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Potentiation of triclabendazole action *in vivo* against a triclabendazole-resistant isolate of *Fasciola hepatica* following its co-administration with the metabolic inhibitor, ketoconazole

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ABSTRACT

An *in vivo* study in the laboratory rat model has been carried out to monitor morphological changes in adult *Fasciola hepatica* over a 4-day period resulting from co-treatment with triclabendazole (TCBZ) and ketoconazole (KTZ), a cytochrome P450 inhibitor. Rats were infected with the triclabendazole-resistant Oberon isolate of *F. hepatica*, dosed orally with triclabendazole at a dosage of 10 mg/kg live weight and ketoconazole at a dosage of 10 mg/kg live weight. Flukes were recovered at 24, 48, 72 and 96 h post-treatment (p.t.) and changes to fluke ultrastructure were assessed using transmission electron microscopy (TEM). Results showed an increase in the severity of changes to the fluke ultrastructure with time p.t. Swelling of the basal infolds and the associated mucopolysaccharide masses became more severe with time. Golgi complexes, if present, were greatly reduced in size and number by 96 h p.t., and sub-tegumental flooding was seen from the 72 h time-period onwards. Some sloughing of the tegumental covering over the spines was observed at 96 h p.t. The results demonstrated that the Oberon isolate is more sensitive to TCBZ action in the presence of KTZ than to TCBZ alone, reinforcing the idea that altered drug metabolism is involved in the resistance mechanism. Moreover, they support the concept that TCBZ + inhibitor combinations (aimed at altering drug pharmacokinetics and potentiating the action of TCBZ) could be used in the treatment of TCBZ-R populations of *F. hepatica*.

1. Introduction

Future control of fasciolosis is faced with two major challenges: a likely increase in disease and the spread of resistance to triclabendazole (TCBZ). There has been a change in the pattern and incidence of disease in recent years, which has been attributed to changes in climate (Kenyon et al., 2009; Van Dijk et al., 2010). This trend is predicted to continue well into the future (Fox et al., 2011). Resistance to TCBZ has been demonstrated in fluke populations in three continents: Australia, Europe and South America (Overend and Bowen, 1995; Coles et al., 2000; Moll et al., 2000; Coles and Stafford, 2001; Gaasenbeek et al., 2001; Walker et al., 2004; McConville et al., 2009a; Mooney et al., 2009; Flanagan et al., 2011a,b; Olaechea et al., 2011). The true extent of resistance is unknown, due to a lack of reliable diagnostic tests and inaccurate reports of resistance (Fairweather, 2011b,c).

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Progress has been made in understanding how flukes have become resistant to TCBZ. While much of the evidence points towards an anti-microtubule action for TCBZ (Fairweather, 2005, 2009), resistance does not appear to involve mutations in β-tubulin (Ryan et al., 2008). There is more evidence for altered drug uptake/efflux and drug metabolism. The uptake of TCBZ and TCBZ sulphoxide (TCBZ.SO) by TCBZ-resistant (TCBZ-R) flukes is significantly lower than in TCBZ-susceptible (TCBZ-S) flukes (Alvarez et al., 2005; Mottier et al., 2006a). This suggests that P-glycoprotein (Pgp)-linked drug efflux pumps are involved in the resistance mechanism. Experiments with Pgp inhibitors have shown that it is possible to "reverse" the condition of the flukes, from resistant to susceptible (Mottier et al., 2006b; Fairweather et al., 2009).

The metabolism of TCBZ and TCBZ.SO is greater in TCBZ-R than TCBZ-S isolates (Robinson et al., 2004; Alvarez et al., 2005). Complementing these pharmacological studies, a series of morphological experiments has been carried out *in vitro* in this laboratory, utilizing inhibitors of the flavin monooxygenase (FMO) and cytochrome P450 (CYP 450) metabolic pathways (Devine et al., 2009, 2010a,b,c, 2011a, in press). The results have been consistent, in that potentiation of drug action only occurred in TCBZ-R flukes. The combined data argue for a role of altered drug metabolism in the development of resistance to TCBZ. Of the three inhibitors used, greatest disruption was observed with ketoconazole (KTZ), a CYP 450 inhibitor. Consequently, this inhibitor was used in a subsequent experiment, to determine whether the potentiation seen *in vitro* could be reproduced under *in vivo* conditions. Progressively severe changes to the tegumental surface over a 4-day period were revealed by scanning electron microscopy (SEM) (Devine et al., 2011b). The result adds further support to the concept of altered drug metabolism within the fluke being involved in the resistance mechanism. Also, they indicate that alteration of TCBZ pharmacokinetics in the host can increase the action of TCBZ, which may open up the use of TCBZ + inhibitor combinations in the treatment of TCBZ-R fluke populations. The present study is concerned with fine structural changes to the tegumental system and somatic musculature of flukes collected in the same *in vivo* experiment. The tegument is the main route of entry for TCBZ compounds into the fluke (Mottier et al., 2006a; Toner et al., 2009, 2010a) and carries out many important functions for the parasite (Fairweather et al., 1999). Consequently, any changes seen are likely to reflect alterations in drug metabolism and so may help to clarify the mechanisms responsible for the gross changes visible externally.

