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ORIGINAL PAPER

Potentiation of triclabendazole sulphoxide-induced tegumental disruption by methimazole in a triclabendazole-resistant isolate of *Fasciola hepatica*

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Abstract A study has been carried out to investigate whether the action of triclabendazole (TCBZ) against *Fasciola hepatica* is altered by inhibition of drug metabolism. The flavin monooxygenase system (FMO) was inhibited using methimazole (MTZ) to see whether a TCBZ-resistant isolate could be made more sensitive to TCBZ action. The Oberon TCBZ-resistant and Cullompton TCBZ-susceptible isolates were used for these experiments. The FMO system was inhibited by a 2-h pre-incubation in methimazole (100 μM), then incubated for a further 22 h in NCTC medium containing either MTZ; MTZ+nicotinamide adenine dinucleotide phosphate (NADPH) (1 nM); MTZ+NADPH+TCBZ (15 μg/ml); or MTZ+NADPH+triclabendazole sulphoxide (TCBZ.SO) (15 μg/ml). Changes to fluke ultrastructure following drug treatment and metabolic inhibition were assessed using transmission electron microscopy. After treatment with either TCBZ or TCBZ.SO on their own, there was greater disruption to the TCBZ-susceptible than triclabedazole-resistant isolate. However, co-incubation with MTZ+TCBZ, but more particularly MTZ+TCBZ.SO, led to more severe changes to the TCBZ-resistant isolate than with each drug on its own, with severe swelling of the basal infolds and mucopolysaccharide masses in the syncytium, accompanied by a reduction in numbers of secretory bodies. The synthesis and production of secretory bodies in the tegumental cells was severely affected as well. With the TCBZ-susceptible Cullompton isolate, there was limited potentiation of drug action. The results support the concept of altered drug metabolism in TCBZ-resistant flukes, and this process may play a role in the development of drug resistance.

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Introduction

Livestock animals are exposed to a large number of xenobiotic agents and within the organism these compounds are metabolised by enzyme systems into more polar metabolites that are more easily excreted (Lanusse and Prichard 1993; González Canga et al. 2009). The most important enzyme systems involved in drug metabolism are the Phase 1 mixed function oxidases. These systems are highly versatile and can adapt to metabolise a range of substrates; they are also important in determining the persistence of therapeutically used drugs in target species (Virkel et al. 2006). Triclabendazole (TCBZ) is an example of an antiparasitic xenobiotic. It is the current drug of choice used for treating *Fasciola hepatica* infections, but its continuing use is being compromised by the development of resistance to it (Fairweather 2005, 2009). Sulphoxidation and sulphonation appear to be the main metabolic reactions involved in TCBZ biotransformation in sheep, and work by Virkel et al. (2006) has shown that the flavin monooxygenase and cytochrome P450 enzyme systems are involved in these reactions.

In addition to drug metabolism by its host, a parasite like *F. hepatica* has also been shown to have the ability to

metabolise TCBZ to its sulphoxide and sulphone metabolites (Mottier et al. 2004; Robinson et al. 2004). Moreover, TCBZ-resistant isolates have been shown to carry out the metabolism of TCBZ at a significantly higher rate than that by TCBZ-susceptible isolates (Robinson et al. 2004; Alvarez et al. 2005). It is this enhancement of oxidative metabolism that has been suggested as a possible mechanism of resistance within the fluke. The FMO system represents the main metabolic pathway for the metabolism of TCBZ to triclabendazole sulphoxide (TCBZ.SO) by *F. hepatica* (Alvarez et al. 2005). A previous scanning electron microscopical study showed that inhibition of this system by methimazole (MTZ) leads to more severe surface changes in a TCBZ-resistant isolate when incubated with TCBZ and TCBZ.SO than when incubated in the drug alone (Devine et al. 2009). More significantly, this potentiation of drug action by MTZ was not seen in a TCBZ-susceptible isolate. Consequently, the present study was carried out to assess internal ultrastructural changes to the same TCBZ-susceptible and TCBZ-resistant fluke isolates following incubation in MTZ, TCBZ and TCBZ.SO. Changes to fluke ultrastructure were assessed using transmission electron microscopy with a focus on the tegumental system. The tegument is the main route of entry for TCBZ compounds into the fluke (Mottier et al. 2006; Toner et al. 2009, 2010) and carries out many important functions for the parasite (Fairweather et al. 1999); therefore, it is likely to manifest the result of any altered drug metabolism. As a result, this study should be seen as a continuation of previous morphological (Devine et al. 2009) and biochemical investigations (Alvarez et al. 2005) involving MTZ which have been carried out to determine the role of altered drug metabolism in the development of resistance to TCBZ in TCBZ-resistant flukes.

Materials and methods

Adult male Sprague–Dawley rats were each infected with 20 metacercarial cysts of either the Oberon TCBZ-resistant or Cullompton TCBZ-susceptible isolate of *F. hepatica*. The Oberon isolate originated on a farm where TCBZ resistance was suspected, in Oberon, New South Wales, Australia in 1999, and has since been maintained in the laboratory. The isolate has been shown to be resistant to TCBZ action as the drug has limited efficacy against Oberon isolates in vivo and in vitro (Walker et al. 2004; Keiser et al. 2007). The Cullompton isolate originated from a field isolate in Cullompton, Devon, England. This isolate has been shown to be susceptible to TCBZ action in vivo and to its sulphoxide metabolite in vitro (Robinson et al. 2002; McCoy et al. 2005; McConville et al. 2006, 2009a; Meaney et al. 2006, 2007; Halferty et al. 2008, 2009a; Devine et al. 2009, 2010; Toner et al. 2009, 2010).

Adult flukes (at least 12 weeks old) were removed from the bile ducts of rats under sterile conditions in a laminar flow cabinet and washed repeatedly in warm (37°C) NCTC 135 culture medium (pH 7.4) containing antibiotics (penicillin 50 IU/ml; streptomycin 50 μg/ml).

