

Drug transfer into target helminth parasites

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The pharmacokinetics of an anthelmintic drug includes the time course of drug absorption, distribution, metabolism and elimination from the host and determines the concentration of the active drug that reaches the location of the parasite. However, the action of the anthelmintic also depends on the ability of the active drug to reach its specific receptor within the target parasite. Thus, drug entry and accumulation in target helminths are important issues when considering how best to achieve optimal efficacy. Passive drug transfer through the external helminth surface is the predominant entry mechanism for most widely used anthelmintics and is discussed in this article. Despite the structural differences between the external surface of nematodes (the cuticle) and the external surface of cestodes and trematodes (the tegument), the mechanism of drug entrance into both types of helminth depends on the lipophilicity of the anthelmintic and this is the major physicochemical determinant for the drug to reach a therapeutic concentration in the target parasite. Understanding the processes that regulate drug transfer into helminth parasites is an important aspect in improving the control of parasites in human and veterinary medicine.

Pharmacological basis of anthelmintic action

Helminth infections are the most important cause of productivity loss worldwide in livestock [1] and a major cause of human morbidity [2]. Benzimidazoles (BZDs), imidazothiazoles (levamisole) and macrocyclic lactones (ivermectins and milbemycins) are the most important chemical families used to control helminth infections. The potency of most anthelmintics is dependent on their affinity for a specific receptor (i.e. BZDs bind to parasite β -tubulin and this disrupts the tubulin–microtubule dynamic equilibrium) and also on the kinetic properties that affect effective drug concentrations at the site of action [3]. The pharmacokinetic events that occur after drug administration and their relationship to the anthelmintic effect (pharmacodynamics) are illustrated in Box 1. Drugs can reach target helminths by oral ingestion, diffusion through the external surface of the parasite or a combination of both routes [4]. Oral ingestion is an obvious route of drug entry in roundworms and in mature stages of the trematode *Fasciola hepatica*. However, the transcuticular

route of drug passage seems to be more important than oral entry [3], which is a key issue in understanding drug action. If drug entry into target parasites occurs mainly through the external surface, then knowledge of the mechanism (passive diffusion or specialized transport) is pivotal to understand the drug–parasite relationship. The transport mechanisms and the factors affecting drug penetration into helminth parasites are addressed in this article.

The process of drug transfer into helminths can be characterized using either *in vitro*, *ex vivo* or *in vivo* approaches. The transport of different substances has been investigated *in vitro* using isolated nematode cuticle because it offers some advantages over the use of intact organisms, particularly for interpretation of permeability data [3]. *Ex vivo* assays use live intact parasites in a closed perfusion system, which enables study of the relative contribution of the transcuticular and oral pathways in addition to the influence that underlying tissues, such as the somatic muscles, have on cuticular drug transport [5]. *Ex vivo* characterization of drug transfer offers technical advantages and reliable results [5–8]. Most current information has been obtained using *ex vivo* approaches, which might be indicative of the *in vivo* situation. It is possible that the drug–parasite interaction could differ *in vivo* because worms are exposed to changing drug concentration over time in a variable physiological environment. However, the assessment of the entry of BZDs into parasites using both *ex vivo* and *in vivo* approaches has provided complementary and useful data and this has enabled characterization of the pattern of drug transfer into target parasites [9–11].

Drug entry into nematode parasites

The external surfaces of helminths (Table 1) serve as a barrier that shields the organism from external conditions. These surfaces are also vital for nutrient uptake, osmoregulation, immunoprotection and structural support. The tegument in flatworms is a simply structured membrane-bound syncytia [4]. By contrast, the cuticle of the nematode is considered to be a barrier that limits the entry of large molecules into the parasite [12,13]. Two main issues are crucial to the comprehension of the process of drug accumulation in nematodes, the oral versus transcuticular entrance routes and identification of the main drug-transport mechanism involved in the transfer process (i.e. active transport versus passive diffusion). Although the oral route is important, there is evidence that transcuticular

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Box 1. Pharmacokinetic barriers in anthelmintic action

The activity of an anthelmintic drug depends not only on its binding to the specific receptor (pharmacodynamics) but also on its ability to reach high and sustained concentrations at the location of the parasite to enable the delivery of effective drug concentrations at the receptor in the parasite cells and in sufficient time to induce the anthelmintic effect [3]. There is a close relationship between drug pharmacokinetic behaviour in the host and the observed final anthelmintic efficacy. The drug needs to bypass different 'barriers' to reach its target receptor in a parasite (Figure 1). Dissolution of the drug in gastrointestinal (GI) fluids is an important factor for drugs administered as suspensions by the oral route (e.g. benzimidazole compounds, morantel and pyrantel). Dissolution is a crucial step because the drug must dissolve in the enteric fluids to enable absorption through the GI mucosa. The undissolved drug then passes down the GI tract and is excreted in faeces without exerting its action. Anthelmintic compounds formulated as drug solutions for parenteral injection (i.e. intramuscular, subcutaneous) in domestic animals (e.g. macrocyclic lactones and levamisole) do not require dissolution before

systemic absorption. Drug absorption is the main limiting factor that determines the amount of drug that reaches the systemic circulation (systemic availability). The reversible exchange between the bloodstream and tissues enables the drug to achieve concentrations that are anthelmintically active in the parasite tissues. The overall pharmacokinetic process, including drug absorption, tissue distribution and its biotransformation and elimination, is crucial to enable the drug to reach the target parasites that are located in different tissues at sufficient concentrations to exert its anthelmintic effect. Finally, the access of anthelmintic molecules to intracellular sites of action depends upon their ability to penetrate the parasite external cuticular (nematodes) or tegumental (cestodes and trematodes) structure (Box 2). Lipophilicity and concentration of the active drug, the physicochemical features of the medium that surrounds the parasite and the structure of the external surface of the parasite are among the factors that affect the transfer (diffusion) and accumulation of the active drug in the target parasite and that determine the anthelmintic effect (pharmacodynamic behaviour) of the drug.

