Xenobiotic-Metabolizing Enzymes in Trematodes

Subjects: Infectious Diseases

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Trematode infections occur worldwide causing considerable deterioration of human health and placing a substantial financial burden on the livestock industry. The hundreds of millions of people afflicted with trematode infections rely entirely on only two drugs (praziquantel and triclabendazole) for treatment. An understanding of anthelmintic biotransformation pathways in parasites should clarify factors that can modulate therapeutic potency of anthelmintics in use and may lead to the discovery of synergistic compounds for combination treatments.

Keywords: liver fluke ; Trematoda ; ATP-binding cassette ; cytochrome P450

1. Introduction

The xenobiotic metabolism, which exists in all species, including parasitic ones, is responsible for homeostasis and the protection of the internal environment of the body when exposed to exogenous compounds, including various xenobiotics and anthelmintics.

Trematoda is a class within the phylum Platyhelminthes. There are no free-living trematodes, and all these species are internal parasites of mollusks and vertebrates. Most trematodes have a complex life cycle with two or three hosts. The life cycle is heterogonic with alternating sexual and asexual reproduction and with changes in generations and hosts. The definitive host, where the flukes sexually reproduce, is a vertebrate. The intermediate host, in which asexual reproduction occurs, is usually a snail. The class Trematoda or flukes includes plenty of species. The most epidemiologically important representatives include lung flukes *Paragonimus* spp.; liver flukes *Opisthorchis* spp., *Clonorchis sinensis*, and *Fasciola* spp.; and blood flukes *Schistosoma* spp. An estimated 200 million people may have a blood fluke schistosomal infection and 56.2 million people are infected with food-borne trematodes, which affect the lungs, liver, or intestines [1]. The hundreds of millions of people afflicted with schistosomiasis and other flatworm infections rely entirely on only two drugs (praziquantel and triclabendazole) for treatment. An understanding of anthelmintic biotransformation pathways in parasites should clarify the factors that can modulate the therapeutic potency of anthelmintics currently in use and may lead to the discovery of synergistic compounds for combination treatments.

2. Composition of the Xenobiotic-Metabolizing System in Trematodes

2.1. Phase I Enzymes

Phase I oxidation reactions are carried out by cytochrome P450 (CYP) enzymes, flavin-containing monooxygenases (FMOs), and epoxide hydrolases [2]. Other enzymes, such as aldo–keto reductases, aldehyde dehydrogenases, and alcohol dehydrogenases, are also involved in the first phase of xenobiotic metabolism.

CYPs in many species are the most extensively studied of all enzymes of xenobiotic metabolism because they are responsible for the metabolism of the vast majority of therapeutic drugs. CYPs are diverse in terms of the regulation of their gene activity and their catalytic activity. The number of genes in the genomes encoding CYP enzymes (CYPome) varies, ranging from a single isoform of an enzyme in some species of bacteria and fungi to dozens of isoforms in mammals and hundreds of CYP genes in plants. Currently, 102 functionally active CYP genes are known in mice and 57 in humans. CYPs contain a heme molecule as a cofactor. A heme is an oxygen-binding molecule that contains one iron atom at the center of the hydrocarbon structure. A heme performs the function of oxygen binding in the active site of the enzyme. CYP enzymes use an oxygen molecule, as well as an H^+ ion, derived from NADPH to carry out the oxidation of substrates. H^+ is supplied by the enzyme NADP-cytochrome P450 oxidoreductase, which works as a partner for cytochromes P450. Substrate metabolism by a CYP proceeds via the consumption of one molecule of oxygen and the formation of an oxidized substrate and a water molecule as a byproduct. The term P450 is derived from the spectrophotometric peak at the maximum absorption wavelength of the enzyme (450 nm) when it is in the reduced state as a complex with carbon monoxide.

Among the various reactions carried out by CYPs, the most well-known are the reactions of N-dealkylation, O-dealkylation, hydroxylation of aromatic hydrocarbons, N-oxidation, S-oxidation, and deamination. CYPs are involved in the metabolism of xenobiotics, as well as the synthesis of endogenous compounds, such as steroids and bile acids, which are byproducts of cholesterol breakdown. Mammalian xenobiotic-metabolizing CYPs have the ability to modify a large number of structurally different chemicals. This property is due to both the multiple forms of CYP and to the capacity of a single CYP to modify many structurally different compounds.