The flukes were transferred to fresh culture medium containing MTZ (1×10^{-4} M) for 2 h at 37°C. After the pre-incubation period, the flukes were transferred to fresh culture medium for 22 h at 37°C containing one of a number of drug and inhibitor combinations (Table 1). A stock solution of methimazole was initially prepared at a concentration of 1×10^{-1} M in distilled water. A stock solution of nicotinamide adenine dinucleotide phosphate (NADPH) was initially prepared at a concentration of 1×10^{-3} M in distilled water. The concentrations of MTZ and NADPH used were chosen to be similar to those used by Alvarez et al. (2005) in a previous study on *F. hepatica*. The concentrations of TCBZ and TCBZ.SO used correspond to the maximum blood level of TCBZ.SO reached in vivo (13.3 μg/ml following a therapeutic dose of 10 mg/kg TCBZ in sheep) (Hennessy et al. 1987). The two compounds were initially prepared as stock solutions in dimethyl sulphoxide (DMSO) and added to the culture medium to give a final solvent concentration of 0.1% (v/v). Controls were prepared by incubating whole flukes in NCTC 135 medium for 24 h at 37°C. Controls at 0 h were also prepared. After incubation, the flukes were fixed and processed for transmission electron microscopy (TEM). A minimum of four flukes were prepared for each treatment.

Tissue preparation for transmission electron microscopy

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

Table 1 Drug and inhibitor combinations

MTZ (100μM)	NADPH (1nM)	TCBZ (15μg/ml)	TCBZ.SO (15μg/ml)
✓			
✓	✓		
✓	✓	✓	
✓	✓		✓
		✓	
			✓

MTZ methimazole, *NADPH* nicotinamide adenine dinucleotide phosphate, *TCBZ* triclabendazole, *TCBZ.SO* triclabendazole sulphoxide

(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.

Results

After incubation in all drug and inhibitor combinations for 24 h in vitro, the flukes were alive prior to fixation. Sections for TEM were taken from the midbody region of the flukes.

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. (2009a, Fig. 4a–c) and Fairweather et al. (1999, Figs. 3.3 and 3.4).

Oberon and Cullompton isolates treated with MTZ and MTZ+NADPH

The tegumental syncytium appeared relatively normal (Fig. 1). Beneath the apical plasma membrane, T1 and T2 secretory bodies were present in normal numbers (Fig. 2). Very minor swelling of the mucopolysaccharide masses was observed just above the basal lamina at the base of the syncytium (Fig. 3). The sub-tegumental muscle blocks remained unchanged (Fig. 1). The tegumental cells appeared normal. Active Golgi complexes and numerous T1 secretory bodies were observed in the Type-1 cells (Fig. 4). Within Type-2 tegumental cells, numerous secretory bodies were present and the nuclei remained unchanged (Fig. 5).

Cullompton isolate treated with TCBZ and TCBZ.SO

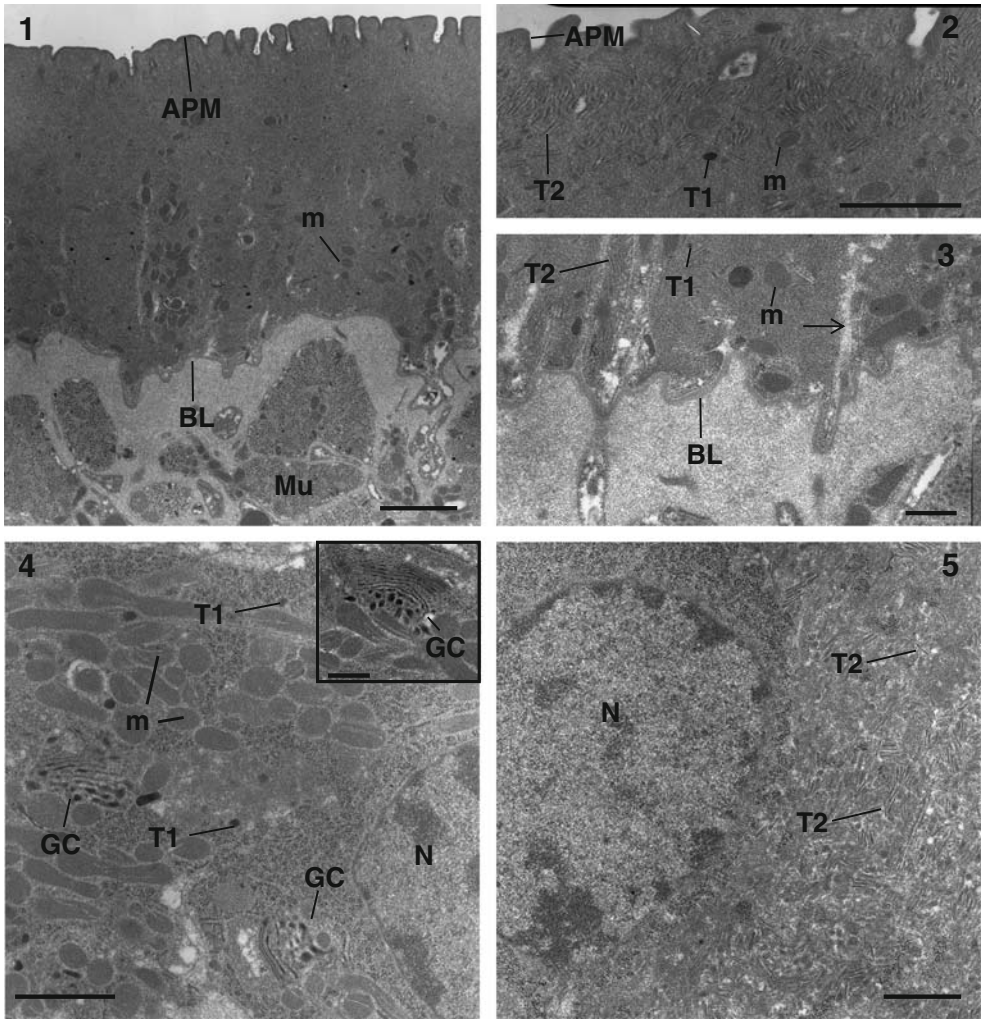
Descriptions of ultrastructural changes brought about by treatment with the two drugs have been published elsewhere and will not be repeated here. The changes observed in the present study matched these descriptions. Therefore, the reader is referred to the paper by Halferty et al. (2009a) (Fig. 4d–f for TCBZ and Fig. 5a–c for TCBZ.SO). Within the tegumental syncytium, the basal infolds were swollen, particularly in the basal region. Few secretory bodies were present throughout the syncytium. Swollen mitochondria were a common feature, both in the syncytium and the tegumental cells. Within T1-type tegumental cells, secretory bodies were scarce, especially after incubation in TCBZ.SO. Golgi complexes were reduced in number and, when present, were reduced in size. The cisternae of granular endoplasmic reticulum (GER) were severely swollen, particularly after treatment with TCBZ.SO.

