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Review

Triclabendazole progress report, 2005–2009: an advancement of learning?

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Abstract

Triclabendazole (TCBZ) remains the drug of choice for treating infections of the liver fluke, *Fasciola hepatica* in livestock and has become the main drug used to treat human cases of the disease as well. Cases of resistance in livestock continue to be reported, suggesting that the problem is increasing. In order to address the problem, there is a need for better understanding of drug action. A 'state-of-play' review on different aspects of TCBZ activity was published by the present author in 2005. The main purpose of the current review is to assess what progress has been made in the past four years towards understanding the main aspects of drug activity, including drug pharmacokinetics and pharmacodynamics and an understanding of the mechanism(s) of resistance. Also, what advances have been made in identifying alternative compounds and using drug combinations to enhance TCBZ activity. Stemming from a number of *in vivo* studies, it has become evident that fluke isolates of differing sensitivity to TCBZ differ in some of their biological parameters, and information on this interesting phenomenon will be presented. An update on the use of TCBZ for human fascioliasis is also given. The review will indicate what progress has been made, but will also highlight areas that remain inadequately understood and require greater research focus.

Introduction

Fasciolosis, caused by the liver fluke, *Fasciola hepatica*, is an extremely important disease of livestock in temperate areas of the world. In recent years, the disease has undergone a sharp rise, which has been attributed to climate change. The human form of the disease has also become a major public health problem in several parts of the world. Triclabendazole (TCBZ) (marketed as Fasinex®) has become established as the main drug used to treat fluke infections in ruminants since its introduction in the early 1980s, due to its high efficacy against all stages of infection in the mammalian host. It is now the drug of choice for human fascioliasis as well, marketed as Egaten®. This apparently rosy scenario is being threatened by the development of resistance among fluke populations, first reported in Australia, but now present in several countries in western Europe. The situation is one of concern, given the over-reliance on a single drug and the zoonotic potential of the disease. There is a need to learn more about the activity of TCBZ and the epidemiology of the disease, to counter the threat of resistance.

Following the Second Ken Mott Symposium at EMOP IX in Valencia, 2004, I wrote a review on TCBZ (Fairweather, 2005). The current review, stemming from last year's Symposium (the Third Ken Mott Symposium) held at EMOP X in Paris, 2008, is intended primarily to provide an update in our understanding of several aspects of TCBZ activity, based largely on research published since 2004. For earlier studies, the reader is referred to my previous review (Fairweather, 2005) and the review by Keiser *et al.* (2005). The main topics that will

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be discussed below are: TCBZ pharmacokinetics; the mechanisms of action and resistance; biological differences between fluke isolates of known susceptibility to TCBZ; the development of alternative drugs and control strategies; and the use of TCBZ for the treatment of human fascioliasis. Information on the unusual chemical structure and narrow spectrum of activity of TCBZ was covered previously and the information will not be repeated here.

TCBZ pharmacokinetics

The basic pattern of biotransformation of TCBZ in the ruminant host was established by Hennessy *et al.* (1987). Briefly, TCBZ is completely removed from the portal blood by the liver and cannot be detected in the plasma. It is oxidized to the sulphoxide (TCBZ.SO) and sulphone (TCBZ.SO_2) metabolites, which are the main metabolites present in the plasma. Hydroxylation of TCBZ and its two metabolites takes place in the liver, too, giving rise to the corresponding hydroxy metabolites, which are excreted in the bile (Hennessy *et al.*, 1987). The flavin monooxygenase (FMO) pathway is the main pathway involved in the conversion of TCBZ to TCBZ.SO, while it contributes equally with the cytochrome P450 (P450) enzyme system to the sulphonation of TCBZ.SO to TCBZ.SO_2 (Mottier *et al.*, 2004; Virkel *et al.*, 2006). It has been shown that the rumen microflora are capable of carrying out the sulphoreduction of TCBZ.SO and OH-TCBZ.SO to TCBZ and OH-TCBZ, respectively, suggesting that the rumen can act as a reservoir of TCBZ compounds. This could serve as a slow-release system for the further availability of TCBZ in the digestive tract, from where it could be absorbed and passed to the liver (Virkel *et al.*, 2006). TCBZ can also be oxidized to TCBZ.SO by digestive microflora prior to its absorption or by the intestinal wall during absorption (Mestorino *et al.*, 2008). It is evident, then, that the mechanisms of TCBZ metabolism are complex, but serve (together with the strong binding to plasma proteins) to maintain active concentrations of TCBZ compounds in the host for considerable periods of time, and this undoubtedly enhances drug efficacy.

A recent study compared the pharmacokinetics of TCBZ in sheep and cattle (Mestorino *et al.*, 2008). Parameters for TCBZ.SO were similar in the two species, although maximum blood levels were reached later in cattle (30 h as against 22 h in sheep). However, the data were very different for TCBZ.SO_2, which reached a higher peak concentration and persisted at higher levels for longer in cattle (Mestorino *et al.*, 2008).

The studies on enzyme pathways cited above (Mottier *et al.*, 2004; Virkel *et al.*, 2006) were carried out *in vitro* with liver microsomes. A follow-up experiment has been conducted by the same group, to determine whether co-administration of TCBZ with metabolic inhibitors would alter the systemic availability of TCBZ metabolites in a natural host (the sheep). Methimazole (MTZ, an FMO inhibitor) did not affect TCBZ disposition kinetics *in vivo*, although it inhibited both TCBZ.SO and TCBZ.SO_2 formation *in vitro* (Virkel *et al.*, 2009). This may have been due to its rapid elimination from the body after the intravenous administration route used in the study, rather than the more typical oral route. In contrast, co-administration with the P450 inhibitors, piperonyl butoxide (PB) and ketoconazole (KTZ) lead to an increase in the maximum blood level of TCBZ.SO and greater plasma availability of this metabolite. The maximum plasma concentration and bioavailability of TCBZ.SO_2 were enhanced following co-administration of TCBZ with PB, but not with MTZ or KTZ (Virkel *et al.*, 2009). So, the experiment showed that it is possible to enhance the availability of TCBZ metabolites, which would extend the exposure of the fluke to the drugs and lead to an improvement in the efficacy of TCBZ.

Triclabendazole is marketed in combination with other anthelmintics and there may be interactions between the drugs that affect its pharmacokinetics. One such combination is TCBZ plus ivermectin. Ivermectin itself has no activity against trematodes such as *Fasciola* (Shoop *et al.*, 1995), but a recent study has shown how it can affect the disposition of TCBZ and its metabolites (Lifschitz *et al.*, 2009). Thus, the systemic availability of TCBZ was reduced, but the maximum plasma levels of TCBZ.SO and TCBZ.SO_2 were enhanced and the plasma availability of the two metabolites was increased for the first 12 and 24 h, respectively (Lifschitz *et al.*, 2009). The relevance of this observation to dealing with the problem of drug resistance will be dealt with later in this review.

Impairment of drug metabolism in heavily fluke-infected animals has been advanced as a possible explanation for product failure and mis-diagnosis of TCBZ resistance. This idea has not been tested in livestock, but a study on patients in Egypt has shown that fluke infection did not affect either TCBZ pharmacokinetics or drug efficacy (El-Tantawy *et al.*, 2007). The level of infection was not determined, so it is not possible to know how much liver damage can be tolerated before drug metabolism is compromised.

Pharmacodynamics and drug action

The extensive metabolism of TCBZ by the host means that (potentially) the adult fluke is exposed to a number of different forms of TCBZ. Moreover, each of the compounds is capable of entering the fluke via diffusion, entry being closely related to their lipophilicity. TCBZ, TCBZ.SO and TCBZ.SO_2 demonstrate a similar ability to diffuse into the fluke and their level of diffusion is higher than that for the corresponding hydroxyl compounds (Mottier *et al.*, 2004, 2006a).

Entry of TCBZ compounds into the fluke has been shown to take place principally by means of diffusion across the tegument, rather than by oral ingestion, a result that is surprising, perhaps, given the strong binding of the metabolites to plasma proteins. Two approaches have been used to confirm this idea, one pharmacological, one morphological. Both made use of flukes that had been ligatured to prevent oral entry of drug. Following incubation in TCBZ.SO, its concentration in the fluke was similar, irrespective of whether the fluke had been ligatured or not. When an excess of bovine serum albumin (BSA) was added to the incubation medium, in order to allow most of the drug to bind to it, the concentration of TCBZ.SO was reduced (by 85%) in both

ligatured and non-ligatured flukes (Mottier *et al.*, 2006a). A parallel morphological study has been carried out, to compare drug-induced changes to the tegument and gut following incubation with TCBZ.SO. Disruption to the tegument, as assessed by scanning electron microscopy (SEM), was similar in ligatured and non-ligatured flukes, indicating that restricting the oral uptake of drug does not affect the ability of TCBZ.SO to enter the fluke and exert its effect (Toner *et al.*, 2009). Incubation with TCBZ.SO in the presence of an excess of BSA led to a reduction in the level of tegumental disruption. In all experiments, the gut remained unaffected by TCBZ.SO action, suggesting that the oral uptake of drug plays only a (very) minor role in drug entry (Toner *et al.*, 2009). The results of the two studies complement each other.