2. Materials and methods

2.1. Experimental protocol

The protocol for this investigation is the same as that employed for the SEM study using TCBZ + KTZ *in vivo* (Devine et al., 2011b). Briefly, adult male Sprague-Dawley rats were each infected orally, under light anaesthesia via a stomach tube, with 20 metacercarial cysts of the Oberon TCBZ-resistant isolate. For details of the provenance of the Oberon isolate, see the review by Fairweather (2011a). At 12 weeks post-infection (p.i.), rats were weighed individually and dosed orally at 10 mg/kg body weight with KTZ and at 10 mg/kg body weight TCBZ ("Fasinex"; 5%, w/v). The dosage of KTZ used was chosen to be similar to those used in a previous *in vivo* study (Virkel et al., 2009). The TCBZ dose rate is the recommended one and has been used in a number of studies (Hennessy et al., 1987; Virkel et al., 2006; McConville et al., 2009a; Hanna et al., 2010; Toner et al., 2010b,c, 2011, in press; Flanagan et al., 2011a,b). Flukes were recovered at four time-points p.t. (24, 48, 72 and 96 h). A second group of rats was treated with triclabendazole alone (10 mg/kg) and sacrificed at 96 h p.t. Flukes from untreated rats were recovered at 96 h p.t. Removal of flukes from all of the rats was carried out under sterile conditions in a laminar flow cabinet and the flukes were washed repeatedly in warm (37 °C) NCTC 135 culture medium (pH 7.4) containing antibiotics (penicillin 50 IU/ml; Streptomycin 50 μg/ml). Specimens were fixed and prepared for transmission electron microscopy (TEM). At least 7 flukes were collected at each time period.

2.2. Tissue preparation for TEM

Specimens were lightly flat-fixed for 0.5 h at room temperature in 4% (w/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and 3% (w/v) sucrose. The flukes were dissected into three body regions: apical cone (including ventral sucker), midbody and tail. The midbody was further divided into transverse sections approximately 2 mm in width. The sections were then free-fixed for a further 3 h at 4 °C, after which they were washed in 0.1 M sodium cacodylate buffer (pH 7.4) containing 3% (w/v) sucrose and left overnight at 4 °C. After post-fixation in 1% osmium tetroxide for 1 h, the tissues were washed several times in fresh buffer, dehydrated through an ascending series of ethanol and infiltrated and embedded in Agar 100 resin. Ultrathin sections, 60–70 nm in thickness, were cut on a Reichert Ultracut E ultramicrotome, mounted on bare 200-mesh copper grids, double-stained with alcoholic uranyl acetate (9 min) and aqueous lead citrate (5 min) and viewed in a FEI CM100 transmission electron microscope operating at 100 keV.

3. Results

3.1. Visual observations

The flukes were alive and had full gut contents at each time period following treatment with KTZ + TCBZ. At 24 h and 48 h p.t., the flukes recovered were active; however, at 72 h and 96 h p.t. the flukes appeared sluggish in their movement.

3.2. Transmission electron microscopy

3.2.1. Controls

The tegumental ultrastructure of the control specimens was normal. For images of normal morphology, the reader is referred to the papers by Halferty et al. (2009, Figs. 4A–C) and Fairweather et al. (1999, Figs. 3.3 and 3.4).

3.2.2. Twenty-four hours post-treatment

Within the tegumental syncytium, the mucopolysaccharide masses were swollen towards their proximal end (Fig. 1A). Secretory bodies were present at the apex of the syncytium, with T2 secretory bodies seeming to accumulate just beneath the apical plasma membrane (Fig. 1B). At the base of the syncytium, swelling of the basal infolds was not observed, although the associated mucopolysaccharide masses were slightly swollen. T1 secretory bodies were present in large numbers in the basal region and the mitochondria were swollen in appearance (Fig. 1C). The muscle fibres within the sub-tegumental muscle blocks seemed fewer in number and more loosely packed than normal (Fig. 1D). Type-1 tegumental cells contained active Golgi complexes and T1 secretory bodies were also present within the cell, but they were few in number (Fig. 1E). The mitochondria present retained a relatively normal morphology. Within Type-2 tegumental cells, a Golgi complex and a small number of secretory bodies were present (Fig. 1F). The mitochondria in the cells appeared normal.

3.2.3. Forty-eight hours post-treatment

Swelling of the mucopolysaccharide masses surrounding the basal infolds was evident in the tegumental syncytium. This swelling extended towards the distal end of the masses, while the basal infolds remained closed (Fig. 2A). Blebbing of the apical plasma membrane was observed at higher magnifications (Fig. 2B). T1 and T2 secretory bodies were present throughout the tegumental syncytium, and seemed to accumulate below the apical plasma membrane (Fig. 2B). Mitochondria within the syncytium were fewer in number than normal and were swollen and rounded in appearance. T1 secretory bodies accumulated towards the base of the syncytium (Fig. 2C). The sub-tegumental muscle blocks contained fewer muscle fibres than normal and the fibres were less tightly packed than normal (Fig. 2D). Within T1-type tegumental cells, active well-developed Golgi complexes were present, secretory bodies were scarce and swollen in appearance and the mitochondria were rounded with distinct cristae (Fig. 2E). Within Type-2 tegumental cells, numerous secretory bodies were present. The mitochondria present within the cell were swollen in appearance and possessed distinct cristae (Fig. 2F).