Flavin-containing monooxygenases (FMOs) are another phase I group of enzymes involved in xenobiotic metabolism. Similar to CYPs, FMOs are highly expressed and associated with the endoplasmic reticulum, the site in the cell where hydrophobic substrates interact and are metabolized. Epoxide hydrolases catalyze the hydrolysis of epoxides produced by CYP reactions. Epoxides are highly reactive and electrophilic; they can bind to cellular structures, proteins, RNA, and DNA, thereby leading to undesirable consequences for both the cell and organism. Epoxide hydrolases participate in the inactivation of potentially toxic derivatives.

Although CYPs are present in all kingdoms of living organisms, for a long time—due to unsuccessful attempts to identify them—it has been believed that parasitic flatworms lack both CYPs and the ability to oxidize xenobiotics [3][4]. Nevertheless, a CYP-like activity was later identified in some species of parasitic Platyhelminthes, the conversion of a number of drugs into their inactive metabolites has been reported [5][6][7], and evidence has been obtained about the role of such an activity in parasite drug resistance [3][8]. In particular, albendazole can be oxidized to albendazole sulfoxide in tissues of nematodes *Haemonchus contortus* [9], trematodes *Dicrocoelium dendriticum* and *Fascioloides vogae*, and the cestode *Moniezia expansa* [9]. *Fasciola hepatica* can metabolize triclabendazole to triclabendazole sulfoxide and triclabendazole sulfone, whereas the amount of metabolites is significantly increased in resistant strains [8]. Moreover, if resistant strains of *Fasciola* are incubated with ketoconazole (an inhibitor of CYPs), then these helminths become sensitive to the anthelmintics. All these data indirectly indicated the presence of a CYP system in parasitic flatworms.

Analysis of the available data on the genomes and transcriptomes of parasitic and nonparasitic flatworms revealed that the CYPome drastically differs between these two groups. Although the free-living species have dozens of weakly homologous diverged CYP genes (for example, 39 CYPs in *Schmidtea mediterranea*), parasitic species (Schistosomatidae, Opisthorchiidae, Taeniidae, and Fasciolidae) have only one cytochrome P450 [10][11][12].

Analysis of the coding region of the P450 sequence revealed the presence of a functional Pfam00067 CYP domain (E-value = 3.75×10^{-29}) characteristic of eukaryotic microsomal type II CYPs [10]. This type of CYP is widespread among many organisms and is directly involved in the biotransformation of exogenous compounds: xenobiotics and drugs. This type of CYP is located in the membrane of the endoplasmic reticulum and functions in tandem with NADPH CYP reductase, which is necessary for electron transfer to cytochrome P450 from NADPH. The gene-encoding NADPH CYP reductase has been found in genomes of parasitic Platyhelminthes [3]. In the *O. felineus* (Of) CYP structure, a transmembrane domain was also found in the N-terminal region [10].

OfCYP shares high homology (91%) with CYP of *C. sinensis* and *O. viverrini*, lower homology with that of Schistosomatidae (29–37%), and low homology with CYPs of free-living flatworm species (23–24%) [10][11]. Similar results indicating low homology (20–24%) with Eumetazoa CYPs were also reported for *S. mansoni* CYP [13]. Despite the low homology of the primary sequences, CYPs have a conserved folding of small regions, which implements the function of monooxygenase catalysis. Conserved regions are located predominantly in the C-terminal region of the protein and form a four-helical globule (D, E, I, L), J and K α-helices, two β-fold regions, and a loop called a “meander”. This region contains a heme-binding loop, with the characteristic P450 consensus motif Phe-X-X-Gly-X-Arg-X-Cys-X-Gly (Phe400-Ser-Leu-Gly-Ala-Arg-Ser-Cys-Pro-Gly409 in OfCYP) [10].

In addition, it has been demonstrated that *O. felineus* detoxification phase I is also represented by genes encoding aldo–keto reductases, aldehyde dehydrogenases, and alcohol dehydrogenases [12]. The expression of all aldehyde dehydrogenases is higher in the adult stage than in metacercariae, while the expression of aldo–keto reductases is almost at the same level in both stages [12].

