
7 Mechanisms of Fasciolicide Action and Drug Resistance in *Fasciola hepatica*

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Introduction

The purpose of this chapter is to review our understanding of the mechanism of action of fasciolicides used for the treatment of liver fluke infections. Also, to assess the extent to which resistance has developed to existing fasciolicides and strategies that can be adopted to overcome the problem. The fasciolicides in common use today (or in the more recent past) fall into five main chemical groupings:

1. *Halogenated phenols*, e.g. bithionol ('Bitin', 'Actamer'), hexachlorophene (formerly 'Bilevon', now obsolete), niclofolan ('Bilevon'), nitroxylnil ('Trodx').
2. *Salicylanilides*, e.g. brotianide ('Dirian'), closantel ('Flukiver', 'Seponver', 'Supaverm', 'Cosicare'), oxyclozanide ('Nilzan', 'Zanil'), rafoxanide ('Flukanide', 'Ranizole').
3. *Benzimidazoles*, e.g. albendazole ('Valbazen'), mebendazole ('Telmin', 'Vermox', 'Supaverm'), triclabendazole ('Fasinex').
4. *Sulphonamides*, e.g. clorsulon ('Curatrem', 'Ivomec F', 'Ivomec Plus').
5. *Phenoxyalkanes*, e.g. diamphenethide ('Coriban').

The chemical structures of these fasciolicides are presented in Fig. 7.1.

The experimental data for each group of fasciolicides will be collected together to determine how close we are to defining their primary mode of action. Wherever possible, concentrations of fasciolicides used in *in vitro* studies have been expressed in terms of $\mu\text{g ml}^{-1}$, in order to permit direct comparison with the maximum blood levels of drug circulating *in vivo*. Due to the constraints of space, only the most salient points will be made. For a more detailed discussion of fasciolicidal action, the reader is referred to Fairweather (1997).

Before discussing the mode of action of individual fasciolicides, brief consideration will be given to the chemotherapy of *Fasciola* infections.

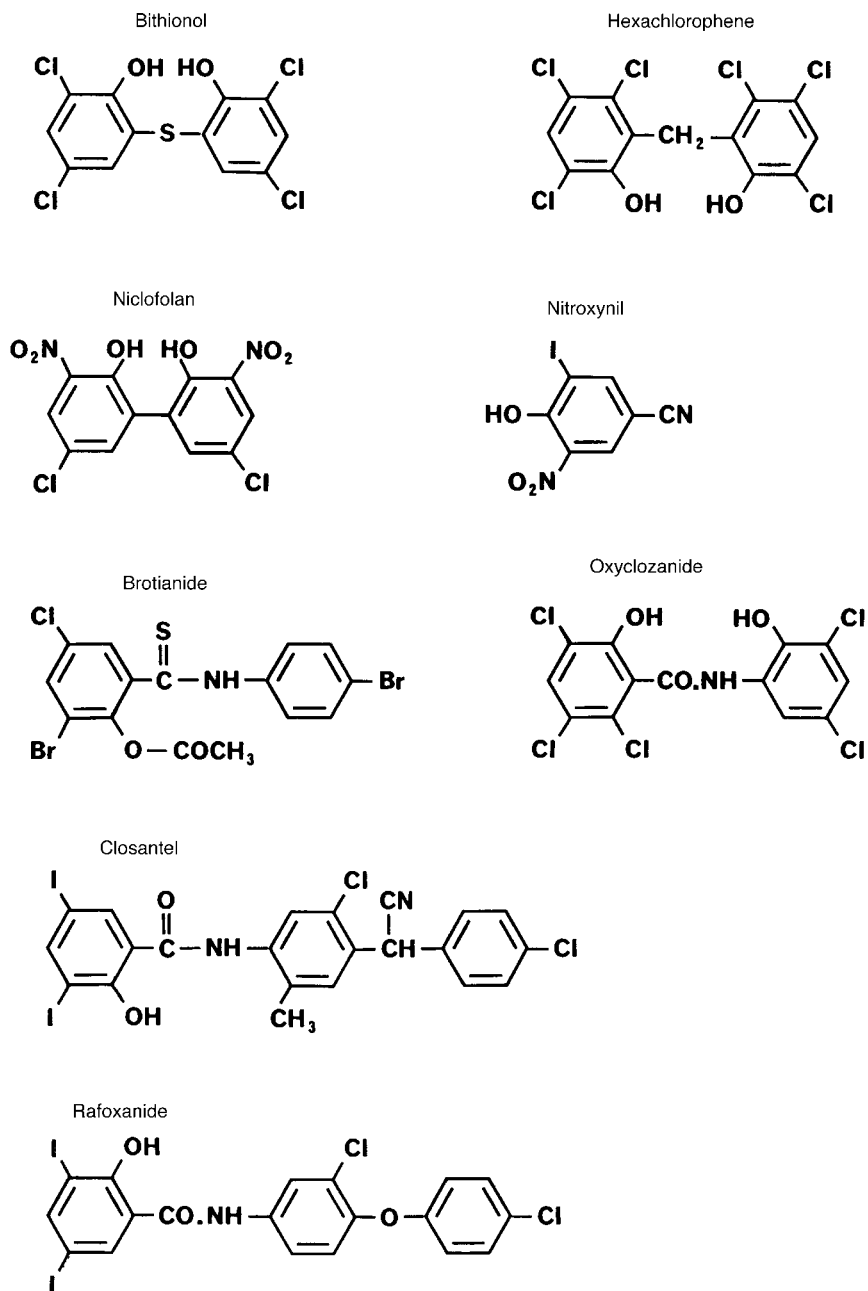


Fig. 7.1 (and opposite). Chemical structures of fasciolicides.

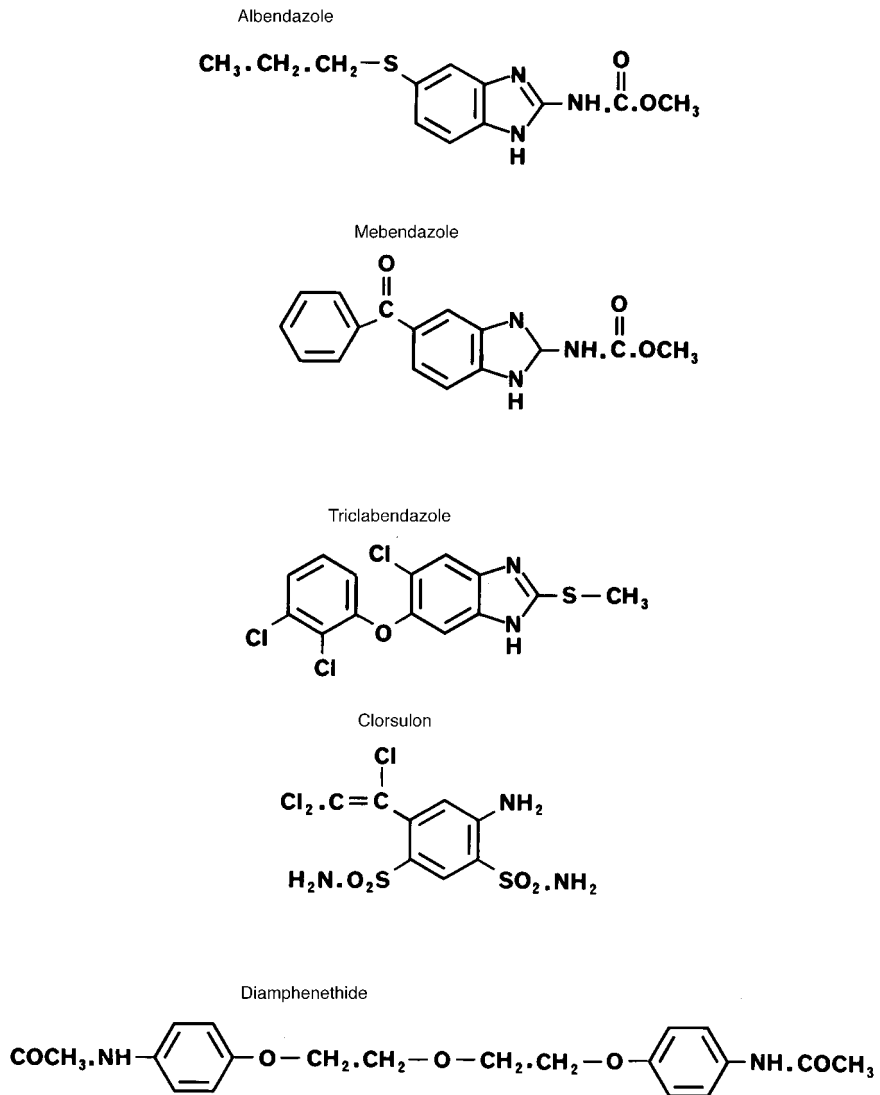


Fig. 7.1 (continued).

Chemotherapy

Data pertaining to the efficacy and safety of fasciolicides are presented in Tables 7.1 and 7.2. The use of the most effective drugs with efficacy against both mature and early immature fluke is essential. It is of particular importance in an efficient strategic control programme, with a minimum number of annual treatments and an expected seasonal elimination of pasture

Table 7.1. Comparative efficiency of drugs against *Fasciola*.

Anthelmintic	Route of application	Recommended dose rate (mg kg ⁻¹)		Maximum tolerated dose in sheep (mg kg ⁻¹)	Safety index at recommended dose rate in sheep	Minimum age of fluke in weeks, efficiency ≥90%	
		Sheep	Cattle			Sheep	Cattle
Hexachlorophene	Oral	15	20	40	2.6	12	12
Bithionol	Oral	75	30	75	1	>12	>12
Oxyclozanide	Oral	15	13–16	60	4.0	12	>14
Niclofolan	Oral	4	3	12	3.0	12	>12
	sc	NR	0.8			NR	>12
Nitroxylin	sc	10	10	40	4.0	8	10
Brotianide	Oral	5.6	NR	27	4.8	12	NR
Rafoxanide	Oral	7.5	7.5	45	6.0	6	12
	sc	NR	3			NR	12
Closantel	Oral	7.5–10	NR	40	4.0	6–8	NR
	sc	NR	3			NR	>12
Diamphenethide (acemidophene, CIS)	Oral	80–120	100	400	3.3–5.0	1 day to 6 wk	1 day to 7 wk
Albendazole	Oral	4.75	10	30	8	>12	>12
Triclabendazole	Oral	10	12	200	20–40	1	1
Clorsulon	Oral	—	7	100	5		8
	sc	—	2				>12

sc = subcutaneous; NR = not recommended.

Table 7.2. Efficiency spectrum of drugs at recommended dose rates against *Fasciola hepatica* in sheep.

Drug	Age of fluke in weeks													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Bithionol, Hexachlorophene, Oxyclozanide, Niclofolan, Albendazole, Clorsulon+Ivermectin (inj)										50-70%			80-99%	
Clorsulon (oral)											90-99%			
Nitroxynil, Closantel								50-90%				91-99%		
Rafoxanide						50-90%					91-99%			
Triclabendazole			90-99%						99-100%					
Diamphenethide				100-91%									80-50%	

contamination for extended periods. The reduction of treatment frequency may prevent development of drug resistance, which will be discussed later. Drugs which only work against adult fluke, such as albendazole, oxclozanide and closulon at the low dose rate combined with ivermectin, are unsuitable for effective chemoprophylaxis. Strategies for the treatment and prophylaxis of *Fasciola* infections based on epidemiological data have been discussed by Boray (1997) and Malone (1997).

Suppression of *F. hepatica* infections has been attempted using treatments every 6 weeks with rafoxanide from spring to autumn for 2 years resulting in 90% reduction of infection and reduction of the infection rate in snails (Armour *et al.*, 1973). Good results were achieved with increased dose rates of rafoxanide given five times between June and January/February in a heavily infected area in Scotland and the infection level remained very low for some time after the programme. It was predicted that virtual eradication of fasciolosis could be achieved using the regimen for 3 to 4 years (Whitelaw and Fawcett, 1977, 1981).

In a field experiment in New Zealand, sheep suffering from heavy acute, subacute and chronic infections were treated with triclabendazole at 10 mg kg⁻¹, with an efficacy of 99.8%. Subsequently, all sheep, cattle and horses were treated on the property every 8 to 11 weeks for a period of 14 months. No patent infection could be detected and the contamination of the pastures was reduced to a negligible level for a period of 12 months after the last treatment (Boray, 1986). It was concluded that effective treatment during the prepatent period for an extended duration could eliminate *Fasciola* infection or reduce contamination to a very low level, requiring less frequent treatments for a considerable time.

Frequent treatments of sheep were carried out in the field with triclabendazole by Fawcett (1990) in a strategic pattern, between June and January, for a period of 5 years and reduced the prevalence of infection from 49% to 1%. Eight-week treatments with triclabendazole between April and October did not reduce infection in the first year but achieved a 70–75% reduction when the treatments commenced in February and were carried out four times a year for 2 years (Taylor *et al.*, 1994).

A small flock of sheep, initially suffering from clinical subacute fasciolosis, was treated every 8 weeks for a period of 3 years with 10 mg kg⁻¹ triclabendazole in a pasture contaminated only by the surviving flukes reaching early maturity. The infection was reduced to a negligible level but the surviving fluke population developed a degree of resistance to triclabendazole (Boray, 1990, 1997).

In comparative efficacy trials it has been shown that triclabendazole, rafoxanide and closantel exert an appreciable retardation of the development of immature flukes in treated sheep. The level of retardation was directly related to the level of efficacy of the drugs against early immature flukes and considerably extended the prepatent period of the surviving fluke populations (Boray, 1997). This phenomenon has a great influence on the success of strategic control programmes. Since triclabendazole is highly effective against flukes aged 1 week or older, the drug is most suitable for reducing the

pasture contamination for extended periods. Retarded development of flukes after treatment with closantel has been reported (Maes *et al.*, 1985, 1990) and a lower degree of retardation also observed in cattle after treatment with clorsulon (Malone *et al.*, 1984; Yazwinski *et al.*, 1985).

Retardation of immature flukes which survive treatment appears to be applicable to all anthelmintics and the degree of retardation depends on the efficacy of the drugs against the immature stages. This phenomenon has a great advantage in strategic control by reducing early pasture contamination with eggs. Unfortunately, recent studies to be discussed later have demonstrated that resistance may develop to most anthelmintics, mainly affecting the flukes aged 6 weeks or younger (Boray, 1990). It appears that during the early immature stages selection for resistance will occur rapidly if eradication of *Fasciola* spp. is attempted with frequent treatments and this method of control is not desirable. Less frequent strategic treatments with a possible yearly rotation of anthelmintics or anthelmintic combinations which are effective against both immature and adult flukes will provide the best method of successful control of fasciolosis.