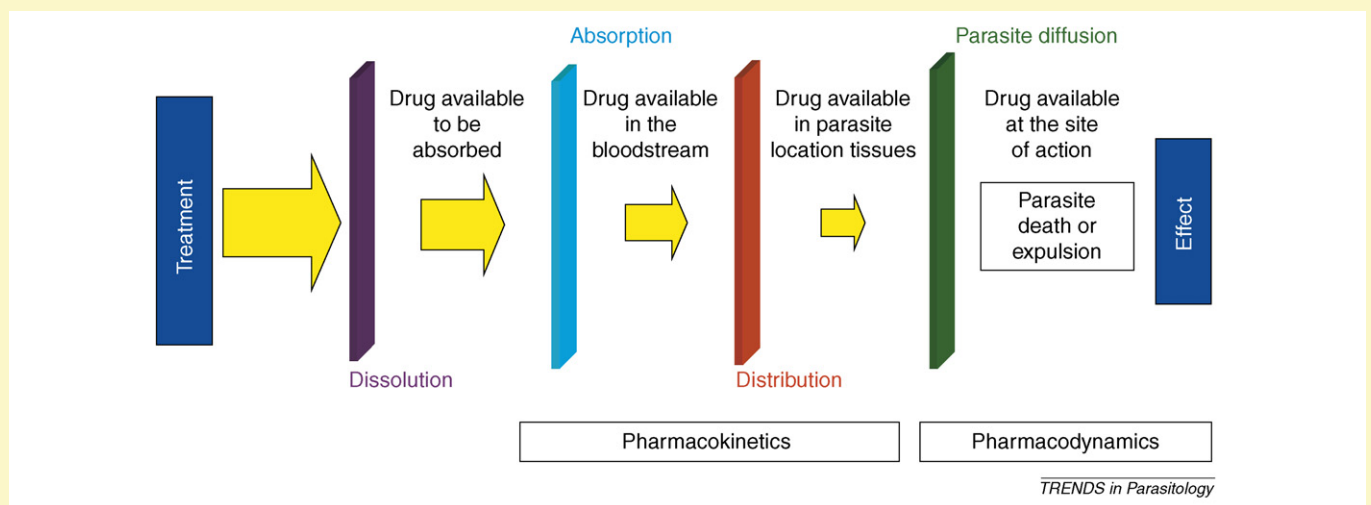


Figure 1. Diagram of barriers that an anthelmintic drug has to pass through in order to reach its specific site of action.

diffusion is the predominant route of access for non-nutrient and nonelectrolyte substances in nematodes [14]. Drug absorption across the nematode cuticle is restricted by lipid barriers in the hypodermis and collagen matrix [3]. The rate of transfer across the cuticle depends mainly on lipophilicity and, in the case of acidic or basic drugs, on the ionized and nonionized (lipid-permeable) fractions of the drug, which are determined by the relationship between drug pK and pH of the aqueous environment within the cuticle [3]. Lipid components in the hypodermis and cuticle complex form the diffusion-rate-limiting barrier for lipophilic molecules of molecular weight <2000 [3] and the hypodermic tissue might be the rate-limiting barrier to peptide transport across the cuticle [15].

Albendazole (ABZ) is a broad-spectrum BZD anthelmintic [16]. Higher concentrations of ABZ, compared with its sulfoxide (ABZSO) metabolite, were measured in *Haemonchus contortus* that were recovered from infected treated sheep [10]. Because ABZ is not found in peripheral plasma, only ABZ from the pool found in the fluid and mucosal tissue of the abomasum (the 'true' stomach of ruminants) is available to reach the target nematode through its external cuticle. It is also probable that *H. contortus* feeds on portal blood. However, the low ABZ

concentrations recovered in portal blood in catheterized ABZ-treated sheep would not explain the large amount of ABZ recovered from this abomasal parasite. These findings could confirm the relevance of the transcuticular diffusion process, even in a blood-sucking parasite such as *H. contortus* [10], in which the higher lipophilicity of ABZ (octanol–water partition coefficient: 3.83) [17] might account for its greater penetration compared with its sulfoxide derivative (partition coefficient: 1.24) (Figure 1b, Box 2).