2.2. Phase II

Phase II enzymes are responsible for the elimination of xenobiotics and drugs from the body and for the inactivation of electrophilic and potentially toxic metabolites produced by phase I enzymes. Phase II reaction products are metabolites with improved water solubility and increased molecular weight. The conjugation reactions that these enzymes perform occur when a substrate has an oxygen-containing group (hydroxyl or epoxy groups), an amino group, or sulfur atoms that serve as an acceptor for a hydrophilic moiety of such molecules as glutathione, glucuronic acid, or sulfate or an acetyl

group that is covalently conjugated to an acceptor site on the xenobiotic molecule. Phase I oxidation by enzymes either adds or modifies a functional group, allowing the resulting reaction products to serve as substrates for phase II conjugating enzymes. As a consequence, the metabolite, which is at this point more soluble in water and has higher molecular weight, is excreted in urine or bile. Enzymes associated in phase II of xenobiotic metabolism include several superfamilies of conjugation enzymes. The most important of these are glutathione S-transferase (GST), sulfotransferase (SULT), UDF-glucuronosyl transferase (UGT), arylamine N-acetyltransferases, glycine-N-acyltransferase (GLYAT), methyl transferase, and glutathione peroxidase.

GSTs catalyze glutathionylation by adding glutathione (GSH) to an electrophilic center of their substrates [14]. They can also reduce lipid peroxidation products formed by a free-radical attack on water-soluble compounds [15]. GSTs are categorized into four major classes based on substrate specificity: cytosolic GSTs, kappa-class GSTs (mitochondrial), membrane-associated proteins of eicosanoid, and glutathione metabolism [16]. The cytosolic GSTs are more abundant and can be further subdivided into several classes, such as mu, alpha, pi, theta, sigma, zeta, omega, nu, lambda, phi, tau, delta, epsilon, iota, chi, and rho [14][17][18]. Structurally, most of the GSTs are dimeric and can be either a homodimer or heterodimer.

The general reaction consists of conjugating the reduced GSH to molecules with an electrophilic center (1), including alkyl and aryl halides, carboxylates, sulphate and phosphate esters, epoxides, organic nitrates, lactones, quinones thiocyanates, and hydroperoxides [4][16].

$$GSH + RX \rightarrow GSR + HX \quad (1)$$

Genes encoding phase II enzymes are widespread in the genomes of flat and round parasitic worms [19][20][21]. The main cytosolic GST classes identified in helminth parasites are mu, pi, and sigma, along with some alpha and omega class GSTs [19][20][21][22].

Sixteen GST genes have been identified in *A. suum* nematodes, and 46 genes in *C. elegans*, while 5–7 GST genes have been identified in opisthorchiids, and three genes in *S. mansoni*. The *O. felineus* genome contains six GST genes, which have the highest expression among all detoxification genes. The 28 kDa GST sigma is especially highly expressed; its mRNA abundance in the adult worm is by two–three orders of magnitude higher as compared with the other detoxification genes [12]. UGTs (common phase II xenobiotic metabolism enzymes in vertebrates enhance hydrophilicity and availability of substrates to efflux transporters. The UGT superfamily comprises over 20 isozymes.

A comparative study on the composition of conjugation phase II enzymes in various parasitic worms as well as free-living worms has been aimed at identifying sequences corresponding to conjugation phase II proteins in the available data on genomes and transcriptomes of Opisthorchiidae and *S. mansoni* flatworms as well as in roundworms: parasitic species *A. suum* and free-living *C. elegans* [12]. It turned out that UDP-glucuronosyl transferases, which are the main enzymes of phase II, both in parasitic and free-living roundworms, are completely absent in flatworms. Currently, 34 UGT genes are known to be present in the nematode *H. contortus* genome [23], and 72 UGT genes in the *C. elegans* genome [24].

2.3. Identification of Phase III Genes

The removal of xenobiotics and drugs from the cell is implemented by the cell's excretory system. The main xenobiotic efflux proteins are ATP-binding proteins (ABC, i.e., ATP-binding cassette) and OATP2 proteins (organic anion transporting polypeptide 2) [25]. ATP-binding proteins have been found in all animal and plant species from prokaryotes to eukaryotes [26]. ABC proteins have a conserved structure, several transmembrane domains, and broad substrate specificity. In mammals, these proteins carry out an ATP-dependent transport of toxic compounds and drugs from the cell to the extracellular space across the membrane.