Mode of Action Data

Group 1: halogenated phenols

These drugs contain a phenolic group and bear a structural similarity to 2,4-dinitrophenol (2,4-DNP), a known uncoupler of oxidative phosphorylation in mammalian systems. This fact has been used to support the idea that they act in a similar way in fluke tissues (Van Miert and Groeneveld, 1969; Corbett and Goose, 1971; Yorke and Turton, 1974; Campbell and Montague, 1981). However, as is evident from Table 7.3, experimental studies were carried out in systems (such as isolated tapeworm or mammalian mitochondria) that are not related to the fluke or at concentrations far higher than those which occur *in vivo*. Consequently, the results may have little bearing on the *in vivo* activity of the drugs. Moreover, oxidative phosphorylation makes a minor contribution to energy production by flukes, accounting for only 10% of the total carbohydrate metabolized (Lloyd and Barrett, 1983). It can generate a greater percentage of ATP production – up to 20% in aerobic conditions in the adult fluke *in vitro* (Tielens *et al.*, 1984). Therefore, most of ATP synthesis in the mitochondria will take place by anaerobic processes, even under aerobic conditions.

Phenolic fasciolicides have also been shown to inhibit certain key enzymes in the pathway from phosphoenolpyruvate to propionate: malate dehydrogenase (hexachlorophene; Lwin and Probert, 1975) and succinic dehydrogenase (bithionol, hexachlorophene and niclofolan; Panitz and Knapp, 1970). Again, the concentrations used were far higher than those attained *in vivo* following drug treatment and so it is difficult to assess the true physiological significance of these findings (see Table 7.3).

Perhaps of greater importance is the fact that the phenols induce a rapid (<3 h) spastic paralysis of the fluke at concentrations comparable to effective

Table 7.3. A comparison of studies on the mode of action of halogenated phenols against the liver fluke, *Fasciola hepatica* (studies *in vitro* unless stated otherwise; concentrations expressed as $\mu\text{g ml}^{-1}$).

Parameter tested	Niclofolan	Bithionol	Hexachlorophene	Nitroxylin
Maximum blood level <i>in vivo</i>	4.5 ¹ (dose 3 mg kg ⁻¹ in sheep and cattle)			80 ² (dose: 10 mg kg ⁻¹ in sheep)
Minimum concentration for paralysis within 3 h ³	1.0 (spastic paralysis)	1.0 (spastic paralysis)	1.0 (spastic paralysis)	50 (spastic paralysis)
Stimulation of oxygen uptake by <i>Fasciola hepatica</i> ⁴			0.41–4.07	2.90–29.0
Induction of ATPase activity of rat liver mitochondria ⁵	0.35–34.51		0.41–40.69	0.29–29.0
Minimum concentration for uncoupling of rat liver mitochondria ^{4,7}			0.24–0.33 ⁴ 1.22–4.07 ⁷	7.83–10.15 ⁴ 8.70–29.0 ⁷
Minimum concentration for maximum uncoupling of <i>Hymenolepis diminuta</i> mitochondria ⁶				130.5
Inhibition of malate dehydrogenase activity in <i>F. hepatica</i> ⁸			406.9	None at 290.0
Inhibition of succinic dehydrogenase activity in <i>F. hepatica</i> ⁹	20.71–153.57	20.30–203.0		
Disruption of spermatogenesis <i>in vivo</i> ¹⁰	Within 4 h (dose: 5.4 mg kg ⁻¹ in rabbit)		Within 4 h (dose: 4.0 mg kg ⁻¹ in rabbit)	Within 4 h (dose: 20 mg kg ⁻¹ in rabbit)
Decrease in egg production <i>in vivo</i> ^{11,12}		After 3 or more single doses of 10 mg kg ⁻¹ in rat ¹¹		dose: 10 mg kg ⁻¹ in sheep ¹²

References: 1. Flucke *et al.* (1969); 2. Parnell (1970); 3. Fairweather *et al.* (1984); 4. Corbett and Goose (1971); 5. Van Miert and Groeneveld (1969); 6. Yorke and Turton (1974); 7. Campbell and Montague (1981); 8. Lwin and Probert (1975); 9. Panitz and Knapp (1970); 10. Stammers (1975b); 11. Dawes (1966); 12. Stammers (1976).

blood levels *in vivo* (see Table 7.1). It is believed that the action is not due to uncoupling *per se*, but rather to changes in the permeability of the muscle membrane to certain ions, particularly K^+ : for a more detailed explanation of the rationale behind this view, see Holmes and Fairweather (1985) and Fairweather (1997). However, the precise nature of this neuromuscular action remains to be defined.

Few studies have been carried out on morphological changes in the fluke induced by phenolic compounds. Vacuolation of the cuticle (*viz.* tegument) leading to widespread stripping of the tegument has been observed in histological sections following treatment with bithionol *in vivo*; the dorsal surface was more seriously affected than the ventral surface (Dawes, 1966). A posterior–anterior spread of surface damage has been described for bithionol and for hexachlorophene (Gusel'nikova, 1974).

Disruption of the reproductive system – in particular the testes – has been described for each of the phenols *in vivo*. With nitroxylnil, niclofolan and hexachlorophene, disruption is rapid, occurring within 4 h (Stammers, 1975b). For both nitroxylnil and bithionol, the testis was more severely affected than other reproductive organs, such as the ovary and vitellaria, although in each case egg production declined (Dawes, 1966; Stammers, 1976). The cause of the latter effect differs for the two drugs, being due to disruption of oogenesis for nitroxylnil (Stammers, 1976) and disruption of vitellogenesis for bithionol (Dawes, 1966). The precise mechanism behind the disruption of spermatogenesis and other reproductive activities is not known, although for nitroxylnil it has been linked to its potential uncoupling action leading to less energy being available for cell division (Stammers, 1975a). However, for these fasciolicides it may simply be an illustration of the axiom advanced by Dawes (1968) that in a stress situation 'flukes which are threatened by drug action on the host will tend first to sacrifice non-vital organs and to conserve vital organs', presumably in an attempt to save energy and therefore aid survival. So, for these compounds, changes to the reproductive organs may be an indirect or secondary effect of some other primary drug action.

Group 2: salicylanilides

Metabolic and motility data for the salicylanilides rafoxanide, oxyclozanide and closantel are summarized in Table 7.4. For these fasciolicides there is more direct evidence for an uncoupling of oxidative phosphorylation in the fluke. With oxyclozanide, for example, some metabolic changes induced *in vitro* are characteristic of an uncoupler – stimulation of oxygen consumption, increases in respiratory intermediates, increase in oxaloacetate:malate ratio and a rise in succinate production (Edwards *et al.*, 1981a; see also Table 7.4). However, there was no indication of any decline in ATP levels in the fluke and the authors concluded that the primary action may be a neurotoxic one (Edwards *et al.*, 1981a). Oxyclozanide causes a rapid (within 0.5 h) spastic paralysis of the fluke at a concentration comparable to maximum blood level attained *in vivo* following drug treatment (Fairweather *et al.*, 1984). This point will be returned to later.

Table 7.4. A comparison of studies on the effects of salicylanilides against motility and energy metabolism of *Fasciola hepatica* (concentrations expressed as $\mu\text{g ml}^{-1}$).

Parameter tested	Rafoxanide	Oxyclozanide	Closantel
Maximum blood level <i>in vivo</i>	18.6 (dose: 7.5 mg kg ⁻¹ in sheep)	10–15 ² (dose: 10 mg kg ⁻¹ in cattle)	45–55 ³ (dose: 10 mg kg ⁻¹ in sheep and cattle) 35.7 ⁴ (dose: 7.5 mg kg ⁻¹ in sheep)
Minimum concentration for paralysis <i>in vitro</i> within 3 h ^{5,6}	1.0 ⁵ (spastic paralysis)	1.0 ⁵ (spastic paralysis)	1.0 ⁶ (spastic paralysis)
Stimulation of oxygen uptake by <i>Fasciola hepatica in vitro</i> ^{7,8}		20–40% at 0.40–4.02 ⁷ 23% at 0.5 ⁸	
Induction of ATPase activity of rat liver mitochondria <i>in vitro</i> ⁹		318% at 4.02 ⁹	
Minimum concentration for uncoupling of rat liver mitochondria <i>in vitro</i> ^{7,10}	0.63–1.88 ¹⁰	1.21–1.61 ⁷ 0.40–1.20 ¹⁰	
Stimulation of rat liver mitochondrial respiration <i>in vitro</i> ⁹		222% at 40.2	
Minimum concentration for maximum uncoupling of <i>Hymenolepis diminuta</i> mitochondria <i>in vitro</i> ¹¹	43.82	96.36	
Increased glucose uptake ^{12,13}	<i>In vitro</i> : not after 3 h at 400 ¹²		<i>In vivo</i> (dose: 5 mg kg ⁻¹) <i>In vitro</i> : not after 6 h at 1.32 ¹³
Decreased glycogen content ^{1,8,12–14}	<i>In vivo</i> : 54% after 24 h (dose: 7.5 mg kg ⁻¹); significant decrease within 4 h (dose: 9 mg kg ⁻¹) ¹⁴ ; <i>in vitro</i> : not after 48 h at 400 ¹²	<i>In vitro</i> : not after 7 h at 0.1–0.5 ⁸	<i>In vivo</i> (dose: 5 mg kg ⁻¹) <i>In vitro</i> : not after 6 h at 1.32 ¹³ <i>In vivo</i> (dose: 5 mg kg ⁻¹) <i>In vitro</i> : 20% after 6 h at 1.32 ¹³

Changes in levels of respiratory intermediates ^{1,8,13-15}	<p><i>In vivo</i>: decreased pyruvate (63%) after 24 h; other intermediates increase within 4 h but return to normal by 24 h (dose: 7.5 mg kg⁻¹)¹</p> <p><i>In vivo</i>: increased oxaloacetate and decreased malate after 24 h; other intermediates increase but return to normal by 24 h (dose: 9 mg kg⁻¹)¹⁴</p> <p><i>In vivo</i> (dose: 9 mg kg⁻¹)¹⁴</p>	<p><i>In vitro</i>: increased pyruvate and oxaloacetate, also glucose 6-phosphate (G6P) and phosphoenolpyruvate (PEP) after 3–5.5 h at 0.25⁸</p>	<p><i>In vivo</i>: decreased G6P (42%) and malate (73%) after 12 h (dose: 5 mg kg⁻¹)¹³</p> <p><i>In vitro</i>: increased pyruvate (473%), decreased G6P (35%) and malate (66%) after 3 h at 1.32¹³</p> <p><i>In vitro</i>: decreased G6P (27%) after 4 h at 3.3 (39%) after 1 h at 33.0¹⁵</p> <p><i>In vitro</i>: 513% after 12 h at 1.32¹³</p>
Increase in oxaloacetate/malate ratio ^{8,13,14}	<p><i>In vivo</i> (dose: 9 mg kg⁻¹)¹⁴</p>	<p><i>In vitro</i>: 67% after 5.5 h at 0.25⁸</p>	<p><i>In vitro</i>: 513% after 12 h at 1.32¹³</p>
Changes in levels of respiratory end products ^{1,8,12,13}	<p><i>In vivo</i>: increased succinate (71%) after 24 h but total unchanged (dose: 7.5 mg kg⁻¹)¹</p> <p><i>In vitro</i>: increased succinate and decreased propionate: total decreased (11%) after 9 h at 400¹²</p>	<p><i>In vitro</i>: increased succinate after 3–5.5 h at 0.25⁸</p>	<p><i>In vivo</i>: increased succinate and acetate (dose: 5 mg kg⁻¹)¹³</p> <p><i>In vitro</i>: increased succinate (491%) and lactate (55%) after 3 h at 1.32¹³</p>
Reduction in ATP and total nucleotide levels ^{1,8,12-17}	<p><i>In vivo</i>: 28–40% and 14–15%, respectively, after 24 h (dose: 7.5 mg kg⁻¹).^{1,6}; 18% decrease in ATP levels after 4 h and 61% decrease after 24 h (dose: 9 mg kg⁻¹)¹⁴</p> <p><i>In vitro</i>: 27–29% decrease in ATP levels after 3–9 h at 400^{12,16}</p>	<p><i>In vitro</i>: not after 5.5 h at 0.25⁸</p>	<p><i>In vivo</i>: 53% and 28%, respectively, after 12 h (dose: 5 mg kg⁻¹)¹³; 41% decrease in ATP levels after 4 h (dose: 5 mg kg⁻¹)¹⁷</p> <p><i>In vitro</i>: 20% decrease in ATP levels after 3 h at 1.32; 25% decrease in ATP levels after 20 h at 3.3 and 20% after 4 h at 33¹⁵</p>

Table 7.4 (continued).

Parameter tested	Rafoxanide	Oxyclozanide	Closantel
Stimulation of ATPase activity of fluke mitochondria <i>in vitro</i> ¹⁷			0.33 (50% inhibition at 66.31)
Increase in cytochemically demonstrable mitochondrial ATPase activity ¹⁹			<i>In vivo</i> (time and dose unknown)
Inhibition of malate dehydrogenase activity in <i>F. hepatica in vitro</i> ¹⁸	none at 626.0	80–100% at 401.5	
<i>References:</i>			
	1. Cornish <i>et al.</i> (1977)	11. Yorke and Turton (1974)	
	2. D.A.D. McIntosh, Macclesfield, 1982 (personal communication)	12. Cornish and Bryant (1976)	
	3. Closantel Information Booklet (Janssen Animal Health Ltd) (1986)	13. Kane <i>et al.</i> (1980)	
	4. Mohammed-Alli and Bogan (1987)	14. Prichard (1978)	
	5. Fairweather <i>et al.</i> (1984)	15. Rohrer <i>et al.</i> (1986)	
	6. Skuce (1987)	16. Bryant <i>et al.</i> (1976)	
	7. Corbett and Goose (1971)	17. Van den Bossche <i>et al.</i> (1979)	
	8. Edwards <i>et al.</i> (1981a)	18. Lwin and Probert (1975)	
	9. Veendaal and De Waal (1974)	19. Verheyen <i>et al.</i> (1979)	
	10. Campbell and Montague (1981)		

Comparable data have been obtained for rafoxanide from both *in vitro* and *in vivo* studies: viz. changes in respiratory intermediates, increase in oxaloacetate:malate ratio, changes in end products and decreased ATP synthesis (Bryant *et al.*, 1976; Cornish and Bryant, 1976; Cornish *et al.*, 1977; Prichard, 1978; see also Table 7.4). The major changes are essentially long term in nature and coincide with (or follow the onset of) removal of the flukes *in vivo* (Cornish *et al.*, 1977; Prichard, 1978). There are some differences between *in vivo* and *in vitro* observations: e.g. depletion of glycogen reserves *in vivo* but not *in vitro* (Cornish and Bryant, 1976; Cornish *et al.*, 1977). This has been attributed to the presence of a ready glucose supply in the culture medium and the passive diffusion of glucose into the fluke obviating the need to draw on glycogen reserves *in vitro*. In contrast, *in vivo*, detachment of flukes and reduced activity lead to cessation of feeding and a greater necessity to call on glycogen deposits (Behm and Bryant, 1979). The effects of rafoxanide on ATP and other nucleotide levels, while important in their own right, also impact on respiratory metabolism because of the modulatory actions of nucleotides on respiratory enzymes and consequently the flux through the metabolic pathways (Behm and Bryant, 1979).

