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Review

Current advances on ABC drug transporters in fish

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A B S T R A C T

Most members of the large ATP-binding cassette (ABC) gene family are transporters involved in substrate translocation across biological membranes. In eukaryotes, ABC proteins functioning as drug transporters are located in the plasma membrane and mediate the cellular efflux of a wide range of organic chemicals, with some transporters also transporting certain metals. As the enhanced expression of ABC drug transporters can confer multidrug resistance (MDR) to cancers and multixenobiotic resistance (MXR) to organisms from polluted habitats, these ABC family members are also referred to as MDR or MXR proteins. In mammals, ABC drug transporters show predominant expression in tissues involved in excretion or constituting internal or external body boundaries, where they facilitate the excretion of chemicals and their metabolites, and limit chemical uptake and penetration into "sanctuary" sites of the body. Available knowledge about ABC proteins is still limited in teleost fish, a large vertebrate group of high ecological and economic importance. Using transport activity measurements and immunochemical approaches, early studies demonstrated similarities in the tissue distribution of ABC drug transporters between teleosts and mammals, suggesting conserved roles of the transporters in the biochemical defence against toxicants. Recently, the availability of teleost genome assemblies has stimulated studies of the ABC family in this taxon. This review summarises the current knowledge regarding the genetics, functional properties, physiological function, and ecotoxicological relevance of teleostean ABC transporters. The available literature is reviewed with emphasis on recent studies addressing the tissue distribution, substrate spectrum, regulation, physiological function and phylogenetic origin of teleostean ABC transporters.

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1. Introduction

When considering the interaction of organisms with the surrounding chemosphere, central questions regard the mechanisms of chemical uptake and elimination, as well as that of chemical distribution between different body compartments (Van Aubel et al., 2002). It is well documented that biotransformation crucially affects chemical fate in fish (Schlenk et al., 2008). In contrast, the impact of active transport across cellular membranes on chemical fate is still incompletely understood in teleosts. While such transport mechanisms likely affect bioaccumulation and toxicity of pollutants in fish (Nichols et al., 2007), an understanding of the specific interactions of environmental chemicals with transport proteins and the ecotoxicological relevance of such interactions is currently only beginning to emerge.

In eukaryotes, ABC (ATP-binding cassette) proteins comprise an important group of transporters that control the movement of compounds between the external environment and the internal milieu, and between body compartments. These proteins were first described as biochemical factors conferring multidrug resistance (MDR) in cancer, i.e., the resistance of tumour cells against structurally and functionally unrelated cytostatic drugs (Roninson et al., 1984; Gros et al., 1986). These MDR conferring proteins are localised in the cell membrane and function as ATP-dependent biochemical pumps mediating the cellular efflux of a diverse array of organic chemicals and some metals (Gottesman et al., 2002). ABC drug transporters also show high expression levels in normal tissues involved in excretion (e. g., kidney, liver) or acting as barriers (gut epithelium, capillary endothelia forming the blood–brain barrier) (Fojo et al., 1987; Thiebaut et al., 1987). ABC drug transporters often localise to the apical side of polarised epithelial cells, suggesting their role in limiting chemical uptake and enhancing chemical elimination, thus contributing to the biochemical defence against toxicants (Leslie et al., 2005). In support of such a role, animals lacking certain ABC drug transporters as the result of natural or targeted mutations usually are healthy and viable, but can show marked hypersensitivity to specific toxicants or mild pathophysiological changes reflecting the impaired excretion of endogenous toxicants (Schinkel et al., 1994, 1995; Wijnholds et al., 1997; Kruh et al., 2007; Lagas et al., 2009). Kurelec and co-workers were the first to report the induction of ABC transporters in marine invertebrate populations from polluted habitats (Kurelec and Pivcevic, 1991; Kurelec et al., 1995). In analogy to the phenomenon of MDR in cancer cells, Kurelec and colleagues coined the term "multixenobiotic resistance (MXR) proteins" for ABC drug efflux transporters in aquatic animals, reflecting the role of the cellular pumps as protective factors against pollutant toxicity (Kurelec and Pivčević, 1989; Kurelec and Pivcevic, 1991; Kurelec, 1992). While the term "MXR proteins" is well established in aquatic toxicology, the present review will employ the more general term "ABC drug transporters" in order to avoid using different terminologies when referring to aquatic and terrestrial animal models.

First evidence for the ABC drug transporter Abcb1[1] (P-glycoprotein) in teleost was provided by a molecular genetic study in winter flounder (*Pleuronectes americanus*) (Chan, 1992). The presence of Abcb1-like proteins in fish was subsequently confirmed in an immunohistochemical study in guppy (*Poecilia reticulata*) (Hemmer et al., 1995). Moreover, P-glycoprotein-like transport activities were measured in isolated proximal tubules from winter flounder and killifish (*Fundulus heteroclitus*) (Miller, 1995; Schramm et al., 1995; Sussman-Turner and Renfro, 1995). Subsequently, ABC drug transporters have been studied in a number of tissues of different teleosts, using immunochemical detection (Hemmer et al., 1998; Cooper et al., 1999; Kleinow et al., 2000; Albertus and Laine, 2001) and transport assays (Doi et al., 2001; Sturm et al., 2001b; Miller et al., 2002). Recently, cDNA sequences have been obtained for various ABC drug transporters (Zaja et al., 2008b; Paetzold et al., 2009; Fischer et al., 2010, 2011, 2013; Loncar et al., 2010; Popovic et al., 2010; Sauerborn Klobučar et al., 2010; Costa et al., 2012; Diaz de Cerio et al., 2012), and the ABC gene family has been annotated and analysed phylogenetically in zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*) (Annilo et al., 2006; Liu et al., 2013). Moreover, a teleost cell line showing enhanced expression of a specific teleost ABC drug transporter has been generated, which enables the identification of environmentally relevant compounds that interact with this efflux pump (Caminada et al., 2008; Zaja et al., 2008a, 2011; Smital et al., 2011).