Cullompton isolate treated with MTZ+NADPH+TCBZ and MTZ+NADPH+TCBZ.SO

The ultrastructure of the tegumental syncytium and its underlying musculature and the tegumental cells was similar after 24-h incubations in either drug and inhibitor combination. Within the tegumental syncytium, mild swelling of the mucopolysaccharide masses was observed (Fig. 6). In the apical region of the syncytium, T1 and T2 secretory bodies were present in normal numbers (Fig. 7). Swelling of the mucopolysaccharide masses was most evident in the basal region of the syncytium; the basal infolds were not swollen (Fig. 8). The mitochondria in the syncytium appeared rounded and slightly swollen (Fig. 8). The muscle blocks remained unchanged after treatment (Fig. 9). Within the T1 tegumental cells, the nucleus and mitochondria retained a normal morphology (Fig. 10). T1 secretory bodies were sparse, and the Golgi complexes were reduced in size (Fig. 10). The T2 tegumental cells appeared to contain accumulations of T2 secretory bodies, and the cisternae of the GER were swollen (Fig. 11).

Oberon isolate treated with TCBZ

The tegumental syncytium appeared relatively normal, with only very minor swelling of the mucopolysaccharide masses observed (Fig. 12). The apical region of the tegument contained a few ‘open’ bodies (Fig. 13). T1 and T2 secretory bodies were present throughout the syncytium in normal numbers, and the mitochondria appeared swollen (Figs. 13 and 14). T2 secretory bodies seemed to have accumulated within the Type-2 tegumental cells (Fig. 15). Type-1 tegumental cells contained active Golgi complexes, and T1 secretory bodies were also present within the cell, but were few in number (Fig. 16). Some mitochondria present took on an abnormal circular appearance, and the cisternae of the GER appeared swollen (Fig. 16).



Figs. 1–5 Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Cullompton and Oberon isolates) treated in vitro with MTZ and MTZ+NADPH for 24 h

1 TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (*APM*) to the basal lamina (*BL*). The muscle blocks (*Mu*) beneath the basal lamina, like the syncytium, retain a normal morphology. *m* mitochondrion. *Bar* 2 μm

2 A high-power micrograph of the apex of the tegumental syncytium, showing the presence of numerous T1 (*T1*) and T2 (*T2*) secretory bodies in this region. *APM* apical plasma membrane; *m* mitochondrion. *Bar* 1 μm

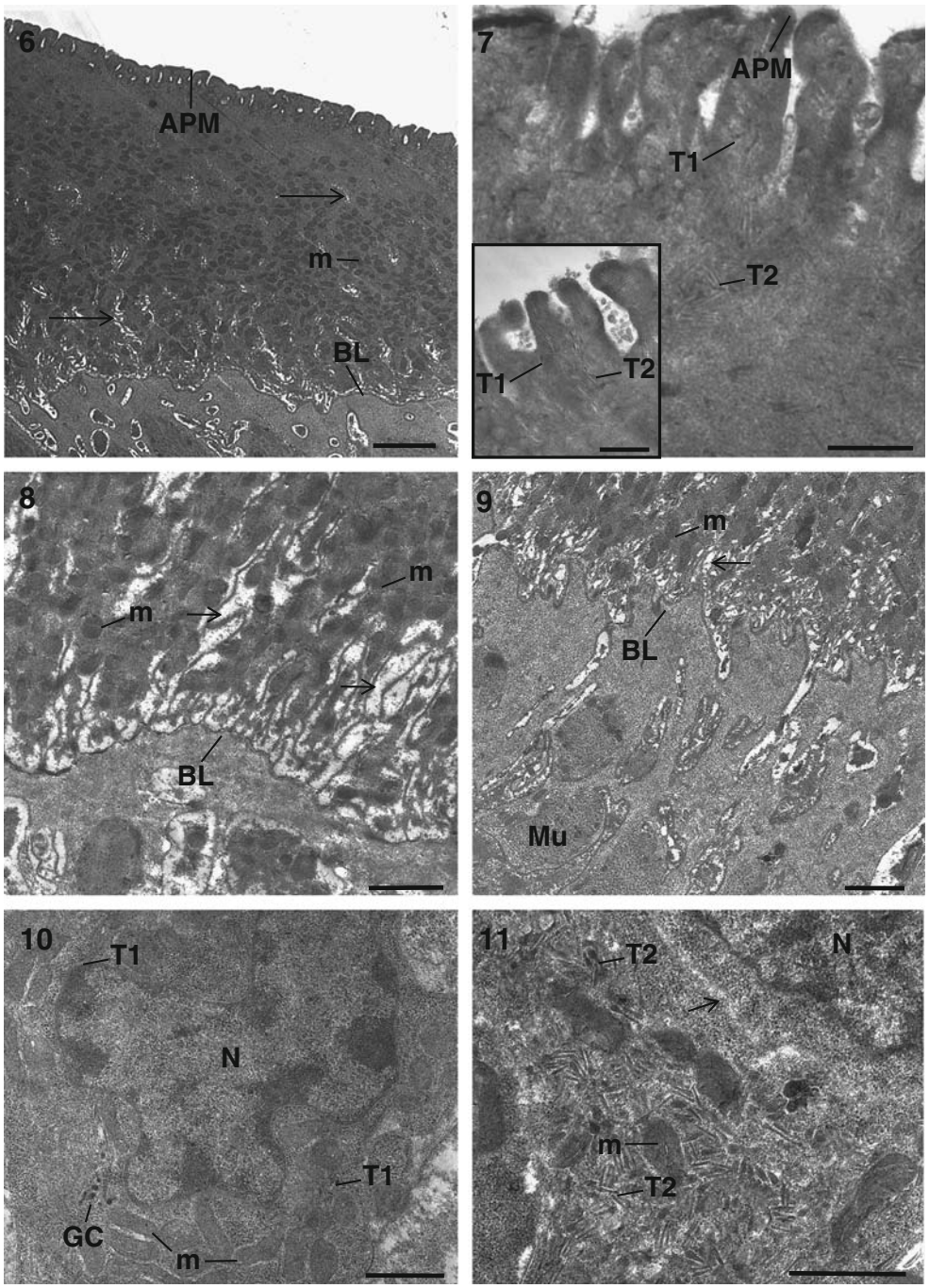
3 Basal region of the tegumental syncytium showing limited swelling of the mucopolysaccharide masses (*arrow*) above the basal lamina (*BL*). T1 (*T1*) and T2 (*T2*) secretory bodies are present in the syncytium. *m* mitochondrion. *Bar* 500 nm