In terms of drug action, the fluke is known to play a more active role than simply being subject to the passive uptake of drug and its diffusion to the site of action, as it has been shown to be capable of metabolizing TCBZ to TCBZ.SO andTCBZ.SO to TCBZ.SO_2 (Mottier *et al.*, 2004; Robinson *et al.*, 2004a).

It is clear from what has been described above, that the fluke is exposed to a number of different forms of TCBZ. Since TCBZ.SO is the main metabolite present in both blood plasma and bile, it has been assumed to be the active form of TCBZ and even the *only* active metabolite. However, after the initial (24–30 h) exposure to TCBZ.SO, the fluke will be exposed to TCBZ.SO_2 for a prolonged period of time (Hennessy *et al.*, 1987). Moreover, TCBZ.SO_2 has been shown to have some activity in its own right *in vivo*: it caused a 41% reduction in worm burden against a juvenile fluke infection in sheep (Büscher *et al.*, 1999). In addition, TCBZ.SO_2 has been shown to be capable of binding to fluke tubulin, in a colchicine binding assay (Fetterer, 1986). A recent study has been carried out *in vitro* to compare the action of TCBZ, TCBZ.SO and TCBZ.SO_2 against *F. hepatica*. It involved the use of SEM (for surface changes) and transmission electron microscopy (TEM, for internal changes) to determine the relative disruption to the tegument caused by the three compounds (Halferty *et al.*, 2009). The level of surface disruption induced by the three compounds varied from region to region, and overall was similar, but that caused by TCBZ was slightly greater than that produced by the two metabolites. Internal changes observed were greatest following treatment with TCBZ.SO_2 and, while TCBZ.SO was also disruptive, TCBZ was far less disruptive. Combining the results for surface and internal changes, the order of severity of disruption was TCBZ.SO_2 > TCBZ.SO > > TCBZ (Halferty *et al.*, 2009). So, TCBZ.SO_2 may well contribute to drug action *in vivo* and is not the inactive metabolite that it was previously thought to be. It may further disrupt flukes already affected by TCBZ.SO. The hydroxy forms of TCBZ.SO and TCBZ.SO_2 have also been shown to be capable of disrupting the tegument of *F. hepatica* (unpublished observations), so drug action may be the combined effect of several metabolites, rather than being due to a single compound.

Some idea of the time-scale of drug action has been provided by recent studies in sheep. Following treatment of a juvenile (4-week) infection with TCBZ (10 mg kg^{-1}), flukes were still active at 48 h post-treatment (p.t.) and displayed limited surface disruption, as observed by SEM (Halferty *et al.*, 2008). By 72 h p.t., all but one of the recovered flukes were dead and they displayed a range of disruption. In most, there was severe swelling over all the body surface, with areas of tegumental sloughing in the tail region. Other flukes were more severely affected, with more widespread loss of the tegument and exposure of the underlying parenchyma. At 96 h p.t., all the flukes were dead and they were grossly disrupted. The tegument had been totally removed and lesions were present in the basal lamina, exposing the internal tissues (Halferty *et al.*, 2008). In adult infections, the posterior end of the fluke's body becomes elongated and exhibits a green discolouration after 72 h p.t.. This phenomenon coincides with the movement of flukes into the gall bladder and their subsequent expulsion from the sheep (personal observations). So, drug action is relatively slow and this would be compatible with a microtubule-based action, rather than one based on energy disruption, for example.

Most of the studies on the mechanism of action of TCBZ have been carried out with TCBZ.SO. The precise mechanism remains to be fully elucidated, but there is more evidence for an action against microtubules and microtubule-based processes than for other possibilities, such as against energy metabolism or neuromuscular co-ordination, for example (for a more complete discussion of the evidence, see Fairweather, 2005).

Mechanism of resistance

Since the previous review in 2005, another report of TCBZ resistance has been published, from north-west Spain (Alvarez-Sánchez *et al.*, 2006). This means that resistance has now been reported in several countries in western Europe, in addition to the original report in Australia in the mid-1990s (for associated references, see Fairweather, 2005). It should be noted that not all reports, anecdotal or otherwise, have been confirmed by rigorous trials. There is convincing evidence for a number of isolates used in TCBZ studies: the Dutch, Oberon and Sligo isolates (previous references are in Fairweather, 2005; see also, Keiser *et al.*, 2007a; McConville *et al.*, 2009a). It is essential that such supporting data are obtained; otherwise, the purported cases could be explained by incorrect (under-) dosing, product failure, reduced metabolism as a result of liver damage, even inadequate and incorrect diagnostic tests. Interestingly, in the Spanish report, the flukes (which we have designated the Leon isolate) were described as being resistant to albendazole and clorsulon (in combination with ivermectin) as well (Alvarez-Sánchez *et al.*, 2006). If validated, this would be the first instance of multiple drug resistance in the liver fluke. The Sligo isolate has now been shown to be resistant at three stages of development in the mammalian host: 3 days, 4 weeks and 12 weeks post-infection (Coles *et al.*, 2000; Coles & Stafford, 2001; McCoy *et al.*, 2005; McConville *et al.*, 2009a).

Since TCBZ is a benzimidazole compound, there is the assumption that its target is tubulin. Immunocytochemical studies using an anti-tubulin antibody have demonstrated that tubulin immunoreactivity in the

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tegument of TCBZ-susceptible (TCBZ-S) Cullompton flukes is abolished by treatment with TCBZ.SO, whereas that in TCBZ-resistant (TCBZ-R) Sligo flukes is unaffected (Robinson *et al.*, 2002; McConville *et al.*, 2006). Another assumption following on from this has been that mutations in the β-tubulin molecule have led to the development of resistance against TCBZ, as is known to be the case for other benzimidazoles in nematode parasites. In the latter, there are three principal substitutions associated with the presumed drug-binding site: the phenylalanine–tyrosine substitution at position 200, the phenylalanine–tyrosine or histidine substitution at position 167 and the glutamic acid–alanine substitution at position 198 (Wolstenholme *et al.*, 2004; Ghisi *et al.*, 2007). Six β-tubulin isotypes have been sequenced in the TCBZ-S Cullompton isolate and in the TCBZ-R Sligo and Oberon isolates (Ryan *et al.*, 2008). However, no differences have been detected between the isotypes in the three isolates. Phenylalanine is present at position 167 and glutamic acid at position 198 in all six isotypes; at position 200, tyrosine is present in three, phenylalanine in two and leucine in one of the isotypes (Ryan *et al.*, 2008). The presence of tyrosine at position 200 in TCBZ-S flukes would render the binding site inaccessible to classical benzimidazoles such as albendazole, and this would go some way towards explaining why *F. hepatica* is refractory to many benzimidazole anthelmintics. In benzimidazole-resistant nematodes, the drug-binding cleft is closed off by the presence of tyrosine at position 200 and this forms the basis of the resistance mechanism (Robinson *et al.*, 2004b). If TCBZ does target tubulin, its binding site may be in a different position on the tubulin molecule, but this remains to be determined.

While, to date, there is no convincing evidence of a role for tubulin mutations in resistance to TCBZ, there is evidence to indicate that altered uptake and metabolism of TCBZ may be involved. Comparison between the Cullompton (TCBZ-S) and Sligo (TCBZ-R) isolates of *F. hepatica* has shown that the diffusion of both TCBZ and TCBZ.SO is significantly lower in TCBZ-R than in TCBZ-S flukes (Alvarez *et al.*, 2005; Mottier *et al.*, 2006b). Interestingly, this was not true for the related benzimidazole, albendazole, whose uptake was similar in both isolates (Mottier *et al.*, 2006b). The results suggest that the mechanism is specific to TCBZ and that P-glycoprotein (Pgp)-linked drug efflux pumps may be involved in the resistance mechanism. Overexpression of Pgp has been linked to resistance in nematodes against different classes of anthelmintics (Kerboeuf *et al.*, 2003; Wolstenholme *et al.*, 2004). Experiments with Pgp inhibitors have shown that it is possible to 'reverse' the condition of the flukes, from resistant to susceptible. For example, co-incubation with ivermectin increased the uptake of TCBZ and TCBZ.SO in TCBZ-R Sligo flukes to levels comparable to those in TCBZ-S Cullompton flukes (Mottier *et al.*, 2006b). In contrast, ivermectin had no impact on the uptake of albendazole in either TCBZ-S or -R flukes (Mottier *et al.*, 2006b). The consequence of Pgp inhibition to the condition of TCBZ-R flukes has been demonstrated in a separate morphological (SEM) study with another Pgp inhibitor, R(+)-verapamil. Co-incubation of R(+)-verapamil with TCBZ.SO led to severe disruption of the tegument of TCBZ-R (Oberon) flukes, whereas treatment with TCBZ.SO on its own (even at a high concentration) caused minimal changes to the tegumental surface (Fairweather *et al.*, 2008). The disruption to the TCBZ-R flukes, which took the form of widespread tegumental sloughing, was greater than that seen in the TCBZ-S Cullompton fluke following treatment with TCBZ.SO. While a change in efflux pump activity may simply represent a non-specific mechanism (although the albendazole result suggests that this is not the case), nevertheless it is likely to play a significant role in the development of resistance.

