated under certain conditions, more factors need to be taken into account during the decision-making process by the pharmaceutical company, as commercialisation is an expensive business. Other factors include safety, quality control, scalability and patent protection. It has been argued that the livestock industry (particularly the sheep sector) is not seen as a sufficiently lucrative market for investment by the multinational drug companies (Besier, 2006), but greater dialogue between

industry and academia may help to develop the new drugs needed to deal with the escalating problem of anthelmintic resistance. Industry has the resources to pick up on ideas and potential new leads generated by academia and test them further.

## 5. Overcoming resistance: management strategies

### 5.1. Anthelmintic-based strategies

In the absence of commercially available vaccines, flukicides will remain in the vanguard of strategies used to control fasciolosis in livestock for the foreseeable future. In one sense, control in cattle is more straightforward than that for sheep, because the acute form of the disease is rarely encountered in cattle. That said, liver fluke infection has a significant impact on cattle health and productivity, in terms of growth rate, fertility and milk production, for example.

#### 5.1.1. Cattle

The choice of which flukicide to use will depend on a number of factors:

- the efficacy spectrum of the drug and what form of the disease is being targeted (that is, acute or chronic);
- whether it is an individual drug or part of a combination product;
- the route and ease of administration of the product (that is, whether it is a drench, an injectable or a pour-on product);
- whether resistance is known or suspected to be present on the farm;
- risk assessment of previous exposure to fluke;
- the withdrawal period for milk and meat; and
- cost (Forbes et al., 2015).

The choice also depends on what the treatment hopes to achieve, whether it is **therapeutic**, to improve the health and productivity of the herd, or **strategic**, to stop egg output and reduce contamination of the pasture when the animals are turned out after housing. Typically, treatment in cattle is linked to housing but, where animals are not housed, treatment strategies are based on the seasons rather than on housing (Boray, 1994, 1997). In relation to housing, when cattle are brought in in late autumn, it might be assumed that the population of fluke present would be a mixture of all ages and stages of development, but this may not be the case as adults may well predominate (MacGillivray et al., 2013). A number of treatment options are available:

- treat with TCBZ 2 weeks after housing. This will remove all stages of fluke present, if susceptible, but not if TCBZ is no longer effective, or if there is a desire to conserve TCBZ; there is a tenable argument that TCBZ should be used only in sheep with acute fasciolosis, to preserve its efficacy (Forbes, 2013, 2017b);
- treat with CLOS or NITROX immediately after animals are housed. This will remove late immature flukes (6–8 weeks old) and adult flukes. Animals should be treated again 8 weeks later, to eliminate any immature flukes that were unaffected by the previous dosing but have subsequently developed to adulthood;
- treat with ABZ or CLORS immediately after animals are housed. These compounds only kill adult flukes, so the treatment needs to be repeated 12 weeks later, to eliminate any immature survivors that have reached maturity. The second treatment should ensure that cattle are not shedding eggs when they are returned to pasture (and so is more important for strategic treatments, in order to protect the pasture); or
- treat with TCBZ immediately at housing, in the hope that some activity remains to remove flukes at all stages, including very early juvenile stages. Animals should be checked about 6 weeks after housing to determine if any eggs remain. If so, a follow-up treatment with CLOS or OXYCLO should clear out late immature flukes from the bile ducts and adults that were missed previously. However, if TCBZ resistance is certain (diagnosed in co-habiting sheep, for example), then it would be best to go with one of the two previous options. There is a strong argument in favour of each farmer knowing accurately what the TCBZ resistance status is on their property, and that is something that should be encouraged.

There is an argument that use of a product with less than 90–95% efficacy still has value, in that it would reduce the fluke burden to a level, or "economic threshold", where it may not affect the overall health and productivity of the animals (Fairweather, 2011b; Forbes, 2013). The threshold has been set at >50 flukes (Vercruysse and Claerebout, 2001; Charlier et al., 2007), but it may be lower (30–40 flukes: Kelley et al., 2016; >10 flukes: Charlier et al., 2008). For more detailed discussion of liver fluke control in cattle, the reader is referred to the excellent articles by Forbes (2013, 2017b) and Forbes et al. (2015).

The choice of drug to treat lactating cows for fasciolosis may be restricted by how the legislation surrounding drug licencing operates in different countries. For example, only ABZ and OXYCLO can be used for lactating and/or dairy cows in the UK and there is a need to discard milk for a few days (Forbes, 2013; Knubben-Schweizer and Torgeson, 2015; Statham, 2015). Up-to-date information for the UK is given on the Veterinary Medicines Directorate website (https://www.cattleparasites.org.uk/app/uploads/2018/04/Joint-NOAH-and-VMD-statement-on-flukicide-use.pdf).

#### 5.1.2. Sheep

For sheep, a potential treatment scheme has been proposed by Hanna et al. (2015). Faced with the lack of an alternative drug to TCBZ that targets acute fasciolosis, the authors consider that it is advisable to continue to use TCBZ in the autumn, in the hope that at least a proportion of the fluke population remains susceptible, and that the fluke burden can be reduced sufficiently to save some animals in an acute outbreak. This can be followed immediately by use of CLOS, to remove adults and late immatures should TCBZ not be fully effective. Dosing twice on the same day would avoid the need for a second "muster" of the flock, but would require the use of separate dosing guns. An alternative would be to follow the cattle pattern and check for eggs 6 weeks after TCBZ treatment, then use CLOS if necessary. However, if solid TCBZ resistance is known to exist on the premises, CLOS in autumn, followed by a second dose in late winter/early spring would be recommended (Hanna et al., 2015). An extra treatment in early summer has also been recommended by Crilly et al. (2015), to reduce pasture contamination later in the year.