3.2.4. Seventy-two hours post-treatment

Within the tegumental syncytium, swelling of the basal infolds and mucopolysaccharide masses was observed (Fig. 3A). Blebbing could be seen at the apex of the syncytium on the apical plasma membrane (Fig. 3B). Secretory bodies were present at the apex of the syncytium, seeming to accumulate just beneath the apical membrane (Fig. 3B). Swelling of the basal infolds was very evident in the basal region of the syncytium and the mitochondria present within the syncytium were swollen and assumed a rounded appearance, rather than the typical cylindrical shape. T1 secretory bodies seemed to accumulate in the basal region (Fig. 3C). Below the tegumental syncytium, the muscle fibres in the muscle blocks were fewer in number and appeared more loosely packed than normal (Fig. 3D). Within the T1-type tegumental cells, the Golgi complexes in the cells retained a normal morphology and T1 secretory bodies were present within the cells, although their numbers were severely reduced (Fig. 3E). The mitochondria within the cell were swollen (Fig. 3E). In the T2 tegumental cells, numerous T2 secretory bodies were present, but they were swollen in appearance, as were the mitochondria (Fig. 3F). The nuclei of both types of tegumental cell retained a normal morphology.

3.2.5. Ninety-six hours post-treatment

Sloughing of the apical plasma membrane was observed at the apex of the tegumental syncytium in the vicinity of the spines (Fig. 4A). In some specimens, the mucopolysaccharide masses were swollen at their proximal and distal ends, but the basal infolds remained tightly closed (Fig. 4A). In other specimens, the major feature of the tegumental syncytium was the severe swelling of the basal infolds (Fig. 4B). Secretory bodies were present at the apex of the syncytium and seemed to accumulate just beneath the apical plasma membrane (Fig. 4C). In the sub-tegumental region, some of the muscle blocks appeared empty and there were spaces between the tegumental cells (Fig. 4D and E). Within T2 tegumental cells, T2 secretory bodies were present but were sparse in number and swollen in appearance (Fig. 4F). Within the T1 tegumental cells, T1 secretory bodies were sparse and swollen in appearance and the Golgi complexes, when present, appeared reduced in size (Fig. 4G). The mitochondria present in both types of tegumental cell were swollen in appearance.

3.2.6. Ninety-six hours post-treatment with TCBZ

Apart from very minor swelling of the mucopolysaccharide masses in the syncytium, the fine structure of the tegumental syncytium and cell bodies was normal following treatment with TCBZ.

3.2.7. Summary of results

The main changes brought about by drug action and the relative severity of these changes are summarised in Table 1.

4. Discussion

The results of this investigation indicate that the TCBZ-resistant Oberon isolate can be made more sensitive to the action of TCBZ when treated *in vivo* in combination with the CYP 450 inhibitor, KTZ. Previous *in vitro* studies have shown that this TCBZ-resistant isolate can be made more susceptible to TCBZ action by combining the drug with metabolic inhibitors and so block its ability to adequately metabolise the drug (Devine et al., 2009, 2010a,b,c, 2011a, in press). What the present investigation (which represents a more natural scenario) has shown is that this phenomenon can be replicated *in vivo* when TCBZ metabolism, both by the host and by the fluke, is manipulated by co-treatment with a metabolic inhibitor.

Changes to the tegumental system followed a pattern of increasing severity over time. There was a typical stress