Similarly, transcuticular transfer seems to be the main route of passage of ivermectin (IVM) into the filarial nematode *Onchocerca ochengi*. The entry of IVM into adult *O. ochengi* occurs by the transcuticular route [7] and because the marked foldings of its cuticle greatly increase the surface area, IVM diffusion is favoured. IVM inhibits the contraction of the pharyngeal muscles, which enable feeding in nematodes, and causes paralysis of the muscles of the nematode [18]. In the absence of pharyngeal pumping, first-stage larvae of *Caenorhabditis elegans* submerged in IVM became paralyzed [19], which reinforces the relevance of IVM transcuticular entry. IVM inhibits pharyngeal pumping more potently than motility in *H. contortus* [18] and it is probable that IVM prevents its own oral entry into worms. However, similar feeding levels

Table 1. Main structural features of the tegument (cestodes and trematodes) and cuticle (nematodes)^{a,b,c}

Cestode tegument	Trematode tegument	Nematode cuticle
Anatomical simplicity. The tegument covers the entire body surface		Rigid but flexible complex structure. Composed of up to six layers (adult stages) that extend into and line the pharynx, rectum, cloaca and other orifices. Aqueous pores, which are negatively charged, traverse the cuticle
Glycocalix (outermost layer): acellular, translucent mucopolysaccharide glycoprotein coat	Glycocalix: a thin coat of glycoproteins with projecting side chains of gangliosides and oligosaccharides. This extends outward from the apical membrane of the tegument	Surface coat (larval stages)
Standard lipid bilayer	Standard lipid bilayer (<i>Fasciola hepatica</i>) or heptalaminated membrane (<i>Schistosoma</i> spp.). Pits and/or invaginations increase the parasite surface area	Epicuticle: trilaminated (three-layered membrane) without a functional lipid barrier. Composed primarily of carbohydrates, some lipids and elastin-like proteins
Tegumental syncytium. The surface is evaginated into microtriches (microvilli-like structures), which increases the functional surface	Tegumental syncytium. Presence of spines (predominantly on the dorsal surface) help with parasite attachment	Cortical layer. Inner and outer cortex – amorphous, electron-dense layer. It contains abundant keratin-like proteins and cuticulin
	N/A	Medial layer: aqueous compartment that contains fine collagenous fibers (in two to three different sublayers), which constitute >80% of the soluble cuticular proteins
	N/A	Fiber layer
	Fibrillar basal lamella	Composite basal layer: contains crosslinked collagen fibers
Subtegumental cells that contain nuclei synthesize the cytoplasm and organelles within the syncytium	Subtegumental cells that contain nuclei, mitochondria and ribosomes are connected to the syncytium through cytoplasmic channels that contain numerous microtubules	Hypodermis: anatomical syncytium with typical cytoplasmic constituents specialized for transport and secretion, which synthesizes the molecules that form the cuticle
	Somatic muscle cells	Somatic muscle cells
The tegument contains different proteins and glycoproteins (actin, tubulin, collagen and keratin), glycolipids and phospholipids	The tegument contains different proteins and glycoproteins (tubulin, collagen, keratin, paramyosin, actin), phospholipids and cerebroside glycolipids	The nematode cuticle contains at least one glycoprotein, small amounts of hyaluronic acid and lipids, chondroitin sulfate and sulfated mucopolysaccharides

^aAbbreviation: N/A, not applicable.

^bExternal parasite surfaces are structurally adapted for immune evasion, nutrient absorption, ion transport and communication with the underlying neuromuscular system [4].

^cTable adapted from Ref. [4].

(measured by ³H-inulin uptake) were observed in *H. contortus* collected from both untreated and IVM-treated sheep [20]. Roundworms have sensory neurons (amphidial neurons) in their cephalic end. They are located in a pair of channels (the amphids) on either side of the pharynx [21]. The amphid dye filling defective (*Dyf*) gene, *osm-1*, and other *Dyf* genes, might work additively to regulate IVM uptake in *C. elegans* [22]. Furthermore, *Dyf* mutations confer low-level resistance to IVM. Interestingly, the structure of the amphids is altered in IVM-resistant *H. contortus* [21], which could implicate the involvement of the amphids on IVM entrance. However, this hypothesis requires confirmation.

Lipophilicity and concentration gradient are major determinants of the ability of a drug to reach therapeutic concentrations within a target parasite. Fenbendazole (FBZ) accumulates inside *Ascaris suum* down a concentration gradient (Figures 1c and 1d, Box 2) [8]. In addition, total ABZ availability in *A. suum* over 180 minutes of incubation was significantly higher compared with the more polar ABZSO metabolite (Figure 1b, Box 2) [11]. Differences in lipophilicity help to explain the greater availability of ABZ, which agrees with the assumption that passive diffusion across the lipid components of the

cuticle is the rate-limiting step in the process of drug entry [23]. Furthermore, the involvement of an active transport mechanism in the entry of IVM into adult *O. ochengi* has been ruled out [7]. Similarly, work on ³H-levamisole uptake by *A. suum* demonstrated that levamisole accumulation also occurs through a transcuticular mechanism [6], reaching its nicotinic site of action at the parasite neuromuscular system (as has been shown in electrophysiology studies) [24].