There is much evidence, too, from both *in vivo* and *in vitro* studies, that closantel acts as an uncoupler in the fluke. An increased carbon flow along energy-producing pathways is indicated by increased glucose uptake, decreased glycogen content, increased end-product formation (especially succinate), changes in respiratory intermediates, increase in oxaloacetate:malate ratio, decreased ATP synthesis and changes in mitochondrial ATPase activity (Van den Bossche *et al.*, 1979; Kane *et al.*, 1980; Rohrer *et al.*, 1986; see also Table 7.4). The *in vitro* studies on closantel are a welcome rarity among *in vitro* studies involving fasciolicides in that they were carried out at concentrations well below the maximum blood level attained *in vivo* following drug treatment (for details see Table 7.4). An alternative suggestion has been put forward for the action of closantel, namely, that the initial effect is not on oxidative phosphorylation, but rather on glycolysis, specifically glucose 6-phosphate accumulation, since decreases in the level of the latter precede any drop in ATP levels. It was also suggested that the reductions in ATP levels may be correlated with the 'death' of the fluke (Rohrer *et al.*, 1986). However, the fall in glucose 6-phosphate accumulation may be due to acidification of the tegument: a rapid (within 10–20 min) decrease in pH and lowering of membrane potential occurs at a closantel concentration of $0.66 \mu\text{g ml}^{-1}$ (Pax and Bennett, 1989).

The three major salicylanilides cause a rapid spastic paralysis of *F. hepatica* at very low levels (Fairweather *et al.*, 1984; Skuce, 1987; Table 7.4). The effect is very similar to that induced by uncouplers such as CCCP and FCCP (Holmes and Fairweather, 1985). However, the raised muscle tone may not be due directly to uncoupling, but may simply reflect an increase in calcium ions in the muscle cells. This may result from the release of Ca^{2+} from internal stores, from an ionophore effect across the plasma membrane or organelle membrane, or from a membrane-perturbing effect that alters membrane permeability to ions (for an additional discussion of these ideas see

Fairweather *et al.*, 1984; Holmes and Fairweather, 1985; Fairweather, 1997). The rapidity of the neuromuscular actions of the salicylanilides may make them more significant than any disruption of energy metabolism because the knock-on effect of paralysis, leading to detachment *in vivo*, is a cessation of feeding. Consequently, the fluke enters a state of starvation and this will impose a severe metabolic stress on it. The fluke has to draw on its energy reserves in an attempt to survive, and this may account, at least in part, for some of the biochemical changes observed and the altered fluxes along respiratory pathways; hence, they may be secondary to a more direct neuromuscular effect.

Morphological studies on the changes induced by salicylanilides are restricted to closantel. The SEM data on the *in vivo* effects are confusing: a posterior–anterior spread of damage has been observed in flukes from closantel-treated sheep (and attributed to a posterior accumulation of drug in the fluke's gut) (Verheyen *et al.*, 1980), whereas loss of tegument was observed over large areas of the anterior and posterior regions of flukes from closantel-treated rats, with the intervening midbody region remaining normal (Skuce and Fairweather, 1990). Internally within the tegument a swelling of the basal infolds has been observed in studies of both the *in vivo* and *in vitro* effects of closantel (Verheyen *et al.*, 1980; Skuce, 1987; Skuce and Fairweather, 1990). Further swelling of the infolds leading to their detachment from the basal plasma membrane will account for the loss of tegument observed with SEM. The swelling may have a metabolic basis, resulting from restriction of the energy supply to the ATPase-driven ion pumps associated with the tegumental membranes (Skuce and Fairweather, 1990). A number of morphological changes evident in the tegumental syncytium and underlying cell bodies are compatible with an uncoupling type of metabolic inhibition, viz. deformed mitochondria, vesiculation and reduction of the Golgi complex, dilation of the GER cisternae and reduction in numbers of secretory bodies (Verheyen *et al.*, 1980; Skuce, 1987; Skuce and Fairweather, 1990). Similar changes take place in the gut, together with increased autophagy, shedding of gut lamellae and eventually 'complete desquamation of the necrotized epithelium' (Verheyen *et al.*, 1980) which indicate a state of stress and/or starvation. Increased autophagic activity is also evident in the vitelline cells, and a marked reduction of glycogen deposits occurs in them and in the parenchymal cells (Skuce and Fairweather, 1990). The latter observation suggests that carbohydrate reserves have been diverted into the glycolytic pathway to maintain energy production in the face of uncoupling of oxidative phosphorylation. Uncoupling leads to an increased carbon flux along energy-producing pathways, in an attempt by the fluke to produce more energy. Furthermore, the hydrolysis of ATP is promoted and electron transfer will continue at an uncontrolled rate until the respiratory substrate is exhausted. Consequently, many of the morphological data support the concept of a metabolic action for closantel, though they may also indicate the consequences of a stress (starvation) response induced by an as yet undefined neuromuscular action. There may even be a synergistic association between the two effects, each magnifying the extent of the other. For more

detail on the timing of the morphological changes induced by closantel see Fairweather (1997).

Apart from the vitelline changes induced by closantel, the only other study showing the effects of salicylanilides on the reproductive system was that describing the disruption of spermatogenesis by rafoxanide, an effect that was evident within 4 h *in vivo* (Stammers, 1975b).

Group 3: benzimidazoles

Comparative data for triclabendazole, albendazole and mebendazole is presented in Table 7.5. Triclabendazole (TCBZ) has an unusual structure for a benzimidazole in that it contains a chlorinated benzene ring but has no carbamate group (Bennett and Köhler, 1987; Lipkowitz and McCracken, 1991). It has an unusual activity, too, in that unlike other broad-spectrum benzimidazoles which show only marginal activity against the liver fluke, its efficacy appears to be restricted to *F. hepatica*, *F. gigantica* and *Fascioloides magna*. Triclabendazole lacks activity against nematodes and cestodes and against other trematodes, including *Dicrocoelium dendriticum*, *Schistosoma mansoni* and *Paramphistomum* spp. (Wolff *et al.*, 1983; Guralp and Tinar, 1984; Coles, 1986). It is a significant fasciolicide because it displays high efficacy against both adult and juvenile flukes (Boray *et al.*, 1983; Smeal and Hall, 1983; Turner *et al.*, 1984). Whether this unusual activity of TCBZ is achieved via a mechanism different from that of other benzimidazoles will be discussed below.

The effects of benzimidazoles on fluke motility are essentially long term in nature and require concentrations far higher than those which are effective *in vivo*, so they probably have little relevance to their mode of action. There are interesting differences between certain benzimidazoles and their active metabolites. For example, albendazole produces a prolonged stimulation of motility before movement finally declines, while albendazole sulphoxide induces a gradual suppression of activity (Fairweather *et al.*, 1984). A number of studies have shown that both TCBZ and its active sulphoxide metabolite (TCBZ-SX) induce a gradual suppression of activity (Fairweather *et al.*, 1984; Coles, 1986; Bennett and Köhler, 1987). Immature flukes are more sensitive to TCBZ than adults (Bennett and Köhler, 1987). The effects on motility are paralleled by a gradual hyperpolarization of the tegumental membrane potential, although this was shown not to be due to an inhibition of ATPase-driven ion pumps (Bennett and Köhler, 1987). Surface membrane changes as observed by SEM occur fairly quickly (within 3 h) following TCBZ-SX treatment (albeit at higher concentrations) and it is difficult to envisage how the ion pumps would remain unaffected by prolonged exposure to the drug given the severe disruption evident (Stütt and Fairweather, 1993a).

With regard to potential disruption of respiratory pathways, there is some evidence for an uncoupling action of mebendazole. *In vitro*, end-product formation (especially succinate) is increased by 12 h and remains so through to 36–48 h, although glucose uptake and glycogen mobilization are not affected. Levels of a number of respiratory intermediates are decreased

Table 7.5. A comparison of studies on the mode of action of benzimidazoles against the liver fluke, *Fasciola hepatica* (concentrations expressed as $\mu\text{g ml}^{-1}$).

Parameter tested	Triclabendazole	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Maximum blood level <i>in vivo</i> ¹⁻⁶	27 ¹ (dose: 10 mg kg ⁻¹ in sheep)	13.3 ² (dose: 10 mg kg ⁻¹ in sheep)	0.05 ³ (dose: 10 mg kg ⁻¹ in sheep)	3.2 ³ (dose: 10 mg kg ⁻¹ in sheep) 0.35 ⁴ (dose: 7.5 mg kg ⁻¹ in cattle)	0.006-0.117 ⁵ (dose: 40 mg day ⁻¹ in man) 0.4 ⁶ (dose: 40 mg kg ⁻¹ in rat) 0.224 ⁷ (dose: 100 mg kg ⁻¹ in sheep)
High activity (>87.5%) against flukes from 1 to 12 weeks in age ⁸⁻¹⁰	Dose: 5-10 mg kg ⁻¹				
Activity against flukes as young as one-day-old in sheep ¹⁰	Dose: 15 mg kg ⁻¹				
Total immobilization of fluke <i>in vitro</i> ¹¹					
(i) adult	(i) 24 h at 9.0-18.0	(i) —			
(ii) 3-week-old juvenile	(ii) 24 h at 3.6; 6 h at 9.0	(ii) 24 h at 3.6			
Total immobilization of adult fluke <i>in vitro</i> ¹²	24 h at 2.5				
Total immobilization of adult fluke <i>in vitro</i> ¹³	>30 h at 100	>18 h at 100	>21 h at 100	>36 h at 100	12 h at 500
Lowering of resting tegumental membrane potential in 3-week-old juvenile flukes <i>in vitro</i> ¹¹	4 h at 3.6 24 h at 0.36-1.08	24 h at 0.36-3.6			

Inhibition of Na ⁺ , K ⁺ , Mg ²⁺ - and Ca ²⁺ -ATPases in adult fluke <i>in vitro</i> ¹¹	No inhibition at 18.0	No inhibition at 18.0	
Uncoupling of rat liver mitochondria <i>in vitro</i> ¹⁴	6.48	3.38 (sulphone metabolite 0.39)	0.165–6.6
Respiratory control index effective concentration (RCI I ₅₀) for uncoupling of rat liver mitochondrial oxidative phosphorylation <i>in vitro</i> ¹⁵			
Changes in glucose uptake ¹⁶			<i>In vitro</i> : no change after 3 h at 400
Change in glycogen content ^{16,17}			<i>In vitro</i> : no change after 48 h at 400 ¹⁶ <i>In vivo</i> : 44.8% decrease after 30 h (dose: 100 mg kg ⁻¹) ¹⁷
Changes in levels of respiratory intermediates ^{16,17}			<i>In vitro</i> : decreases in various intermediates (e.g. G6P, malate) after 48 h at 400 ¹⁶

Table 7.5 (continued).

Parameter tested	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Changes in levels of respiratory intermediates ^{16,17}				<i>In vivo</i> : decreases in G6P (30%) and malate (17%) after 30 h (dose: 100 mg kg ⁻¹) ¹⁷
Changes in levels of respiratory end products ^{11,16-18}	<i>In vitro</i> : increased acetate (157%) and propionate (164%) after 24 h at 3.6 ¹¹	<i>In vitro</i> : no change after 24 h at 1.65 ¹⁸	<i>In vitro</i> : no change after 24 h at 1.81 ¹⁸	<i>In vitro</i> : increased succinate (14%) and decreased lactate (7%) after 36-48 h at 400 ¹⁶ <i>In vivo</i> : increased lactate (83%) after 30 h (dose: 100 mg kg ⁻¹) ¹⁷ <i>In vitro</i> : 33-60% and 19-37%, respectively, after 36-48 h at 400 ^{16,19} <i>In vivo</i> : 28% and 13%, respectively, after 30 h (dose: 100 mg kg ⁻¹) ¹⁷ 36-43% at 38.39
Reduction in ATP and total nucleotide levels ^{16,17,19}				
Inhibition of succinate decarboxylase system in <i>F. hepatica in vitro</i> ²⁰				
Inhibition of colchicine binding to tubulin from adult flukes ^{11,21}	(i) <i>In vitro</i> : no inhibition at 3.6 ²¹ (ii) <i>In vitro</i> : 86% inhibition at 3.6 ¹¹	<i>In vitro</i> : inhibition at 1.65 <i>In vivo</i> : no inhibition after 12, 24 h (dose: 20 mg kg ⁻¹) ²¹	<i>In vitro</i> : 25-75% after 5-15 min at 1.81 ²¹	<i>In vitro</i> : inhibition at 2.95 ²¹

- Inhibition of protease enzyme secretion *in vitro*¹
- (i) adult
 - (ii) 3-week-old juvenile
- Evidence of tegumental surface membrane changes *in vitro*²
- (i) adult
 - (ii) 3-week-old juvenile
- Evidence of internal tegumental changes *in vitro*³
- (i) adult
 - (ii) 3-week-old juvenile
- Disruption of secretory activity in tegumental cells *in vitro*³
- (i) adult
 - (ii) juvenile
- Disruption of spermatogenesis *in vitro*⁴
- (i) 12 h at 3.6–9.0
(ii) 6 h at 1.08–9.0
- (i) 6 h at 50.0
(ii) 3 h at 20.0
- (i) 6 h at 15.0–50.0
(ii) 3 h at 20.0
- (i) 6 h at 15.0–50.0
(ii) 3 h at 20.0
6 h at 15.0; 3 h at 50.0

Table 7.5 (continued).