Although there are differences in their function and types of substrates, they share high structural homology. Human P-glycoprotein (ABCB1), which is encoded by the *MDR1* gene, is the best-known and best-studied ABC transporter among human ABC proteins. ABCB1 was discovered in 1976 [27] because of its involvement in the multidrug resistance of cancer cells to chemotherapy, which is how it got its name (multidrug resistance gene, MDR). The protein is made up of two parts that share high similarity. Each homologous region contains six hydrophobic transmembrane domains and a hydrophilic intracellular region encoding an adenosine triphosphate (ATP)-binding site (nucleotide-binding domain, NBD).

The genes of this family are common in genomes of parasitic flat and round worms. In particular, 20 genes in the genome have been detected in the parasitic flatworm *S. mansoni* [28], and 23 genes of transporters of the ABC family were identified in the *O. felineus* genome [29]. In particular, the genome contains four genes of the A-subfamily, eight genes of

the B subfamily, five ABCC genes (multidrug resistance associated protein), one ABCD, two ABCF, and three ABCG genes.

The organization of conserved regions in the coding regions of the ABC transporters differs among the subfamilies of these proteins. In particular, complete transporters composed of two successively alternating transmembrane and nucleotide-binding domains are present only in the ABCC subfamily (five proteins) and ABCB subfamily (six proteins). This domain organization is conserved and is present in mammals, plants, and roundworms. The structure of the predicted semitransporters of the ABCG and ABCB6 subfamilies, which include only one transmembrane domain and one NBD, also matches the structure of homologs in mammals. The structure of transporters of the ABCA subfamily (four proteins), which do not contain transmembrane domains, is noteworthy, while in mammals these are complete transporters. Their function is the transport of lipids and signaling molecules. Obviously, in trematodes, due to the absence of transmembrane domains in ABCA, the transport of lipid compounds is performed by transporters of other subfamilies.

All four *MDR* genes (ABCB1) of *O. felineus* have homologs in other species of liver flukes. The degree of homology with those of *C. sinensis* and *O. viverrini* is 94–97% [29]. A 3D model of the *O. felineus* Pgp4 (MDR, ABCB1) protein built using Phyre2 multi-array modeling indicates conserved structure of this protein. The matrices selected by the Phyre algorithm for modeling were P-gp from *C. elegans* (4F4CA) and P-gp1 from *Mus musculus* (3G5U). The structure of a region of 1202 amino acid residues (95% of the P4 sequence) was modeled at a 100.0% match [29].

3. Xenobiotic-Metabolizing Enzymes as Targets for Anthelmintic Therapy

3.1. Phase I Enzymes

Some proteins of the detoxification system are important pharmacological targets because they are critical for their own metabolism and the survival of pathogens. In particular, this topic primarily concerns cytochromes P450. More than 20 years ago, based on a fungal cytochrome P450 inhibitor, fungicide drugs were developed that are effective as fungicidal agents and are currently used in medicine [2]. These are azole compounds, such as ketoconazole, fluconazole, and other imidazole derivatives. It is known that *Trypanosoma cruzi* CYP51 is an important pharmacological target, and clinical trials of compounds based on *T. cruzi* CYP inhibitors are currently underway [30]. Previously, these compounds have been successfully tested in the treatment of trypanosomiasis in model animals. Lately, the search for new drugs against trematodiases has been actively conducted [1], both by the screening of libraries of chemical compounds and by researching parasitic proteins as new molecular targets for the development of targeted drugs.

From this point of view, cytochrome P450 can be a promising target for the development of drugs against trematodiases. From the evidence of a decrease in the survival rate of liver flukes subjected to a CYP gene knockdown [11][13], it can be concluded that this gene is important for parasite viability. Because all parasitic flatworms, including liver flukes (Opisthorchiidae and Fasciolidae), blood flukes (Schistosomatidae), and cestodes (Taeniidae) have only one CYP gene [10], it is possible to suppress CYP monooxygenase activity by means of selective inhibitors.