There is a marked difference in the ability of TCBZ-S and TCBZ-R isolates to metabolize TCBZ. Thus, TCBZ-R (Sligo) flukes have been shown to carry out the metabolism of TCBZ to TCBZ.SO, and TCBZ.SO to $TCBZ.SO_2$, at a significantly higher rate than that achieved by TCBZ-S (Cullompton) flukes (Robinson *et al.*, 2004a; Alvarez *et al.*, 2005). Methimazole, an FMO inhibitor, had a significantly greater inhibitory effect on TCBZ sulphoxidation in TCBZ-R than -S flukes, reducing it to a level comparable to that in TCBZ-S flukes (Alvarez *et al.*, 2005). By comparison, the cytochrome P450 inhibitor, PB had a lesser effect on TCBZ.SO formation and the effect was similar in the two isolates (Alvarez *et al.*, 2005). These experiments were carried out on microsomal fractions of flukes. A subsequent study on intact flukes *in vitro* has shown that it is possible to reverse the TCBZ-R condition of a fluke (in this case, the Oberon isolate) by co-incubation of TCBZ with MTZ (Devine *et al.*, 2009). Treatment with either TCBZ or TCBZ.SO on their own resulted in more severe disruption to the TCBZ-S Cullompton isolate than the TCBZ-R Oberon isolate, as visualized by surface changes to the tegument (Devine *et al.*, 2009). Methimazole alone had no effect on either isolate, but when it was included alongside TCBZ or TCBZ.SO, disruption to the Oberon isolate was greater than that to the Cullompton isolate, and greater than that in both isolates after either drug on its own. Severe swelling and blebbing of the tegument occurred all over the body and stripping of the apical plasma membrane was observed in the oral cone and midbody regions (Devine *et al.*, 2009). The study showed the morphological manifestation of what the inhibition of drug metabolism by the fluke can lead to in terms of the whole fluke.

Biological differences between isolates

Studies on the various isolates of *F. hepatica* have revealed interesting differences between them, in relation to their fitness, which have implications for the spread of resistance in the field. For example, in a snail and rat study on the Oberon (TCBZ-resistant) and Fairhurst (TCBZ-susceptible) isolates, the Oberon isolate was shown to be faster to egg hatch (by 2 days: 12 days as against 14 days); faster to produce cercariae (by 4 days: 49 days as against 53 days); and it produced more cercariae (>4 times as many). Moreover, the metacercariae were more infectious to the rat hosts and the flukes reached patency more quickly (by 11 days: 59 days as against 70 days) (Walker *et al.*, 2006). Across the life cycle, the Oberon isolate could be gaining an approximately 2.5 week advantage over the Fairhurst isolate if the

isolates were competing with each other. From egg hatch, it would be infecting the mammalian host ~1 week before the Fairhurst isolate and it would be releasing eggs ~1.5 weeks earlier. This would give it a considerable advantage. The results also indicated that the development of drug resistance by the Oberon isolate has not led to a reduction of fitness by comparison with the Fairhurst isolate. In fact, the data showed that the Oberon isolate maintained a higher level of fitness throughout the life cycle. This goes against the general rule that resistance to benzimidazoles results in reduced fecundity (Maingi *et al.*, 1990), although this rule is not absolute (Kelly *et al.*, 1978; Elard *et al.*, 1998). This is important, because if resistant isolates can maintain fecundity, there will be no reversion to a drug-susceptible status; this idea is supported by field data from The Netherlands, which showed no reversion of TCBZ resistance after a 3-year period when TCBZ was not used for treatment (Borgsteede *et al.*, 2005).

A separate comparison has been made between infections of the Cullompton (TCBZ-susceptible) and Sligo (TCBZ-resistant) isolates in sheep. Sligo flukes were smaller than their Cullompton counterparts, but migrated more quickly, reaching the bile ducts 1 week earlier (week 7 post-infection, as against week 8), and they produced eggs more quickly (60 days as against 75 days). On the other hand, Sligo flukes produced relatively fewer eggs (approximately one-third as many as the Cullompton flukes) and they were less infectious to sheep (24% of the metacercarial dose reached maturity, as against 57%) (McConville *et al.*, 2009a). The more rapid egg production would be an advantageous quality, but the Sligo isolate appears to have sacrificed a number of physiological attributes in order to survive TCBZ treatment. The Cullompton flukes are known to be aspermic and triploid, so perhaps they can devote more energy to growth, resulting in their larger size (Hanna *et al.*, 2008). Spermatogenesis in Cullompton flukes does not proceed beyond the primary spermatocyte stage, presumably due to a failure of meiosis. Despite this, Cullompton flukes produce normal-looking eggs which are capable of hatching and undergoing parthenogenic development (Hanna *et al.*, 2008). Sligo flukes show two different phenotypes: in one, the testis contains fully developed sperm, whereas in the other, spermatogenesis is halted at the spermatid stage, due to the failure of nuclear elongation that leads to sperm formation. The two phenotypes are present in the same animal and cross-fertilization between the two takes place (Hanna *et al.*, 2008). Other fluke isolates (including the Oberon isolate) undergo full sperm development, are diploid and produce normal eggs (Hanna *et al.*, 2008). The Cullompton result shows that, in the field, it would be possible for there to be a rapid evolution of clonal populations following selection for resistance. So, the limited data show the variation between fluke populations and this needs to be taken into account when understanding fluke population dynamics and the epidemiology of fascioliasis.

As well as fluke isolates having differing sensitivities to TCBZ, studies have shown that they differ in their response to other anthelmintics. For example, the activity of nitroxynil (a fasciolicide) has been compared against four isolates of *F. hepatica*: the TCBZ-S Cullompton and Fairhurst isolates and the TCBZ-R Oberon and Sligo isolates. The impact of nitroxynil action was assessed by fine structural changes to the tegument and gut. In terms of the severity of disruption observed, the isolates were ranked in the following order: Cullompton > Sligo > Oberon > Fairhurst (McKinstry *et al.*, 2007, 2009). Interestingly, this ranking does not coincide with their susceptibility to TCBZ, which was: Cullompton > Fairhurst > Oberon > Sligo. The Sligo isolate appears to be particularly susceptible to nitroxynil, whereas the Fairhurst isolate is more refractory. Such variations may need to be taken into account in the field when designing control strategies, although the data on nitroxynil will only apply to adult flukes as it is not active against juveniles.

Dealing with resistance

This topic was discussed in the previous review (Fairweather, 2005). Strategies include the better use of existing fasciolicides, the use of drug combinations and the development of new drugs.

Use of current drugs

A number of current drugs have been shown to be active against the Sligo TCBZ-R isolate of *F. hepatica*: albendazole, clorsulon (in combination with ivermectin), closantel, nitroxynil and oxyclozanide (Coles *et al.*, 2000; Moll *et al.*, 2000; Coles & Stafford, 2001). In terms of the response to albendazole and clorsulon, the data for Sligo is at odds with that for the Leon isolate, which indicated that the isolate was resistant to these compounds. Perhaps the drug status of fluke populations from different geographical regions varies, a point that needs to be taken into account when considering alternative therapies. The value of using existing flukicides would be restricted to treatment of adult fluke infections, as they are not effective against the juvenile stages. However, there is evidence that, because of the perceived (but not necessarily proven) problem of TCBZ resistance, farmers are turning away from the use of TCBZ to older compounds, such as closantel and nitroxynil (Hanna, personal communication).

One way to enhance the efficacy of TCBZ would be to modulate its pharmacokinetics. As described above, this can be achieved by co-administration of TCBZ with metabolic and Pgp inhibitors (Lifschitz *et al.*, 2009; Virkel *et al.*, 2009). The feasibility of adopting this approach has been demonstrated in studies on a number of anthelmintics: e.g. albendazole, ivermectin and oxfendazole (Lanusse & Prichard, 1991, 1992; López-Garcia *et al.*, 1998; Alvinerie *et al.*, 1999; Sánchez *et al.*, 2002; Merino *et al.*, 2003; Ballent *et al.*, 2006, 2007; see also reviews by Alvarez *et al.*, 2006; Lespine *et al.*, 2008). More significantly, co-administration of anthelmintic-plus-inhibitor has been shown to lead to greater efficacy against drug-resistant nematodes (Benchaoui & McKellar, 1996; Molento & Prichard, 1999). TCBZ is marketed in combination with ivermectin and this combination needs to be examined further, to determine whether it possesses activity against TCBZ-R fluke infections.