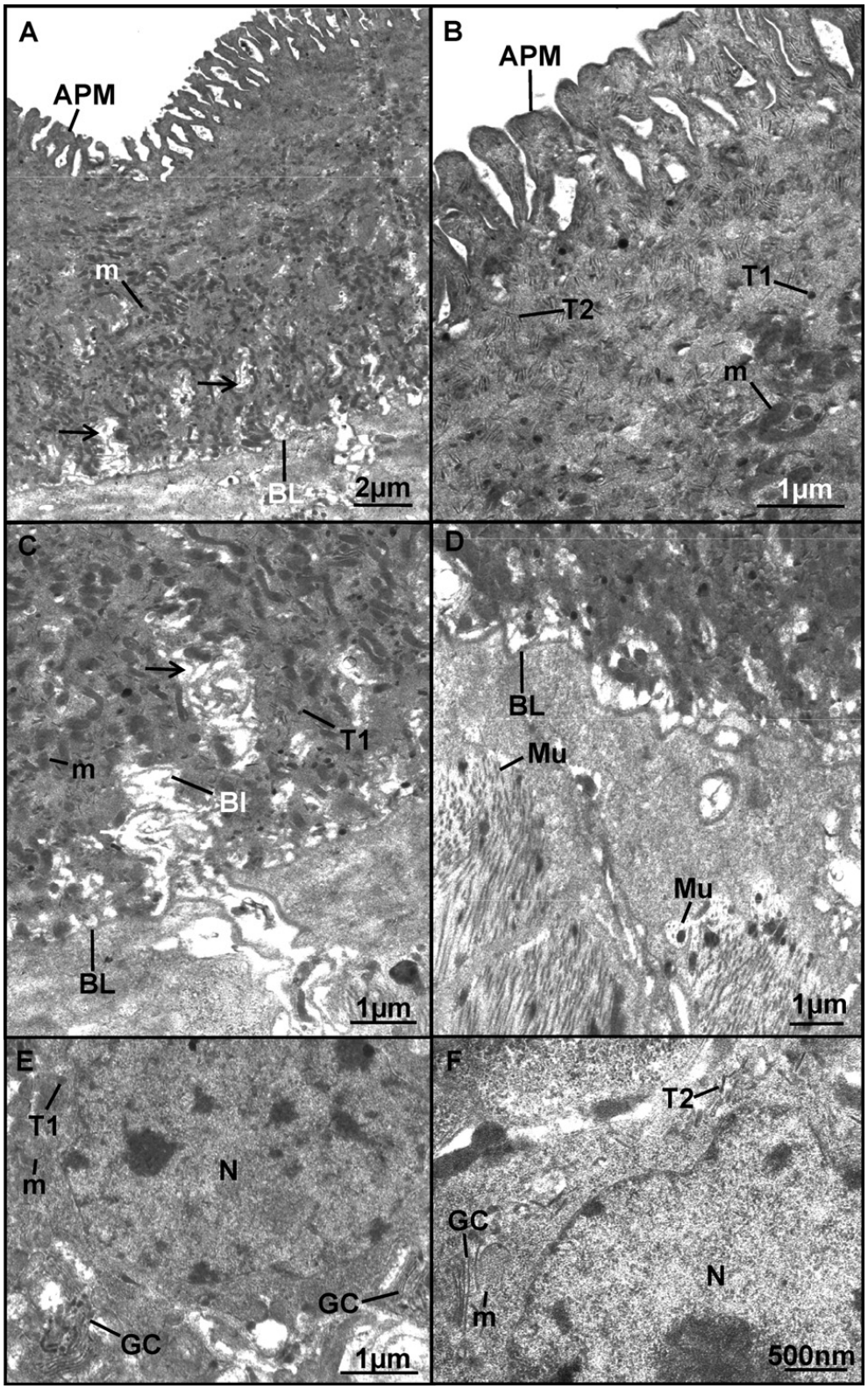

Fig. 1. Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) following 24 h *in vivo* treatment with KTZ + TCBZ. (A) TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (APM) to the basal lamina (BL). Some swelling of the mucopolysaccaride masses (arrows) can be seen. m, mitochondrion. (B) A high-power micrograph of the apical region of the tegumental syncytium. T1 (T1) and T2 (T2) secretory bodies are present just below the apical plasma membrane. There is an accumulation of secretory bodies, mainly T2 secretory bodies (T2), below the apical plasma membrane (APM). m, mitochondrion. (C) TEM showing the basal region of the tegumental syncytium. Above the basal lamina (BL), the mucopolysaccharide masses (arrow) are swollen, but the basal infolds (BI) remain tightly closed. Some of the mitochondria (m) are swollen in appearance. Numerous T1 secretory bodies (T1) are present in this region. (D) A high-power micrograph of the underlying muscle layer. The fibres within the sub-tegumental muscle blocks (Mu) are fewer in number and appear more loosely packed than normal. BL, basal lamina. (E) TEM of a T1-type of tegumental cell. Well-developed Golgi complexes (GC), but few T1 secretory bodies (T1), are present within the cell. The mitochondria (m) retain a relatively normal morphology. N, nucleus. (F) A high-power TEM of a T2-type of tegumental cell. A small number of T2 secretory bodies (T2) are present within the cell. A Golgi complex (GC) is also present. The mitochondria (m) appear normal. N, nucleus.