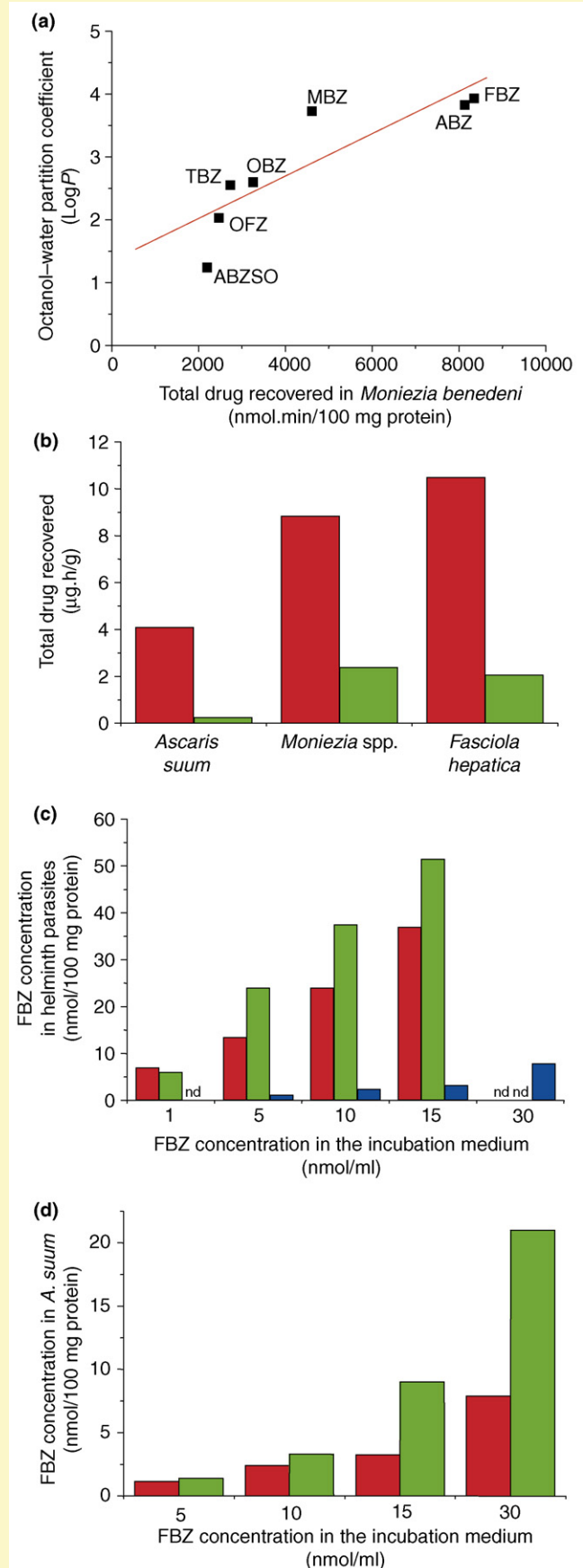
The complex structure of the nematode cuticle compared with the flatworm tegument (Table 1) could explain the observed differences in BZD accumulation in *A. suum*, *F. hepatica* and *Moniezia benedeni* (Figures 1b and 1c, Box 2). The diffusion of ABZ and ABZSO into *A. suum* was markedly lower than that observed in the trematode and cestode parasites [11]. The cuticle of *A. suum* can be breached by drugs and the limiting barrier for passive transport is the lipoidal hypodermis, in which the rate-determining factors are the intrinsic lipid–water partition coefficient, the pH and p*K*_a relationship and molecular size [12]. Drug transcuticular transport is mostly controlled by the pH at this surface because, in the absence of facilitated transport, only nonionized molecules can partition across a lipoidal surface. Parasites excrete several volatile fatty acids and

Box 2. Passive drug entry into different helminth parasites

Different drug-diffusion studies performed with helminth parasites demonstrate that passive diffusion is the main transport mechanism in the entry of benzimidazole (BZD) anthelmintics through the external surface of nematodes (cuticle) and cestodes and trematodes (tegument). Mottier *et al.* [17] showed that the higher the lipophilicity, the greater the ability of the BZD compounds to cross the tegument of tapeworms. A correlation between the octanol–water partition coefficients for different BZD anthelmintics and their ability to diffuse through the tegument of *Moniezia benedeni* has been shown [17]. Higher concentrations of the most lipophilic BZD compounds [fenbendazole (FBZ), albendazole (ABZ), mebendazole] (partition coefficients >3.7) compared with those drugs with the lowest lipid–water partition values [oxfendazole, ABZ-sulfoxide (ABZSO), thiabendazole] were recovered from the incubated tapeworms (Figure 1a). In agreement, the amounts of ABZ recovered from roundworms and flatworms after *ex vivo* incubations were higher than those measured for the more-polar ABZSO metabolite (Figure 1b) [11].

The diffusion and accumulation of different BZD molecules in nematodes were markedly reduced compared with those observed in cestodes and trematodes. A lower diffusion of ABZ and ABZSO (Figure 1b) and FBZ (Figure 1c) into *Ascaris suum* compared with *M. benedeni* and *Fasciola hepatica* has been documented [8,11]. Nematode parasites maintain a strongly buffered environment in the aqueous spaces of the cuticle structure. This local environment has a pH value of ~ 5.0 and results from the accumulation of organic acid byproducts of carbohydrate metabolism [25]. BZD molecules are weak bases that are typically found in their ionized form ($\sim 99\%$) because of the acidic environment of the nematode surface, which might limit their diffusion across the cuticle. This ionization-mediated impairment of drug diffusion and the complex structure of the nematode cuticle compared with the cestode or trematode tegument might explain the drug penetration differences observed between nematode and flat helminth parasites [8,11]. The lack of an acidic barrier in dead worms enabled the increased entry of FBZ molecules into *A. suum* (Figure 1d). FBZ concentrations recovered from tissues of dead cestodes and nematodes over time were significantly higher compared with those measured in living parasites [8]. In addition, FBZ accumulated within the three parasites down a concentration gradient (Figure 1c). Transcuticular and tegumental absorption of BZD molecules was linear over the range of concentrations assayed. High correlation coefficients (>0.99) were obtained between initial drug concentrations in the incubation medium and those measured inside the cestode, trematode and nematode parasites (Figure 1c), which demonstrates that concentration is an important factor that determines drug penetration. The experimental results summarized in Box 2 demonstrate that passive drug transfer through the external helminth surface is a major transport mechanism that accounts for accumulation of lipophilic anthelmintic molecules into target parasites.