Parameter tested	Triclabendazole	Triclabendazole sulphoxide	Albendazole	Albendazole sulphoxide	Mebendazole
Disruption of secretory activity in vitelline cells <i>in vitro</i> ²⁵		6 h at 50.0			
Disruption of vitellogenesis <i>in vitro</i> ²⁵		6 h at 50.0			
Inhibition of protein synthesis by adult flukes <i>in vitro</i> ²⁶		6 h at 15.0–50.0			
Inhibition of embryonation of fluke eggs ²⁷			LD ₅₀ 0.004		LD ₅₀ 0.005
(i) degenerative changes in reproductive organs <i>in vivo</i> ²⁸			(i) In ovary, testis and vitellaria after 39–40 days (dose: 10 mg kg ⁻¹)		
(ii) decreased hatchability of eggs ²⁸			(ii) After 1–7 days (dose: 10 mg kg ⁻¹)		

References: 1. R.J. Richards, Cambridge, 1983 personal communication; 2. Hennessy *et al.* (1987); 3. Marriner and Bogan (1980); 4. Prichard *et al.* (1985); 5. Karlaganis *et al.* (1979); 6. Van den Bossche *et al.* (1982); 7. Behm *et al.* (1983); 8. Boray *et al.* (1983); 9. Smeal and Hall (1983); 10. Turner *et al.* (1984); 11. Bennett and Köhler (1987); 12. Coles (1986); 13. Fairweather *et al.* (1984); 14. McCracken and Stillwell (1991); 15. Carr *et al.* (1993); 16. Cornish and Bryant (1976); 17. Rahman *et al.* (1977); 18. Fetterer and Rew (1984); 19. Bryant *et al.* (1976); 20. Köhler *et al.* (1978); 21. Fetterer (1986); 22. Stitt and Fairweather (1993a); 23. Stitt and Fairweather (1994); 24. Stitt and Fairweather (1992); 25. Stitt and Fairweather (1996); 26. Stitt *et al.* (1995); 27. Coles and Briscoe (1978); 28. Lang *et al.* (1980).

(Cornish and Bryant, 1976) (see Table 7.3). ATP levels show a reduction of 33–40% after 36 h, but total nucleotide levels do not fall until after 48 h *in vitro* (Bryant *et al.*, 1976; Cornish and Bryant, 1976). The studies were carried out at a concentration of 400 $\mu\text{g ml}^{-1}$, which is approximately 4000 times the maximum blood level *in vivo* (see Table 7.5). *In vivo*, changes in intermediary metabolites and end products do not occur until after 18 h; ATP levels do not fall until after 30 h, total nucleotide levels showing a decrease of 13% at this time (Rahman *et al.*, 1977). So, these changes are long term in nature and may follow detachment and removal of the flukes *in vivo* (Chevis, 1980; Rahman *et al.*, 1977).

There are interesting differences between *in vitro* and *in vivo* studies: for example, glycogen depletion occurs *in vivo* but not *in vitro* and lactate production decreases *in vitro* but increases *in vivo* (see Table 7.5). For glycogen, the difference has been attributed to a protected environment *in vitro* as against a more stressful situation *in vivo* which requires the mobilization of glycogen reserves; this point has already been made with regard to the action of rafoxanide. There is little evidence for disruption of energy metabolism in *F. hepatica* by albendazole, although it has been suggested that it, along with other 'classical' benzimidazoles, is capable of uncoupling oxidative processes in rat liver mitochondria (McCracken and Stillwell, 1991).

The impact of TCBZ on energy-producing pathways in the fluke has yet to be resolved. On the one hand, a stimulation of acetate and propionate production has been observed, the increase surprisingly coinciding with a decrease in motility (Bennett and Köhler, 1987). Prolonged incubations and higher concentrations were required before any major drop in ATP levels takes place, even when the fluke is immobile (Bennett and Köhler, 1987). On the other hand, experiments with rat liver mitochondria have shown that not only is triclabendazole capable of uncoupling oxidative phosphorylation in the mitochondria, but so too (and to a greater extent) are its sulphoxide and sulphone metabolites (Carr *et al.*, 1993).

It is generally accepted that benzimidazole anthelmintics bind to, and cause depolymerization of, cytoplasmic microtubules, thus disrupting microtubule-based processes in helminths (Lacey, 1988; Lubega and Prichard, 1990, 1991). Colchicine-binding data to support such a role for TCBZ are contradictory, Fetterer (1986) reporting a lack of inhibition of [^3H]colchicine binding to tubulin from homogenized flukes, while Bennett and Köhler (1987) demonstrated an inhibition of [^3H]colchicine binding to purified fluke tubulin. The discrepancy between the two studies has been attributed by Bennett and Köhler (1987) to non-specific binding of TCBZ to fluke protein in the study of Fetterer (1986), thus reducing drug availability to microtubular protein. However, a variety of other, more typical benzimidazoles – including mebendazole and albendazole (together with its sulphoxide and sulphone metabolites) – were shown to inhibit colchicine binding in the *in vitro* study by Fetterer (1986). Having said that, the picture for albendazole is confused, in that no inhibition of colchicine binding was evident in flukes recovered from sheep 12 h and 24 h after treatment with albendazole (Fetterer, 1986; see also Table 7.5). Morphological data in support of a microtubule-targeted

action for mebendazole and albendazole in *F. hepatica* itself are lacking, but mebendazole treatment has been shown to disrupt microtubule-dependent movements of secretory vesicles in the intestine of nematodes (e.g. Borgers *et al.*, 1975a) and the tegument of cestodes (e.g. Borgers *et al.*, 1975b). Microtubules are involved in many processes within cells and their disruption could have wide-ranging effects on parasites, thus accounting for some of the long-term metabolic changes observed.

In contrast to mebendazole and albendazole, morphological data regarding the action of triclabendazole on *F. hepatica* are available. The ultrastructural changes induced by TCBZ-SX *in vitro* are compatible with a microtubule-based action. Thus, there is a block in the transport of secretory bodies from the tegumental cell bodies to the apical surface of the tegument. The block occurs at their site of formation by the Golgi complex in the cell body, in their movement through the cytoplasmic connections to the syncytium, and in their movement from the base to the apex of the syncytium (Stitt and Fairweather, 1994). The disruption of secretory activity occurs fairly rapidly (3–6 h) in both adult and juvenile flukes (Table 7.5). The presence of a microtubule system in the tegument of *F. hepatica* has been demonstrated by Stitt *et al.* (1992). Since maintenance of the integrity of the tegumental surface membrane is dependent on the continual turnover of secretory bodies, it is reasonable to assume that the disruption of secretory activity evident in the tegument leads to the progressively severe surface damage visible externally, culminating in the total loss of the tegument (by 24 h in the adult) (Stitt and Fairweather, 1993a). Inhibition of proteolytic enzyme secretion in *F. hepatica* by TCBZ has also been attributed to disruption of microtubule-based secretory processes (Bennett and Köhler, 1987).

TCBZ-SX has been shown to inhibit the mitotic division of spermatogenic cells, in particular the early spermatogonial stages (Stitt and Fairweather, 1992). Fragmentation and autophagy of the central cytophore region of the rosette stages takes place, leading to disruption of the spermatocyte and spermatid stages. Spermiogenesis is completely inhibited and few spermatozoa are formed (Stitt and Fairweather, 1992). Again, the changes occur quite quickly (3–6 h *in vitro*: see Table 7.5). Division of the stem vitelline cells is also inhibited by TCBZ-SX, preventing them from undergoing their normal developmental sequence, which involves the production of eggshell material and the laying down of glycogen reserves for the developing embryo. Inhibition is fairly rapid: 6 h *in vitro* (Table 7.5) (Stitt and Fairweather, 1996).

The changes observed in the tegument, testis and vitelline cells of *F. hepatica* following TCBZ-SX treatment are what might be expected following microtubule inhibition. Moreover, they bear close similarity to those induced by microtubule inhibitors, especially tubulozole-C (Stitt and Fairweather, 1992, 1993b). The liver fluke is less susceptible to colchicine, relatively high concentrations (1×10^{-3} M) being required to bring about any morphological changes (Stitt and Fairweather, 1993b). The differential sensitivity to the two microtubule inhibitors may have an important bearing on the mode of action of TCBZ in relation to other benzimidazoles. Thus, the latter are

known to act by binding to the colchicine-binding site on the tubulin molecule (Lacey, 1988). Colchicine binds to tubulin heterodimers and inhibits their polymerization, thereby progressively depolymerizing microtubules (Mareel and De Mets, 1984). In contrast, tubulozole-C inhibits tubulin polymerization by inducing the formation of aggregates of the tubulin monomers (Mareel and De Mets, 1984; De Brabander *et al.*, 1986). So it is possible that TCBZ-SX binds to a separate (non-colchicine) binding site on the tubulin molecule of the fluke, a site that it shares with tubulozole-C. The colchicine-binding site may be less sensitive to disruption, explaining why more typical benzimidazoles are poorly active against *F. hepatica*, while TCBZ lacks activity against helminth parasites other than *F. hepatica*. It may be of significance that the TCBZ-SX molecule is U-shaped rather than the L-shape of other benzimidazoles (Lipkowitz and McCracken, 1991). Only *F. hepatica* among helminth parasites may possess a binding site on the tubulin molecule that can accommodate such a shape of drug. So, the combined data argues for disruption by TCBZ-SX of microtubule-based processes in the fluke, a conclusion that disagrees with the views of Coles (1986), Fetterer (1986) and Guralp and Tinar (1984).

The ultrastructural changes induced by TCBZ-SX have indicated an additional possible mode of action, namely, inhibition of protein synthesis. In the tegumental cells, for example, there is a decline in the number of secretory bodies, a reduction in the amount of GER and a gradual disappearance of the Golgi complex in the cytoplasm and a condensation of chromatin and disappearance of the nucleolus in the nucleus (Stitt and Fairweather, 1994). Similar nuclear changes were evident in the vitelline cells, which also showed a reduction in shell protein production, as evidenced by a decrease in the number of shell protein globules produced, together with a swelling of the GER cisternae and a decrease in their ribosomal covering (Stitt and Fairweather, 1996). In a separate study, it has been shown that TCBZ-SX causes a marked inhibition of the incorporation of [¹⁴C]leucine into *F. hepatica* proteins over a 6 h period, thus confirming the conclusion from the morphological studies (Stitt *et al.*, 1995). Moreover, the same study showed that TCBZ-SX is a more potent inhibitor of protein synthesis than tubulozole, suggesting that it inhibits protein synthesis in a manner that is not based on microtubule inhibition (Stitt *et al.*, 1995). Which aspect of protein synthesis is inhibited by TCBZ-SX is not known, although it has been suggested that the action is directed against RNA synthesis (Stitt *et al.*, 1995).

In conclusion, many of the available data support the notion that TCBZ acts against microtubule-based processes in the liver fluke, albeit in a different way from other, more typical, benzimidazole anthelmintics. A second action is directed against protein synthesis. The two actions may be separate, though interrelated because disruption of the microtubule-dependent organization of organelles such as the GER and Golgi complex that are involved in synthetic mechanisms is likely to affect protein synthesis. Effects on energy metabolism are likely to be secondary as they are very long term in nature, occurring long after effects on the cytoskeleton and protein synthesis become apparent and at a time when morphological 'damage' is very severe. A direct action on the

neuromuscular system is also unlikely as the suppression of motility is very gradual.

The severe disruption of spermatogenesis and vitellogenesis by TCBZ-SX has already been described. Albendazole also causes marked damage to the reproductive system of *F. hepatica*. Thus, flukes recovered from cattle 5–6 weeks after treatment showed gross degenerative changes to the testes, ovary and vitelline cells (as well as to the gut) and reduced numbers of eggs in the uterus were observed. In addition the eggs were abnormal and exhibited reduced hatchability for 3 weeks post-treatment (Lang *et al.*, 1980). It remains to be resolved whether these changes represent a direct action of albendazole or whether they are simply a manifestation of the axiom proposed by Dawes (1968) and discussed previously in relation to bithionol. In a separate study, it has been demonstrated that both albendazole and mebendazole inhibit the embryonation of fluke eggs (Coles and Briscoe, 1978).

Group 4: sulphonamides

Clorsulon is the only fasciolicide believed to act against glycolysis, which is the main energy-producing pathway in the fluke (Coles, 1975; Barrett, 1976, 1981; Van Vugt, 1979/80). It has been shown to cause inhibition of the glycolytic enzymes 3-phosphoglycerate kinase and phosphoglyceromutase (Schulman and Valentino, 1980). However, the inhibition of the former was 40% at a concentration of $399.53 \mu\text{g ml}^{-1}$, 47.7 times that of the maximum blood level attained *in vivo* ($8.37 \mu\text{g ml}^{-1}$ in the rat following a dose of 12.5 mg kg^{-1} ; Schulman *et al.*, 1979). Inhibition of phosphoglyceromutase was 43% at a concentration of $197.86 \mu\text{g ml}^{-1}$, 23.6 times that of the maximum blood level. Consequently, the disruption of glycolysis *in vivo* may not be very great. Inhibition of glycolysis is potentially very serious for the fluke because it would block the early steps in the respiratory pathway and the downstream reactions would become inoperable; energy production would become severely, if not completely, impeded. In support of this idea, flukes treated for 1 h in clorsulon at a concentration of $500 \mu\text{g ml}^{-1}$ show a 60% decrease in glucose utilization, a 54% and 85% inhibition of the formation of the metabolic end products acetate and propionate, respectively, and a 67% reduction in ATP levels (Schulman and Valentino, 1980).

Clorsulon causes a gradual suppression of motility, leading to paralysis of the fluke in a flaccid condition (Fairweather *et al.*, 1984). This might be expected to occur with the depletion of energy reserves following glycolysis inhibition; established inhibitors of glycolysis such as sodium fluoride and sodium iodoacetate produce a similar suppression of motility (Holmes and Fairweather, 1985). The motility data agree with pharmacokinetic studies on clorsulon, in which a cessation of feeding was seen to coincide with, or just precede, the onset of elimination of the drug from the fluke, a process which begins 8–12 h following drug administration (Schulman *et al.*, 1979). It is possible that the cessation of feeding coincides with the sudden drop in activity that occurs after 10–12 h *in vitro* (Fairweather *et al.*, 1984).