The aim of the present review article is to summarise recent insights into ABC transporters in teleost fish, focusing on data that have become available since earlier reviews on the subject (Bard, 2000; Sturm and Segner, 2005). Molecular, physiological and in vitro studies on ABC transporters are reviewed focusing on teleosts, but also taking into account studies on elasmobranchs where available. Since the annotation of ABC drug transporters in zebrafish (Annilo et al., 2006), genome assemblies have become available in further teleost species, allowing to draw a more complete picture of the complement of ABC drug transporters present in this economically and ecologically important vertebrate taxon. To this end, evolutionary relationships between ABC drug efflux transporters from selected tetrapods and the presently seven teleost species with available genome assemblies are presented.

1.1. Teleosts and other fishes

"Fish" have been defined as aquatic vertebrates having gills and fin-shaped limbs (Nelson, 2006). This definition comprises more than half of the extant vertebrates, and includes taxa as diverse as jawless (Agnatha), cartilaginous (Chondrichthyes) and bony fishes (Osteichthyes) (Helfman et al., 2009). The first fossil evidence for the Chondrichthyes, which include the elasmobranchs (sharks and rays), dates from the early Devonian (418 mya). Osteichthyes are divided

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[1] Our designation of gene and protein names is based on the Zebrafish Nomenclature Guidelines (http://zfin.org/zfinfo/nomen.html); fish: *shh*/Shh, human: *SHH*/SHH, mouse: *Shh*/SHH (gene/protein).

into actinopterygians (ray-finned fish) and sarcopterygians (lobe-finned fish), with the latter division containing the coelancanths, lungfish and tetrapods (Bone and Moore, 2008). The split between actinopterygians and sarcopterygians has been recently dated to have taken place before 419 mya (Zhu et al., 2009). The teleost fish (Teleostei) are the most advanced actinopterygian division containing ~27,000 living species and account for the majority (~96%) of all living fishes, including important fishery species (Helfman et al., 2009). Teleosts have probably emerged in the Late Triassic (~200 mya) from a neopterygian ancestor (Bone and Moore, 2008), and underwent four radiations, producing the osteoglossomorphs (bony tongues), elopomorphs (tarpons and true eels), ostarioclupeomorphs (herrings and minnow relatives) and finally the Euteleostii, which constitute the most advanced and species-rich teleost subdivision (Helfman et al., 2009).

Based on the observation that mammals often possess multiple copies of genes present only once in invertebrates, it has been suggested that whole genome duplications played an important role in vertebrate evolution (Ohno, 1970). For instance, mammals possess four clusters of Hox genes whereas only one cluster is found in the primitive chordate amphioxus (Garcia-Fernandez and Holland, 1994). According to current models, the ancestral chordate genome has undergone at least two rounds of duplication in the lineage leading to the jawed vertebrates (Aparicio, 2000; Escriva et al., 2002). While no further genome duplications appear to have occurred in the sarcopterygian and Chondrichthyes lineages, a third whole genome duplication took place in a common ancestor of extant teleosts (Amores et al., 1998; Taylor et al., 2001; Jaillon et al., 2004; Steinke et al., 2006). As a result, teleost fish frequently possess duplicate copies of genes present in mammals only once (Robinson-Rechavi et al., 2001). Complicating the situation further, additional lineage-specific genome duplications have occurred in particular teleost groups (Johnson et al., 1987).

Teleosts inhabit almost every imaginable marine or freshwater habitat and pursue a wide range of trophic strategies, feeding on zooplankton, benthic invertebrates, other fishes, mammals, carrion, detritus, phytoplankton, macroalgae or vascular plants (Helfman et al., 2009). In comparison, elasmobranchs are typically large predators and the majority of species is marine (Helfman et al., 2009). Ecotoxicological, physiological and/or genomic data are available only for a limited number of teleosts and a few elasmobranchs, usually species that can be bred in captivity or be obtained easily. The aim of the present article is to review the current knowledge on ABC transporters in teleost fish. Where available, data from elasmobranchs are also considered. While at points general patterns seem to emerge, it should be kept in mind that only a small fraction of teleost species have been studied, and prudence is advised regarding generalisations.