Figure 1. (a) Correlation (r) between the total drug recovered in *Moniezia benedeni* (expressed as area under the concentration versus time curve) after 210 minutes of incubation and the octanol–water partition coefficient ($\text{Log } P$) of different benzimidazole (BZD) anthelmintic compounds. Adapted, with permission, from Ref. [17]. (b) Comparative pattern of *ex vivo* diffusion of ABZ (red) and ABZSO (green) into *Ascaris suum*, *Moniezia* spp. or *Fasciola hepatica* after 180 minutes of incubation. Values represent drug availability in the parasite tissue and are expressed as area under the concentration versus time curve. The total availability for both molecules in *A. suum* was significantly lower ($P < 0.05$) than those measured in the trematode and cestodes parasite. ABZ diffusion into the three helminth parasites was significantly higher ($P < 0.05$) than that observed for its ABZSO metabolite. Adapted, with permission, from Ref. [11]. (c) Comparative *ex vivo* diffusion of fenbendazole (FBZ) into *Moniezia benedeni* (red), *Fasciola hepatica* (green) and *Ascaris suum* (blue). Results represent mean FBZ concentrations (nmol/100 mg protein) ($n = 4$) in a drug-gradient concentration. Adapted, with permission, from Ref. [8]. (d) Comparative *ex vivo* diffusion of fenbendazole (FBZ) into living (red) and dead (green) specimens of *A. suum*. Results represent mean FBZ concentrations measured in *A. suum* after 90 minutes of incubation in a drug-gradient concentration. Concentration values obtained for FBZ in living parasites were significantly lower ($P < 0.05$) than those measured in the dead parasites. Adapted, with permission, from Ref. [8]. Abbreviations: ABZ, albendazole; ABZSO, ABZ-sulfoxide; FBZ, fenbendazole; MBZ, mebendazole; nd, not determined; OBZ, oxiabendazole; OFZ, oxfendazole; TBZ, thiabendazole.