#### 5.1.3. Other management practices related to anthelmintic use

Practices include the timing of treatment, the frequency of treatment, product rotation and quarantine treatment. A recent study in Northern Ireland showed that treatment had moved earlier in the year, perhaps in response to climate change or to a change in emphasis to adult fluke control earlier in the season, so as to reduce pasture contamination; that there had been an increase in drug rotation; and that there had been a marked fall in quarantine treatments (McMahon et al., 2016). The survey results also revealed that there had been a move away from TCBZ use, with it being replaced by CLOS. This shift may represent a response by farmers to anecdotal accounts of treatment failure amongst other flock owners, without any definitive evidence that TCBZ resistance actually existed on their farms.

#### 5.1.4. Quarantine

It is important to prevent the importation of TCBZ-resistant liver fluke onto the farm. Obtaining information on the efficacy status of drugs used on the seller's farm is very useful, but not always possible, so a well thought-out quarantine protocol should be in place. Treatment with a non-TCBZ flukicide, such as CLOS or NITROX, repeated after 6–7 weeks, combined with grazing on quarantine or low-risk pasture, is recommended (Crilly et al., 2015). Advice is given on the Sustainable Control of Parasites in Sheep (SCOPS) website: https://www.scops.org.

uk/internal-parasites/liver-fluke/fluke-quarantine/

### 5.2. Farm management practices

In addition to the practices outlined above, other steps that can be taken include weighing animals before dosing, to avoid under- or over-dosing; calibrating drenching equipment (to ensure that the correct dose is administered); correct storage of products; and pasture rotation. In relation to the danger of over-dosing, several cases of neurotoxicity have been described following CLOS treatment (eg Van der Lugt and Venter, 2007; Rivero et al., 2015). Studies in Switzerland have shown that the adoption of individual farm schemes involving pasture rotation systems (of the kind recommended by Boray, 1971) can lead to a significant reduction in fluke prevalence (Knubben-Schweizer et al., 2010; Knubben-Schweizer and Torgerson, 2015).

While the logical view would be to avoid grazing animals on pastures with high metacercarial populations in the late summer/early autumn, it may be acceptable to graze adult cattle on the high-risk areas at this time, when it would be dangerous to graze sheep. This is due to the relatively greater resilience of cattle than sheep to the immature stage of infection. Young cattle are susceptible to infection and, while acute disease is rare in cattle, it is not unheard of. Nevertheless, cattle grazed in this way should be treated before the flukes become adult (Forbes, 2017a, b).

The cost of infection can be high, so efficient control schemes can be cost-effective. However, precise figures are difficult to come by and can be extremely variable, depending on what parameter is being measured and used in the calculations. The parameters include reduction in milk yield, reduction in fertility, liver condemnation, reduction in weight gain and carcase weight. For example, for dairy cattle infected with *F. hepatica*, figures of €299, €6, €7.95 and US$430.7 per infected animal have been quoted in the literature (Schweizer et al., 2005; Charlier et al., 2012; Fanke et al., 2017; and Sariözkan and Yalçin, 2011, respectively). A comparative cost for *F. gigantica* infection is $12.11 (Wamae et al., 1998). For sheep, the cost of infection with *F. hepatica* has been estimated at £8.73 per infected animal (Sargison and Scott, 2011) and with *F. gigantica* at US$4.26 (Mungube et al., 2006).

### 5.3. Intermediate host control

As a result of asexual reproduction in the snail intermediate host, high numbers of cercariae can be produced from a single miracidium. This will undoubtedly promote the evolution and spread of clonal drug-resistant fluke isolates and populations (Fairweather, 2011b). Control strategies focused on the snail host aim to block transmission of disease. Options include the drainage of wet pasture, fencing off potential snail habitats, and use of molluscicides. Each option suffers from one or more drawbacks of being expensive, ineffective or environmentally unacceptable, and may fall foul of environmental protection directives relating to wildlife conservation and preservation of biodiversity, for example, also the run-off of fertiliser or slurry, as slurry may contain fluke eggs (Fairweather, 2011b; Knubben-Schweizer and Torgerson, 2015; Forbes, 2017a).

Environmental DNA (eDNA) assays have been developed for the detection of *F. hepatica* and snail hosts in potential snail habitats on farms in the UK (Jones et al., 2018) and Australia (Rathinasamy et al., 2018). The assays are highly specific, sensitive and allow for the independent detection of fluke and snail. They can be used to identify and monitor likely transmission zones in water bodies on farms and so inform the farmer as to infection risk. Strategies can then be implemented to minimise the exposure of livestock to infection. In turn, this may help to lower the dependence on drugs and help to conserve the usefulness of current drugs. The technique could be used to monitor risk of human infection as well (Jones et al., 2018; Rathinasamy et al., 2018). However, it should be acknowledged that the source of the eDNA is not clear, that is, whether it comes from fluke eggs, cysts or miracidia, nor does the test provide information on the viability of the infection.

### 5.4. Summary of section

A number of regimes have been recommended for the treatment of fasciolosis, in both cattle and sheep and, for the latter in particular, with the aim of protecting TCBZ activity as much as is practically possible. The switching of drugs (especially from TCBZ to CLOS: McMahon et al., 2016) when there is no evidence of TCBZ resistance and no rational reason for doing so, argues for better education of farmers.