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Use of drug combinations

Drug combinations are a routine part of parasite control in livestock, often being used to treat mixed infections. For example, TCBZ is marketed with levamisole, oxfendazole, ivermectin and abamectin, to provide fluke and nematode control. Drug combinations are also considered to be the most effective way of slowing down the development of resistance and extending the life span of the drugs (Barnes *et al.*, 1995; Sangster, 2001). This is particularly true if the drugs are from different chemical groupings and possess different mechanisms of action, because this opens up the possibility of producing additive or synergistic effects. Moreover, the latter would permit the use of lower quantities of drugs, with the added advantage of reducing drug residues in host tissues and in the environment. There are reports of synergistic interactions between drugs used for schistosome and soil-transmitted helminth infections in humans (see reviews by Albonico, 2003; Utzinger & Keiser, 2004). Synergistic combinations have also been described for veterinary infections (e.g. Bennet *et al.*, 1980; Hopkins & Gyr, 1991). Synergism between TCBZ and clorsulon or luxabendazole (at greatly reduced dose rates) has been demonstrated for *F. hepatica* (data in Fairweather & Boray, 1999). Two recent studies have examined the morphological effects of a TCBZ + clorsulon combination against adult flukes (Meaney *et al.*, 2006, 2007). The two drugs have different mechanisms of action, with clorsulon targeting energy metabolism and TCBZ (presumably) microtubules; also, they have different routes of entry into the fluke – clorsulon oral and TCBZ trans-tegumental. The combination of the two drugs at half-normal dose rates induced greater disruption than either drug on its own (at reduced and normal levels). Surface changes observed with the combination treatment were: stripping of the apical plasma membrane in the anterior half of the fluke, spine loss, blebbing and swelling (Meaney *et al.*, 2006). Among the internal changes seen with the combination were a reduction in the production of secretory bodies in the tegumental cells, swelling of the basal infolds and autophagy in the syncytium, flooding of the internal tissues and disruption to the spines (Meaney *et al.*, 2007). Such changes are likely to lead to the surface changes just described. The results pointed to additive or synergistic effects of the two drugs when used together and support the concept of employing drug combinations against fluke infections. The studies were carried out on the Cullompton (TCBZ-S) isolate of *F. hepatica*; it remains to be seen whether the phenomenon can be replicated in TCBZ-R flukes. In a separate study in sheep, no synergism was demonstrated between TCBZ and nitroxynil (at normal dose rates) against juvenile (4-week-old) TCBZ-R (Sligo) flukes (McCoy *et al.*, 2005). The study showed that the Sligo isolate was resistant to TCBZ at a juvenile stage; all other studies have been concerned with the adult fluke.

Development of new drugs

In relation to new compounds, information on the TCBZ derivative, compound alpha, was presented in the previous review. It showed promise as an alternative to TCBZ, since it possesses a spectrum of activity similar to that of TCBZ itself. A number of studies on compound alpha have been carried out since 2005. Treatment of both adult and juvenile TCBZ-S Cullompton flukes *in vivo* (in sheep) led to progressive disruption over time: the disruption took the form of tegumental loss, degeneration of the sub-tegumental tissues, internal flooding and disruption of the muscle bundles (McConville *et al.*, 2008, 2009b). The most significant changes occurred between 48 and 72 h p.t., indicating a slow action, even though maximum blood levels are reached quite quickly – after only 10–14 h p.t. (Rivero *et al.*, 1998; Ramírez *et al.*, 2009). The effect was more rapid with juvenile than adult flukes: after 72 h treatment, almost 90% of juvenile flukes were dead, whereas only 23% of adult flukes were dead at this time. However, ~50% of the flukes displayed a discolouration in the midbody region, which coincided with the loss of the tegument (McConville, unpublished observations). Compound alpha causes a significant reduction in tubulin immunostaining in TCBZ-S flukes (McConville *et al.*, 2006), suggesting that it may share a target and mode of action similar to those of TCBZ. Experiments carried out *in vitro* with adult and juvenile stages of the Sligo TCBZ-R isolate showed that compound alpha affects tegumental structure, and more severely than that induced by TCBZ.SO, although the changes were not accompanied by any loss of tubulin immunoreactivity (McConville *et al.*, 2006, 2007). Unfortunately, when tested *in vivo* (in sheep) against the Sligo isolate, compound alpha treatment did not result in a reduction of fluke burden at 3 days, 4 weeks and 12 weeks post-infection (McConville *et al.*, 2009a). So, the *in vitro* data did not translate into *in vivo* efficacy. It is possible that the flukes can survive any initial impact of drug action and recover: they are known to be able to mount a stress response to drug action (McConville *et al.*, 2008; Halferty *et al.*, 2008). As a consequence of this result, the potential of compound alpha to replace TCBZ for the treatment of TCBZ-R fluke infections may be limited. A carbamate derivative of compound alpha has been synthesized and shown to possess a high level of efficacy against the gut paramphistome, *Calicophoron calicophorum* (Reyes *et al.*, 2008); unfortunately, its activity against flukes has not been tested. Tribendimidine is an anthelmintic that is effective against soil-transmitted helminths and the intestinal trematode, *Echinostoma caproni*. When tested against the Cullompton (TCBZ-S) isolate of *F. hepatica*, it had no impact on fluke burdens in rats. Nor was it effective against *Schistosoma mansoni*, although it showed activity against *Clonorchis sinensis* and *Opisthorchis viverrini* (Keiser *et al.*, 2007b).

Recently, there has been an upsurge of interest in making use of natural plant products that have been used as traditional medicines in developing countries (Hammond *et al.*, 1997; Iqbal *et al.*, 2003; Kayser *et al.*, 2003; Anthony *et al.*, 2005; Crump, 2006; Stepek *et al.*, 2007). The marketing of 'Mirazid', derived from the myrrh tree *Commiphora molmol* was discussed in the previous review and the reader is referred to that review for a discussion of the controversy surrounding its use as an anti-schistosomal drug (Fairweather, 2005). Other natural products with reported efficacy against *F. hepatica* include extracts of the fern *Matteuccia orientalis* (Shiramizu *et al.*, 1993); the

black-fruited galangal *Alpinia nigra* (Roy & Tandon, 1999); the fineleaf fumitory *Fumaria parviflora*, the nickernut *Caesalpinia crista* and the black cumin *Nigella sativa* (Akhtar *et al.*, 2000); the silk tree *Albizia anthelmintica* and the soapberry tree *Balanites aegyptiaca* (Koko *et al.*, 2000); the toothache tree *Zanthoxylum alatum* (Tagboto & Townson, 2001); persimmon *Albizia anthelmintica*, the coral tree *Diospyrus*, henna *Erythrina*, *Lawsonia* and katigua pyta *Trichilla* (Iqbal *et al.*, 2003).

Another natural product is genistein. It is an isoflavone derivative of *Flemingia vestita*, known as Soh-Phlang in north-east India, and extracts of the plant are eaten raw as a cure for various helminth infections, including that caused by the trematode, *Fasciolopsis buski* (Rao, 1981). The activity of genistein has been tested *in vitro* against *F. hepatica*. Incubation of intact flukes at a concentration of 0.27 mg ml^{-1} (= 1 mM) led to a rapid loss of movement (in less than 3 h), while exposure of fluke muscle strips led to significant increases in the frequency and/or amplitude of muscle contractions at concentrations of 10 μM to 10 mM (Toner *et al.*, 2008). Genistein is believed to affect Ca^{2+} homeostasis as a result of modulating nitric oxide activity, via changes to cyclic guanosine monophosphate (cGMP) levels (Das *et al.*, 2007, 2009). Within the short time-frame of 3 h, genistein caused marked surface changes to the tegument of *F. hepatica*: the changes included widespread blebbing and swelling of the tegument, and spine loss (Toner *et al.*, 2008). The internal tissues were severely affected, too: there was reduced secretory activity together with autophagy in the tegumental and gastrodermal cells and inhibition of cell development and differentiation in the testis and vitelline follicles (Toner *et al.*, 2008). In other organisms, genistein is known to inhibit mitosis, induce apoptosis and interfere with signalling pathways, as it is an inhibitor of tyrosine-specific protein kinases. The pathways are present in *Schistosoma* and *Echinococcus* and the changes observed in *F. hepatica* could have a similar basis (for the relevant references on genistein action and signalling in helminths, see Toner *et al.*, 2008).

Propolis, or bee glue, is a resinous hive product that has been used for a number of medicinal purposes and has anti-protozoal activity (Higashi & de Castro, 1994). Incubation of *Fasciola gigantica* in propolis *in vitro* led to severe disruption of the surface tegument: there was swelling, blebbing, loss of spines, formation of lesions and (in extreme cases) loss of the tegument (Hegazi *et al.*, 2007a). It was described as being relatively more disruptive than TCBZ itself. A separate study showed that propolis inhibits the development and hatching of fluke eggs (Hegazi *et al.*, 2007b). It is a very complex mixture of components, so it may be extremely difficult to identify the active constituent(s).

Although the results of studies on natural products as discussed above are interesting, it remains to be seen whether their use will make a significant contribution to fluke therapy in the future. Much further evaluation and testing will be required before their true usefulness will be known. It is relatively easy to find compounds that are active *in vitro*, but less so to translate that to efficacy *in vivo*.