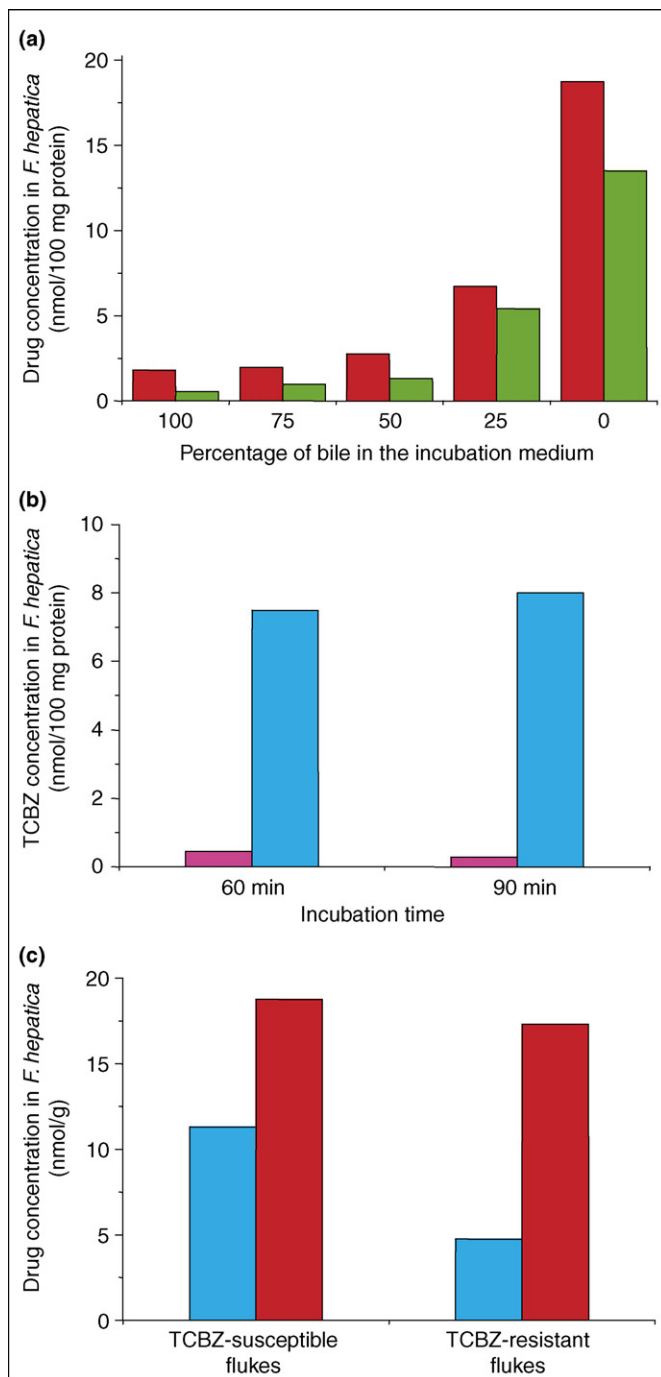


Figure 1. Assessment of drug transfer into *Fasciola hepatica*. **(a)** Comparative concentrations of albendazole (ABZ; red) and fenbendazole (FBZ; green) recovered from adult *F. hepatica* incubated with different proportions of ovine bile after 60 minutes of incubation. The amounts of both compounds measured in *F. hepatica* incubated in the absence of bile were significantly greater ($P < 0.05$) than those obtained with media containing different percentages of bile (100, 75 and 50%). Adapted, with permission, from Ref. [35]. **(b)** Diffusion of triclabendazole (TCBZ) into *F. hepatica*. Results show TCBZ concentrations measured in adult flukes after 60 and 90 minutes of incubation with bile (pink) and without bile (blue) in the incubation medium. TCBZ concentration values were significantly higher in the absence of bile ($P < 0.05$). Adapted, with permission, from Ref. [35]. The results summarized here demonstrate that the composition and physicochemical characteristics of the incubation medium drastically affect the diffusion of benzimidazole (BZD) anthelmintics into *F. hepatica*. The 'environment' at the location of the parasite and the physicochemical properties of the drug should be considered together to understand the access of a drug molecule to the site of action within a target helminth. **(c)** Comparative accumulation of TCBZ (blue) and ABZ (red) into adult specimens of TCBZ-susceptible and TCBZ-resistant *F. hepatica*. TCBZ concentrations were significantly lower than those of ABZ measured in TCBZ-susceptible and -resistant flukes ($P < 0.05$). Adapted, with permission, from Ref. [42]. The greater ABZ accumulation in both susceptible and

nonvolatile organic acids (end products of carbohydrate metabolism) through the transcuticular route at sufficient rates to establish and maintain a buffered microenvironment (pH \sim 5.0) in the aqueous space of the pores of the cuticle [25]. Most drugs are weak bases or weak acids and exist in solution, depending on the pH of the medium, as different proportions of both the nonionized and ionized forms. Although the poor lipophilicity of ionized molecules excludes them from passive diffusion, lipophilic, nonionized moieties passively diffuse across cell membranes until an equilibrium is established. ABZ and ABZSO are weak bases ($pK_a = 7-8$) [26] that mostly exist in their ionized forms in the acidic environment of the nematode cuticle, thereby limiting their entrance. The pH of the medium in which the nematode is incubated does not influence the rate of absorption of weak acids or bases across the nematode cuticle [27]. Thus, the acidic microenvironment within the water-filled pores of the cuticle and the lipoidal hypodermal membrane are probably the main barriers for transcuticular drug absorption. Furthermore, flatworms also excrete organic acids by the transtegumental route, thus forming an acidic microenvironment in the immediate vicinity of the tegument [28]. FBZ (a weak base) [26] mostly exists as its ionized form in the acidic environment of the helminth surface, which helps to explain its limited diffusion across the cuticle or tegument in intact live parasites. The lack of this acidic barrier might explain the increased entry of FBZ into dead *A. suum* (Figure Id, Box 2) and *M. benedeni* [8] compared with that observed in living specimens. This *ex vivo* experimental work using either live or dead helminths has been useful in demonstrating how the cuticular and tegumental transfer of weak basic molecules (BZD compounds) could be affected by the excretion of organic acid metabolites.

Closantel (CLS) is a salicylanilide drug that is active against *F. hepatica*, *H. contortus*, certain arthropod parasites (e.g. mites and ticks) and *Oestrus ovis*. CLS is active against IVM-, BZD-, levamisole-, morantel- and radoxanide-resistant strains of *H. contortus* [29]. It is a highly lipophilic compound that is extensively ($>99\%$) bound to plasma proteins and has long half-life (14.5 days) [30]. The accumulation of ^{14}C -CLS in adult *H. contortus* was measured in both the absence and presence of IVM (used to prevent CLS oral uptake) [29]. CLS lipophilicity might explain its transcuticular entry into CLS-susceptible and -resistant *H. contortus*, even in the presence of ovine serum albumin and when oral ingestion was abolished by IVM [29]. Nevertheless, its extensive binding to albumin could facilitate oral ingestion of CLS in hematophagous parasites such as *H. contortus* and *F. hepatica*. However, the work summarized in this opinion article indicates that passive drug transfer through the external helminth

resistant flukes might be linked to the higher lipophilicity of ABZ compared with TCBZ [17]. The amount of TCBZ recovered from resistant flukes was significantly lower (by $\sim 50\%$) ($P < 0.05$) than that measured in susceptible parasites. The same pattern has been observed for TCBZ-sulfoxide (data not shown) [41,42]. Increased TCBZ oxidative metabolism by the fluke [41] and enhanced drug efflux mediated by ATP-dependent transmembrane transporters, such as P-glycoprotein [42], might account for the reduced drug accumulation observed in resistant flukes and have been proposed as potential mechanisms of TCBZ resistance in *F. hepatica* (Box 3). By contrast, ABZ accumulation was similar in both susceptible and resistant flukes.

surface is, in most cases, the main entry mechanism for the most widely used anthelmintics.