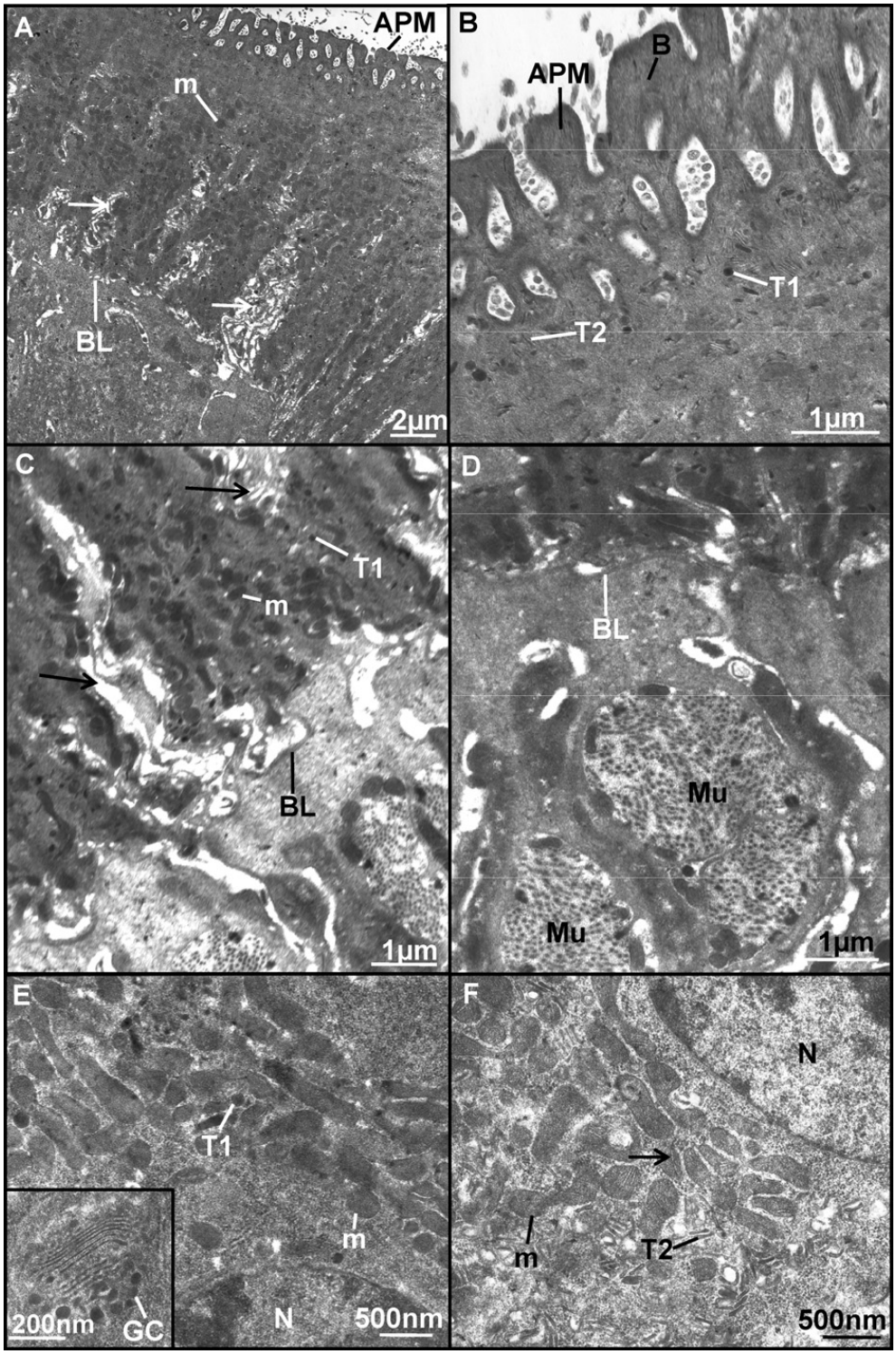

Fig. 2. Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) following 48 h *in vivo* treatment with KTZ + TCBZ. (A) TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (APM) to the basal lamina (BL). The mitochondria (m) in the syncytium are rounded in appearance. Slight swelling of the mucopolysaccharide masses (arrows) can be seen at their proximal and distal ends. (B) A high-power micrograph of the apex of the tegumental syncytium. Blebbing (B) of the apical plasma membrane (APM) is evident and T1 (T1) and T2 (T2) secretory bodies are present just below the membrane. (C) TEM showing the basal region of the tegumental syncytium. Above the basal lamina (BL), the mucopolysaccharide masses (arrows) are swollen. The mitochondria (m) are also swollen. Numerous T1 secretory bodies (T1) are present in this region. (D) TEM showing the underlying muscle layer. The fibres within the sub-tegumental muscle blocks (Mu) are fewer in number and appear more loosely packed than normal. BL, basal lamina. (E) TEM showing a T1-type of tegumental cell. A few swollen T1 secretory bodies (T1) can be seen within the cell. The mitochondria (m) are rounded in appearance, with distinct cristae, but the nucleus (N) retains a normal morphology. Inset shows a well-developed Golgi complex (GC). (F) A high-power TEM of a T2-type of tegumental cell. T2 secretory bodies (T2) are present within the cell. The mitochondria (m) present are rounded with swollen cristae (arrow).