One group of drugs derived from natural products that has attracted considerable attention in recent years is the artemisinins. Artemisinin itself was originally isolated from the wormwood plant *Artemisia annua*; extracts of the plant have been used in China for more than 2 millennia as traditional herbal remedies for the treatment of various illnesses (Li & Wu, 2003; Woodrow *et al.*, 2005). Semi-synthetic derivatives of artemisinin were isolated in the 1970s and are well-established anti-malarial drugs: they include artemether, artesunate, arteether and their principal metabolite, dihydroartemisinin (Borstnik *et al.*, 2002; Woodrow *et al.*, 2005). Further modification of the compounds led to the development of the synthetic 1,2,4-trioxolanes, which retain the peroxide moiety essential for antiparasitic activity, yet are simpler molecules, easier to synthesize and have improved pharmacokinetic properties (e.g. greater stability, better absorption, longer half-life). One of the compounds, OZ277, has gone to clinical trials as part of the Medicines for Malaria Venture (Vennerstrom *et al.*, 2004). In addition to their use as anti-malarials, artemisinins are used for the treatment of schistosome infections, especially in combination with praziquantel. The combination is valuable as the artemisinins have activity against juvenile stages, whereas praziquantel targets the adult worms (Xiao, 2005; Utzinger *et al.*, 2007). Recent studies have shown that artemisinin compounds are active against other trematode parasites, such as *C. sinensis*, *O. viverrini* and *E. caproni*, both *in vitro* and *in vivo* in rodent models (Keiser *et al.*, 2006a, b, c, 2007c; Shu-Hua *et al.*, 2008; see also the reviews by Keiser & Utzinger, 2007a, b). In contrast, artemether (and tribendimidine) lacks activity against *Paragonimus westermani* (Xue *et al.*, 2008). Little is known about the activity of artemisinins against tapeworm parasites. In one study, artesunate and dihydroartemisinin (but not artemether) were effective against the protoscoleces of *Echinococcus granulosus in vitro*, but were ineffective against *Echinococcus multilocularis* metacestodes in an *in vivo* mouse model (Spicher *et al.*, 2008).

A number of artemisinin compounds have been tested against *F. hepatica*, both *in vitro* and in the rat model: artemether, artesunate and the synthetic trixolane, OZ78 (Keiser *et al.*, 2006c, d, 2007a; Keiser & Morson, 2008a, b; O'Neill *et al.*, 2009). They displayed high levels of efficacy against both adult and juvenile flukes, with relatively greater activity against the adult stage. The compounds were also shown to be capable of inducing marked changes to the surface tegument. The disruption became progressively more severe over time following treatment *in vivo*, leading to the death and expulsion of flukes after approximately 72–96 h (Keiser & Morson, 2008a, b; Keiser *et al.*, 2006c, d). Of particular interest is that artemether and OZ78 showed an extremely high level of efficacy against the TCBZ-R Oberon isolate in a rodent model (Keiser *et al.*, 2007a). If that activity could be repeated in a ruminant animal, this would be a promising breakthrough and the result warrants further investigation.

Greater disruption to the tegument was observed when haemin was incorporated in the culture medium. This result ties in with the idea that activation of artemisinin-type compounds depends on the presence of an iron-containing compound (as would be derived from haemoglobin *in vivo*). Activation leads to cleavage of the peroxide bridge in the drug and the generation of free radicals. These free radicals are damaging to flukes

(Xiao *et al.*, 2003). It has been suggested that artemisinins need to be ingested by the blood fluke, *Schistosoma* in order for activation to occur (Xiao *et al.*, 2003), and this may be true for *F. hepatica* as well, since it is an haematophagous feeder. In a recent study on the action of artemether against *F. hepatica*, the gut was seen to be more severely affected than the tegument following treatment *in vivo*, and this result supports the idea that oral ingestion is the main route of entry into the fluke (O'Neill *et al.*, 2009).

A pilot study in human patients has been carried out in Vietnam, to compare the impact of artesunate and TCBZ on relieving the symptoms (abdominal pain) of fascioliasis. The initial response in the artesunate-treated group was better than in the TCBZ-treated group, but 3 months after treatment the response was lower (Hien *et al.*, 2008). The study needs to be followed up by a more rigorous assessment of the efficacy of artemisinin compounds, before any conclusion can be reached as to what role they might play in controlling fluke infections in humans. The extremely high value placed on these compounds in malarial areas may limit their use against other parasites, due to the risk of promoting drug resistance.

Use of TCBZ for the treatment of human fascioliasis

In recent years, fascioliasis has emerged as a major zoonotic disease, with an increase in the number of human cases, and it is a serious health problem in a number of countries (Mas-Coma *et al.*, 2005; WHO, 2007). TCBZ is the drug of choice for treating human fascioliasis: a summary of its use was given in my previous review (Fairweather, 2005; see also Keiser *et al.*, 2005; WHO, 2007). The success of a selective treatment programme targeted to schoolchildren in Egypt has been presented by Curtale *et al.* (2005). Despite this success, there was a real concern that Novartis would stop production of Egaten®, the human formulation of TCBZ (Curtale, 2006). Fortunately, that decision was rescinded and Novartis resumed production. Moreover, the company decided to donate 600,000 tablets to WHO, to ensure that the drug was available in endemic countries (Curtale, 2008). Egypt, Iran, Bolivia, Peru, Vietnam, Georgia and the Yemen have benefited from this scheme, made possible by the generosity of Novartis.

TCBZ is also effective against lung infections of human paragonimiasis (Calvopiña *et al.*, 2003; Keiser *et al.*, 2005), but showed no activity against the human blood fluke, *Schistosoma mansoni* in patients co-infected with *Fasciola* spp. (Barduagni *et al.*, 2008).

Conclusions

It is fair to say that a substantial amount of work has been carried out since the previous review. We have learned a lot about the pharmacokinetics and pharmacodynamics of TCBZ, although identification of its target molecule remains tantalizingly elusive. Some progress has been made on clarifying the mechanism(s) of resistance and that information may be of use in designing new strategies to deal with resistance to TCBZ. But has the knowledge gained actually led to a greater *understanding* of these topics? Probably not entirely, not yet, but the results have opened up new lines of enquiry to pursue, to edge us closer to a more complete understanding of TCBZ action in all its facets. In terms of addressing the problem of resistance, evaluation of novel compounds and drug combinations has attracted a lot of interest, but it is uncertain whether this will translate into marketable therapies. However, one area highlighted in the previous review remains neglected and that concerns the development of reliable tests, not just for diagnosis of fluke infection, but for detection of resistance. Until tests are standardized, apparent cases of resistance may continue to be reported that turn out not to be the case at all and the true extent of resistance will remain confused.

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References

Akhtar, M.S., Iqbal, Z., Khan, M.N. & Lateef, M. (2000) Anthelmintic activity of medicinal plants with particular reference to their use in animals in the Indo-Pakistan subcontinent. *Small Ruminant Research* **38**, 99–107.

Albonico, M. (2003) Methods to sustain drug efficacy in helminth control programmes. *Acta Tropica* **86**, 233–242.

Alvarez, L.I., Solana, H.D., Mottier, M.L., Virkel, G.L., Fairweather, I. & Lanusse, C.E. (2005) Altered drug influx/efflux and enhanced metabolic activity in triclabendazole-resistant liver flukes. *Parasitology* **131**, 501–510.

Alvarez, A.I., Merino, G., Molina, A.J., Pulido, M.M., McKellar, Q.A. & Prieto, J.G. (2006) Role of ABC transporters in veterinary drug research and parasite resistance. *Current Drug Delivery* **3**, 199–206.

Alvarez-Sánchez, M.A., Mainar-Jaime, R.C., Pérez-García, J. & Rojo-Vázquez, F.A. (2006) Resistance of *Fasciola hepatica* to triclabendazole in sheep in Spain. *Veterinary Record* **159**, 424–425.

Alvinerie, M., Dupuy, J., Eeckhoutte, C. & Sutra, J.F. (1999) Enhanced absorption of pour-on ivermectin formulation in rats by co-administration of the multidrug-resistant-reversing agent verapamil. *Parasitology Research* **85**, 920–922.

Anthony, J.-P., Fyfe, L. & Smith, H. (2005) Plant active components – a resource for antiparasitic agents? *Trends in Parasitology* **21**, 462–468.

Ballent, M., Lifschitz, A., Virkel, G., Sallovitz, J. & Lanusse, C. (2006) Modulation of the P-glycoprotein-mediated intestinal secretion of ivermectin: *in vitro* and

in vivo assessments. *Drug Metabolism and Disposition* **34**, 457–463.

Ballent, M., Lifschitz, A., Virkel, G., Sallovitz, J. & Lanusse, C. (2007) Involvement of P-glycoprotein on ivermectin kinetic behaviour in sheep: itraconazole-mediated changes on gastrointestinal disposition. *Journal of Veterinary Pharmacology and Therapeutics* **30**, 242–248.

Barduagni, P., Hassanein, Y., Mohamed, M., El Wakeel, A., El Sayed, M., Hallaj, Z. & Curtale, F. (2008) Use of triclabendazole for treatment of patients co-infected by *Fasciola* spp. and *S. mansoni* in Behera Governate, Egypt. *Parasitology Research* **102**, 631–633.

Barnes, E.H., Dobson, R.J. & Barger, I.A. (1995) Worm control and anthelmintic resistance: adventures with a model. *Parasitology Today* **11**, 56–63.

Benchaoui, H. & McKellar, Q.A. (1996) Interaction between fenbendazole and piperonyl butoxide: pharmacokinetic and pharmacodynamic implications. *Journal of Pharmacy and Pharmacology* **48**, 753–759.