Drug entry into cestode and trematode parasites

The absence of a digestive system in cestodes simplifies the interpretation of the functional properties of the external surface. The tegument of cestodes is structurally adapted to interact with the surrounding environment and to perform all functions normally associated with intestinal tissue [4]. Movement of solutes across the tegumental surface in trematodes and cestodes is essentially a matter of transepithelial transport.

Although ABZ is not detected in plasma, its metabolites ABZSO and ABZ-sulfone are found in the bloodstream of treated sheep [31] and cattle [32]. However, ABZ and ABZSO (both active anthelmintic molecules) have been

recovered from abomasal and intestinal contents [9]. These molecules can only reach intestinal tapeworms from the gut content in which they are immersed. Equivalent ABZ and ABZSO concentrations were measured in intestinal fluid after ABZ administration [9]. However, the amount of ABZ recovered in *Moniezia* spp. collected from treated sheep was greater than that of ABZSO. Because ABZ is more lipophilic than ABZSO, this finding was considered as indirect evidence that passive diffusion could be the mechanism by which BZDs enter tapeworms. Studies performed to clarify this issue have confirmed that lipophilicity aids drug diffusion through the external surface of cestode and trematode parasites. There is a close correlation between molecular lipophilicity (expressed as the octanol–water partition coefficient) and the amount of drug recovered within *M. benedeni* (expressed as area under the

Box 3. Pharmacological mechanisms involved in triclabendazole accumulation in *Fasciola hepatica*

Drug accumulation influences the amount of drug available to interact with a specific receptor within a target parasite. The accumulated experimental evidence to explain the availability of the flukicidal compound triclabendazole (TCBZ) and its metabolites within *F. hepatica* and the proposed mechanisms of parasite resistance to

TCBZ are schematically summarized in Figure 1. Overall, the pharmacological mechanisms described here are important to understand TCBZ activity in *F. hepatica*. The altered drug influx and efflux and enhanced metabolic capacity identified in TCBZ-resistant liver flukes might contribute to the development of resistance to TCBZ.