Morphological studies have shown that changes in the gastrodermis induced by clorsulon occurred more rapidly than those in the tegument and were of greater severity, in both *in vivo* (following an oral dose of 12.5 mg kg⁻¹) and *in vitro* (10 µg ml⁻¹) studies. This may be a reflection of the means by which the drug enters the fluke: clorsulon binds to red blood cell carbonic anhydrase and enters the fluke with ingested erythrocytes (Schulman *et al.*, 1979). Surface damage to the fluke, as visualized by SEM, was confined primarily to the oral cone region and was more severe following *in vivo* treatment. By 48 h *in vivo*, the tegument was completely sloughed off the anterior region of the fluke. After 24 h, the tegumental cells appeared to be synthetically inactive and this may have caused the tegumental loss. Also, the gastrodermal cells showed signs of necrosis within 24 h (Fairweather and McDowell, 1995). Within the gut, the apical region of the gastrodermal cells showed signs of vacuolation and disruption after 12 h treatment *in vitro*; by 24 h the cells were showing signs of breakdown (Fairweather and McDowell, 1995). Some of the morphological changes observed may account for the postulated biochemical effects of clorsulon, which were only apparent at extremely high concentrations. For example, once paralysed the fluke ceases to feed and enters a state of starvation. The severe disruption of the gut and, to a lesser extent, that of the tegument then exacerbates the damaging situation for the fluke. Thus, for clorsulon, with its unusual mechanism of uptake, it may well be a case of disruption from the inside out rather than from the outside in, as occurs with other compounds.

Group 5: phenoxyalkanes

Diamphenethide is the only member of this group. It is a unique fasciolicide in that it is more active against juvenile than adult flukes, even against flukes as young as 1 day old (Annen *et al.*, 1973; Rowlands, 1973). As such, it offers the possibility of preventing much of the liver damage caused by the migrating juvenile flukes. Diamphenethide itself is rapidly metabolized by deacetylation in the liver of the host to an amine compound (Harfenist, 1973). This deacetylated (amine) metabolite of diamphenethide (DAMD) is responsible for the flukicidal activity of diamphenethide, its locally high concentration in the liver explaining why diamphenethide is so effective against juvenile flukes. Despite its significance, diamphenethide has remained something of an enigma, because little is known about its precise mode of action. The results of *in vitro* studies involving diamphenethide and DAMD are summarized in Table 7.6. Perhaps more is known of what it does *not* do than what it does.

A number of possible actions have been eliminated. Thus, there is general agreement that it does *not* disrupt energy metabolism in the fluke, in contrast to the postulated action of many commonly used fasciolicides, as discussed previously (Campbell and Montague, 1981; Edwards *et al.*, 1981a). On the basis of elevated internal Na⁺ levels in the fluke, it has been suggested that DAMD acts as an inhibitor of Na⁺/K⁺-ATPase activity or as a sodium ionophore (Rew *et al.*, 1983). There is no pharmacological evidence to support the former

Table 7.6. A comparison of studies on the mode of action of diamphenethide against the liver fluke, *Fasciola hepatica* (concentrations expressed as $\mu\text{g ml}^{-1}$) (studies carried out *in vitro* unless stated otherwise).

Parameter tested	Diamphenethide	Deacetylated (amine) metabolite of diamphenethide	Reference
Maximum blood level <i>in vivo</i> in sheep	30 Dose: 400 mg kg ⁻¹	12 Dose: 100 mg kg ⁻¹	R.C. Parker, Berkhamsted, 1981 (personal communication)
Greater activity against juvenile than adult <i>F. hepatica</i> in sheep	Dose: 100 mg kg ⁻¹	—	Armour and Corba (1972); Kingsbury and Rowlands (1972); Annen <i>et al.</i> (1973); Kendall and Parfitt (1973); Rew <i>et al.</i> (1978)
Activity against flukes as young as one-day-old in sheep	Dose: 100 mg kg ⁻¹	—	Annen <i>et al.</i> (1973); Rowlands (1973)
Inhibition of malate dehydrogenase activity in adult <i>F. hepatica</i>	None at 372.4	—	Lwin and Probert (1975)
Minimum concentration for maximum uncoupling of <i>Hymenolepis diminuta</i> mitochondria	No uncoupling at 74.48	—	Yorke and Turton (1974)
Minimum concentration for uncoupling of rat liver mitochondria	372.4–∞	288.4–∞	Campbell and Montague (1981)
Reduction of ATP levels in adult <i>F. hepatica</i>	—	47% at 28.84 after 24 h	Rew and Fetterer (1984)
Changes in levels of ATP, respiratory intermediates and end products in adult <i>F. hepatica</i>	—	Only increased malate at 2.0 (after 3 h)	Edwards <i>et al.</i> (1981a)
Changes in respiratory end products in adult <i>F. hepatica</i>	—	Increase in acetate, propionate and lactate between 6 and 24 h at 28.84	Rew and Fetterer (1984)
Inhibition of glucose transport in juvenile (3–5-weeks-old) and adult <i>F. hepatica</i>	—	39% in juvenile at 28.84 (after 2 h). None in adult	Rew <i>et al.</i> (1983)

Inhibition of glucose transport in adult <i>F. hepatica</i>	—	30% at 10 (after 2 h)	Edwards <i>et al.</i> (1981a)
Minimum concentration for paralysis of adult <i>F. hepatica</i>	No paralysis within 12 h at 100	10 (flaccid paralysis within 1.5 h)	Fairweather <i>et al.</i> (1984)
Change in motility of 4-week-old juvenile and adult <i>F. hepatica</i>	No change in either adult or juvenile over 2 h period (concentration not given)	Spastic paralysis within 2 h at 28.84	Rew <i>et al.</i> (1983)
Inhibition of cholinesterase activity in adult <i>F. hepatica</i>	20.8% at 372.4	—	Durrani (1980)
Protection of <i>F. hepatica</i> by dopamine against flukicidal action	—	5.0	Edwards <i>et al.</i> (1981b)
Depolarization of tegumental membrane potential of 4-week-old juvenile <i>F. hepatica</i>	—	Within 0.5 h at 28.84	Rew <i>et al.</i> (1983)
Change in ion content of adult <i>F. hepatica</i>	—	15% increase in Na ⁺ concentration at 28.84 (after 3 h)	Rew <i>et al.</i> (1983)
Change in ion levels in adult <i>F. hepatica</i>	—	Only initial and short-lived drop in Na ⁺ over a 6 h period at 10	Caseby <i>et al.</i> (1991)
Change in wet weight of adult <i>F. hepatica</i>	—	12% increase at 28.84 (after 3 h)	Rew <i>et al.</i> (1983)
Evidence of tegumental surface changes (leading to sloughing of tegument)	—	Adult: 3 h (24 h) 5-week-old: 3 h (6 h) 3-week-old: 1.5 h (9 h) Freshly-excysted metacercaria (day 0): 1 h (all at 10)	Anderson and Fairweather (1988); Fairweather <i>et al.</i> (1987)

Table 7.6 (continued).

Parameter tested	Diamphenethide	Deacetylated (amine) metabolite of diamphenethide	Reference
Inhibition of tegumental Na ⁺ /K ⁺ -ATPase activity	—	No inhibition after 18 h at 10	Skuce <i>et al.</i> (1987)
Evidence of internal tegumental changes (and onset of flooding)	—	Adult: 6 h (9 h) 5-week-old: 3 h (3 h) 3-week-old: 1.5 h (3 h) 0-day: 0.5 h (1 h) (all at 10)	Anderson and Fairweather (1995); Fairweather <i>et al.</i> (1986)
Disruption of secretory activity in tegumental cells	—	Adult: 9 h 5-week-old: 3 h 3-week-old: 3 h 0-day: 1 h (all at 10)	Anderson and Fairweather (1995); Fairweather <i>et al.</i> (1986)
Disruption of secretory activity in gut cells	—	Adult: 6 h 5-week-old: 6 h 3-week-old: 3 h 0-day: — (all at 10)	Anderson (1989)
Disruption of secretory activity in vitelline cells	—	Stem cell: 6 h I11 cell: 6 h I12 cell: 9 h Mature cell: 9 h (all at 10) After 6 h at 10	Fairweather <i>et al.</i> (1988a)
Inhibition of protein synthesis	—	—	Anderson <i>et al.</i> (1993)
Disruption of spermatogenesis <i>in vivo</i>	Within 4 h (dose: 20 mg kg ⁻¹ in rabbit)	—	Stammers (1975b)

suggestion, because ouabain, a known inhibitor of Na^+/K^+ -ATPase activity, induces a spastic rather than flaccid paralysis of the fluke (Fairweather *et al.*, 1988b). A similar neuromuscular response to ouabain has been cited for *S. mansoni* (Fetterer *et al.*, 1980). The increased muscle tension is probably the result of the elevated intracellular sodium concentration following sodium pump inhibition; this acts on the membrane $\text{Na}^+/\text{Ca}^{2+}$ exchange to increase the intracellular level of calcium (Allen and Navran, 1984; Repke and Schönfeld, 1984; Allen *et al.*, 1985).

There is some evidence to support an ionophore role for DAMD, although the data are not straightforward. Monensin, a sodium ionophore, also induces a flaccid paralysis of the fluke (Fairweather *et al.*, 1988b). This is the opposite of what might be expected: the influx of sodium ions should lead to a depolarization of the membrane potential, triggering muscle contraction and leading to a spastic paralysis. However, the paradox of membrane hyperpolarization by monensin has been observed in a variety of cell types, including muscle cells, and has been attributed to the stimulation of sodium pump activity by raised intracellular Na^+ levels, leading to their reduction (Brock and Smith, 1982). Prior inhibition of pump activity by ouabain allows subsequent monensin treatment to generate the anticipated depolarization (Fahim *et al.*, 1983). The same is true for *F. hepatica*, both monensin – and, more significantly, DAMD – elicit a rapid spastic paralysis following ouabain pretreatment (Fairweather *et al.*, 1988b).

DAMD induces a swelling of the infoldings of the basal plasma membrane of the tegument of *F. hepatica*; the swelling leads to flooding and eventual sloughing of the tegument (Fairweather *et al.*, 1986; Anderson and Fairweather, 1995). The changes are suggestive of a disruption of the osmoregulatory role of the tegument, with the swelling of the basal infolds being what might be expected of an ionophore action. Thus, the large influx of Na^+ ions might overwhelm the capacity of the Na^+/K^+ -ATPase-driven ion pumps located along the apical plasma membrane, the ions would penetrate deeper into the syncytium and be pumped into the basal infolds by the ion pumps situated along their membranes. The lumen of the infolds would become hypertonic with respect to the surrounding cytoplasm and water would be drawn into the infolds from the cytoplasm, making them swell and thus causing the flooding. Unfortunately for this idea, the sodium ionophore monensin does not cause any swelling of the fluke basal infolds, even after prolonged (24 h) incubation *in vitro* (Skuce and Fairweather, 1989). This unexpected result may be due to stimulation of the ion pumps because of increased internal levels of Na^+ following monensin treatment, the activity of the pump thus effectively masking the normal action of monensin. 'Unmasking' of monensin activity by prior inhibition of the pumps with ouabain does cause vacuolation of the tegument of *F. hepatica*, but this has nothing to do with the basal infolds (Skuce and Fairweather, 1989). Ouabain could mimic the effect of monensin by inhibiting the ion pumps, thus allowing Na^+ to enter down its concentration gradient. However, ouabain does not induce any swelling of the basal infolds (Skuce and Fairweather, 1989), although it has been shown to inhibit Na^+/K^+ -ATPase activity in the

tegument (Skuce *et al.*, 1987). With regard to a potential role as an ATPase inhibitor, it has been shown that, while DAMD does induce swelling of the basal infolds, it does not affect the ATPase activity associated with the tegumental ion pumps (Skuce *et al.*, 1987).

Additional analytical studies have demonstrated that DAMD causes little change in internal ion levels in *F. hepatica* over a 6 h period (Caseby *et al.*, 1991). The sodium ionophore monensin induces a decrease in K^+ levels to a value below that of Na^+ , the reverse of the normal condition. There is a short-lived drop in Na^+ levels early on, but otherwise there is no difference from controls. Ouabain, an inhibitor of Na^+/K^+ -ATPase activity, causes a marked reduction in K^+ levels; Na^+ and Ca^{2+} levels also fall. Pretreatment with ouabain followed by monensin does not affect the decline in K^+ levels, but prevents the short-lived Na^+ decline observed with monensin alone (Caseby *et al.*, 1991). The results with monensin and ouabain were somewhat unexpected and consistent with the results of the pharmacological and morphological studies discussed previously. Neither pattern matched that produced by DAMD, so the ion analysis data add further weight against a Na^+ ionophore or Na^+/K^+ -ATPase inhibitor role for DAMD.

DAMD induces a rapid flaccid paralysis of the fluke, but the basis of this action remains unclear (Fairweather *et al.*, 1984). DAMD may have a neuropharmacological action, although a cholinesterase inhibitor role appears unlikely because there is only a 20.8% inhibition at a concentration of $372.4 \mu\text{g ml}^{-1}$, although this result was obtained with the parent compound (Durrani, 1980). There is a possibility of disruption of dopaminergic mechanisms because dopamine, an excitatory transmitter in *F. hepatica* (Holmes and Fairweather, 1984), can (at $200 \mu\text{g ml}^{-1}$) protect the fluke against the metabolic effects (elevated malate and succinate levels) of DAMD ($5.0 \mu\text{g/ml}^{-1}$) (Edwards *et al.*, 1981b). This possibility needs to be explored further.

DAMD causes extensive damage to the tegumental surface as evident with SEM. Studies involving different developmental stages, from freshly excysted metacercaria to mature adult, have shown that the early stages are more severely and more rapidly affected than the adult (Fairweather *et al.*, 1987; Anderson and Fairweather, 1988). There are also interesting regional differences in the pattern of drug-induced surface damage: in the adult, it is the dorsal surface that is more severely affected, whereas it is the ventral surface in the case of the earlier stages. These differences have been linked to developmental changes in tegumental architecture and Na^+/K^+ -ATPase-driven ion pumps (Fairweather *et al.*, 1986). In the adult fluke, there is an anterior-posterior spread of damage (Fairweather *et al.*, 1987; Anderson and Fairweather, 1988).