1.2. The ABC protein family

Members of the large ABC (ATP-binding cassette) gene superfamily are characterised by conserved nucleotide binding domains and are found in all biota (Higgins, 1992). Typical ABC proteins are ATP-dependent transporters mediating the trafficking of diverse substrates across biological membranes (Dean et al., 2001). Some ABC proteins function as drug transporters and have central relevance in the biochemical defence against toxic chemicals (Schinkel and Jonker, 2003; Leslie et al., 2005). Other ABC superfamily members are not transporters, but have roles as ion channels, receptors or factors involved in transcription and translation (Dean et al., 2001). The 48 human ABC transporters known to date are divided into seven subfamilies characterised by a high degree of sequence homology and designated ABCA/Abca to ABCG/Abcg (Dean et al., 2001). In addition, some teleosts possess members of an eighth subfamily called Abch initially identified in the fruitfly (Dean et al., 2001; Annilo et al., 2006; Popovic et al., 2010). Systematic names for ABC transporters consist of the subfamily name (ABCA/Abca, ABCB/Abcb, ABCC/Abcc, …) followed by a number denoting the protein (e.g., ABCB1/Abcb1, ABCC1/Abcc1). In some cases

a further letter is added to distinguish multiple paralogues. In addition, earlier non-systematic names given upon first description of ABC proteins are still common. ABC full transporters (FT) possess two nucleotide-binding domains (NBD) and two transmembrane domains (TMD) arranged in the N- to C-terminal order TMD_1-NBD_1-TMD_2-NBD_2. ABC FTs are found in subfamilies A/a, B/b, and C/c, with some ABCC/Abcc members showing an additional N-terminal TMD (termed TMD_0) (Deeley et al., 2006). ABC half transporters (HT) have only one TMD and one NBD which are arranged in the N- to C-terminal order TMD-NBD (subfamilies B/b and D/d) or NBD-TMD (subfamilies G/g and h) (Dean et al., 2001). HTs are generally assumed to form homo- or heterodimers to constitute a functional pump (Schinkel and Jonker, 2003). The members of subfamilies E/e and F/f are not transporters and lack TMDs (Dean et al., 2001).

1.3. ABC drug transporters

The contribution of ABC drug transporters to the biochemical defence against toxicants is illustrated in a schematic fashion in Fig. 1. After the passive uptake of hydrophobic organic chemicals by a hypothetical epithelial cell, a first line of defence is provided by ABC transporters that pump out hydrophobic substrates (Fig. 1). Biotransformation enzymes constitute a second level of defence by converting organic chemicals to products that are usually less toxic (Fig. 1). Phase I biotransformation reactions involve the introduction of polar groups into the chemical, while phase II reactions comprise conjugations with endogenous moieties such as glutathione, sulfate or glucuronic acid. The products of biotransformation metabolism are removed from the cell by drug transporters accepting less hydrophobic substrates, which include other types of ABC proteins (Fig. 1). In polarised epithelia or endothelia, ABC drug transporters generally show a predominantly apical localisation, resulting in directional transport into excreta or away from sanctuary sites (Fig. 1).

All eukaryotic ABC drug transporters are active efflux pumps, i.e., substrate translocation occurs from the cytosol or the cell membrane to the extracellular space and is energetically linked to the cleavage of ATP (Ambudkar and Cardarelli, 1997; Van Veen et al., 2001; Bouige et al., 2002). This enables ABC pumps to transport substrates against a concentration gradient. In contrast, drug transporters of the solute carrier (SLC) superfamily depend on facilitated diffusion, exchange or co-transport for transport (El-Sheikh et al., 2008; Oostendorp et al., 2009). While certain SLCs and ABC transporters can show overlaps in substrate specificity, SLC proteins are beyond the scope of the present review.