Many of the farm management steps outlined above are fairly simple and straightforward to implement. It is important that farmers have a sound understanding of the biology and epidemiology of the disease and of the spectra of activity of the flukicides they use, together with the ability to apply that knowledge in the field, so that they can determine whether any treatment has been effective. The ultimate aim is to use the right flukicide at the right dose at the right time and for the right reason, in order to achieve fluke control. In an ideal world, we would look to get the best efficacy out of any treatment and using synergised flukicides offers one potential way to do that. Therefore, farmers need to seek advice from vets, so that they can design effective farm health plans that will address on-farm risk factors that are selective for resistance. Such factors cover drug use, the importation of drug-resistant flukes and intermediate host control. In relation to drug use, several practices have already been mentioned: the right choice of drug, its administration at the correct dosage, the timing of drug treatment, the frequency of drug treatment, drug rotation, the accurate weighing of animals to prevent under- or over-dosing, the storage of drugs and the calibration of dosing equipment. The movement of livestock may spread infection and drug-resistant parasites, so it is essential to have an effective quarantine strategy in place to prevent the importation of drug-resistant flukes. Restriction of access to snail habitats is also important. Implementation of a fluke monitoring system, with egg checks every 2 months, may help to determine the timing of dosing, in conjunction with fluke forecast information from organisations such as NADIS (National Animal Disease Information Service: https://www.nadis.org.uk/parasite-forecast.aspx) in the UK (Crilly et al., 2015). Advice on fluke control is available on the COWS (Control of Worms Sustainably) https://www.cattleparasites.org.uk and SCOPS (Sustainable Control of Parasites in Sheep) https://www.scops.org.uk websites.

Of course, all of these strategies and advice depend on accurate diagnosis and determination of drug efficacy, which leads into the next section.

## 6. Diagnosis

It is convenient to divide diagnosis of liver fluke infections into three categories: diagnosis of infection *per se*, determination of drug efficacy and detection of drug resistance. A number of tests are available, including faecal egg counts (FECs), serological and coprological methods, egg hatch assays, molecular techniques and histology; they will be discussed below.

### 6.1. Diagnosis of fasciolosis in livestock

#### 6.1.1. Faecal egg counts (FECs)

Historically, FECs and the faecal egg count reduction test (FECRT) have been the tests most widely used for the diagnosis of infection and for the determination of drug efficacy and drug resistance. Drug treatment is regarded as successful if there is a 90–95% or greater reduction in fluke FECs (typically 3 weeks pt) (cf Section 6.3). However, the test suffers from a number of limitations: it only detects patent infections (fluke do not lay eggs until they are ~8–10 weeks of age in the definitive ruminant host); egg shedding can be irregular; eggs may be stored for some time in the host gall bladder and their delayed release could lead to false positive FECs pt, even when the flukes have been removed by successful drug action; and FECs are not related to fluke

numbers (for references on individual points, see Flanagan et al., 2011a). Moreover, when fluke burdens are small and FECs low, inaccuracies of sampling could have a very big effect on the outcome, with a miscounting of just 1 or 2 eggs having an undue influence on the pre- or post-FEC.

Nevertheless, FEC tests are used because they are simple, convenient and are applicable to all anthelmintic classes. However, there is no standard protocol (for *Fasciola*) – for example, whether a flotation (in zinc sulphate) or sedimentation (in water) method should be used, or whether individual or composite samples should be examined. A more sensitive and accurate copromicroscopic method, the FLOTAC technique, has been developed for the detection and diagnosis of a wide range of helminth and protozoan infections of animals and humans. It has a sensitivity of 1 epg and can be used with individual or pooled samples and with fresh or preserved samples (Cringoli et al., 2010, 2017). With respect to *F. hepatica*, a modified zinc sulphate FLOTAC system has been used for large-scale on-farm surveys (Rinaldi et al., 2015) and for the assessment of drug efficacy (Cringoli et al., 2006; Keiser et al., 2008, 2010a; Duthaler et al., 2010; Meister et al., 2013). In a study with experimentally infected rats, the FLOTAC double technique (which allows two samples to be examined simultaneously) was shown to be more sensitive than a sedimentation method and sample processing time 5–6 times faster (Duthaler et al., 2010).

#### 6.1.2. Serodiagnosis

The serodiagnosis of *F. hepatica* infection has been thoroughly covered by the review of Rojas et al. (2014), so the reader is referred to that publication for details. Enzyme-Linked Immunosorbent Assays (ELISAs) can detect flukes at an early stage of infection – as early as 2 weeks pi, if not sooner – which is earlier than that for the coproantigen ELISA (cELISA) and much sooner than that for FECs. However, antibodies can continue to persist for some time after successful termination of infection, so the serological tests are unable to differentiate between a current and a previous infection. This factor makes them unsuitable for efficacy and resistance testing. Also, there are issues of cross-reactivity with circulating antigens from other helminth parasites, for example, the rumen fluke, *Calicophoron daubneyi* (Mazeri et al., 2016), and other infections.

Serodiagnosis also covers methods to determine the level of liver enzymes such as glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (GGT) and other blood parameters. This information will help to assess the impact of infection on the health of the individual animal and help in determining whether treatment is required.

A bulk tank milk ELISA has been used to survey for fluke prevalence in dairy herds across regions and across seasons (eg Byrne et al., 2018). It may have greater value in areas of relatively low fluke prevalence, than in areas where the levels of fluke infection are high.