Internal changes within the tegumental syncytium initially take the form of a 'stress' reaction (an apical concentration and increased exocytosis of secretory bodies, formation of microvilli and blebbing of the surface membrane), leading to the swelling of the basal infolds described previously. It is the latter that is responsible for the sloughing of the tegument. Age-related differences in the time sequence of tegumental changes are apparent and consistent with the SEM observations (Fairweather *et al.*, 1986; Anderson and Fairweather, 1995). The trends parallel the results of *in vivo* efficacy

studies (see Table 7.6). The flooding of the tegument spreads internally to involve the tegumental cell bodies. A number of distinct changes in cell structure take place and will be discussed below in relation to the mode of action of DAMD. Here, perhaps, it is relevant to point out that the changes observed are *not* indicative of an ionophore or ATPase inhibitor role. The sodium ionophore monensin causes osmotic dilation of the *cis* Golgi cisternae, whereas the action of DAMD is directed more against the *trans* cisternae. Unmasking of monensin activity by ouabain pretreatment does lead to a typical swelling of the Golgi cisternae, although ouabain alone only causes the complex to become diffuse, even after 18 h incubation (Fairweather *et al.*, 1986; Skuce and Fairweather, 1989).

DAMD induces marked disruption of the gut of *F. hepatica*: changes include reduced numbers of secretory bodies, increased autophagy, vesiculation of the GER, swelling of the mitochondria, accumulation of lipid, and cellular necrosis in extreme cases. There are clear age-related trends in the onset and extent of the changes, with juvenile flukes being more susceptible than adult flukes (Anderson, 1989). The trends mirror those observed in the tegument. Again, the changes observed are not compatible with either an ionophore or ATPase inhibitor action (Skuce, 1987).

Diamphenethide treatment leads to disruption of the reproductive system of *F. hepatica*. The parent compound, diamphenethide itself, affects spermatogenesis *in vivo* fairly rapidly (within 4 h) (Stammers, 1975b). Treatment with the active metabolite, DAMD, *in vitro* causes changes to the vitelline cells, particularly the undifferentiated stem cells and the intermediate cells in the early stages of protein synthesis; the changes occur fairly quickly (within 6 h) and will be discussed in more detail later (see Fairweather *et al.*, 1988a). The vitelline cells appear to become inactive, being prevented from proceeding with their normal developmental sequence: as time progresses, there is a change in the cell population within the follicle, with relatively more stem, early It1 and mature cells and a decline in numbers of characteristic It1 and It2 cells (Fairweather *et al.*, 1988a). With regard to a possible ionophore or ATPase inhibitor action for DAMD, the profile of monensin- and ouabain-treated cells is very different from that for DAMD, adding further morphological evidence against such roles (for details, see Skuce and Fairweather, 1988a).

The morphological data from studies on the vitelline, tegumental and gut cells of *F. hepatica* indicate an alternative action for DAMD, namely, inhibition of protein synthesis in the fluke. In the vitelline cells, for example, there is condensation of chromatin within the nucleus and disappearance of the nucleolus, a progressive loss of ribosomes from the GER cisternae and reduction in the number of eggshell protein globules produced (Fairweather *et al.*, 1988). The action of DAMD is directed against the stem and early It1 cells, preventing them from proceeding with their normal developmental sequence. Similar, though less dramatic, changes are evident in the tegumental and gut cells (Fairweather *et al.*, 1986; Anderson, 1989).

In order to test this idea further, the effect of DAMD on the uptake and incorporation by adult *F. hepatica* of radioactively labelled precursors of DNA, RNA and protein synthesis has been determined by a liquid scintillation

counting technique. DAMD caused a significant decrease in the overall uptake and incorporation of [^3H]uridine after 6 h incubation *in vitro*, decreased the incorporation of [^3H]leucine from 6 h onwards and also caused a significant decrease in the overall protein content of the fluke, although it had no significant effect on the uptake or incorporation of [^3H]thymidine (Anderson *et al.*, 1993). The results indicate that DAMD inhibits protein synthesis in *F. hepatica* by inhibition of RNA synthesis. Inhibition of protein synthesis would have serious consequences for the fluke. This is not only with regard to processes such as egg production and the maintenance of tegumental integrity (which is essential for nutrient uptake, immunoprotection and osmoregulation). It would also affect the synthesis of enzymes involved in metabolic pathways within the fluke. This could account for the metabolic changes which are known to occur following treatment with DAMD, but are not considered to be the primary effect of the drug (Edwards *et al.*, 1981a; Rew *et al.*, 1983; Rew and Fetterer, 1984; see also Table 7.4).

An inhibition of protein synthesis might explain the high activity of DAMD against juvenile flukes. Juveniles are in a very active phase of growth and differentiation and require higher rates of production of tegumental secretory bodies and glycocalyx turnover than the adult flukes to protect them from the immune response in the host liver (Hanna, 1980). Therefore, the juvenile flukes might be expected to be more susceptible to a drug which inhibits protein synthesis.

Furthermore, inhibition of protein synthesis would be a novel mode of action for a fasciolicide, although emetine dihydrochloride, an inhibitor of protein synthesis, has been used to treat liver fluke infections in rodents, sheep and man (Duriez *et al.*, 1964; Grant and Jagers, 1969). It is only effective against intrahepatic juvenile flukes, not against adult flukes in the bile duct, so it shows some similarity with DAMD. Emetine also shows greatest activity against juvenile flukes in *in vitro* screens (Ibarra and Jenkins, 1984). However, the antischistosomal drug hycanthonone is known to act against protein synthesis in *S. mansoni*. Hycanthonone treatment brings about a change in the population of cells within the vitelline follicle as a result of inhibition of division of the stem cells. The number of stem cells declines and mature cells accumulate, while intermediate stages disappear. This was interpreted as being due to inhibition of nucleic acid synthesis (Erasmus and Popiel, 1980). Initial studies indicated that hycanthonone inhibited RNA synthesis *in vitro* (Pica-Mattocchia *et al.*, 1981). *In vivo*, however, hycanthonone action is directed predominantly towards DNA synthesis (Pica-Mattocchia and Cioli, 1983). More recent work has shown that hycanthonone-sensitive schistosomes are able to convert the drug to a reactive ester, which spontaneously dissociates to an electrophilic species which is capable of alkylating macromolecules, preferentially DNA (Cioli *et al.*, 1985; Pica-Mattocchia *et al.*, 1988). Deoxyguanosine is the site of covalent binding and alkylation in the DNA molecule (Archer *et al.*, 1990). Oxamniquine is believed to act in the same way against schistosomes (Pica-Mattocchia and Cioli, 1985; Pica-Mattocchia *et al.*, 1989; Archer *et al.*, 1990). Similar studies involving labelled DAMD may represent a possible future step in the elucidation of the mode of action of diamphenethide.

Finally, the morphological data from studies on the tegument and gut of *F. hepatica* have highlighted interesting age-related trends in the onset and severity of changes induced by DAMD (Fairweather *et al.*, 1986, 1987; Anderson and Fairweather, 1988, 1995; Anderson, 1989). Thus, the fluke becomes less susceptible to DAMD action with increasing age. The trend parallels the situation *in vivo*, efficacy studies showing that diamphenethide is more active against immature pre-bile duct stages than against adult flukes (for references, see Table 7.6).

From the data available, it is possible to establish a time course of events during DAMD treatment *in vitro*. Taking the adult fluke as an illustration, the fluke is paralysed within 1.5–2.0 h, surface alterations are evident from 3 h onwards and internal tegumental changes after 6 h. Flooding of the tegument begins after 9 h and leads to the tegumental sloughing observed after 24 h. Inhibition of secretory activity in the vitelline and gut cells is evident after 6 h and that in the tegument after 8 h. Inhibition of protein synthesis has been recorded from 6 h onwards. These changes occurred at a concentration of $10 \mu\text{g ml}^{-1}$, which corresponds very closely to the maximum blood level *in vivo*. Of the metabolic changes observed, malate levels were elevated after 3 h (at $2.0 \mu\text{g ml}^{-1}$), levels of the end products acetate, propionate and lactate were increased between 6 and 24 h (at $28.84 \mu\text{g ml}^{-1}$) and ATP levels had dropped by 47% after 24 h (at $28.84 \mu\text{g ml}^{-1}$).

The time sequence indicates that the fluke enters a state of starvation fairly quickly, as a result of the induced paralysis. This condition is compounded by the tegumental damage and gut changes observed and may account for some of the longer-term metabolic changes. Inhibition of protein synthesis will certainly exacerbate the situation even further and can be considered as a major target for the drug. However, it is probably not possible to conclude that this is the primary mode of action of DAMD. The neuromuscular effect, being the most immediate, may be of paramount importance, and this action remains to be resolved. Consequently, DAMD remains something of an enigma in terms of its mechanism of action.

Drug Resistance in *Fasciola hepatica*

Resistance to anthelmintics among helminth parasites is a global problem of increasing concern. It is particularly prevalent in nematode parasites (see reviews by Jackson, 1993; Shoop, 1993; Prichard, 1994), but is also evident in other trematodes such as schistosomes (Cioli *et al.*, 1993; Brindley, 1994). Drug resistance in *F. hepatica* is not yet a major problem, but resistance to a number of fasciolicides has been identified in the field and in the laboratory (Boray and De Bono, 1989; Boray, 1990, 1997). Data pertaining to individual compounds are discussed below.

Salicylanilides

It has been shown that long and regular use of salicylanilide compounds, particularly radoxanide and closantel for the treatment of fasciolosis in sheep,

has selected resistant strains of *Fasciola hepatica* in endemic areas of New South Wales. These two compounds are also used for the control of the nematode *Haemonchus contortus* and there is evidence for resistance in the worm to them (Van Wyk and Malan, 1988; Rolfe *et al.*, 1990). The fluke strains retained their resistant status in cattle and through several passages in sheep. In about 60% of the properties, and from different geographical regions, surveyed in New South Wales, flukes showed resistance to rafoxanide at recommended dose rates in *F. hepatica* and side resistance to closantel was evident (Fig. 7.2). There was also cross-resistance to nitroxylnil, a halogenated phenol (Boray, 1997). Resistance manifested against immature, but rarely against adult, fluke. A degree of salicylanilide resistance has also been reported in western England and Wales, respectively (Boray, J.C. and W.M. Allen, unpublished data). Fluke strains resistant to rafoxanide and closantel show no side resistance to another salicylanilide, oxyclozanide. This may be due to differences in the age-related susceptibility of the fluke to the drugs (Coles, 1975) or to the pharmacokinetic characteristics of the latter. Oxyclozanide quickly reaches its peak concentration in the blood after treatment and its excretion is equally rapid. Rafoxanide and particularly closantel are strongly bound to plasma protein and persist in the blood at subtherapeutic concentrations for up to 90 days and are more likely to select for resistance.

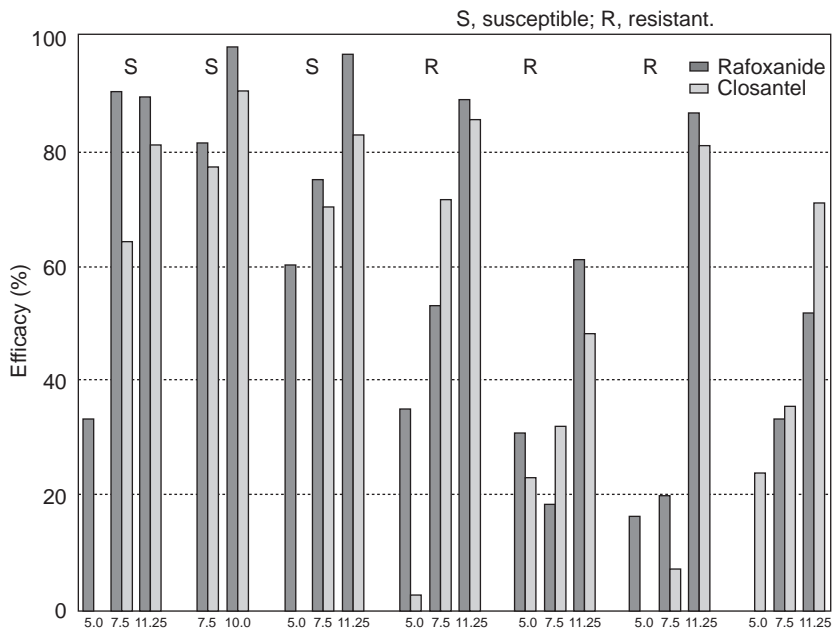


Fig. 7.2. Efficacy of rafoxanide and closantel against fluke aged 6 weeks.

Triclabendazole

An *F. hepatica* isolate was selected by drenching every 8 weeks with triclabendazole in the field for a period of 3 years and further selected by discriminative doses in the laboratory. Efficacy of the drug at comparable dose rates against the strain 4 weeks after infection was appreciably lower than against strains never exposed to triclabendazole (Fig. 7.3). Further selections at the recommended dose rate of 10 mg kg⁻¹ significantly reduced the efficacy of the drug against flukes aged 2 weeks from 98% to 60% (Fig. 7.4). Recent studies confirmed the occurrence of serious triclabendazole resistance of an undefined intensity in the field in Australia (Overend and Bowen, 1995). In one sheep farm in the same area, triclabendazole at the recommended dose rate of 10 mg kg⁻¹ was ineffective against the fluke isolate aged 2, 4, 6 and 12 weeks. Increased dose rates of 20, 45 and 67 mg kg⁻¹ were ineffective against flukes aged 4 to 6 weeks and treatment at the dose rate of 100 mg kg⁻¹ resulted in less than 80% efficacy against flukes aged 6 weeks (Boray *et al.*, 1997: Fig. 7.5). Anecdotal evidence suggests the occurrence of resistance to the drug in Counties Sligo and Cavan in Ireland (Anon., 1995).