Bennet, E.V., Behm, C., Bryant, C. & Chevis, R.A.F. (1980) Synergistic action of mebendazole and levamisole in the treatment of a benzimidazole-resistant *Haemonchus contortus* in sheep. *Veterinary Parasitology* **7**, 207–214.

Borgsteede, F.H.M., Moll, L., Vellema, P. & Gaasenbeek, C.P.H. (2005) Lack of reversion in triclabendazole-resistant *Fasciola hepatica*. *Veterinary Record* **156**, 350–351.

Borstnik, K., Paik, I.-H., Shapiro, T.A. & Posner, G.H. (2002) Antimalarial chemotherapeutic peroxides: artemisinin, yinghaosu A and related compounds. *International Journal for Parasitology* **32**, 1661–1667.

Büscher, G., Bowen, F.L., Strong, M.B. & Crowfoot, P.D. (1999) Efficacy of triclabendazole, its metabolites and other compounds in sheep. *17th International Conference of the WAAVP*, Copenhagen, 15–19 August, abstract c7.40.

Calvopiña, M., Guderian, R.H., Paredes, W. & Cooper, P.J. (2003) Comparison of two single-day regimens of triclabendazole for the treatment of human pulmonary paragonimiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **97**, 451–454.

Coles, G.C. & Stafford, K.A. (2001) Activity of oxyclozanide, nitroxynil, clorsulon and albendazole against adult triclabendazole-resistant *Fasciola hepatica*. *Veterinary Record* **148**, 723–724.

Coles, G.C., Rhodes, A.C. & Stafford, K.A. (2000) The activity of closantel against adult triclabendazole-resistant *Fasciola hepatica*. *Veterinary Record* **146**, 504.

Crump, A. (2006) New medicines from nature's armamentarium. *Trends in Parasitology* **22**, 51–54.

Curtale, F. (2006) Treatment of fascioliasis in human infections: a reply. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **100**, 187–188.

Curtale, F. (2008) Treatment of human fascioliasis with triclabendazole: good news. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102**, 508.

Curtale, F., Hassanein, Y.A.E.W. & Savioli, L. (2005) Control of human fascioliasis by selective chemotherapy: design, cost and effect of the first public health, school-based intervention implemented in endemic areas of the Nile Delta, Egypt. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **99**, 599–609.

Das, B., Tandon, V. & Saha, N. (2007) Genistein from *Flemingia vestita* (Fabaceae) enhances NO and its mediator (cGMP) production in a cestode parasite, *Raillietina echinobothridia*. *Parasitology* **134**, 1457–1463.

Das, B., Tandon, V., Lyndem, L.L., Gray, A.I. & Ferro, V.A. (2009) Phytochemicals from *Flemingia vestita* (Fabaceae) and *Stephania glabra* (Menispermeaceae) alter cGMP concentration in the cestode *Raillietina echinobothrida*. *Comparative Biochemistry and Physiology part C*, **149**, 397–403.

Devine, C., Brennan, G.P., Lanusse, C.E., Alvarez, L.I., Trudgett, A., Hoey, E. & Fairweather, I. (2009) Effect of the metabolic inhibitor, methimazole on the drug susceptibility of a triclabendazole-resistant isolate of *Fasciola hepatica*. *Parasitology* **136**, 183–192.

Elard, L., Sauve, C. & Humbert, J.F. (1998) Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Parasitology* **117**, 571–578.

El-Tantawy, W.H., Salem, H.F. & Mohammed Safwat, N.A.S. (2007) Effect of fascioliasis on the pharmacokinetic parameters of triclabendazole in human subjects. *Pharmacy World and Science* **29**, 190–198.

Fairweather, I. (2005) Triclabendazole: new skills to unravel an old(ish) enigma. *Journal of Helminthology* **79**, 227–234.

Fairweather, I. & Boray, J.C. (1999) Fasciolicides: efficacy, actions, resistance and its management. *Veterinary Journal* **158**, 81–112.

Fairweather, I., Meaney, M., Savage, J., Brennan, G., Hoey, E. & Trudgett, A. (2008) The role of drug efflux pumps in the development of resistance to triclabendazole in *Fasciola hepatica*. *Xth European Multicolloquium of Parasitology (EMOP X)*, Paris, August, abstract SY21/01–03.

Fetterer, R.H. (1986) The effect of albendazole and triclabendazole on colchicine binding in the liver fluke *Fasciola hepatica*. *Journal of Veterinary Pharmacology and Therapeutics* **9**, 49–54.

Ghisi, M., Kaminsky, R. & Mäser, P. (2007) Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. *Veterinary Parasitology* **144**, 313–320.

Halferty, L., Brennan, G.P., Hanna, R.E.B., Edgar, H.W., Meaney, M., McConville, M., Trudgett, A., Hoey, L. & Fairweather, I. (2008) Tegumental surface changes in juvenile *Fasciola hepatica* in response to treatment *in vivo* with triclabendazole. *Veterinary Parasitology* **155**, 49–58.

Halferty, L., Brennan, G.P., Trudgett, A., Hoey, L. & Fairweather, I. (2009) Relative activity of triclabendazole metabolites against the liver fluke, *Fasciola hepatica*. *Veterinary Parasitology* **159**, 126–138.

Hammond, J.A., Fielding, D. & Bishop, S.C. (1997) Prospects for plant anthelmintics in tropical veterinary medicine. *Veterinary Research Communications* **21**, 213–228.

Hanna, R.E.B., Edgar, H., Moffett, D., McConnell, S., Fairweather, I., Brennan, G.P., Trudgett, A., Hoey, E.M., Cromie, L., Taylor, S.M. & Daniel, R. (2008) *Fasciola hepatica*: histology of the testis in egg-producing adults of several laboratory-maintained

isolates of flukes grown to maturity in cattle and sheep and in flukes from naturally infected hosts. *Veterinary Parasitology* **157**, 222–234.

Hegazi, A.G., Abd El Hady, F.K. & Shalaby, H.A. (2007a) An *in vitro* effect of propolis on adult worms of *Fasciola gigantica*. *Veterinary Parasitology* **144**, 279–286.

Hegazi, A.G., Abd El Hady, F.K. & Shalaby, H.A. (2007b) Inhibitory effect of Egyptian propolis on *Fasciola hepatica* eggs with reference to its effect on *Clostridium oedematiens* and correlation to chemical composition. *Pakistan Journal of Biological Sciences* **10**, 3295–3305.

Hennessy, D.R., Lacey, E., Steel, J.W. & Prichard, R.K. (1987) The kinetics of triclabendazole disposition in sheep. *Journal of Veterinary Pharmacology and Therapeutics* **10**, 64–72.

Hien, T.T., Truong, N.T., Minh, N.H., Dat, H.D., Dung, N.T., Hue, N.T., Dung, T.K., Tuan, P.Q., Campbell, J.I., Farrar, J.J. & Day, J.N. (2008) A randomised controlled pilot study of artesunate versus triclabendazole for human fascioliasis in Central Vietnam. *American Journal of Tropical Medicine and Hygiene* **78**, 388–392.

Higashi, K.O. & de Castro, S.L. (1994) Propolis extracts are effective against *Trypanosoma cruzi* and have an impact on its interaction with host cells. *Journal of Ethnopharmacology* **43**, 149–155.

Hopkins, T.J. & Gyr, P. (1991) Synergism of a combination of febantel and pyrantel emboate against *Ancylostoma caninum* on dogs. *Veterinary Medical Review* **61**, 3–9.

Iqbal, Z., Akhtar, M.S., Sindhu, Z., Khan, M. & Jabbar, A. (2003) Herbal dewormers in livestock – a traditional therapy. *International Journal of Agriculture and Biology* **2**, 199–206.

Kayser, O., Kiderlen, A.F. & Croft, S. (2003) Natural products as antiparasitic drugs. *Parasitology Research* **90**, S55–S62.

Keiser, J. & Morson, G. (2008a) *Fasciola hepatica*: tegumental alterations in adult flukes following *in vitro* and *in vivo* administration of artesunate and artemether. *Experimental Parasitology* **118**, 228–237.

Keiser, J. & Morson, G. (2008b) *Fasciola hepatica*: surface tegumental responses to *in vitro* and *in vivo* treatment with the experimental fasciolicide OZ78. *Experimental Parasitology* **119**, 87–93.

Keiser, J. & Utzinger, J. (2007a) Food-borne trematodiasis: current chemotherapy and advances with artemisinins and synthetic trioxolanes. *Trends in Parasitology* **23**, 555–562.

Keiser, J. & Utzinger, J. (2007b) Artemisinins and synthetic trioxolanes in the treatment of helminth infections. *Current Opinion in Infectious Diseases* **20**, 605–612.

Keiser, J., Engels, D., Buscher, G. & Utzinger, J. (2005) Triclabendazole for the treatment of fascioliasis and paragonimiasis. *Expert Opinion in Investigational Drugs* **14**, 1513–1526.

Keiser, J., Xiao, S.-H., Jian, X., Chang, Z.-S., Odematt, P., Tesana, S., Tanner, M. & Utzinger, J. (2006a) Effect of artesunate and artemether against *Clonorchis sinensis* and *Opisthorchis viverrini* in rodent models. *International Journal of Antimicrobial Agents* **28**, 370–373.