#### 6.1.3. The coproantigen ELISA (cELISA)

This is based on the monoclonal (mAB) MM3 assay first described by Mezo et al. (2004). It has been developed into a commercial kit – the BIO K201 ELISA kit (Bio-X Diagnostics, Jemelle, Belgium) – and the kit has been used in numerous studies since its commercialisation in 2007. The antibody probably recognises a cathepsin-type enzyme, as immunolabelling is restricted to the gastrodermal cells of the fluke (Flanagan et al., 2011a; Muiño et al., 2011; Kajugu et al., 2012; Gordon et al., 2013). The ELISA is very specific for *F. hepatica*, not cross-reacting with other trematode species, cestodes, gastrointestinal nematodes or coccidian infections (Kajugu et al., 2012, 2015; Gordon et al., 2013). It is highly sensitive, being capable of detecting infections of as few as one fluke in sheep and cattle (Mezo et al., 2004; Martínez-Sernández et al., 2016). The antigens only persist for the lifetime of the infection, so are indicative of current infection. Other advantages of the cELISA are that it is non-invasive and farmers can submit samples without requiring a visit from the vet. Coproantigens can be detected from 5 to 6 weeks pi, a time that corresponds to the entry of the flukes into the bile ducts (Mezo et al., 2004; Flanagan et al., 2011a, b; Brockwell et al., 2013; Calvani et al., 2018). Although this is later than the reported detection of circulating antigens or detection by molecular methods, it is earlier than the detection of eggs at patency. The difference may be 2 weeks (Flanagan et al., 2011a, b; Brockwell et al., 2013; Calvani et al., 2018) but can be longer, up to 5 weeks (Valero et al., 2009; Martínez-Pérez et al., 2012). The cELISA has proved to be a more convenient and sensitive assay than FECs and serological tests (Flanagan et al., 2011b; Gordon et al., 2012b; Brockwell et al., 2013; Robles-Pérez et al., 2013; Arifin et al., 2016; Calvani et al., 2018). There is a strong correlation between cELISA data and fluke burdens (Mezo et al., 2004; Brockwell et al., 2013, 2014; Elliott et al., 2015; George et al., 2017). Although it is preferable to use the cELISA with individual samples, it can also be used with bulk pooled faecal samples, which would reduce the cost of sampling for the farmer (Brockwell et al., 2013; Elliott et al., 2015). The assay is suitable for use with sheep, cattle and deer (French et al., 2016), but not horses (Palmer et al., 2014).

The assay has been validated with experimental infections (Flanagan et al., 2011a, b; Brockwell et al., 2013; Robles-Pérez et al., 2013; George et al., 2017; Calvani et al., 2018). It also works well under natural (field) conditions (Gordon et al., 2012a; Novobilský et al., 2012, 2016; Robles-Pérez et al., 2013; Brockwell et al., 2014; Elliott et al., 2015; Hanna et al., 2015; Novobilský and Höglund, 2015; Arifin et al., 2016). There has been increasing use of the cELISA as a diagnostic tool, and not simply for diagnosis of current infection, as it has been applied to the determination of drug efficacy and, by extension, to the diagnosis of drug resistance. While (understandably) the test has been used mainly for the determination of TCBZ efficacy, it has also been used to evaluate the efficacy of ABZ, CLOS, CLORS, NITROX, OXYCLO (Flanagan et al., 2011a, b; Novobilský et al., 2012, 2016; Brockwell et al., 2013, 2014; Robles-Pérez et al., 2013; Elliott et al., 2015; Hanna et al., 2015; Novobilský and Höglund, 2015; George et al., 2017, 2019). It has also assisted in the diagnosis of resistance to TCBZ (Flanagan et al., 2011a, b; Gordon et al., 2012a, b; Robles-Pérez et al., 2013; Brockwell et al., 2014; Elliott et al., 2015; Hanna et al., 2015), ABZ (Novobilský et al., 2012, 2016; Robles-Pérez et al., 2013) and CLOS (Novobilský and Höglund, 2015).

Modifications and improvements have been made to the ELISA kit produced commercially and to the technique in individual laboratories; they include overnight antigen extraction and host species-specific cut-off values (Brockwell et al., 2013, 2014; Palmer et al., 2014; Elliott et al., 2015; Martínez-Sernández et al., 2016). Further work may be required to optimise the protocol for field use. A recent study has raised concerns about the reliability of the cELISA under field conditions, in situations where mixed age infections are likely to be present, and especially if the fluke population is largely immature (George et al., 2017). This potential limitation can be overcome by carrying out a second test at least 6 weeks after the first one; this would allow the immature flukes to develop and be recognised. A recent field investigation by George et al. (2019) has shown that the diagnostic sensitivity for epidemiological studies can be increased if the cELISA and FEC methods are used together and ideally in parallel.