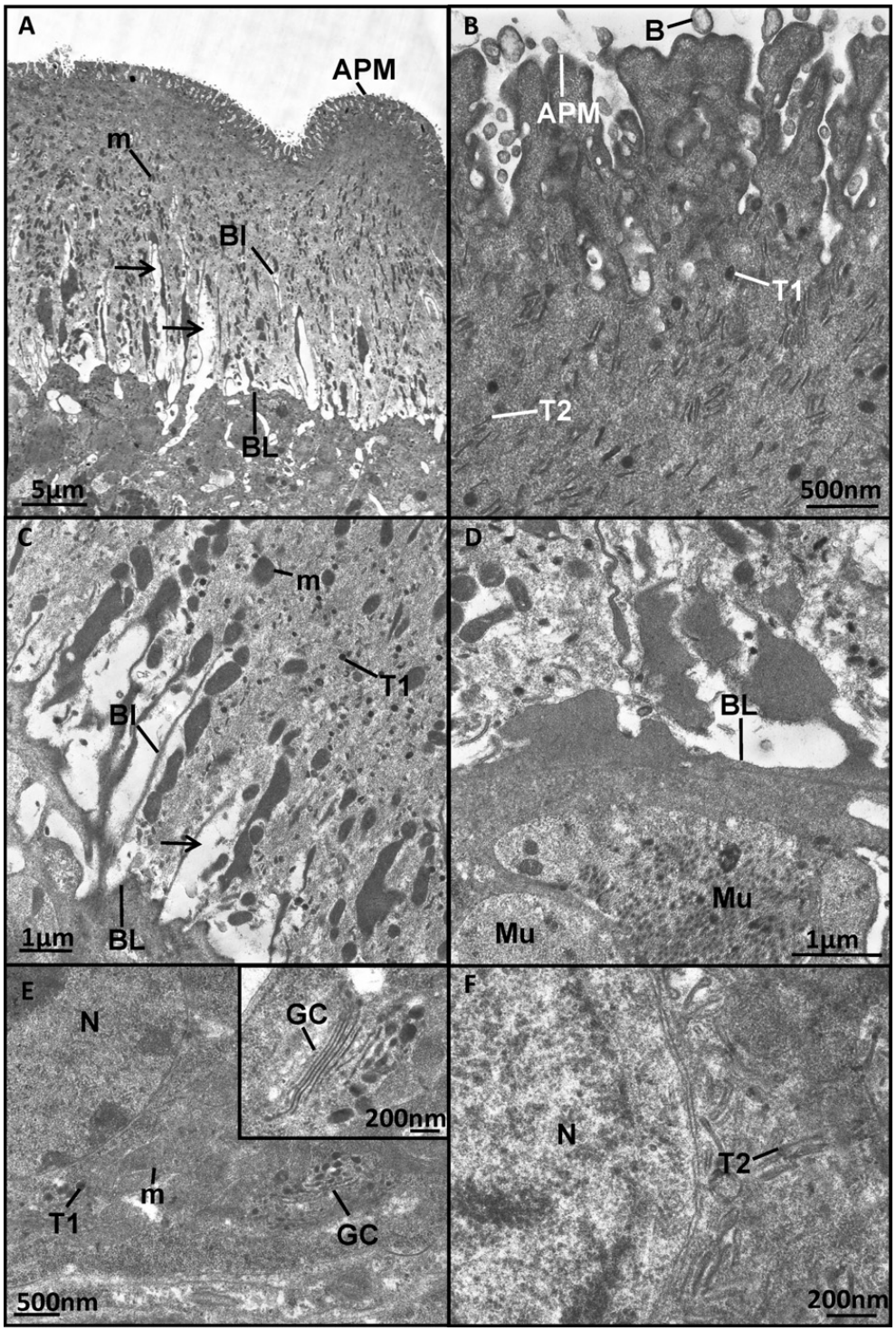

Fig. 3. Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) following 72 h *in vivo* treatment with KTZ + TCBZ. (A) TEM of the tegumental syncytium from the apical plasma membrane (APM) to the basal lamina (BL). Swelling of the mucopolysaccaride masses (arrows) and basal infolds (BI) can be seen. The mitochondria (m) in the syncytium are rounded in appearance. (B) A high-power micrograph of the apical plasma membrane (APM) of the tegumental syncytium. Blebs (B) are present on the membrane. T1 (T1) and T2 (T2) secretory bodies have accumulated below the apex. (C) TEM showing the basal region of the tegumental syncytium. Above the basal lamina (BL), the basal infolds (BI) and mucopolysaccharide masses (arrow) are swollen. The mitochondria (m) are also swollen. Numerous T1 secretory bodies (T1) are present in this region. (D) A high-power micrograph of the sub-tegumental muscle blocks. The fibres within the muscle blocks (Mu) are fewer in number and more loosely packed than normal. BL, basal lamina. (E) TEM showing a T1-type of tegumental cell. T1 secretory bodies (T1) are present within the cell, but are few in number. The Golgi complexes (GC) present retain a relatively normal morphology. Some mitochondria (m) have become swollen in appearance. N, nucleus. Inset shows a well-developed Golgi complex (GC). (F) A high-power micrograph of a T2-type of tegumental cell. The T2 secretory bodies (T2) appear slightly swollen. N, nucleus.