4 TEM of a T1-type of tegumental cell. Well-developed Golgi complexes (*GC*) and numerous T1 secretory bodies (*T1*) are present within the cell. The mitochondria (*m*) retain a relatively normal morphology. *N* nucleus. *Bar* 1 μm. *Inset* shows a high-power image of a Golgi complex (*GC*) which retains a normal morphology. *Bar* 500 nm

5 A high-power micrograph of a T2-type of tegumental cell containing large numbers of T2 secretory bodies (*T2*). *N* nucleus. *Bar* 1 μm

Oberon isolate treated with TCBZ.SO

The changes to the tegumental syncytium following incubation in TCBZ.SO have been described by McKinstry (2008; Chapter 7, Figs. 15–20) and will not be repeated in detail here. Briefly, there was blebbing of the apical plasma membrane, and 'open bodies' were observed below the apex of the tegument. T2 secretory bodies were present in high numbers below the apical plasma membrane. 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. Swelling of the basal infolds was not observed, though the associated mucopolysaccharide masses were slightly swollen. Within the tegumental cells, secretory bodies were observed in normal numbers within their respective cells. The Golgi complexes within the T1-type tegumental cells appeared reduced in size and number.



Figs. 6–11 Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Cullompton isolate) treated in vitro with MTZ +NADPH+TCBZ and MTZ+NADPH+TCBZ.SO for 24 h

6 TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (*APM*) to the basal lamina (*BL*). *m* mitochondrion. Some swelling of the mucopolysaccharide masses (*arrows*) can be seen in the basal region. *Bar* 2 μm

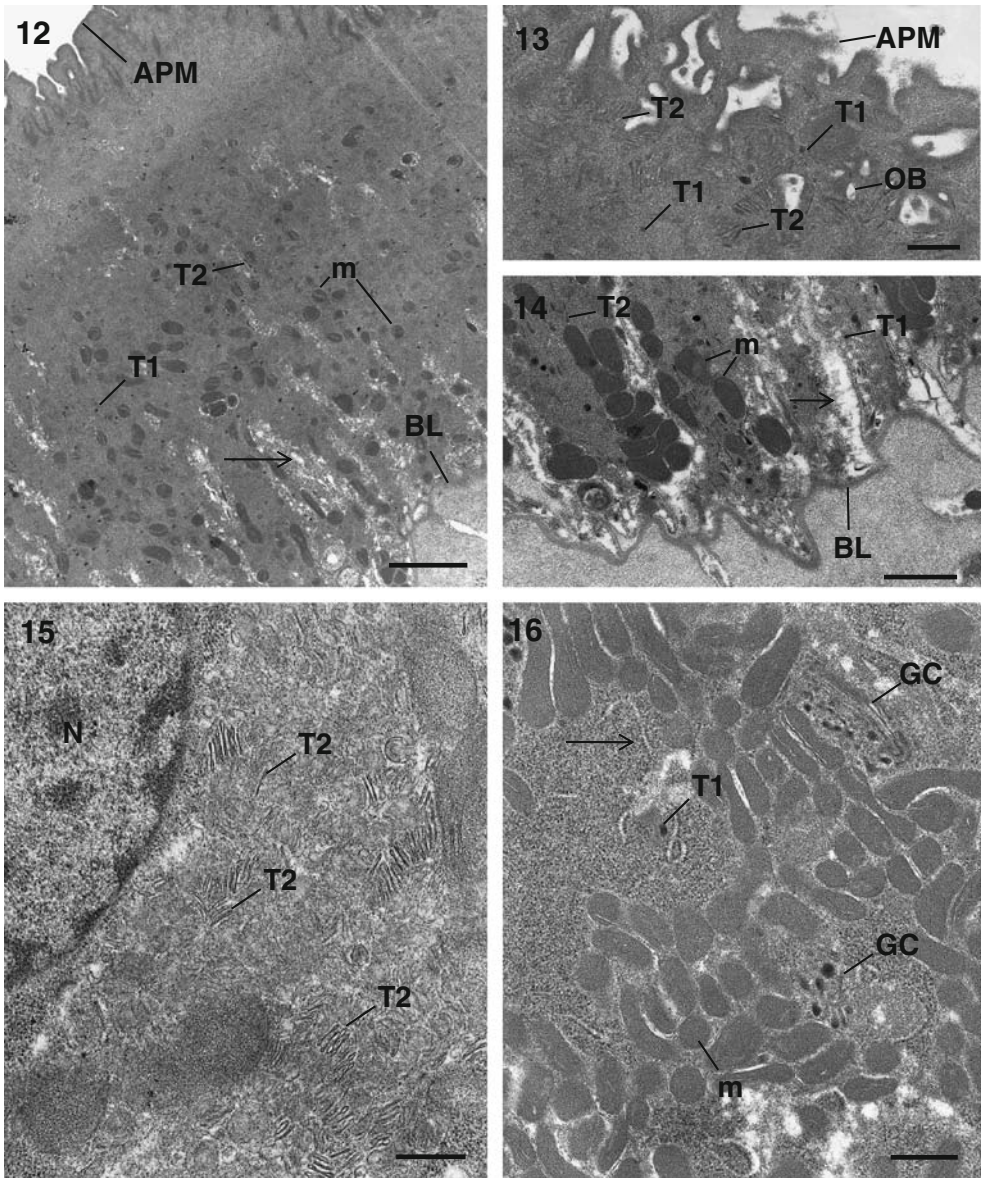
7 A high-power micrograph of the apex of the tegumental syncytium. Numerous T1 (*T1*) and T2 (*T2*) secretory bodies are present below the apical plasma membrane (*APM*). *Bar* 500 nm. *Inset* highlights the abundance of T1 (*T1*) and T2 (*T2*) secretory bodies just below the apical plasma membrane. *Bar* 500 nm