The testing of the anthelmintic activity of universal azole CYP inhibitors—ketoconazole, miconazole, triadimenol, clotrimazole, 4-phenyl imidazole, and others—on *O. felineus* and *S. mansoni* trematodes indicates that some of these compounds actually have promising anthelmintic activity against both juvenile and adult parasites. Such an activity has been documented in vitro using micromolar drug concentrations in both motility and survival tests [13][31]. The most effective drugs in this regard are miconazole and clotrimazole [13][29]. At the same time, miconazole has proven to be as effective as praziquantel against *O. felineus* and *S. mansoni* and causes 100% mortality of worms at a dose of 5–10 μM [13][31]. In addition, other inhibitors of heme-containing enzymes with non-azole structures were tested: disulfiram, metyrapone, benzyl isothiocyanate, and ticlopidine [31]. The evaluation of these substances by standard motility tests on excysted metacercariae showed that some of the compounds are quite promising.

Moreover, a synergistic interaction was demonstrated for a praziquantel–clotrimazole (CI = 0.68) in vitro combination and for a praziquantel–miconazole (CI = 0.68) in vitro combination against adult helminths [32]. Praziquantel and miconazole (CI = 0.30) had a strongly synergistic effect against newly excysted *O. felineus* metacercariae [32]. Unfortunately, the synergistic effects of the praziquantel–clotrimazole combination and praziquantel–miconazole combination observed in vitro were not confirmed in vivo.

The treatment of hamsters infected with juvenile worms (1 day postinfection) resulted in a worm burden reduction of 37.5%, with 100 mg/kg clotrimazole killing 31.25% of the worms [32]. At 5–6 weeks postinfection, which corresponded to the infection with adult worms, the treatment of hamsters with miconazole yielded a worm death rate of 23.8% and 21.4%,

respectively. The administration of praziquantel together with clotrimazole or with miconazole killed worms slightly more frequently than praziquantel alone did (59.5% and 54.7% versus 50% for praziquantel).

3.2. Phase II Enzymes

Despite the clear proof of the involvement of GST in drug resistance in nematodes, there is currently no evidence that GST expression and activity are linked with drug resistance in trematodes. For instance, RNA interference targeting the σ class of GSTs in the liver fluke *F. gigantica* causes the robust transcriptional silencing of σGST for up to 15 days of observation without any measurable changes in worm viability [33]. Because a GST knockdown in trematodes has not been shown to have any significant effect on survival in either juvenile or adult individuals, GST enzymes are not regarded as potential targets for the development of human anthelmintic drugs against trematodes.

Nevertheless, helminth GSTs are considered an important target for vaccine development [34][35][36]. In particular, a cytosolic GST from *S. haematobium* (Sh28GST) has passed phase I of clinical trials [37]. Similarly, glutathione S-transferase (SjGST) is regarded as a basis for a DNA vaccine against murine *S. japonicum* infection [36]. A SjGST DNA vaccine delivered using the nanoparticle gene delivery system exerts an antifecundity effect on female adult schistosomes and is a promising candidate for anthelmintic therapy and transmission-blocking applications [36].

3.3. Phase III Proteins

The inhibitors of P-glycoproteins enhance the therapeutic anthelmintic effect of drugs in vitro. In addition, it was shown that mammalian P-glycoprotein inhibitors retain their effectiveness at suppressing the helminth efflux system and even raise the sensitivity of resistant isolates. In particular, the disruption of vitellogenesis and spermatogenesis by triclabendazole (TCBZ) was revealed in a TCBZ-resistant isolate of *F. hepatica* following incubation in vitro with a P-glycoprotein inhibitor [38]. P-glycoprotein inhibitor R(+)-verapamil increases the drug susceptibility of a triclabendazole-resistant isolate of *F. hepatica* [39].

By contrast, the treatment of murine schistosomiasis with P-glycoprotein inhibitors does not improve the therapeutic effect against schistosomes. In particular, systemic treatment with ivermectin, even in the presence of the pharmacological inhibition of P-glycoprotein or cytochrome P450 3A, does not result in effective prophylaxis of *S. mansoni* infection in an experimental murine model [40].

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