Three ABC subfamilies, namely ABCB/Abcb, ABCC/Abcc and ABCG/Abcg, are known to contain drug transporters, as well as proteins having roles unrelated to drug transport. In human, the most important ABC drug efflux pumps are ABCB1 (also called MDR1 or P-glycoprotein), ABCC1 (also known as the multidrug resistance-associated protein, MRP1), ABCC2 (also known as MRP2 and the canalicular multispecific organic anion transporter cMOAT) and ABCG2 (also known as the breast cancer resistance protein, BCRP) (Schinkel and Jonker, 2003; Leslie et al., 2005). ABCB1 shows high levels of expression in hepatocytes, proximal renal tubules, enterocytes and brain capillary endothelial cells, and adopts an apical localisation in polarised cells (Ambudkar et al., 1999; Gottesman et al., 2002). ABCC members acting as drug transporters are called multidrug resistance proteins (MRPs) (Deeley et al., 2006; Slot et al., 2011). ABCC1 is found in many tissues, showing high levels in lung, testis, kidney, placenta and skeletal and heart muscles (Deeley et al., 2006; Bakos and Homolya, 2007). The localisation of ABCC1 in polarised cells is variable, being basolateral in most cell types, but apical in others, such as the capillary endothelial cells of the blood–brain barrier (Deeley et al., 2006; Bakos and Homolya, 2007). ABCC2 always shows an apical localisation in polarised cells and is predominantly expressed in liver, kidney, small intestine, colon, gallbladder, placenta and lung (Nies and Keppler, 2007). Compared to ABCC1 and ABCC2,
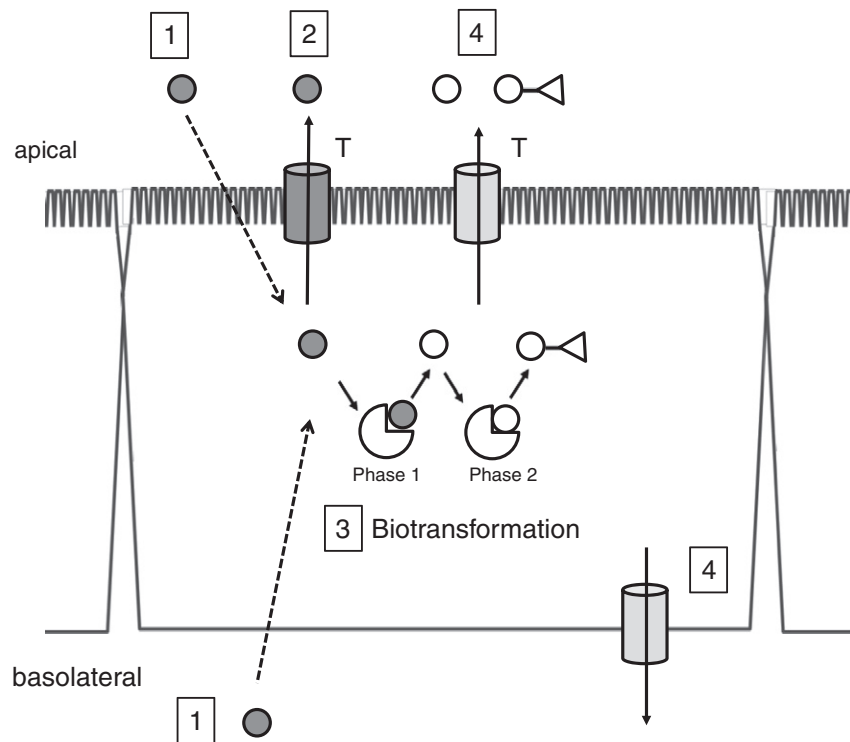
Fig. 1. ABC drug efflux and biotransformation metabolism in a hypothetical epithelial cell. (1) Hydrophobic chemicals (filled circles) can enter cells by passive diffusion (dashed arrows). (2) Hydrophobic chemicals may be extruded from the cell by the active transport activity (filled arrows) of ABC transporters (T) such as ABCB1 (MDR1 P-glyoprotein) or ABCG2 (breast cancer-related protein), which in polarised epithelia usually localise to the apical side. (3) Hydrophobic chemicals may undergo biotransformation metabolism, in which phase I usually leads to more polar products (open circles), while phase II involves conjugation with endogenous anionic moieties (triangles). (4) ABC drug transporters of the ABCC subfamily (multidrug resistance-associated proteins) mediate the cellular efflux of organic anions and conjugated metabolites. ABCC pumps can show an apical or basolateral localisation, depending on the isoform. The schematic is based on the mammalian intestine and was modified from Chan (2004).

less is known about the remaining human MRPs. Data available to date suggest that ABCC3, 4, and 5 may contribute to the biochemical defence against toxicants (Borst et al., 2007; Kruh et al., 2007; Lagas et al., 2009). ABCC4 parallels ABCC1 in that its localisation in polarised cells depends on the cell type, showing a basolateral expression in hepatocytes but an apical expression in renal proximal tubules and endothelial cells of brain capillaries (Van Aubel et al., 2002; Leggas et al., 2004). ABCG2 shows significant levels in liver, kidney and intestine, as well as in the blood–brain and blood–placenta barriers, where it adopts an apical localisation (Robey et al., 2009; Vlaming et al., 2009).

ABC drug transporters generally show a broad substrate selectivity, and substrate spectra significantly overlap among different types of ABC drug pumps (Schinkel and Jonker, 2003; Leslie et al., 2005). In mammals, ABCB1 accepts a broad range of typically uncharged or moderately basic amphipathic substrates (Ambudkar et al., 1999). Typical substrates of ABCC subfamily transporters are organic anions and include chemical conjugates with glutathione, glucuronic acid or sulphate produced in phase II biotransformation metabolism (Deeley et al., 2006; Slot et al., 2011). Despite the low degree of sequence homology between ABCB1 and ABCC1 (~19% amino acid identity) (Cole et al., 1992), MDR phenotypes associated with ABCB1 and ABCC1 widely overlap, and include resistance to anthracyclins, Vinca alkaloids and epipodophyllotoxins (Gottesman et al., 2002). However, ABCC1 differs from ABCB1 in that it provides only very limited protection against taxanes and confers resistance to metalloid oxyanions (Deeley et al., 2006). Both ABCC1 and ABCC2 transport free glutathione (GSH), and GSH can stimulate the transport of other substrates by ABCC1 and ABCC2 (Borst et al., 2006; Deeley and Cole, 2006). Moreover, ABCC1, ABCC2 and homologous proteins from invertebrates can transport metals such as cadmium, mercury and platinum, probably as complexes with GSH (Broeks et al., 1996; Ishikawa et al., 1996; Bridges et al., 2008; Bosnjak et al., 2009). ABCC4 and 5 are able to transport cyclic

nucleotides, as well as drugs that are nucleoside and nucleotide analogues (Deeley et al., 2006; Borst et al., 2007). ABCG2 overlaps in substrate selectivity with both ABCB1 and ABCCs (Krishnamurthy and Schuetz, 2006; Robey et al., 2009). In addition, ABCG2 mediates the biliary excretion of porphyrin precursors, limits the gut uptake of photo-toxic chlorophyll breakdown products (Jonker et al., 2002) and contributes to renal urate secretion (Woodward et al., 2011).