8 TEM showing the basal region of the syncytium. The mucopolysaccharide masses (*arrows*) are slightly swollen just above the basal lamina (*BL*). The mitochondria (*m*) present in the syncytium are slightly rounded in appearance. *Bar* 2 μm

9 Basal region of the syncytium. Slight swelling of the mucopolysaccharide masses (*arrow*) can be seen. Below the basal lamina (*BL*), the muscle blocks (*Mu*) retain a normal morphology. *Bar* 2 μm

10 TEM of a T1-type tegumental cell. Golgi complexes (*GC*) present appear reduced in size. The nucleus (*N*) and mitochondria (*m*) present within the cell retain normal morphology. T1 secretory bodies (*T1*) are sparse within the cell. *Bar* 500 nm

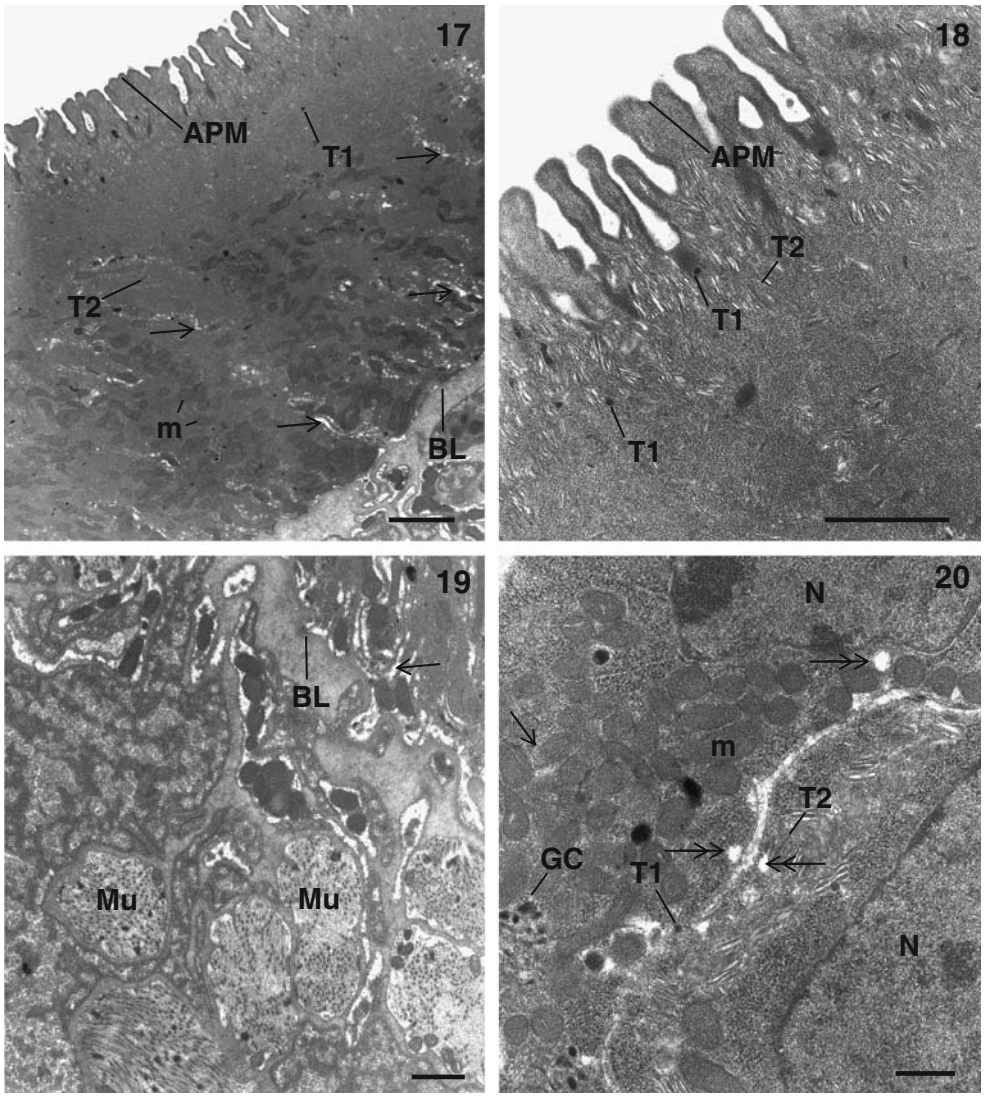
11 A high-power micrograph of a T2-type tegumental cell. T2 secretions (*T2*) are present within the cell. The cell's nucleus (*N*) and mitochondria (*m*) are normal in appearance. The cisternae (*arrow*) of the granular endoplasmic reticulum appear swollen. *Bar* 500 nm



Figs. 12–16 Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) treated in vitro with TCBZ for 24 h
12 TEM showing the full depth of the tegumental syncytium, from the apical plasma membrane (*APM*) to the basal lamina (*BL*). T1 (*T1*) and T2 (*T2*) secretory bodies and mitochondria (*m*) are present throughout the syncytium. Slight swelling of the mucopolysaccharide masses (*arrow*) can be seen above the basal lamina. *Bar* 2 μm
13 A high-power micrograph of the apex of the tegumental syncytium. Numerous T1 (*T1*) and T2 (*T2*) secretory bodies are present below the apical plasma membrane (*APM*). A few 'open bodies' (*OB*) are present just below the apical plasma membrane. *Bar* 1 μm
14 TEM showing the basal region of the syncytium. Above the basal lamina (*BL*), the mucopolysaccharide masses (*arrow*) are swollen. The mitochondria (*m*) appear slightly swollen and T1 (*T1*) and T2 (*T2*) secretory bodies are present in the syncytium. *Bar* 1 μm
15 TEM of a T2-type of tegumental cell in which the nucleus (*N*) retains a normal morphology. Numerous T2 secretory bodies (*T2*) have accumulated within the cell. *Bar* 500 nm
16 TEM showing a T1-type tegumental cell. T1 secretory bodies (*T1*) are few in number. Some mitochondria (*m*) have become swollen in appearance, as have the cisternae of the granular endoplasmic reticulum (*arrow*). The Golgi complexes (*GC*) present retain a relatively normal morphology. *Bar* 500 nm