Keiser, J., Brun, R., Fried, B. & Utzinger, J. (2006b) Trematocidal activity of praziquantel and artemisinin derivatives: *in vitro* and *in vivo* investigations on adult *Echinostoma caproni*. *Antimicrobial Agents and Chemotherapy* **50**, 803–805.

Keiser, J., Utzinger, J., Tanner, M., Dong, Y. & Vennerstrom, J.L. (2006c) The synthetic peroxide OZ78 is effective against *Echinostoma caproni* and *Fasciola hepatica*. *Journal of Antimicrobial Chemotherapy* **58**, 1193–1197.

Keiser, J., Shu-Hua, X., Tanner, M. & Utzinger, J. (2006d) Artesunate and artemether are effective fasciolicides in the rat model and *in vitro*. *Journal of Antimicrobial Chemotherapy* **57**, 1139–1145.

Keiser, J., Utzinger, J., Vennerstrom, J.L., Dong, Y., Brennan, G.P. & Fairweather, I. (2007a) Activity of artemether and OZ78 against triclabendazole-resistant *Fasciola hepatica*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **101**, 1219–1222.

Keiser, J., Shu-Hua, X., Chollet, J., Tanner, M. & Utzinger, J. (2007b) Evaluation of the *in vivo* activity of tribendimidine against *Schistosoma mansoni*, *Fasciola hepatica*, *Clonorchis sinensis* and *Opisthorchis viverrini*. *Antimicrobial Agents and Chemotherapy* **51**, 1096–1098.

Keiser, J., Xiao, S.H., Dong, Y., Utzinger, J. & Vennerstrom, J.L. (2007c) Clonorchicidal properties of the synthetic trioxolane OZ78. *Journal of Parasitology* **93**, 1208–1213.

Kelly, J.D., Whitlock, H.V., Thompson, H.V., Hall, C.A., Campbell, N.J., Martin, I.C.A. & Lejambre, L.F. (1978) Physiological characteristics of the free-living and parasitic stages of strains of *Haemonchus contortus* susceptible or resistant to benzimidazole anthelmintics. *Research in Veterinary Science* **25**, 376–385.

Kerboeuf, D., Blackhall, W., Kaminsky, R. & von Samson-Himmelstjerna, G. (2003) P-glycoprotein in helminths: function and perspectives for anthelmintic treatment and reversal of resistance. *International Journal of Antimicrobial Agents* **22**, 332–346.

Koko, W.S., Galal, M. & Khalid, H.S. (2000) Fasciolicidal efficacy of *Albizia anthelmintica* and *Balanites aegyptiaca* compared with albendazole. *Journal of Ethnopharmacology* **71**, 247–252.

Lanusse, C.E. & Prichard, R.K. (1991) Enhancement of the plasma concentration of albendazole sulphoxide in sheep following coadministration of parenteral netobimin and liver oxidase inhibitors. *Research in Veterinary Science* **51**, 306–312.

Lanusse, C.E. & Prichard, R.K. (1992) Methimazole increases the plasma concentrations of the albendazole metabolites of netobimin in sheep. *Biopharmaceutics and Drug Disposition* **13**, 95–103.

Lespine, A., Alvinerie, M., Vercruysse, J., Prichard, R. & Geldhof, P. (2008) ABC transporter modulation: a strategy to enhance the activity of macrocyclic lactone anthelmintics. *Trends in Parasitology* **24**, 293–298.

Li, Y. & Wu, Y.-L. (2003) An over four millennium story behind qinghaosu (artemisinin) – a fantastic antimalarial drug from a traditional Chinese herb. *Current Medicinal Chemistry* **10**, 2197–2230.

Lifschitz, A., Virkel, G., Ballent, M., Sallovitz, J. & Lanusse, C. (2009) Combined use of ivermectin and triclabendazole in sheep: *in vitro* and *in vivo* characterisation of their pharmacological interaction. *Veterinary Journal*, in press.

López-Garcia, M.L., Torrado, S., Torrado, S., Martinez, A.R. & Bolás, F. (1998) Methimazole-mediated enhancement of albendazole oral bioavailability and anthelmintic effects against parenteral stages of *Trichinella spiralis* in mice: the influence of the dose-regime. *Veterinary Parasitology* **75**, 209–219.

Maingi, N., Scott, M.E. & Prichard, R.K. (1990) Effect of selection pressure for thiabendazole resistance on fitness of *Haemonchus contortus* in sheep. *Parasitology* **100**, 327–335.

Mas-Coma, S., Bargues, M.D. & Valero, M.A. (2005) Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* **35**, 1255–1278.

McConville, M., Brennan, G.P., McCoy, M., Castillo, R., Hernández-Campos, A. & Fairweather, I. (2006) Adult triclabendazole-resistant *Fasciola hepatica*: surface and subsurface tegumental responses to *in vitro* treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. *Parasitology* **133**, 195–208.

McConville, M., Brennan, G.P., McCoy, M., Castillo, R., Hernández-Campos, A., Ibarra, F. & Fairweather, I. (2007) Immature triclabendazole-resistant *Fasciola hepatica*: tegumental responses to *in vitro* treatment with the sulphoxide metabolite of the experimental fasciolicide compound alpha. *Parasitology Research* **100**, 365–377.

McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W.J., McCoy, M., Castillo, R., Hernández-Campos, A. & Fairweather, I. (2008) Surface and internal tegumental changes in juvenile *Fasciola hepatica* following treatment *in vivo* with the experimental fasciolicide, compound alpha. *Veterinary Parasitology* **153**, 52–64.

McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W.J., Hanna, R.E.B., McCoy, M., Gordon, A.W., Castillo, R., Hernández-Campos, A. & Fairweather, I. (2009a) An evaluation of the efficacy of compound alpha and triclabendazole against two isolates of *Fasciola hepatica*. *Veterinary Parasitology*, in press.

McConville, M., Brennan, G.P., Flanagan, A., Edgar, H.W.J., Castillo, R., Hernández-Campos, A. & Fairweather, I. (2009b) TEM ultrastructural changes to the tegumental system and the gastrodermal cells in mature flukes following *in vivo* treatment with the experimental fasciolicide, compound alpha. *Parasitology*, in press.

McCoy, M.A., Fairweather, I., Brennan, G.P., Kenny, J.M. & Forbes, A.B. (2005) The efficacy of nitroxynil and triclabendazole administered synchronously against juvenile triclabendazole-resistant *Fasciola hepatica* in sheep. *Research in Veterinary Science* **78**, 33.

McKinstry, B., Brennan, G.P., Halferty, L., Forbes, A.B. & Fairweather, I. (2007) Ultrastructural changes induced in the tegument and gut of *Fasciola hepatica* following *in vivo* and *in vitro* drug treatment with nitroxynil (Trodax). *Parasitology Research* **101**, 929–941.

McKinstry, B., Halferty, L., Brennan, G.P. & Fairweather, I. (2009) Morphological response of triclabendazole-resistant isolates of *Fasciola hepatica* to treatment *in vitro* with nitroxynil (Trodax). *Parasitology Research* **104**, 645–655.

Meaney, M., Allister, J., McKinstry, B., McLaughlin, K., Brennan, G.P., Forbes, A.B. & Fairweather, I. (2006) *Fasciola hepatica*: morphological effects of a combination of triclabendazole and clorsulon against mature fluke. *Parasitology Research* **99**, 609–621.

Meaney, M., Allister, J., McKinstry, B., McLauglin, K., Brennan, G.P., Forbes, A.B. & Fairweather, I. (2007) *Fasciola hepatica*: ultrastructural effects of a combination of triclabendazole and clorsulon against mature fluke. *Parasitology Research* **100**, 1091–1104.

Merino, G., Molina, A.J., Garcia, J.L., Pulido, M.M., Prieto, J.G. & Alvarez, A.I. (2003) Intestinal elimination of albendazole sulfoxide: pharmacokinetic effects of inhibitors. *International Journal of Pharmaceutics* **263**, 123–132.

Mestorino, N., Formentini, E.A., Lucas, M.F., Fernandez, C., Modamio, P., Mariño Hernández, E. & Errecalde, J.O. (2008) Pharmacokinetic disposition of triclabendazole in cattle and sheep; discrimination of the order and the rate of the absorption process of its active metabolite triclabendazole sulfoxide. *Veterinary Research Communications* **32**, 21–33.

Molento, M.B. & Prichard, R.K. (1999) Effects of the multidrug-reversing agents verapamil and CL 347, 099 on the efficacy of ivermectin or moxidectin against unselected and drug-selected strains of *Haemonchus contortus* in jirds. *Parasitology Research* **85**, 1007–1011.

Moll, L., Gaasenbeek, C.P.H., Vellema, P. & Borgsteede, F.H.M. (2000) Resistance of *Fasciola hepatica* against triclabendazole in cattle and sheep in the Netherlands. *Veterinary Parasitology* **91**, 153–158.

Mottier, L., Virkel, G., Solana, H., Alvarez, L., Salles, J. & Lanusse, C. (2004) Triclabendazole biotransformation and comparative diffusion of the parent drug and its oxidised metabolites into *Fasciola hepatica*. *Xenobiotica* **34**, 1043–1047.