Luxabendazole

Two isolates were selected in the laboratory by luxabendazole, a new broad-spectrum benzimidazole carbamate. Using discriminative dose rates, the

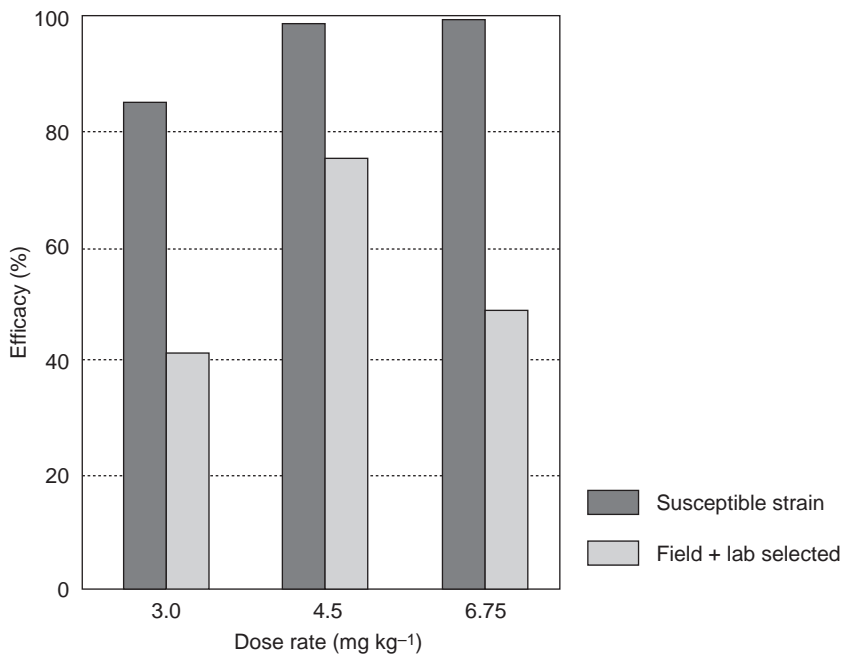


Fig. 7.3. Efficacy of triclabendazole against fluke aged 4 weeks.

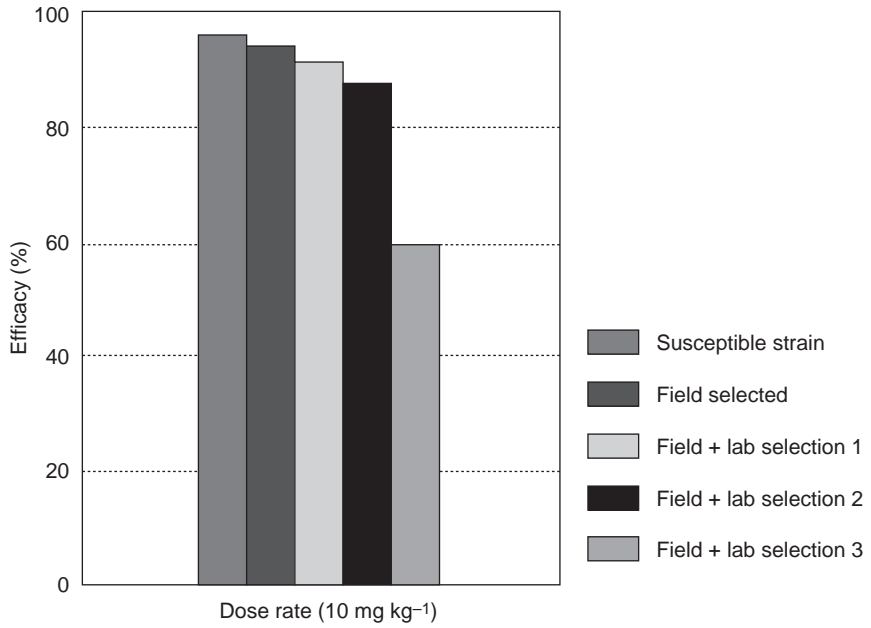


Fig. 7.4. Efficacy of triclabendazole against fluke aged 2 weeks.

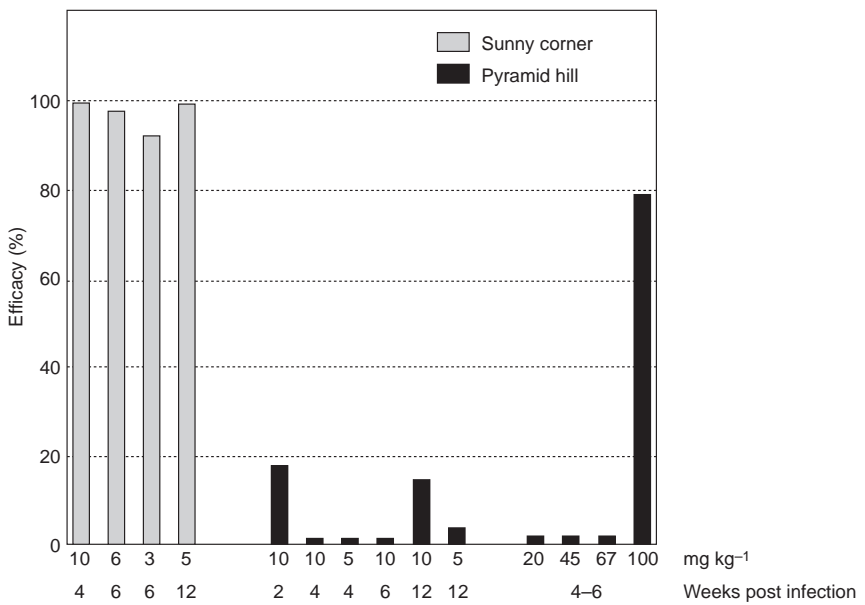


Fig. 7.5. Efficacy of triclabendazole drench against *Fasciola hepatica* in sheep.

efficacy of the drug against the selected strains in sheep 6 weeks after infection was appreciably reduced compared with the usually high efficacy achieved at similar dose rates against susceptible isolates (Boray, 1997).

Clorsulon

Attempts to induce resistance to clorsulon in the laboratory have failed: thus three laboratory selections with clorsulon did not result in reduced efficacy against the strain.

Management of Drug-resistant *Fasciola hepatica*

The resistance of *F. hepatica* to anthelmintics has been shown to be genetically controlled but the selection mechanisms involved are unknown. Through preferential selection for resistance in the immature stage of *F. hepatica*, drugs effective against early immature fluke could lose their advantage in chemoprophylaxis of fasciolosis. Demonstration of an unexpected 'total' resistance to triclabendazole against immature and adult flukes in the field suggests that more serious problems in controlling fasciolosis may occur in the future. Wide usage of a single highly effective anthelmintic is undesirable since resistance may develop against several chemically unrelated drugs.

A strategic programme with alternating drug use and specific farm management with minimum use of drugs should be implemented for the prevention of resistance, when resistance has been established or is suspected. However, this method may prove to be too complicated or not attainable. An alternative strategy is to use a combination of drugs.

Synergistic drug combinations

True synergism occurs when a combined efficacy of two or more chemotherapeutic agents is greater than the sum of their individual activities. The combinations are particularly useful when the efficacy of individual drugs has been reduced by the appearance of resistance, but they are still highly effective in synergistic combinations. In recent studies in Australia the successful use of synergistic combinations of drugs of different chemical groups has been reported for the prevention of resistance and for the treatment of fasciolosis due to resistant strains of *F. hepatica* (Boray, 1993, 1997).

The results of experimental studies have shown that combinations of drugs from different chemical groups, such as triclabendazole and clorsulon or triclabendazole and luxabendazole, achieve high efficacy against susceptible strains of *F. hepatica* aged 6 weeks at a fraction of their respective recommended dose rates by a synergistic effect. The combination of clorsulon and luxabendazole showed strong synergistic action. These or similar combinations would be useful for the prevention of resistance (Fig. 7.6). The combination of triclabendazole and clorsulon at one-fifth of their recommended dose rates or triclabendazole and luxabendazole at one-fifth and one-third of their respective

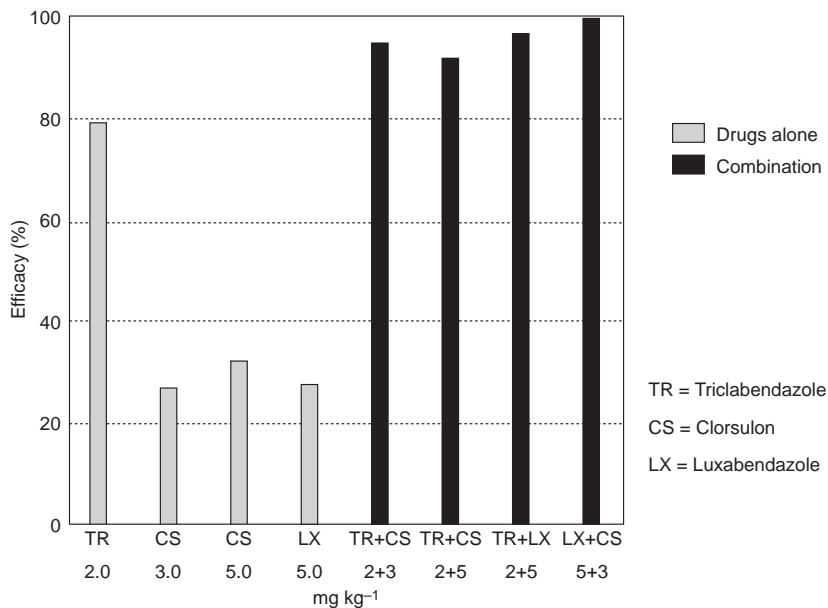


Fig. 7.6. Efficacy of drug combinations against susceptible *F. hepatica* aged 6 weeks.

recommended dose rates were highly effective against triclabendazole-resistant *F. hepatica* aged 6 weeks by a true synergistic action (Fig. 7.7). A strong synergistic effect was demonstrated when closantel at the dose rate of 7.5 mg kg⁻¹, which is lower than the effective dose rate against flukes aged 6 weeks, was combined with either triclabendazole, clorsulon or the benzimidazole carbamate, luxabendazole at reduced dose rates (Fig. 7.8). Nitroxylinil at lower than its recommended dose rate has been used successfully against strains of 6-week-old *F. hepatica* resistant to both closantel and luxabendazole when the drug was combined with low dose rates of closantel or clorsulon in injectable formulations (Fig. 7.9).

When closantel was combined with other benzimidazole carbamates, such as albendazole and fenbendazole, no synergism was observed. Oxfendazole showed a moderate synergistic action but luxabendazole achieved a strong synergistic effect against a salicylanilide-resistant strain aged 6 weeks (Fig. 7.10). Against another salicylanilide-resistant strain, the efficacy of closantel was enhanced by combining the drug with either clorsulon or luxabendazole when the drugs were used at a fraction of their respective recommended dose rates (Fig. 7.11). The combination of a slightly increased dose rate of closantel with a low dose rate of clorsulon showed strong synergistic effect and achieved high efficacy against a salicylanilide-resistant strain of *F. hepatica* aged 4 weeks (Fig. 7.12). With some adjustments of dose rates, combination products can be developed which are highly effective against *Fasciola* spp. aged 2 weeks and older.

In certain combinations, one or both of the active components have additional effect against parasitic infections other than fasciolosis. Some

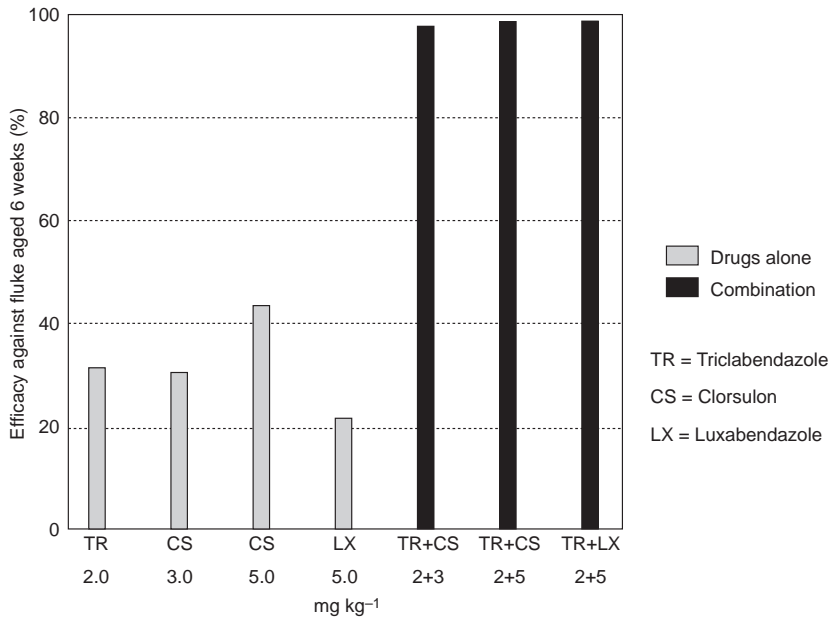


Fig. 7.7. Anthelmintic efficacy against triclabendazole-resistant *F. hepatica*.

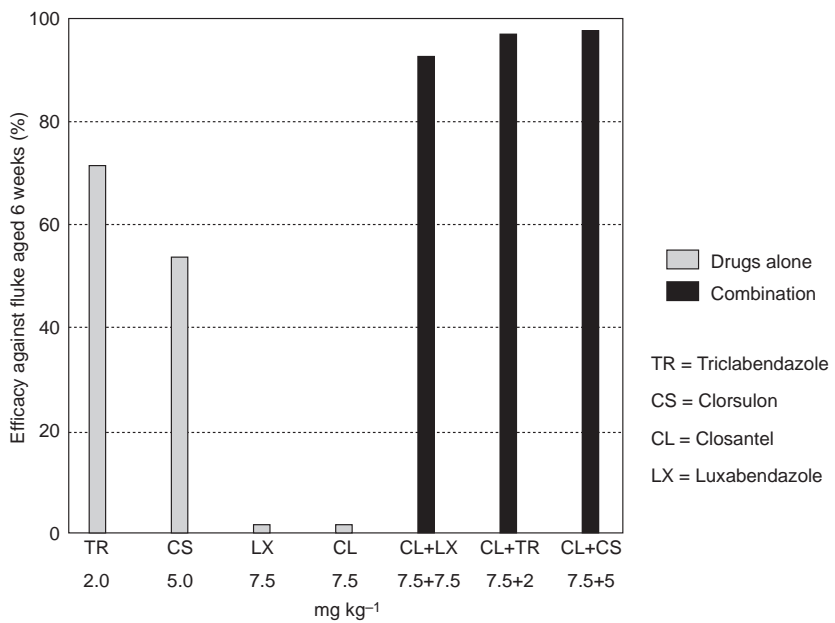


Fig. 7.8. Anthelmintic efficacy against closantel + luxabendazole-resistant *F. hepatica*.