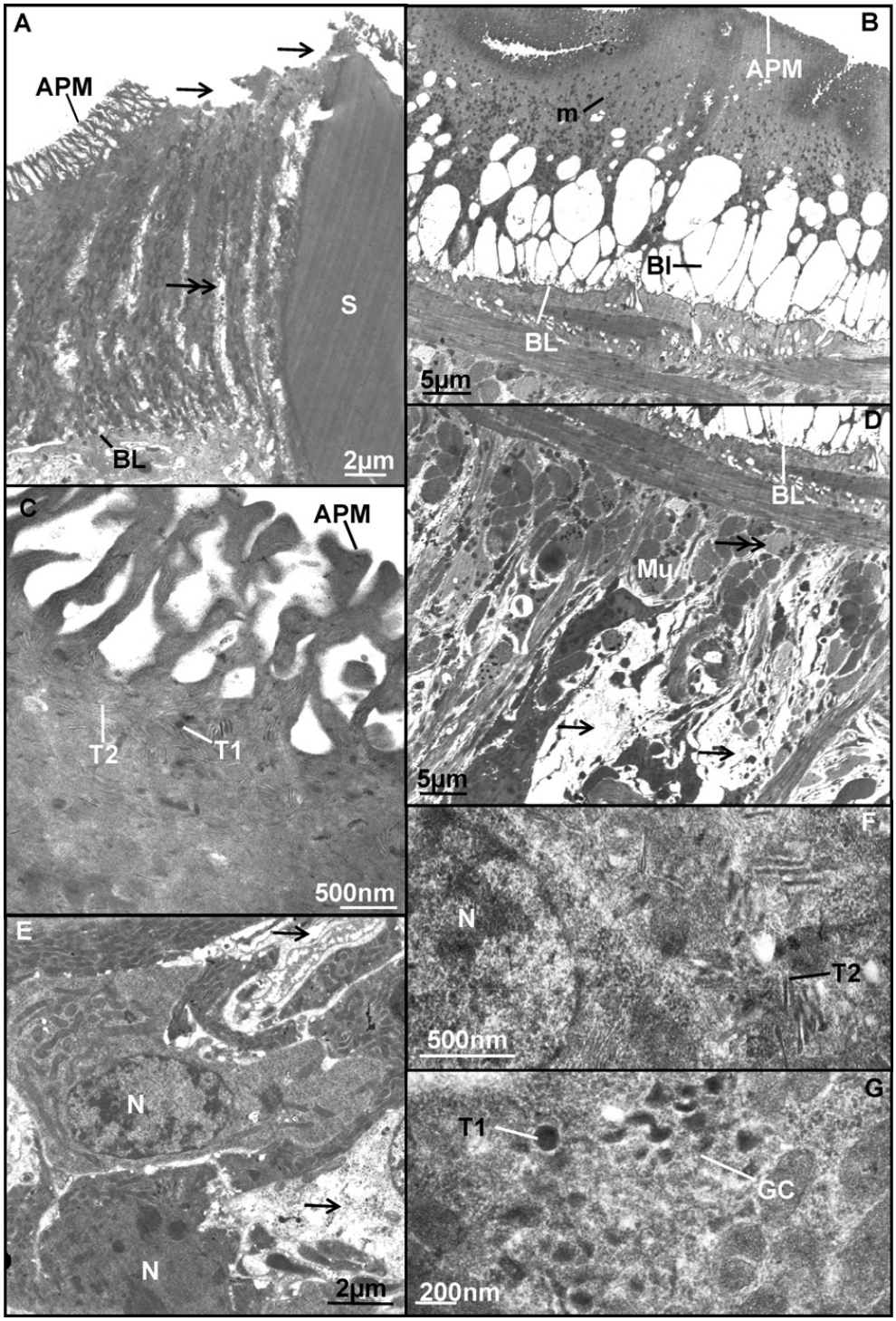

Fig. 4. Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) following 96 h *in vivo* treatment with KTZ + TCBZ. (A) TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (APM) to the basal lamina (BL). Sloughing (arrows) of the apical plasma membrane can be seen around the spines (S). Slight swelling of the mucopolysaccharide masses (double arrow) can be seen throughout the syncytium. (B) TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (APM) to the basal lamina (BL). The basal infolds (BI) are severely swollen. Mitochondria (m) are present throughout the syncytium and appear slightly swollen. (C) A high-power micrograph of the apical region of the tegumental syncytium. Numerous T1 (T1) and T2 (T2) secretory bodies are present below the apical plasma membrane (APM). (D) TEM showing the region below the basal lamina (BL). Some of the sub-tegumental muscle blocks (Mu) appear empty (double arrow). Severe flooding between cells can also be seen (arrows). (E) TEM showing a number of tegumental cells. There is spacing between the cells (arrows). The nuclei (N) of the cells retain a normal morphology. (F) A high-power micrograph of a T2-type of tegumental cell containing T2 secretory bodies (T2), some of which appear swollen. N, nucleus. (G) A high-power micrograph of a Golgi complex (GC) in a T1-type of tegumental cell. The complex appears reduced in size and the T1 secretory bodies (T1) appear swollen.

Table 1
Oberon isolate of *Fasciola hepatica*. Summary of changes to the tegumental system following *in vivo* and *in vitro* treatments.

	In vivo treatment				*In vitro*
	24 h	48 h	72 h	96 h	KTZ + NADPH + TCBZ.SO
Changes in Syncytium					
Blebbing	–	+	++	–	–
Sloughing	–	–	–	+	–
Altered numbers of secretory bodies at apex	+++	+++	+++	+++	++
Swelling of basal infolds	–	–	++	+++	+++
Swelling of mucopolysaccharide masses	+++	+++	+++	+++	+++
Swelling of mitochondria	+	++	+++	+++	+++
Disruption to muscle fibres	+	+	++	+++	–
Changes in Tegumental cells					
Altered numbers of secretory bodies	+	+	++	+++	++
Reduction in number and size of Golgi complexes	–	–	–	+++	+++
Swelling of secretory bodies	–	+	++	+++	++
Swelling of cisternae of GER	–	–	–	–	–
Swelling of mitochondria	–	++	++	+++	+++
Spacing between cells	–	–	–	+++	++
Total	9	14	22	31	23

–, no noticeable disruption; +, general/mild disruption; ++, severe disruption; +++, very severe disruption. KTZ, ketoconazole; NADPH, nicotinamide adenine dinucleotide phosphate; TCBZ, triclabendazole; TCBZ.SO, triclabendazole sulphoxide. GER, granular endoplasmic reticulum.

response, characterised by apical accumulations of secretory bodies in the syncytium and blebbing of the surface membrane. These features were accompanied by swelling of the mucopolysaccharide masses and of the mitochondria, together with a decrease in the number of secretory bodies produced by the tegumental cells. Later, the secretory bodies became abnormal in appearance, the basal infolds started to swell and, eventually, sloughing of the tegument was observed and there was flooding of the internal tissues. The efficacy of the combination was not assessed but, given the degree of disruption observed, it is difficult to envisage how the fluke could have survived treatment if the experiment had been extended beyond 4 days.

The accumulation of secretory bodies beneath the apical tegumental membrane is believed to be a response by the fluke to drug action, in that it represents an accelerated movement of secretory bodies to the surface, in order to repair damage caused by drug action. Blebbing of the surface membrane is another aspect of the stress response, as the fluke attempts to shed membrane that has been damaged by drug action, in an effort to preserve the integrity of the membrane. Extended maintenance of the responses is dependent on the continued production of secretory bodies by the tegumental cells: once this is affected and the secretory bodies become abnormal, then the integrity of the surface is critically impaired and more severe disruption, such as sloughing, will occur and will hasten the death of the fluke. Areas of sloughing were evident around the spines at 96 h p.t., indicating that the fluke was no longer able to maintain the structure of the tegument and was succumbing to TCBZ action. Larger areas of sloughing were observed in the SEM study (Devine et al., 2011b), although the limitations in the area of tissue able to be sampled by TEM could account for these areas being missed.