#### 6.1.4. Egg hatch assay

Egg formation, production, development and viability are vital for the transmission of disease and are known to be sensitive to drug action (eg Toner et al., 2011; McConville et al., 2012; Hanna, 2015; O'Neill et al., 2015). A large proportion of the fluke's body and metabolic budget are given over to reproduction, to maintain a phenomenally high rate of egg production, estimated at 25,000 eggs per fluke per day in a light infection (Happich and Boray, 1969; Fairweather et al., 1999). The egg is probably the most accessible stage in the life cycle for collection and experimentation. A number of protocols have been devised to study the impact of drug action on the development and hatching of fluke eggs, but there is no standardised method. Results of studies have been inconsistent, no doubt due to the methodological variations between them. For example, differences in terms of the source of the eggs (whether from the faeces, gall bladder or directly from the fluke itself); whether the eggs are collected from faeces following treatment *in vivo* or are incubated in drug *in vitro* without any prior exposure to drug; the

length of incubation, either a relatively short, 12 h exposure to the drug, or a longer incubation, between 8 d and 15 d (this is important, as drug sensitivity can change over time: Ceballos et al., 2019); the range of concentrations used; and which form of the drug is used, either the commercial drench diluted with a solvent such as DMSO, or the parent drug or its metabolite(s). The choice of parent compound or metabolite is perhaps most relevant for BZ-type compounds, to best reflect which form of the drug the fluke is likely to be exposed to *in vivo*. Most Egg Hatch Tests (EHTs) have been carried out with BZs: TCBZ/TCBZ.SO (Alvarez et al., 2009; Fairweather et al., 2012; Arafa et al., 2015); ABZ/ABZ.SO (Coles and Briscoe, 1978; Alvarez et al., 2009; Canevari et al., 2014; Robles-Perez et al., 2014; Arafa et al., 2015; Novobilský et al., 2016; Pereira et al., 2016; Nwofor et al., 2018; Ceballos et al., 2019); OXF (Alvarez et al., 2009); and MBZ (Coles and Briscoe, 1978; Alvarez et al., 2009). Other drugs tested are CLOS (Solana et al., 2016; Ceballos et al., 2017), NITROX (Hegazi et al., 2018) and OXYCLO, although OXYCLO was shown to have no effect on the development and hatching of *F. gigantica* eggs (Arafa et al., 2015). EHTs are perhaps most appropriate for BZs, as their lipophilicity facilitates penetration through the eggshell. The assays can discriminate between drug-susceptible and drug-resistant fluke isolates and, therefore, they have the potential to be used for the diagnosis of drug resistance (eg Fairweather et al., 2012; Canevari et al., 2014; Novobilský et al., 2016; Ceballos et al., 2019).

#### 6.1.5. Molecular methods

Molecular methods, including the polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA), have been developed for the detection of *F. hepatica* infections in faecal samples from sheep and cattle. The techniques typically target the cytochrome C oxidase 1 (Cox1) gene or the second internal-transcribed spacer (IST2) region within the *Fasciola* nuclear ribosomal DNA (rDNA).

A nested-PCR, based on faecal samples, has been shown to be more sensitive than a standard PCR in enabling detection of infection at 2 weeks pi, a week earlier than that with the latter technique, though the source of fluke DNA at this time is open to question (Martínez-Pérez et al., 2012). With the LAMP method, the detection time can be even earlier, at 1 week pi (Martínez-Valladares and Rojo-Vázquez, 2016). The LAMP method has been shown to be highly sensitive, being capable of detecting a single *F. hepatica* egg in a spiked faecal sample. It is very specific, too, there being no cross-amplification of *F. gigantica*, *D. dendriticum* or *Taenia saginata* DNA (Ghodsian et al., 2019). The technique is very sensitive, isothermal, doesn't require sophisticated equipment and the results are more consistent than with equivalent PCR; also, it has potential for environmental sampling (Skuce, unpublished observations). The RPA method has advantages over PCR in terms of sensitivity and speed and avoids the need for expensive equipment and highly trained personnel (Cabada et al., 2017). Generally, detection of infection by molecular techniques is earlier than that achieved with other methods (eg FECs and cELISA), but this may not always be the case (Calvani et al., 2018). Also, the sensitivity of molecular methods is greater than that of conventional methods (Martínez-Pérez et al., 2012; Robles-Pérez et al., 2013; Cabada et al., 2017), but there have been some inconsistent results (Arifin et al., 2016; Calvani et al., 2018). The discrepancies may be due to the method of DNA extraction procedure and/or the way in which the faecal material is processed, so further improvement and development of the extraction method may be required to provide a more reliable test (Arifin et al., 2016).

eDNA assays for the detection of fluke have been mentioned previously, in Section 5.3.

#### 6.1.6. Histology

The histological approach is a post-mortem test designed to evaluate changes in the reproductive organs of flukes following drug treatment (Hanna, 2015). The organs occupy much of the fluke's body and display a rapid rate of cellular turnover to meet the demands of a high egg output. This makes them uniquely sensitive to drug action. The histological methods used include the staining of whole fluke preparations and sections, immunocytochemistry and *in situ* hybridisation. With these methods, large numbers of flukes can be processed and examined very easily, all tissues in a sample can be screened simultaneously and quantitative data can be generated for analysis. Although histology by itself cannot be used to establish a diagnosis of drug resistance, it can be used to complement and support the results of other methods (eg FECRT and CRT). For examination of field cases, it is important to collect material within 3 days of treatment; flukes should be collected from freshly dead carcases and fixed straightaway with neutral buffered formalin (Hanna et al., 2010, 2013, 2015). Histology has also been of value in complementing ultrastructural data to understand drug actions and mechanisms of resistance.