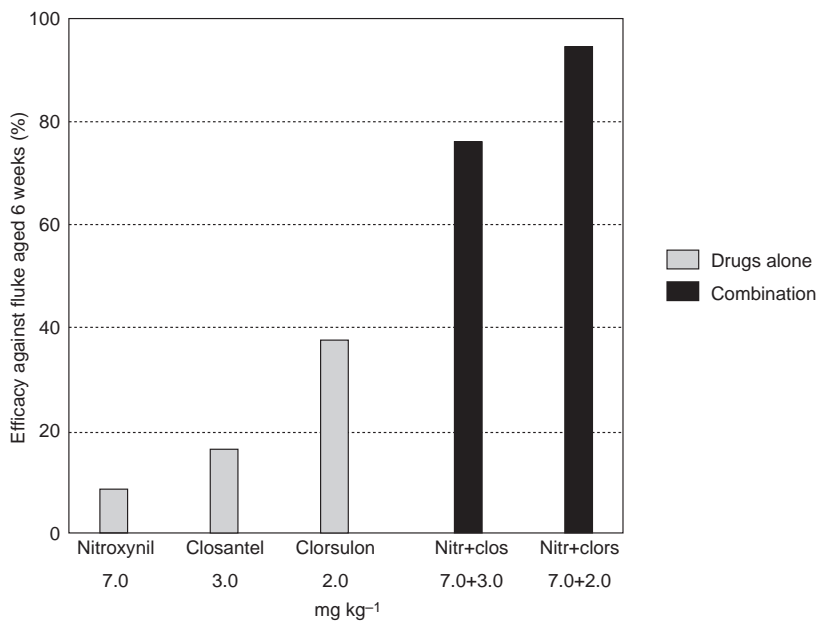


Fig. 7.9. Anthelmintic efficacy against salicylanilide-resistant *F. hepatica*; applied as a subcutaneous injection.

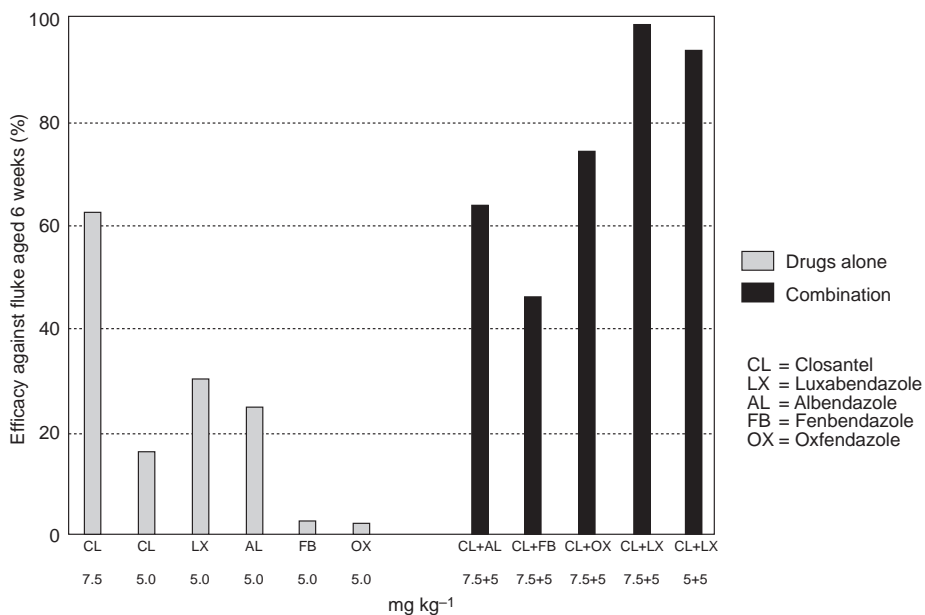


Fig. 7.10. Efficacy of closantel and benzimidazoles against rafoxanide-resistant *F. hepatica*.

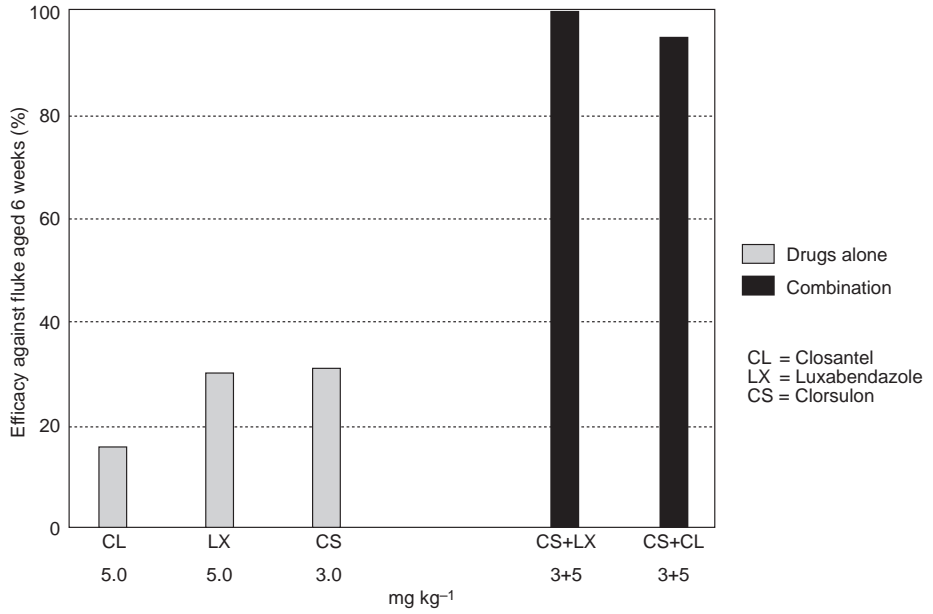


Fig. 7.11. Efficacy of clorsulon + anthelmintics against rafoxanide-resistant *F. hepatica*.

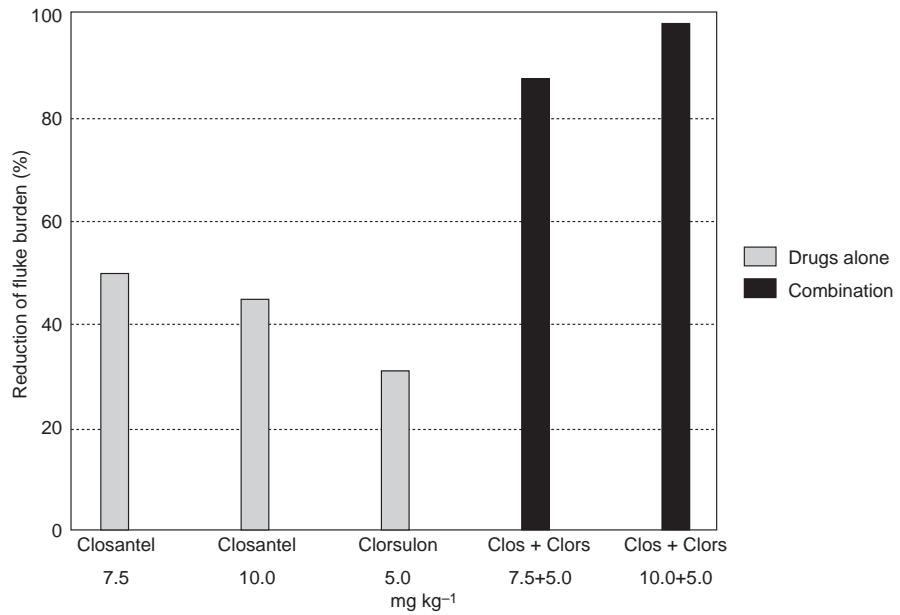


Fig. 7.12. Efficacy of clorsulon and closantel against *F. hepatica* aged 4 weeks; resistant to salicylanilides.

combinations would be suitable for the treatment of resistant and susceptible strains of trematodes (*Fasciola* spp., *Dicrocoelium* spp. and *Eurytrema pancreaticum*) as well as gastrointestinal nematodes, lungworms, tapeworms and *Oestrus ovis* in sheep (see references in Boray, 1990).

Salicylanilides act on both *F. hepatica* and *H. contortus* by uncoupling oxidative phosphorylation and related reactions of the mitochondrial membranes involved in electron transport. In the development of resistance a permeability barrier may operate (for references see Boray, 1990). The regular use of these drugs may play an important role in the development of salicylanilide resistance for both parasites. A level of resistance of *H. contortus* to rafoxanide and closantel has been reported with references to previous investigations by Rolfe *et al.* (1990). Since the mode of action of salicylanilides is similar in either *Fasciola* spp. or *Haemonchus contortus*, the closantel–luxabendazole combination would be effective against the salicylanilide-resistant strains of the two parasites, with an additional broad-spectrum activity against gastrointestinal nematodes. All combinations with closantel would give persistent efficacy against susceptible *H. contortus*.

By using synergistic combinations, three major aims may be achieved:

1. A combination product that is highly effective against both immature and adult *F. hepatica* at reduced dose rates, and which results in reduced tissue residues of the synergistic components and possibly reduced cost of production.
2. A product that would be effective against a variety of fluke strains resistant to one or both components, and which may prevent the development of resistance in susceptible fluke populations.
3. A product which would be suitable for the treatment of resistant and susceptible strains of trematodes (*F. hepatica*, *F. gigantica*, *Fascioloides magna*, *Fasciolopsis buski*, *Dicrocoelium* spp., *Eurytrema pancreaticum*) and some intestinal nematodes, lungworms and tapeworms in sheep and bovines and *Oestrus ovis* in sheep.

Conclusions

When all the disparate data from studies on fasciolicides are collected together, it is evident that they represent a fair body of work. More has been done than might have been imagined. Individual researchers have adopted a variety of approaches and examined different targets and processes within the fluke, namely, energy metabolism, neuromuscular activity, the tegument and tegument-based secretory processes, the osmoregulatory system and ionic regulation, reproduction and egg production and the cytoskeleton. On the basis of the evidence available is it possible to state unequivocally what is the precise action of any particular fasciolicide? The disappointing answer is no. We can say that closantel and possibly other related salicylanilides interfere with energy metabolism, that triclabendazole interacts with the microtubular cytoskeleton and that diamphenethide disrupts protein synthesis. However, they may not be the definitive actions of these drugs and other possibilities exist.

Why is this so? There are a number of reasons. Any fasciolicide will interact with a number of different systems within the fluke and exert a variety of effects. It is unrealistic and probably foolish to focus on any single target to the exclusion of others. An effect on one biochemical system is likely to have a knock-on effect on other systems and so it is very difficult to determine whether fasciolicidal action is due to a single effect or to a combination of effects. For example, a major disturbance of the neuromuscular system may not only cause detachment of the fluke, but also lead to the cessation of feeding. The fluke will enter a state of starvation and this will impose a severe metabolic stress upon it, draining its energy reserves. The paralysis induced by a number of fasciolicides is rapid at physiological concentrations and so may be more important than any long-term metabolic changes. In a similar way, disruption of gut cells and their production of digestive enzymes will impair the uptake of nutrients and trigger a state of starvation, with the consequences just described. A number of fasciolicides will enter the fluke in the ingested blood and thus have a direct impact on the gastrodermal cells. The tegument of *Fasciola* has a number of important roles, including nutrient uptake (sugars and amino acids), immunoprotection and osmoregulation. It represents the first line of defence against drug attack (excretion of the fasciolicide in the bile bringing it into contact with the tegument) and its integrity is essential for the continued viability of the fluke. Once this defence has been breached, the drug may be capable of penetrating to many of the innermost tissues of the fluke and, in the *in vivo* situation, external factors such as bile and an immune response become more significant in inducing severe damage. The surface damage caused by fasciolicides can be extreme and this will have serious consequences for the fluke. Moreover, maintenance of the integrity of the surface membrane is dependent on the turnover of secretory bodies produced by the underlying tegumental cell bodies, so any disruption of secretory processes in the cells would only exacerbate the situation. Finally, the microtubular component of the cytoskeleton is important for many synthetic and secretory processes, as well as for cell division; its disruption would have wide-ranging effects on the parasite.

So, an 'integrated systems' approach rather than an 'isolationist' view is required when trying to understand fasciolicidal action. Having said that, it is true to say that so far no such 'broad-spectrum' study for any single fasciolicide has been carried out under uniform conditions at a single concentration to obtain a complete profile of the morphological, biochemical and physiological effects of the drug both *in vivo* and *in vitro*. Such a study would enable the time course of drug-induced changes to be built up and so help to establish the mechanism of action of the fasciolicide concerned. The nearest attempt along these lines has been achieved for diamphenethide, as discussed above.

Another problem in elucidating fasciolicide actions concerns the difficulty of comparing different studies – not just comparisons between *in vivo* and *in vitro* studies, where the more protected environment *in vitro* may underplay the effect of the drug on the parasite. Studies *in vivo* may involve different hosts, different dosing regimes and different time courses, for example. *In*

vitro, drug concentrations, culture media, solvents and timings are some of the factors that have to be taken into account. Even the test system that is used is important because it may involve non-fluke material. Too many *in vitro* studies have been carried out at concentrations far in excess of maximum blood levels *in vivo* and so the results they generate may have little bearing on the actual mode of action of the fasciolicide concerned.

Having presented a slightly jaundiced view of studies aimed at unravelling the mechanisms of action of different fasciolicides, the reader may be excused for wondering what purpose they have served and whether the data they have generated have any value. After all, the basic motivation for such studies is that a better understanding of drug actions could be used for the development of more effective and perhaps safer drugs in the future. For *F. hepatica* there is a continuing need to develop compounds against the immature, intrahepatic stage that causes the pathology associated with fasciolosis during its migration through the liver parenchyma to the bile duct. Any search for new compounds also has to take into account that *F. hepatica*, *F. gigantica* and *Fascioloides magna* are unusual parasites, seemingly requiring drugs that are specific for themselves – diamphenethide, clorsulon and triclabendazole being cases in point. It is to be hoped that ‘mode of action’-type studies will help to identify potential ‘targets’ within the fluke – perhaps an enzyme or receptor that can be linked to a process – and that such information could be used to generate lead compounds for further evaluation and screening. From an academic point of view, drug studies have the added bonus of stimulating fundamental research into particular aspects of parasite biology, improving our overall knowledge of liver fluke physiology, which in turn may highlight further possibilities for therapeutic exploitation.

In conclusion, the future for fasciolicides as the main form of fluke control is assured, at least in the short term. Even when reliable and effective vaccines come on stream, which is unlikely to materialize in the near future (see Chapter 15 of this volume), there is likely to be a complementary role for drugs. Drug resistance is not a major problem at present, but that is not a reason for complacency; constant vigilance and monitoring are needed to avoid the problems that bedevil the control of nematode parasites. The authors hope that studies on drug mechanisms will continue, generating information for the more rational development of fasciolicides in the future.

Acknowledgement

A particular debt of thanks is due to Mrs Karen Moore who has managed to transform my disorganized manuscript into the final polished article – even to the point of correcting my mistakes!

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