2. Genetic evidence for ABC drug transporters in fish

ABC transporters have previously been annotated from the zebrafish genome, the first fish genome that was comprehensively sequenced (Dean and Annilo, 2005; Annilo et al., 2006). Moreover, the ABC gene family has been analysed in a transcriptome of the catfish (*Ictalurus punctatus*) generated by RNA-seq (Liu et al., 2013). The zebrafish genome contains members of ABC subfamilies A to G known from tetrapods, as well as one transporter of subfamily H (Annilo et al., 2006). While the presence of an *abch* gene has been also confirmed for green spotted puffer (*Tetraodon nigroviridis*), no member of this subfamily has been retrieved in catfish (Liu et al., 2013). The identification of zebrafish homologues to human *ABCB1*, *ABCC1–5* and *ABCG2* confirmed the presence of all major vertebrate ABC drug transporters in teleosts. However, for some tetrapod ABC drug transporters, several isoforms were found in zebrafish, complicating the assignment of function (Annilo et al., 2006). Since the annotation of zebrafish ABC transporters (Annilo et al., 2006), assembled genome sequences have become available for a number of further fish species. In order to base the discussion of the presence or absence of specific ABC transporters in fish on the full range of currently available data, we consider here ABC transporter sequences from seven actinopterygian species with available genome assemblies (zebrafish *Danio rerio*, medaka *Oryzias latipes*, stickleback *Gasterosteus aculeatus*, fugu *Takifugu rubripes*, green spotted puffer

Tetraodon nigroviridis, cod *Gadus morhua* and tilapia *Oreochromis niloticus*) and the sarcopterygian species coelacanth (*Latimeria chalumnae*). Fish sequences of ABC subfamilies containing drug transporters were subjected to phylogenetic analyses together with transporters from human and chicken.

2.1. ABCB/Abcb subfamily

The ABCB/Abcb subfamily contains both FTs and HTs, of which the latter locate to intracellular membranes and have evolutionarily conserved physiological roles unrelated to drug transport (Abele and Tampé, 2006; Burke and Ardehali, 2007; Herget and Tampé, 2007), with evidence for drug transport existing only for ABCB/Abcb FTs (Gottesman et al., 2002; Leslie et al., 2005). Therefore, our evolutionary analyses of vertebrate ABCB/Abcb proteins excluded HTs, focusing on ABCB/Abcb FTs.

In the obtained tree, ABCB1/Abcb1 and ABCB4/Abcb4 sequences form a well-supported clade, within which the teleost sequences group together in a subcluster of high bootstrap support, opposing sarcopterygian ABCB1/Abcb1 and ABCB4/Abcb4 sequences (Fig. 2).
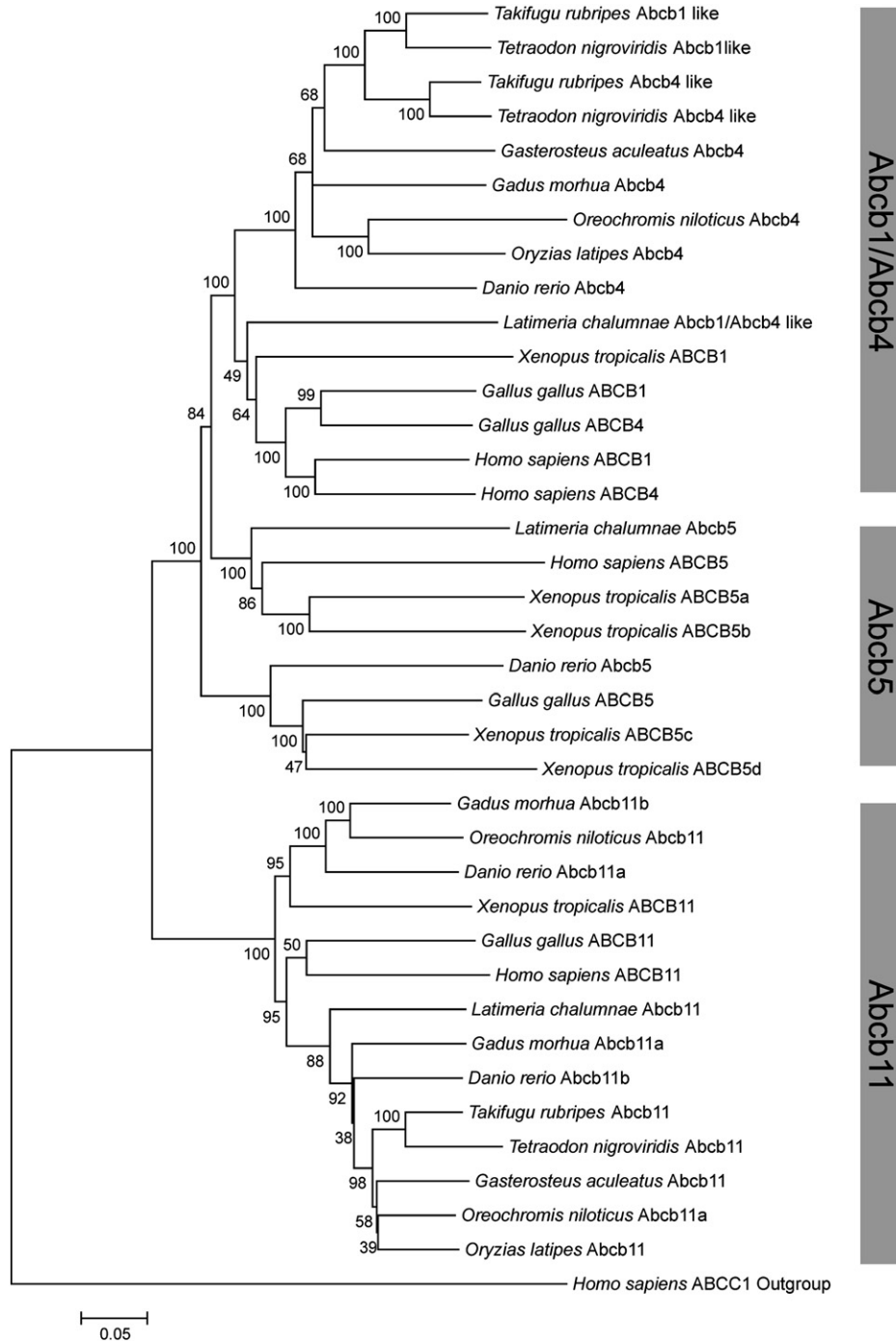
Fig. 2. Phylogenetic analysis of vertebrate ABCB/Abcb subfamily full transporters. Amino acid sequences of transporters from teleosts (*Danio rerio, Gasterosteus aculeatus, Gadus morhua, Oreochromis niloticus, Oryzias latipes, Takifugu rubripes, Tetraodon nigroviridis*) and further vertebrates (*Latimeria chalumnae, Xenopus tropicalis, Gallus gallus* and *Homo sapiens*) (see Table S1 for accession numbers) were aligned using the programme TCoffee (Notredame et al., 2000) and a phylogenetic tree constructed using the neighbor-joining method as implemented in the software MEGA5 (Tamura et al., 2011). The percentage concordance based on 1000 bootstrap iterations is shown at the nodes. Trees obtained with the alternative maximum likelihood and minimum evolution methods had very similar topologies (data not shown), indicating that the results are robust.