Oberon isolate treated with MTZ+NADPH+TCBZ

Within the tegumental syncytium, minimal changes were observed. Swelling of the mucopolysaccharide masses was minimal (Fig. 17). The mitochondria present within the syncytium appeared swollen (Fig. 17). T1 and T2 secretory bodies and mitochondria were abundant throughout the syncytium, with a slight accumulation of the secretory bodies just below the apical plasma membrane (Fig. 18). The muscle blocks lying below the basal lamina retained a



Figs. 17–20 Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon isolate) treated in vitro with MTZ+NADPH+TCBZ for 24 h

17 TEM showing the full depth of the tegumental syncytium from the apical plasma membrane (*APM*) to the basal lamina (*BL*). T1 (*T1*) and T2 (*T2*) secretory bodies are present throughout the syncytium. Very limited swelling of the mucopolysaccharide masses (*arrows*) can be seen. Mitochondria (*m*) are abundant throughout the syncytium, but appear slightly swollen. *Bar* 2 μm

18 A high-power micrograph of the apex 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*). *Bar* 1 μm

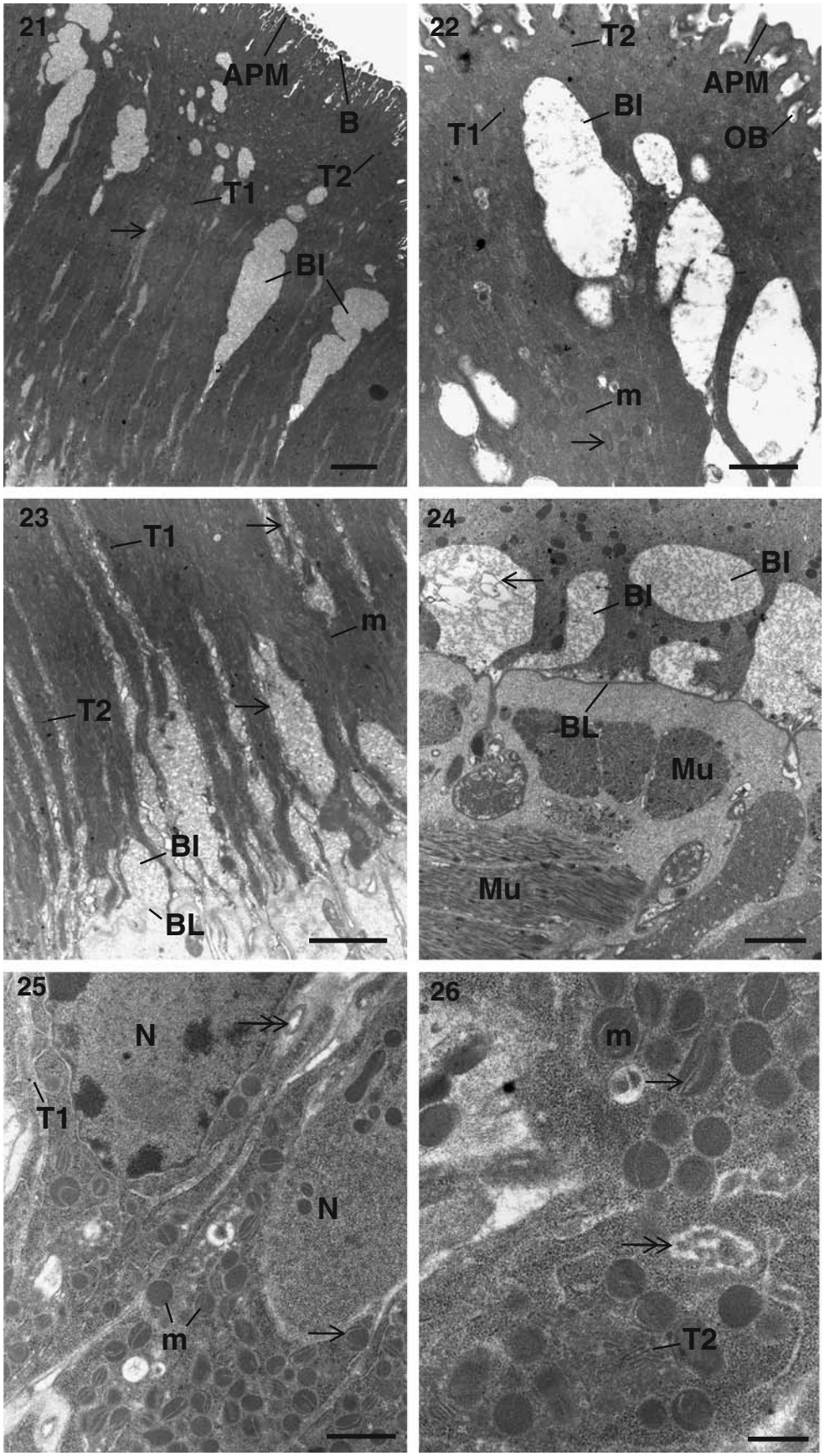
19 TEM showing the basal region of the syncytium. The mucopolysaccharide masses (*arrow*) are slightly swollen just above the basal lamina (*BL*). The muscle blocks (*Mu*) remain unchanged. *Bar* 1 μm

20 TEM showing a Type-1 and a Type-2 secretory cell adjacent to one another. The nucleus (*N*) in each cell appears normal. Few T1 secretory bodies (*T1*) are present in the T1-type tegumental cell. The mitochondria (*m*) (*arrow*) are swollen within the cell. The Golgi complexes (*GC*) present in the cell appear reduced in size. Numerous T2 secretory bodies (*T2*) are present within the T2-type tegumental cell. The cisternae of granular endoplasmic reticulum appear swollen (*double arrow*) in both T1- and T2-type tegumental cells. *Bar* 500 nm

normal morphology (Fig. 19). Within the T1-type tegumental cells, the nuclei retained a normal appearance, although the mitochondria were swollen with distinct cristae, and the Golgi complexes were reduced in size (Fig. 20). T2 secretory bodies seemed to have accumulated within the Type-2 tegumental cell and, while T1 secretory bodies were present within Type-1 tegumental cells, they were few in number (Fig. 20). Within both cell types, the cisternae of the GER were swollen (Fig. 20).