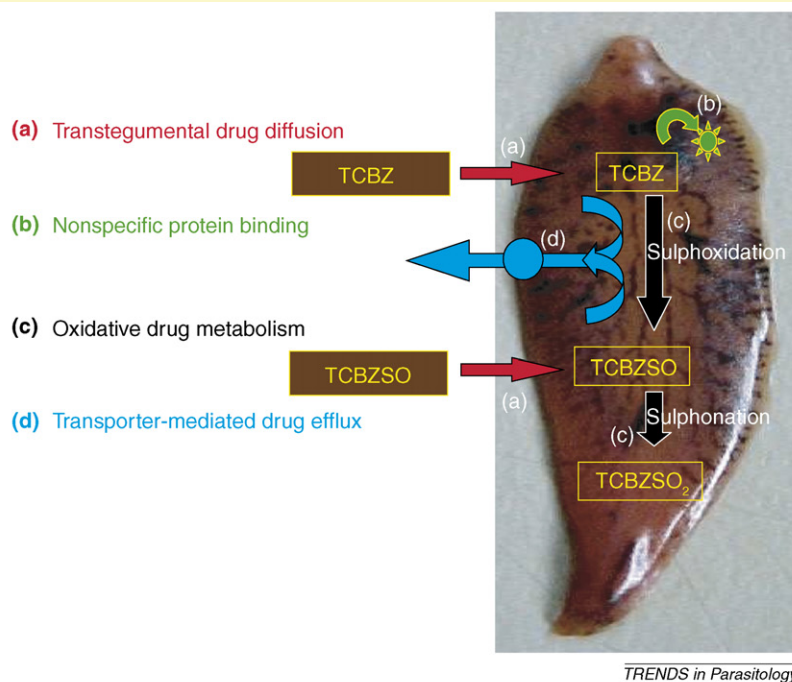


Figure 1. Triclabendazole (TCBZ) accumulation in its main target trematode (*Fasciola hepatica*) parasite is based on different molecular events. (a) TCBZ diffusion. The entry of TCBZ and its active TCBZ-sulfoxide (TCBZSO) metabolite into the fluke have been shown to occur mainly by diffusion across the tegumental syncytium rather than by oral ingestion [8]. The diffusion of both TCBZ and TCBZSO into TCBZ-resistant flukes is significantly lower than in TCBZ-susceptible flukes [41,42]. Interestingly, as seen in Figure 1c in the main text, this is not applicable to albendazole (ABZ), a related benzimidazole (BZD) flukicidal compound whose uptake is similar in both strains [42]. This indicates that drug entrance might not be altered in TCBZ-resistant *F. hepatica*. (b) Nonspecific protein binding. For drug molecules with high protein-binding affinity, such as TCBZ, nonspecific binding within parasite tissues could restrict drug availability at the specific intracellular site of action (still unknown for TCBZ). However, similar total protein contents were measured in TCBZ-susceptible and -resistant isolates of *F. hepatica* (90–94 mg of protein per gram of parasite) [41]. (c) TCBZ oxidative biotransformation. Earlier work showed that adult liver flukes have the ability to oxidize ABZ into ABZ-sulfoxide (ABZSO) and ABZ-sulfone (ABZSO₂) [43]. This finding demonstrates that *F. hepatica* has the enzymatic capacity to biotransform drugs. Furthermore, the sulfone metabolite (TCBZSO₂) has been identified in *F. hepatica* after incubation with TCBZSO [44]. Moreover, the microsomes (a subcellular fraction rich in enzymes) obtained from adult flukes generated TCBZSO (the main product) and TCBZSO₂ after incubation with the TCBZ parent drug [33]. The rate of TCBZ sulfoxidative metabolism into TCBZSO was significantly higher in TCBZ-resistant flukes compared with susceptible flukes [41]. (d) Transporter-mediated TCBZ efflux. Membrane drug transporters participate in the efflux of drugs in many vertebrate and invertebrate organisms as part of a general mechanism of cell protection. In vertebrates, P-glycoprotein (Pgp), among others, is a major transmembrane transporter for different drug substances. Pgp is a member of the ATP-binding cassette (ABC) transporters that function as an ATP-dependent efflux mechanism and that enable substrates to be expelled from cells [45]. Over-expression of Pgp has been implicated in the resistance to macrocyclic lactones (ivermectin, moxidectin) [46,47], closantel and BZD in nematodes, although the exact nature of the role has yet to be established [48,49]. In addition, an ABC transporter has been identified in *F. hepatica* [50]. Overexpression of these transporters in the TCBZ-resistant flukes has been postulated as a possible mechanism, among others, of TCBZ resistance in *F. hepatica* [42].

concentration versus time curve) for BZD compounds (Figure 1a, Box 2) [17]. A similar behavior has also been shown for the halogenated BZD triclabendazole (TCBZ) and its metabolites in *F. hepatica* [33]. The same pattern has been observed for ABZ and ABZSO (Figure 1b, Box 2) [11,34]. The results from those studies demonstrated that the higher the lipophilicity (higher partition coefficient value), the greater the ability of the BZD molecules to cross the helminth external surface.

Furthermore, TCBZ-sulfoxide (TCBZSO), FBZ and oxfendazole accumulated within cestode (*M. benedeni*) and trematode (*F. hepatica*) parasites down a concentration gradient [8]. This transtegumental absorption was linear over the assayed concentrations range (1–20 nmol ml⁻¹) (Figure 1c, Box 2). In addition, the physicochemical composition of the surrounding environment of the parasite (in which it is immersed) has a pivotal role in the process of drug transfer into *F. hepatica* (Figure 1) [35]. Different studies were performed in *F. hepatica* to determine the relative contribution of transtegumental versus digestive absorption of TCBZ [8]. Equivalent TCBZSO concentrations were recovered from mouth-ligated (i.e. unable to ingest anything orally) (21.3 ± 1.9 nmol/100 mg protein) and non-ligated (25.0 ± 3.5 nmol/100 mg protein) adult *F. hepatica* after 45 minutes of *ex vivo* incubation. These results [8] confirmed TCBZ entry into the flukes even when the oral route had been closed off by ligation. By contrast, mouth-ligated flukes had minimal damage caused by the anthelmintic clorsulon on tegumental and gastrodermal surfaces [36,37], which might indicate that the oral ingestion of this flukicidal drug, strongly bound to red blood cells, should be considered as an entrance route in the hematophagous adult liver fluke, as suggested for CLS in *H. contortus* [29]. However, concentration gradient, drug lipophilicity and physicochemical features of the incubation medium are crucial for the penetration of BZDs through helminth external surfaces, which indicates that passive diffusion could also be the main mechanism involved in their transfer into flatworms.

Usually, the higher the concentration achieved at the tissue where the parasite is located, the higher the amount of drug reaching the target parasite. This is strongly supported by the findings from different *in vivo* studies [38–40] in which systemic drug availability and efficacy were simultaneously estimated. The accumulation of active drug at the site of action depends on various factors, including the mechanism of drug entry, the capacity of a parasite to inactivate the drug and drug efflux mediated by transporter proteins. The mechanisms involved in TCBZ accumulation in *F. hepatica*, and some postulated changes occurring in resistant flukes, are shown in Box 3.

Concluding remarks

Understanding the mechanisms of drug diffusion and accumulation in target parasites is a key issue in predicting anthelmintic activity. Determining the capability of different helminth parasites to biotransform (i.e. convert) anthelmintic drugs is another crucial step in identifying their pharmacological activity. The body of scientific information that supports this article demonstrates that

passive drug transfer through the external helminth surface is the main entry mechanism that accounts for accumulation of lipophilic anthelmintic molecules into target parasites. Drug lipophilicity, the physicochemical features of the medium surrounding the parasite and the structure of the external surface of the parasite are among the factors that affect the transfer of the active drug into a target parasite and that determine the anthelmintic effect. Future research should include additional *in vivo* work to complement the available data on *ex vivo* drug transfer and integrated research on the correlation between parasite expulsion kinetics and drug concentrations achieved both in target tissues and in the parasite. Considering the increasing concern for the development of resistance to anthelmintics, it is now accepted that the use of pharmacology-based information is crucial to design successful strategies for parasite control.

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