Human ABCB1 is known to constitute a drug efflux pump also known as MDR1 P-glycoprotein, whereas human ABCB4 is a biliary phospholipid transporter (Ambudkar et al., 1999; Oude Elferink and Paulusma, 2007). The topology of the tree suggests that human ABCB1 and ABCB4 arose from a lineage-specific gene duplication and that the teleost sequences are, despite their names, not one-to-one orthologues to either ABCB1 or ABCB4, but co-orthologues to both (Fig. 2). All teleost species possess at least one transporter in the teleost Abcb1/Abcb4 clade, with individual sequences being named "Abcb1-like," "Abcb4-like" or "Abcb4" by automatic and/or synteny-based annotation. The teleostean Abcb1/Abcb4 clade includes zebrafish Abcb4 (Fischer et al., 2013), previously annotated as Abcb1b (Annilo et al., 2006), but excludes zebrafish Abcb5, previously annotated as Abcb1a (Annilo et al., 2006; Fischer et al., 2013) (see below). Some teleost species, such as japanese pufferfish (*Takifugu rubripes*) and green spotted pufferfish (*Tetraodon nigroviridis*), possess two P-glycoprotein genes that based on synteny have been designated as Abcb1 and Abcb4 (Fischer et al., 2013) (Fig. 2).

An earlier study proposed that ABCB4 arose in the mammalian lineage, and that birds and teleosts lack functional orthologues to ABCB4 (Annilo et al., 2006). Indeed, no phospholipids are detectable in bile fluid from teleosts and elasmobranch fishes, indicating the lack of hepatic ABCB4-like transport activity (Goto et al., 2003; Oude Elferink et al., 2004; Moschetta et al., 2005). Phosphatidylcholine translocation thus appears to be a specific, relatively recent function of mammalian ABCB4, whereas the property of transport of a wide range of toxic compounds by mammalian ABCB1 is more ancient. In support of this notion, it is well documented that teleosts possess ABCB1-like transport activities (Miller, 1995; Schramm et al., 1995; Sussman-Turner and Renfro, 1995), which have been shown to coincide with the expression of *abcb1/4* (Tutundjian et al., 2002; Zaja et al., 2008b; Fischer et al., 2013). Recent studies provide insights into the molecular identity of teleost ABCB1-like transporters. The characterisation of topminnow (*Poeciliopsis lucida*) Abcb1 using a cell line overexpressing the transporter demonstrated that this ABC pump constitutes a multidrug transporter (Zaja et al., 2008a, 2011). Evidence for a similar function of zebrafish Abcb4 was obtained by morpholino knockdown studies in embryos and recombinant expression studies (Fischer et al., 2013).

In the phylogenetic analysis of vertebrate ABCB/Abcb proteins (Fig. 2), ABCB5/Abcb5 proteins formed two clusters. The function of ABCB5, the most recently isolated human ABCB protein, is still unclear (Frank et al., 2003; Frank and Frank, 2009). While an initial report found *ABCB5* homologues to be lacking in zebrafish (Annilo et al., 2006), a later study proposed that the zebrafish gene initially annotated as *abcb1a* is actually an *ABCB5* orthologue, and suggested renaming the gene *abcb5* (Fischer et al., 2013). An *abcb5* gene has further been reported from catfish (Liu et al., 2013), but orthologues to *ABCB5* appear to be absent in medaka, stickleback, fugu, green spotted puffer, cod and tilapia (Fig. 2). While gene knockdown studies in embryos did not provide evidence for a multidrug transporter function of the zebrafish Abcb5 orthologue (Fischer et al., 2013), hepatic transcript expression of *abcb5* is induced by the main zebrafish bile acid cyprinol sulphate (Reschly et al., 2007), suggesting potential roles of Abcb5 related to biliary excretion. On the other hand, mRNA expression of zebrafish *abcb5* in embryo epidermal cells (Thisse and Thisse, 2004) parallels epidermal ABCB5 expression in mammals where it has been suggested that this protein regulates membrane potential and cell fusion of skin progenitor cells (Frank et al., 2003).