Oberon isolate treated with MTZ+NADPH+TCBZ.SO

Blebbing of the apical plasma membrane was observed after incubation in MTZ+NADPH+TCBZ.SO (Fig. 21). At


21
APM
B
T2
T1
Bl
22
T2
APM
Bl
T1
OB
m
23
T1
m
T2
Bl
BL
24
Bl
Bl
BL
Mu
Mu
25
N
T1
N
m
26
m
T2

◀**Figs. 21–26** Transmission electron micrographs (TEMs) of the tegumental syncytium, underlying musculature and tegumental cells of adult *Fasciola hepatica* (Oberon) isolate treated in vitro with MTZ +NADPH+TCBZ.SO for 24 h

21 TEM of the tegumental syncytium. Blebbing (*B*) of the apical plasma membrane (*APM*) can be seen. T1 (*T1*) and T2 (*T2*) secretory bodies are present within the syncytium. The basal infolds (*BI*) are severely swollen, and swelling of the mucopolysaccharide masses (*arrow*) can also be seen. *Bar* 2 μm

22 High-power micrograph of the apical region of the tegument apex showing 'open' bodies (*OB*) below the apical plasma membrane (*APM*). T1 (*T1*) and T2 (*T2*) secretory bodies are present within this region. The basal infolds (*BI*) are severely swollen and rounded mitochondria (*m*) with swollen cristae (*arrow*) can be seen within the syncytium. *Bar* 1 μm

23 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. T1 (*T1*) and T2 (*T2*) secretory bodies are present in the syncytium. *Bar* 2 μm

24 Tegumental syncytium showing severe swelling of the mucopolysaccharide masses (*arrow*) and basal infolds (*BI*). Below the basal lamina (*BL*), the muscle blocks (*Mu*) remain unchanged. *Bar* 2 μm

25 TEM showing two T1-type tegumental cells. The mitochondria (*m*) are rounded with swollen cristae (*arrow*). The nuclei (*N*) appear normal, but the cisternae of granular endoplasmic reticulum (*double arrow*) are severely swollen. A small number of T1 (*T1*) secretory bodies can be seen within one of the cells. *Bar* 1 μm

26 A high-power TEM of a T2-type of tegumental cell. T2 (*T2*) secretory bodies are present within the cell. The mitochondria (*m*) are rounded with swollen cristae (*arrow*), and the cisternae of the granular endoplasmic reticulum are also swollen (*double arrow*). *Bar* 500 nm

higher magnification, "open bodies" were observed below the apex of the tegument (Fig. 22). T1 and T2 secretory bodies were present throughout the tegumental syncytium, and seemed to accumulate below the apical plasma membrane, as were the mitochondria, which appeared swollen, with distinct cristae, and they assumed an abnormal round shape rather than the normal cylindrical form (Fig. 22). The major feature of the syncytium was the severe swelling of the basal infolds (Figs. 21–24); this was accompanied by swelling of the associated mucopolysaccharide masses (Figs. 21 and 23). Below the basal lamina, the muscle blocks remained unchanged (Fig. 24). T1 and T2 secretory bodies were present in their respective tegumental cells, though the number of secretory bodies was fewer than normal in both cell types (Figs. 25, 26). In both Type-1 and Type-2 tegumental cells, the mitochondria were swollen with distinct cristae; the cisternae of the GER was swollen in both types of tegumental cell (Figs. 25, 26). Neither type of tegumental cell contained active Golgi complexes (Figs. 25, 26).

The main changes brought about by drug action and the relative severity of the changes are summarised in Tables 2 and 3.

Discussion

The results of this investigation have demonstrated that co-administration of MTZ with either TCBZ or TCBZ.SO has a greater effect on the Oberon TCBZ-resistant isolate than on the TCBZ-susceptible Cullompton isolate. While treatment with TCBZ and (more particularly) TCBZ.SO caused greater

Table 2 Oberon isolate of *Fasciola hepatica*. Summary of changes to the tegumental system following different drug treatments

Disruption	Treatment					
	MTZ	MTZ+ NADPH	MTZ+NADPH+ TCBZ	MTZ+NADPH+ TCBZ.SO	TCBZ	TCBZ. SO
Changes in syncytium						
Blebbing	−	−	−	++	−	+
Presence of "open" bodies	−	−	−	++	+	+
Altered numbers of secretory bodies at apex	−	−	+	+	−	+
Swelling of basal infolds	−	−	−	+++	−	−
Swelling of mucopolysaccharide masses	+	+	+	+++	+	+
Swelling of mitochondria	−	−	++	+	+	+
Changes in tegumental cells						
Reduction in number of secretory bodies	−	−	++	+++	+	−
Reduction in number and size of Golgi complexes	−	−	+	++	−	+
Swelling of cisternae of GER	−	−	++	++	+	−
Swelling of mitochondria	−	−	+	++	+	−
Total	1	1	10	21	6	6

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

Table 3 Cullompton isolate of *Fasciola hepatica*. Summary of changes to the tegumental system following different drug treatments

Disruption	Treatment					
	MTZ	MTZ+ NADPH	MTZ+NADPH+ TCBZ	MTZ+NADPH+ TCBZ.SO	TCBZ	TCBZ. SO
Changes in syncytium						
Blebbing	–	–	–	–	–	–
Presence of "open" bodies	–	–	–	–	–	–
Altered numbers of secretory bodies at apex	–	–	–	–	+	+
Swelling of basal infolds	–	–	–	–	+	++
Swelling of mucopolysaccharide masses	+	+	+	+	+	+
Swelling of mitochondria	–	–	+	+	+	++
Changes in tegumental cells						
Reduction in number of secretory bodies	–	–	+	++	+	++
Reduction in number and size of Golgi complexes	–	–	++	++	+	+
Swelling of cisternae of GER	–	–	+	+	–	++
Swelling of mitochondria	–	–	–	–	+	++
Total	1	1	6	7	7	13

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

disruption of the Cullompton isolate, potentiation of drug action was only observed with the Oberon isolate and was far more distinct with TCBZ.SO than TCBZ. Incubation in MTZ alone or MTZ+NADPH caused limited disruption to the ultrastructure of flukes belonging to both isolates: only very minor swelling of the mucopolysaccharide masses associated with the basal infolds was observed in a few fluke specimens, but it was not a widespread phenomenon.