Evidence of a stress response was first noted in *in vitro* experiments on flukicide action (*e.g.* Fairweather et al., 1986; Anderson and Fairweather, 1995; Robinson et al., 2002). It was confirmed as a genuine phenomenon by subsequent *in vivo* studies in both rat and sheep (Skuce and Fairweather, 1990; Meaney et al., 2004; McKinstry et al., 2007; McConville et al., 2008, 2009b). The swelling of the basal infolds and flooding of the internal tissues indicate that disruption of the apical membrane and its associated ion pumps has led to perturbation of the fluke's osmoregulatory system and the influx of water (Threadgold and Brennan, 1978). The swelling of the basal infolds will facilitate the sloughing of the tegument as a whole, by causing the detachment of the basal plasma membrane from the underlying basal lamina. Flooding of the syncytium and sub-tegumental tissues is a particular feature of drug action with a number of flukicides (Fairweather et al., 1986; Skuce et al., 1987; Anderson and Fairweather, 1995; Meaney et al., 2004, 2007; McKinstry et al., 2007, 2009; Halferty et al., 2009; Toner et al., 2010c).

The internal changes in the tegument observed in this study are compatible with surface changes seen in the SEM part of this investigation, using the same drug and inhibitor combination (Devine et al., 2011b). The tegumental swelling and furrowing observed on the surface can be linked to the swelling of the basal infolds and mucopolysaccharide masses seen internally. The presence of blebs and microvillus-like projections indicates that the fluke is having problems repairing and replacing surface membrane damaged by drug action. This is probably due to the sharp decrease in the number of secretory bodies and the reduction in size and number of Golgi complexes at 96 h p.t. in the tegumental cells. Also, to the abnormalities of those secretory bodies that are produced, an effect that was evident from 48 h p.t. onwards. The impact on the synthesis, production and transport of secretory bodies would have a major impact on the maintenance of the apical plasma membrane and lead to the blebbing seen in this study and in the previous SEM investigation. Eventually, this would lead to sloughing of the tegumental syncytium: this was first noticed at 48 h p.t., it increased in severity with time and was accompanied by loss of spines (Devine et al., 2011b).

The results of the present study add further weight to the concept that altered drug metabolism by *F. hepatica* is involved in resistance to TCBZ, as potentiation of drug action by the use of inhibitors is seen in TCBZ-R, but not in TCBZ-S flukes (for a summary of the data, see Section 1). Initially, the phenomenon was demonstrated *in vitro*; this TEM study (along with its companion SEM study) has taken the idea forward by showing that manipulation of drug metabolism by the host (in addition to that by the fluke) can affect drug susceptibility. In turn, this may have a practical application in the treatment of TCBZ-R populations of fluke and so help to preserve the efficacy of TCBZ in the short- to medium-term. By enhancing the bioavailability of TCBZ and extending the exposure of the fluke to active metabolites, the aim of using such drug plus metabolic inhibitor combinations is to increase the efficacy of the drug. It is an approach that has been shown to be successful in studies on other parasites with benzimidazole compounds (Benchaoui and McKellar, 1996; López-García et al., 1998; Sánchez-Bruni et al., 2005). These workers and others (Lanusse and Prichard, 1993; Lanusse et al., 1995; Dupuy et al., 2003; Alvinerie et al., 2008) have advocated the use of anthelmintic + inhibitor combinations as a potential option for dealing with drug resistance. It is a strategy that could be applied to the liver fluke as well and merits further investigation in a primary host, *e.g.* sheep. Co-treatment of TCBZ + ketoconazole has been shown to increase the bioavailability of TCBZ in sheep (Virkel et al., 2009). Thus, the peak plasma concentration (C_{max}) for TCBZ.SO was increased by 37% and the area under the concentration–time curve (AUC) by 41%; the equivalent figures for triclabendazole sulphone (TCBZ.SO_2) were 19% and 23%, but they were not significant. Co-treatment with another CYP 450 inhibitor, piperonyl butoxide (PB), led to significant increases in C_{max} and AUC for both TCBZ.SO and TCBZ.SO_2: 62% (for both metabolites) for C_{max} and 99% and 53% for AUC. In contrast, the FMO inhibitor, methimazole (MTZ) did not enhance the bioavailability of TCBZ metabolites (Virkel et al., 2009). The impact of enhanced pharmacokinetics with KTZ and PB on drug efficacy was not tested, but it is a parameter that should be tested. A similar approach could be taken with Pgp inhibitors as well, because altered drug uptake may also be involved in the development of resistance in *F. hepatica* to TCBZ (see Section 1 for discussion of this point).

In conclusion, this study has demonstrated that it is possible to alter the pharmacokinetics of TCBZ by co-treatment with a metabolic inhibitor and so enhance its activity against TCBZ-R flukes. In doing so, it has reinforced the results of previous *in vitro* experiments. Together, the results have shown that the CYP 450 drug metabolism pathway is more susceptible to modification than the FMO pathway, for both fluke and its host. As no new flukicidal compounds are under development and no other flukicide has activity equivalent to that of TCBZ – and in the absence of any commercial vaccines – drug combinations may be one way of allowing continued use of TCBZ. The approach may be hindered by licensing regulations, but the basic concept has been established and is worth pursuing, to determine the most appropriate inhibitor to use and the best balance of drug/inhibitor concentrations. Moreover, the concept should be tested on juvenile flukes, as they represent the most damaging phase of infection.

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