All vertebrates considered in the evolutionary analysis of the ABCB/Abcb subfamily possess at least one *abcb11* gene (Fig. 2). *Abcb11* was originally isolated in winter flounder (Chan, 1992) and regarded a potential drug transporter. However, later studies showed that *ABCB11/abcb11* encodes the hepatic bile salt export pump (BSEP) (Gerloff et al., 1998; Stieger et al., 2007). The cloning and functional characterisation of Abcb11 in the elasmobranch *Raja erinacea* (Cai et al., 2001)

demonstrated transport activity with taurocholate, suggesting the functional conservation of Abcb11 across the vertebrates.

2.2. ABCC/Abcc subfamily

The ABCC/Abcc subfamily is large and complex, containing both transporters and proteins with other roles. ABCC1 and ABCC2 are well-studied drug efflux transporters (Schinkel and Jonker, 2003; Leslie et al., 2005). While ABCC3–5 and ABCC10 and 11 are capable of drug transport in vitro, little further evidence exists for roles of ABCC10 and 11 as factors in the biochemical defence against toxicants (Deeley et al., 2006; Slot et al., 2011). A number of vertebrates possess a further MRP (Mrp10/Abcc13), which has undergone pseudogenisation in human (Annilo and Dean, 2004; Annilo et al., 2006). ABCC proteins that are not transporters include the cystic fibrosis transmembrane conductance regulator (CFTR, also called ABCC7), which is a chloride channel, and the sulfonylurea receptors (SUR1, SUR2, also called ABCC8 and ABCC9, respectively), which are regulators of potassium channels (Riordan et al., 1989; Hibino and Kurachi, 2006; Aleksandrov et al., 2007; Bryan et al., 2007). Moreover, ABCC6 and ABCC12 are likely not drug transporters (Slot et al., 2011). Loss-of-function mutations of human ABCC6 are associated with a rare genetic disorder called PXE (pseudoxanthoma elasticum) (Bergen et al., 2007). ABCC12 is a protein of unknown function expressed in testicular germ cells and sperm (Ono et al., 2007).

An evolutionary analysis of the vertebrate ABCC/Abcc family was first carried out taking into account all members (Fig. S1). In the obtained phylogenetic tree, ABCC/Abcc proteins grouped into distinct clusters corresponding to individual isoforms (Fig. S1), suggesting that the divergence of different *ABCC/abcc* paralogues is likely ancient and has occurred in a common ancestor of vertebrates. All teleosts studied had at least one Abcc6, Abcc7 (CFTR), Abcc8 (SUR1) and Abcc9 (SUR2) member, and orthologues of each of these non-drug transporter ABCCs/Abccs formed well supported clades (Fig. S1). A distinct clade with high bootstrap support was also formed by ABCC10/Abcc10 members, whereas proteins labelled ABCC11/Abcc11 and ABCC12/Abcc12 combined in one clade (Fig. S1). In order to obtain a tree of manageable size, the analysis was re-run with the main drug-transporting ABCC/Abcc isoforms, ABCC1-5/Abcc1-5 (Fig. 3). Each of the available teleost genomes contains one orthologue of each Abcc1-3 and Abcc5, whereas in some teleost species there are multiple isoforms of Abcc4 (Fig. 3). The occurrence of different numbers of isoforms in the different teleost species raises questions about their specific functions, to which extent functions of the isoforms differ and whether functions of certain isoforms are homologous across species. A cDNA encoding an Abcc2 homologue was isolated from rainbow trout liver (Zaja et al., 2008b). mRNA expression profiles of *abcc* isoforms in rainbow trout (*Oncorhynchus mykiss*) are generally comparable to those of their counterparts in mammals (Loncar et al., 2010). Previously, an Abcc2 homologue had been cloned from the elasmobranch small skate (*Raja erinacea*), where it showed apical expression in liver, kidney and intestine, paralleling the tissue distribution and localisation of ABCC2 in mammals (Cai et al., 2003).