### 6.2. Diagnosis of fascioliasis in humans

Coprological and serological methods for the diagnosis of

**Table 4**
Efficacy percentages in reports of drug resistance in *Fasciola hepatica* and *Fasciola gigantica*.

| Efficacy (%) | TCBZ | ABZ | CLOS | CLORS | RAFOX |
|---|---|---|---|---|---|
| 91-100 | | | | | |
| 81–90 | (R13)$^{S}$; (R21)$^{C}$ | (R28)$^{C}$ | | | (R20)$^{C}$ |
| 71–80 | (R4)$^{S}$; (R10)$^{S}$; (R21)$^{C}$; (R26)$^{C}$ | (R20)$^{C}$; (R26)$^{C}$ | (R25)$^{C}$ | (R4)$^{S}$; (R18)$^{S}$ | |
| 61–70 | (R6)$^{S}$; (R13)$^{S}$; (R16)$^{C}$ | (R5)$^{C}$; (R18)$^{S}$; (R27)$^{S}$ | | | (R5)$^{C}$ |
| 51–60 | (R7)$^{S}$; (R13)$^{S}$; (R31)$^{C}$ | | | | |
| 41–50 | | (R4)$^{S}$; (R28)$^{C}$; (R29)$^{S}$ | | (R22)$^{S}$ | |
| 31–40 | (R2)$^{C}$; (R8)$^{S}$; (R12)$^{C}$; (R13)$^{S}$; (R14)$^{S}$; (R17)$^{C}$; (R24)$^{S}$ | | | | |
| 21–30 | (R1)$^{S}$; (R15)$^{S}$; (R17)$^{S}$ | (R19)$^{S}$; (R29)$^{S}$ | | | |
| 11–20 | (R2)$^{S}$; (R9)$^{S}$; (R13)$^{S}$; (R14)$^{S}$; (R21)$^{C}$ | (R8)$^{S}$ | | | |
| 0–10 | (R2)$^{C}$; (R3)$^{S}$; (R11)$^{C}$; F$^{2}$ (R13)$^{S}$; F$^{2}$ (R16)$^{C}$; (R23)$^{C}$; F$^{4}$ (R24)$^{S}$; F$^{9}$ (R30)$^{S}$ | (R8)$^{S}$; (R12)$^{C}$; (R22)$^{S}$; F$^{2}$ (R29)$^{S}$ | (R25)$^{C}$ | | |

ABZ, albendazole; CLORS, clorsulon; CLOS, closantel; RAFOX, rafoxanide; TCBZ, triclabendazole.
F$^{n}$, number of farms involved (NB where F not included, n = 1); (Rn), reference number; $^{C}$, cattle; $^{S}$, sheep; %, percentage.
References: R1, Overend and Bowen (1995); R2, Moll et al. (2000); R3. Borgsteede et al. (2005); R4, Álvarez-Sánchez et al. (2006); R5, Elitok et al. (2006); R6, Oliveira et al. (2008); R7, Mooney et al. (2009); R8, Mamani and Condori (2009); R9, Flanagan (2010); R10, Martínez-Valladares et al. (2010); R11, Olaechea et al. (2011); R12, Chávez et al. (2012); R13, Daniel et al. (2012); R14, Gordon et al. (2012b); R15, Hassell and Chapman (2012); R16, Rojas (2012); R17, Ortiz et al. (2013); R18, Robles-Pérez et al. (2013); R19, Sanabria et al. (2013); R20, Shokier et al. (2013); R21, Brockwell et al. (2014); R22, Martínez-Valladares et al. (2014); R23, Elliott et al. (2015); R24, Hanna et al. (2015), McMahon et al. (2016); R25, Novobilský and Höglund (2015); R26, Venturina et al. (2015); R27, Novobilský et al. (2016); R28, Nzalawahe et al. (2018); R29, Ceballos et al. (2019); R30, Kamaludeen et al. (2019); R31, Romero et al. (2019).

fascioliasis in humans have been comprehensively dealt with by Mas-Coma et al. (2014). It may be useful to highlight just a few examples of techniques that may find greater use in the future in epidemiological and community surveillance surveys:

- the development of a lateral flow immunoassay (LFIA) (the SeroFluke test), based on the use of a recombinant cathepsin L1 protein (Martínez-Sernández et al., 2011). It is a rapid, simple and inexpensive technique that doesn't require highly trained personnel; it has a very high sensitivity and specificity; it can be used with both serum and whole blood samples, and can be applied to both the acute and chronic phases of the disease;
- the development of a recombinant saposin-like protein-2 antigen-proteinA/ProteinG-alkaline phosphatase-conjugate-ELISA (recSAP2-PAG-AP-ELISA) for the routine serodiagnosis of fascioliasis (Gottstein et al., 2014). It is a very specific, sensitive and accurate method;
- the MM3-COPRO ELISA, with its high sensitivity and specificity, has been shown to perform well in a large-scale community survey under field conditions in Bolivia and Peru (Valero et al., 2012); and
- the RPA method, as mentioned above (Section 6.1.5.), has been shown to be a rapid, highly sensitive and specific molecular test for the detection of chronic human *F. hepatica* infection in stool samples (Cabada et al., 2017).

### 6.3. Summary of section

Many tests are available for the diagnosis of liver fluke infections in livestock and humans, each with its own advantages and disadvantages and each with a protocol that varies from laboratory to laboratory. This is sufficient for determining whether an animal or patient is infected or not, but when it comes to evaluating drug efficacy, it means that there is no standardised protocol, which is a matter of concern. Of greater concern is the lack of any guidelines or validated tests when it comes to the diagnosis of drug resistance. This situation needs to be addressed. Perhaps the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) could draw up guidelines for fluke, in the same way that it did for gastrointestinal nematodes (Coles et al., 1992, 2006; Wood et al., 1995).