Mottier, L., Alvarez, L., Ceballos, L. & Lanusse, C. (2006a) Drug transport mechanisms in helminth parasites: passive diffusion of benzimidazole anthelmintics. *Experimental Parasitology* **113**, 49–57.

Mottier, L., Alvarez, L., Fairweather, I. & Lanusse, C. (2006b) Resistance-induced changes in triclabendazole transport in *Fasciola hepatica*: ivermectin reversal effect. *Journal of Parasitology* **92**, 1355–1360.

O'Neill, J.F., Johnston, R.C., Halferty, L., Brennan, G.P., Keiser, J. & Fairweather, I. (2009) Adult triclabendazole-resistant *Fasciola hepatica*: morphological responses to *in vivo* treatment with artemether in the rat model. *Journal of Helminthology* this issue.

Ramírez, N., Mayet, L., Del Rivero, L., Ibarra-Velarde, F., Castillo, R., Hernández-Campos, A. & Jung-Cook, H. (2009) Pharmacokinetic behaviour in sheep and cattle of 5-chloro-2-(methylthio)-6-(1-naphthyloxy)-1*H*-benzimidazole, a new fasciolicide agent. *Journal of Veterinary Pharmacology and Therapeutics*, in press.

Rao, R.R. (1981) Ethnobotany of Meghalaya: medicinal plants used by Khasi and Garo tribes. *Economic Botany* **35**, 4–9.

Reyes, P.M., Ibarra, V.F., Vera, M.Y., Cantó, A.G., Hernández, A., Hernández, C.A., Castillo, R. & Villa, M.A. (2008) Paramphistomicidal efficacy of an experimental compound in sheep. *Parasitology Research* **102**, 705–708.

Rivero, L.M., Jung, H., Castillo, R. & Hernández-Campos, A. (1998) High-performance liquid chromatographic assay for a new fasciolicide agent, α-BIOF10,

in biological fluids. *Journal of Chromatography B* **712**, 237–241.

Robinson, M.W., Trudgett, A., Hoey, E.M. & Fairweather, I. (2002) Triclabendazole-resistant *Fasciola hepatica*: β-tubulin and response to *in vitro* treatment with triclabendazole. *Parasitology* **124**, 325–338.

Robinson, M.W., Lawson, J., Trudgett, A., Hoey, E.M. & Fairweather, I. (2004a) The comparative metabolism of triclabendazole sulphoxide by triclabendazole-susceptible and triclabendazole-resistant *Fasciola hepatica*. *Parasitology Research* **92**, 205–210.

Robinson, M.W., McFerran, N., Trudgett, A., Hoey, L. & Fairweather, I. (2004b) A possible model of benzimidazole binding to β-tubulin disclosed by invoking an inter-domain movement. *Journal of Molecular Graphics and Modelling* **23**, 275–284.

Roy, B. & Tandon, V. (1999) Flukicidal activity of *Alpinia nigra* (Zingiberaceae) against the trematode, *Fasciolopsis buski*, in humans. *Biomedical Letters* **60**, 23–29.

Ryan, L.A., Hoey, E., Trudgett, A., Fairweather, I., Fuchs, M., Robinson, M.W., Chambers, E., Timson, D., Ryan, E., Fetwell, T., Ivens, A., Bentley, G. & Johnston, D. (2008) *Fasciola hepatica* expresses multiple α- and β-tubulin isotypes. *Molecular and Biochemical Parasitology* **159**, 73–78.

Sánchez, S., Small, J., Jones, D. & McKellar, Q.A. (2002) Plasma achiral and chiral pharmacokinetic behaviour of intravenous oxfendazole co-administered with piperonyl butoxide in sheep. *Journal of Veterinary Pharmacology and Therapeutics* **25**, 7–13.

Sangster, N.C. (2001) Managing parasiticide resistance. *Veterinary Parasitology* **98**, 89–109.

Shiramizu, K., Tuchida, T. & Anu, M. (1993) The anthelmintic effect on bovine fascioliasis of a crude preparation containing the root of *Matteuccia orietalis*. *Journal of the Japan Veterinary Medical Association* **46**, 561–564.

Shoop, W.L., Ostlind, D.A., Rohrer, S.P., Mickle, G., Haines, H.W., Michael, B.F., Mrozik, H. & Fisher, M.H. (1995) Avermectins and Milbemycins against *Fasciola hepatica*: *in vivo* drug efficacy and *in vitro* receptor binding. *International Journal for Parasitology* **25**, 923–927.

Shu-Hua, X., Jian, X., Tanner, M., Yong-Nian, Z., Keiser, J., Utzinger, J. & Hui-Qiang, Q. (2008) Artemether, artesunate, praziquantel and tribendimidine administered singly at different dosages against *Clonorchis sinensis*: a comparative *in vivo* study. *Acta Tropica* **106**, 54–59.

Spicher, M., Roethlisberger, C., Lany, C., Stadelmann, B., Keiser, J., Ortega-Mora, L.M., Gottstein, B. & Hemphill, A. (2008) *In vitro* and *in vivo* treatments of *Echinococcus* protoscoleces and metacestodes with artemisinin and artemisinin derivatives. *Antimicrobial Agents and Chemotherapy* **52**, 3447–3450.

Stepek, G., Lowe, A.E., Buttle, D.J., Duce, I.R. & Behnke, J.M. (2007) *In vitro* anthelmintic effects of cysteine proteinases from plants against intestinal helminths of rodents. *Journal of Helminthology* **81**, 353–360.

Tagboto, S. & Townson, S. (2001) Antiparasitic properties of medicinal plants and other naturally occurring products. *Advances in Parasitology* **50**, 198–295.

Toner, E., Brennan, G.P., Wells, K., McGeown, J.G. & Fairweather, I. (2008) Physiological and morphological effects of genistein against the liver fluke, *Fasciola hepatica*. *Parasitology* **135**, 1189–1203.

Toner, E., McConvery, F., Brennan, G.P., Meaney, M. & Fairweather, I. (2009) A scanning electron microscope study on the route of entry of triclabendazole into the liver fluke, *Fasciola hepatica*. *Parasitology*, in press.

Utzinger, J. & Keiser, J. (2004) Schistosomiasis and soil-transmitted helminthiasis: common drugs for treatment and control. *Expert Opinion on Pharmacotherapy* **5**, 263–285.

Utzinger, J., Xiao, S.-H., Tanner, M. & Keiser, J. (2007) Artemisinins for schistosomiasis and beyond. *Current Opinion in Investigational Drugs* **8**, 105–116.

Vennerstrom, J.L., Arbe-Barnes, S., Brun, R., Charman, S.A., Chiu, F.C.K., Chollet, J., Dong, Y., Dorn, A., Hunziker, D., Matile, H., McIntosh, K., Padmanilayam, M., Tomas, J.S., Scheurer, C., Scorneaux, B., Tang, Y., Urwyler, H., Wittlin, S. & Charman, W.N. (2004) Identification of an antimalarial synthetic trioxolane drug development candidate. *Nature* **430**, 900–904.

Virkel, G., Lifschitz, A., Sallovitz, J., Pis, A. & Lanusse, C. (2006) Assessment of the main metabolism pathways for the flukicidal compound triclabendazole in sheep. *Journal of Veterinary Pharmacology and Therapeutics* **29**, 213–223.

Virkel, G., Lifschitz, A., Sallovitz, J., Ballent, M., Scarcella, S. & Lanusse, C. (2009) Inhibition of cytochrome P450 activity enhances the systemic availability of triclabendazole metabolites in sheep. *Journal of Veterinary Pharmacology and Therapeutics* **32**, 79–86.

Walker, S.M., Hoey, E., Fletcher, H., Brennan, G., Fairweather, I. & Trudgett, A. (2006) Stage-specific differences in fecundity over the life-cycle of two characterized isolates of the liver fluke, *Fasciola hepatica*. *Parasitology* **133**, 209–216.

WHO (2007) Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control, held at WHO Headquarters, Geneva, Switzerland, October 2006. 39 pp. Geneva, WHO.

Wolstenholme, A.J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G. & Sangster, N.C. (2004) Drug resistance in veterinary helminths. *Trends in Parasitology* **20**, 469–476.

Woodrow, C.J., Haynes, R.K. & Krishna, S. (2005) Artemisinins. *Postgraduate Medical Journal* **81**, 71–78.

Xiao, S.-H. (2005) Development of antischistosomal drugs in China, with particular consideration to praziquantel and the artemisinins. *Acta Tropica* **96**, 153–167.

Xiao, S.-H., Wu, Y.-L., Tanner, M., Wu, W.-M., Utzinger, J., Mei, J.-Y., Scorneaux, B., Chollet, J. & Zhai, Z. (2003) *Schistosoma japonicum*: *in vitro* effects of artemether combined with haemin depend on cultivation media and appraisal of artemether products appearing in the media. *Parasitology Research* **89**, 459–466.

Xue, J., Utzinger, J., Zhang, Y.-N., Tanner, M., Keiser, J. & Xiao, S.-H. (2008) Artemether and tribendimidine lack activity in experimemtal treatment of *Paragonimus westermani* in the dog. *Parasitology Research* **102**, 537–540.

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