The syncytium of the Oberon isolate treated with TCBZ alone appeared relatively normal, with only slight swelling of the mucopolysaccharide masses and mitochondria observed. "Open" bodies were present below the apical plasma membrane: they are indicative of accelerated release of secretory bodies from the tegumental surface, as would occur in a stress situation such as that induced by drug action (Rogan and Threadgold 1984). Tegumental secretory bodies and active Golgi complexes were observed within the tegumental cells, indicating that production of the secretory bodies was still taking place. Co-incubation of MTZ with TCBZ resulted in an accumulation of secretory bodies just below the apical plasma membrane. This, too, is indicative of a stress response by the fluke and is a common reaction to treatment with other fasciolicidal drugs, as it serves as a survival mechanism to replace damaged tegument (Fairweather et al. 1986; Stitt and Fairweather 1994; Buchanan et al. 2003; Meaney et al. 2004, 2005, 2007; McConville et al. 2006, 2007, 2008, 2009b; McKinstry et al. 2007, 2009; Halferty et al. 2009a, b; O'Neill et al. 2009; Toner et al. 2009, 2010). Swelling of the mitochondria and cisternae of GER was exacerbated but, more significantly, the production of secretory bodies in the tegumental cells appeared to be affected, as evidenced by the reduced size of the Golgi complexes and the reduced number of secretory bodies in the cells.

Treatment of the Oberon isolate with TCBZ.SO led to a similar level of disruption as that induced by TCBZ. However, when flukes were incubated in a combination of TCBZ.SO and MTZ, there was a dramatic increase in the degree of disruption observed, far more so than that seen when MTZ was combined with TCBZ. In particular, there was severe swelling of the basal infolds and their associated mucopolysaccharide masses and a marked reduction in the number of secretory bodies in the syncytium. The latter was probably due to a knock-on effect of the sharp decrease in the number of secretory bodies and the reduction in size and number of the Golgi complexes in the tegumental cells. Clearly, there was a major impact on the synthesis, production and transport of secretory bodies. This would have an effect on the maintenance of the apical plasma membrane and lead to the blebbing seen both in this study and in the previous study with MTZ (Devine et al. 2009). Loss of the apical membrane was observed in the SEM study, but not in the present one: this can be explained by the fact that it occurred in small patches and that TEM cannot sample such large areas as SEM. The severe swelling of the basal infolds would account for the general swelling of the tegument observed in the previous SEM study; if allowed to continue, this would lead to the

sloughing of the tegument. The general swelling of the basal infolds, mucopolysaccharide masses, mitochondria and cisternae of GER is indicative of disruption of the osmoregulatory function of the tegument (Fairweather et al. 1999). Similar observations have been made in previous studies involving TCBZ.SO (Stitt and Fairweather 1994; Halferty et al. 2009a) and various other fasciolicides (Fairweather et al. 1986; Skuce et al. 1987; Skuce and Fairweather 1990; Anderson and Fairweather 1995; Meaney et al. 2004, 2005, 2007; McConville et al. 2006, 2007, 2008, 2009b; McKinstry et al. 2007, 2009; Halferty et al. 2009b).

With the Cullompton isolate, incubation in TCBZ alone caused limited swelling of the basal infolds, mucopolysaccharide masses and mitochondria, together with a decline in the number of secretory bodies in the syncytium and cell bodies and disruption of the Golgi complexes (Halferty et al. 2009a). The only potentiation of drug action observed following treatment with MTZ and TCBZ concerned a further reduction in the size of the Golgi complexes; otherwise, the disruption seen was similar to that induced by TCBZ treatment alone. Treatment of the Cullompton isolate with TCBZ.SO lead to greater disruption than that seen with TCBZ: there was a more marked swelling of the basal infolds, the mitochondria and (in the cell bodies) the cisternae of GER, along with fewer secretory bodies in the cells (Halferty et al. 2009a). When TCBZ.SO was combined with MTZ, there was no potentiation of drug action; in fact, there was less disruption, a result that is difficult to explain.

The internal changes in the tegument observed here are compatible with the surface changes described previously for the same drug and inhibitor combinations (Devine et al. 2009). For example, with the Oberon isolate following TCBZ.SO +MTZ treatment, surface swelling and furrowing were seen. This can be linked to the disruption of the osmoregulatory system in the syncytium (Threadgold and Brennan 1978; Skuce et al. 1987). The inability to adequately repair and replace surface membrane damaged by drug action, as manifested by blebbing, is related to the decline in secretory body production by the cell bodies. Loss of the apical membrane was not seen in the present study, but this may be due simply to a sampling issue, as discussed above.

The combined SEM and TEM results show that inhibition of drug metabolism by the FMO inhibitor, methimazole has a relatively greater effect in TCBZ-resistant than TCBZ-susceptible flukes. Thus, enhancement of the FMO-mediated metabolic activity responsible for the conversion of TCBZ to TCBZ.SO may contribute to the mechanism of resistance in the Sligo and Oberon fluke isolates (Alvarez et al. 2005; Devine et al. 2009; this study). This may be due to the over-expression of the FMO enzyme system in TCBZ-resistant flukes, which would render them particularly sensitive to enzyme inhibition. It has been shown that MTZ has a relatively greater impact on TCBZ sulphoxidation in the TCBZ-resistant Sligo isolate than in Cullompton flukes: 43% compared with 34% inhibition (Alvarez et al. 2005). The TCBZ-resistant isolates may be more reliant on this pathway and therefore are more sensitive to its inhibition. The morphological and biochemical data are consistent with each other.

In conclusion, this study has shown that it is possible to modulate the susceptibility of a TCBZ-resistant isolate of *F. hepatica* and change it to a more TCBZ-susceptible state by co-treatment with the FMO inhibitor, MTZ. FMO pathways play a major role in the metabolism of xenobiotic drugs by livestock (Lanusse and Prichard 1993). The co-administration of MTZ with benzimidazole compounds in livestock has been shown to enhance the efficacy of the anthelmintic drugs, by enhancing their bioavailability via manipulation of their pharmacokinetics (Lanusse and Prichard 1992a, b, 1993; Lanusse et al. 1992, 1995). The strategy has been extended to TCBZ use in sheep (Virkel et al. 2006, 2009). Moreover, it may have benefits for the treatment of drug-resistant parasites, not just due to altered host metabolism, but to altered drug–parasite interaction as well, especially if enhanced metabolism is involved in drug resistance. For TCBZ, which remains the most effective anti-fluke drug, the concept of combined TCBZ+inhibitor treatment represents a possible way of maintaining drug activity in the face of drug resistance. So, the current results are of more than academic interest and could find application in the field situation.

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