It is worthwhile to examine the data in Tables 1 and 2 in this review, along with that in Table 2 in Kelley et al. (2016) and Table 6 in McMahon et al. (2016). The majority of the studies included in the Tables were carried out on 1 or 2 farms, so large-scale prevalence studies have not been the norm, most likely for resource or logistical reasons. Consequently, the true prevalence of TCBZ resistance is unknown. It is notable that the test most widely used was the FECRT, despite its limitations. Indeed, in 41% of the studies, it was the only test used to "diagnose" TCBZ resistance. The veracity of diagnosis can be questioned in some of the studies, despite them appearing in the literature. On their own, FEC data are not entirely reliable, especially when fluke burdens are low, and the results can be inconsistent when set against the results of more accurate tests such as the cELISA (Kajugu et al., 2015). Nor can they be used to unequivocally confirm a diagnosis of drug resistance. More sensitive and accurate tests are available or are being developed and could be used more often. As one example, it is encouraging to see greater application of the coproantigen reduction test (CRT), which has been utilised in just over one-third (35%) of studies since 2010.

The controlled efficacy test (CET), or "dose and slaughter trial", is the most reliable diagnostic method for the detection of resistance and was described as the only test available for fluke by Coles et al. (2006). However, it is not always practicable or economic to perform in the field: it was carried out in less than one-fifth of the studies.

For drug resistance, it is critical to know what you are dealing with, so that appropriate control measures can be put in place. Therefore, for a diagnosis, it is important that it is based on the results of more than one test, that is, any initial indication or suspicion of reduced drug efficacy or treatment failure should be followed up and confirmed by additional tests (Fairweather, 2011a, b, c). That should be the aspiration, though it has to be accepted that there might not always be the time or resources to carry it out in practice.

Perhaps the most significant variable with regard to the evaluation of drug resistance is where to set the cut-off value of reduced efficacy that will indicate a potential case of resistance. Percentage efficacy information from the 39 entries in Tables 1 and 2 has been collated in Table 4. A 95% threshold was used in 7 studies, 90% in another 9, and no level mentioned in the rest (23 studies). Whilst the studies listed in Table 4 have

**BOX 1**
Definitions of anthelmintic efficacy and resistance.

**Anthelmintic Efficacy**.

"A quantitative measure of the effectiveness of a drug intended to produce a desired effect" (Vidyashankar, 2012).

Classification scheme for evaluating efficacy: >98% (highly effective); 90–98% (effective); 80–89% (moderately effective); <80% (insufficiently effective). This scheme is applicable to trematodes (Wood et al., 1995).

**Anthelmintic Resistance**.

Anthelmintic resistance has been defined in a variety of ways, eg:

"Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of a compound than in a normal population of the same species and is heritable" (Prichard et al., 1980; used by Coles et al., 1992, 2006 for the WAAVP methods for detection of anthelmintic resistance in nematodes of veterinary importance).

"Anthelmintic resistance can be defined as a heritable change in susceptibility to an anthelmintic in a population of parasitic nematodes such that a dose which normally provides ≥95% clearance of adult worms provides ≤80% clearance" (WAAVP Guideline for evaluation of anthelmintic combination products targeting nematodes: Geary et al., 2012).

Alternative wording: "Resistance can be attributed to a population of a parasite species that exhibits substantial reductions in efficacy (eg to ≤80%) when treated with a dose of the anthelmintic which is historically ≥95% efficacious against that species (based on adequate evidence from worm counts and/or faecal egg count reductions)" (Geary et al., 2012).

Anthelmintic resistance: "The ability of parasites to survive doses of drugs that would normally kill parasites of the same species and stage. It is inherited and selected for because the survivors of drug treatments pass genes for resistance on to their offspring. These genes are initially rare in the population or arise as rare mutations, but as selection continues, the proportion of resistance genes in the population increases as does the proportion of resistant parasites" (European Medicines Agency, 2017).

https://www.ema.europa.eu/en/documents/scientific-guideline/reflection-paper-anthelmintic-resistance_en-1.pdf.

NB Most, if not all, of the definitions of anthelmintic resistance refer to nematode parasites.

**Cut-off values**.

Nematodes: 95% (Coles et al., 1992, 2006). Values of 90% and 80% have also been applied (Vidyashankar et al., 2012; Kaplan, 2002).

Cestodes and trematodes: no values have been standardised, although a value of 90% is often used for liver flukes (Fairweather and Boray, 1999).

undoubtedly provided very valuable data, it is almost impossible to compare any of them directly due to the variability of experimental design, protocols, data analyses and interpretations. Again, this highlights the need for standardisation of techniques and production of guidelines for investigating drug resistance on farms. This is not a simple task, however, as a number of important factors must be taken into account:

- Some reports of "resistance" shown in Table 4 (e.g. 71–80% and 81–90% efficacy) could be regarded as within the normal efficacy range of the drug, especially the non-TCBZ drugs such as ABZ and OXYCLO. Thus, cut-off values for reporting resistance may need to be tailored for individual drugs.
- What is actually meant by "efficacy"? Efficacy has been defined as "a quantitative measure of the effectiveness of a drug intended to produce a desired effect" (Vidyashankar, 2012). However, the "true" efficacy of a drug (i.e. the effective level when first introduced) versus "observed" efficacy (i.e. the efficacy at the time of treatment) may not be the same.
- What is actually meant by "resistance"? Definitions of anthelmintic resistance are given in Box 1.

If a drug is reported to be 80% effective, does this mean it is removing 80% of flukes in a homogeneous population, or is it fully effective against the 80% sub-population that is susceptible, but not against the other 20% that is resistant? Given the high levels of genetic variation that are known to exist in fluke populations - within the same host and between infra-populations in the same flock/herd and also between populations on different, even closely separated farms - the latter scenario may be more likely. Indeed, fluke populations can change quickly over time and the occurrence of aspermic triploid forms in the wild, with their inherent potential for the parthenogenetic production of eggs, would conceivably allow the rapid evolution of clonal populations and the rapid evolution of anthelmintic resistance (Fletcher et al., 2004; Walker et al., 2007, 2011; Hanna et al., 2011; Beesley et al., 2017).

- What are we actually aiming for by using drugs? Is the total elimination of flukes an unrealistic target? Yes, it is a practical impossibility. A significant reduction in fluke burden might be a more acceptable and achievable goal, especially if this is sufficient to reduce production impacts, ameliorate any pathological effects and reduce subsequent transmission. In this regard, a drug with 80% efficacy (or lower) could still be beneficial.

## 7. Concluding remarks

The ultimate goal of any parasite control programme is to manage the parasite population so that it never exceeds a level that is having major welfare and/or economic impacts. In order to allow animals to be at low risk of disease and to perform well in the face of parasitism, farmers depend on sound advice from veterinarians and other advisors, to design an effective management plan that is tailored to the needs of the individual farm. In turn, this relies on accurate local forecasting systems, monitoring infection sources and reliable diagnosis of infection. Each farm, field and year is potentially different, which makes the task a demanding one. So, training of advisors is key, to avoid farmers following poor management practices, in particular using inappropriate drugs and at the wrong time.

A number of reviewers have raised important questions and identified research needs to stimulate and direct future research (Kelley et al., 2016; Beesley et al., 2018; Sabourin et al., 2018). They have stressed the need to determine the true prevalence of resistance to TCBZ and other flukicides in different areas of the world and to what extent resistance has permeated human populations. Logistically, this would be challenging to accomplish, even on a small scale, especially bearing in mind that surveillance for fluke is not routinely carried out. Given the number of anecdotal reports and unverified claims that exist in the literature, accurate diagnosis is essential. In the absence of a single recognised method, it is probably best to use more than one technique to confirm resistance and so deliver a more robust diagnosis.

This review has dealt with the scientific work relating to the mechanisms of drug action and drug resistance. The mode of action of TCBZ is still not clearly understood, despite there being some credible options, and it may involve more than one target. This concept has been discussed by Geary et al. (2015). It is true for drugs, such as PZ, the drug of choice for the treatment of clonorchiasis and opisthorchiasis infections, as well as for schistosomiasis (Keiser and Utzinger, 2009; Cupit and Cunningham, 2015; Vale et al., 2017; Thomas and Timson, 2018). The situation (for TCBZ) is complicated further by the contribution made by different metabolites to drug action (Fairweather, 2009, 2011b). In turn, the potential multifactorial nature of drug action may hinder attempts to determine the mechanism of resistance, which may have a polygenic basis as well (see Section 3), and this may well impact on efforts to identify and develop molecular markers for resistant fluke populations. However, the real priority here should be the identification and confirmation of genuine resistance to whichever drugs are being used.

Kelley et al. (2016) have identified 3 stages for future fluke control in livestock and that is a constructive way to view the outlook. **Stage 1** (the short-term) entails the better delivery of advice to farmers which, as indicated above, involves the training of expert personnel to pass on good, clear, simple and consistent advice. It is envisaged that **Stage 2** (the medium-term) will lead to the development of a new drug with a similar efficacy range to TCBZ. It is evident from this review that much work has been carried out in academic laboratories on the potential use of drug combinations, on the search for new drugs and on the development of new drug formulations. To further this work, Geary et al. (2015) have argued the need for a far greater understanding of basic parasite biology. It is essential that parasitologists, expert in narrow disciplines and sophisticated technologies, do not lose sight of the parasite as a whole and its interaction with the environment inside its host – and what happens in the field. It is imperative that academic initiatives receive input from the pharmaceutical industry to pick up and develop any promising leads further, in order to bring suitable compounds to market. Finally, development of an effective vaccine is seen as the long-term goal (**Stage 3**).

This review is dedicated to Joe Boray. If we are to learn from his example and focus on improving disease management at the farm level, it is important that any advances made in academic or industry laboratories are communicated down to vets and advisors so that they are better informed. After all, it is they who are in the best position to ensure that farmers make the right decisions to deal with the disease and conserve the efficacy of the anthelmintics at our disposal for as long as possible. This would be a fitting legacy for a truly great parasitologist.

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## Glossary of drug abbreviations used in the review

| | |
|---|---|
| ABZ | albendazole |
| ABZ.SO | albendazole sulphoxide |
| BZ | benzimidazole |
| CLORS | clorsulon |
| CLOS | closantel |
| IVM | ivermectin |
| KTZ | ketoconazole |
| LEV | levamisole |

| | |
|---|---|
| LUX | luxabendazole |
| MBZ | mebendazole |
| ML | macrocyclic lactone |
| MOX | moxidectin |
| MTZ | methimazole |
| NITROX | nitroxynil |
| OXF | oxfendazole |
| OXYCLO | oxyclozanide |
| PZ | praziquantel |
| RAFOX | rafoxanide |
| TCBZ | triclabendazole |
| TCBZ.SO | triclabendazole sulphoxide |
| $TCBZ.SO_2$ | triclabendazole sulphone |

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