2.3. ABCG/Abcg subfamily

The ABCG/Abcg subfamily contains the drug transporter ABCG2/Abcg2, as well as members involved in sterol metabolism (ABCG/Abcg1, 4, 5 and 8) (Wang et al., 2004; Hazard and Patel, 2007). In phylogenetical analyses, all teleost Abcg sequences could be clearly affiliated to one of the above ABCG paralogues (Fig. S2). To obtain a tree of manageable size, the analysis was rerun including ABCG2/Abcg2 homologues only (Fig. 4). Teleosts show duplications of *abcg2*, with two isoforms being present in medaka, stickleback, tilapia, green puffer and pufferfish, and four in cod and zebrafish (Fig. 4). In synteny analyses, human *ABCG2* showed synteny to *abcg2a* of green puffer and to *abcg2d* of zebrafish, but not to the remaining *abcg2* homologues of
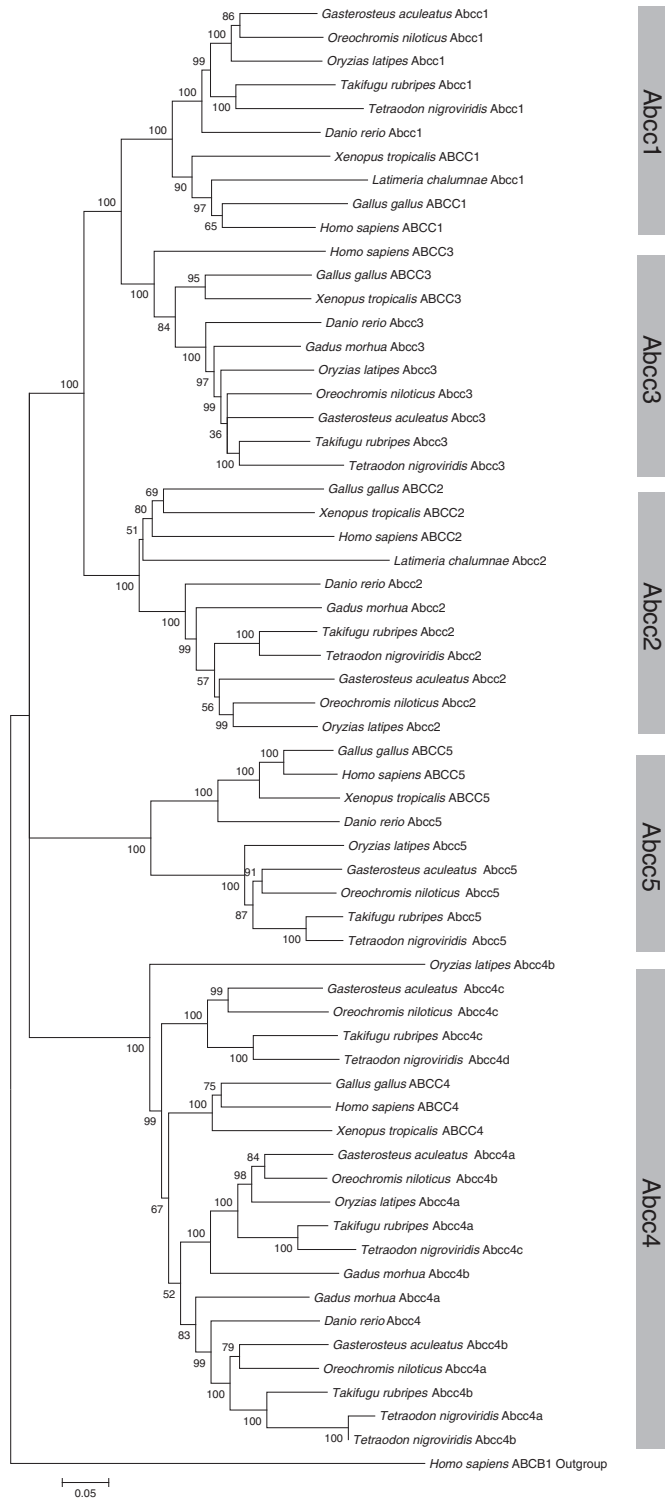
Fig. 3. Phylogenetic analysis of vertebrate ABCC1-5/Abcc1-5 transporters. Included in the analysis are transporter amino acid sequences from teleosts (*Danio rerio, Gasterosteus aculeatus, Gadus morhua, Oreochromis niloticus, Oryzias latipes, Takifugu rubripes, Tetraodon nigroviridis*) and other vertebrates. For further details see the legend of Fig. 2.

3. Functional activity and expression of ABC drug transporters in fish

The measurement of transport activity of ABC drug transporters usually involves monitoring the translocation of a conveniently detectable model substrate in a suitable in vitro model (Calcagno et al., 2007). Parallel treatments with inhibitors are included to confirm the identity of the involved transporters. Results obtained with model substrates and inhibitors need to be interpreted with caution, as different classes of ABC pumps overlap in substrate and inhibitor specificity (Calcagno et al., 2007). Moreover, the assumed specificities of "selective" compounds have usually been established in mammalian systems and may not necessarily apply in fish. The specificities of the most commonly used substrates and inhibitors are briefly reviewed here to provide the background for the understanding of specific findings in fish reviewed below.

A number of substrates initially described as selective for ABCB1 have later been shown to interact with other ABC transporters. For instance, the fluorescent ABCB1 substrates rhodamine 123 and doxorubicin are also transported by ABCC1 (Barrand et al., 1993; Twentyman et al., 1994; Yeheskely-Hayon et al., 2009). Doxorubicin was further shown to be also transported by ABCG2, which further interacts with the ABCB1 substrate Hoechst 33342 (Robey et al., 2004; Krishnamurthy and Schuetz, 2006). Calcein-acetoxymethyl ester (Calcein-AM) is a non-charged non-fluorescent substrate for both ABCB1 and ABCCs. After cellular uptake, calcein-AM undergoes enzymatic hydrolysis to the anionic fluorophore calcein, which is transported by ABCCs but not ABCB1 (Essodaigui et al., 1998). Pheophorbide a is regarded a specific substrate of ABCG2 (Robey et al., 2004). In order to obtain selective probes for particular ABC transporters, fluorescent derivatives of drug substrates have been prepared (Calcagno et al., 2007). BODIPY-FL-verapamil, initially regarded as selective for ABCB1, has been shown to also be transported by ABCC1 (Crivellato et al., 2002). Fluorescein-methotrexate is regarded a specific substrate of ABCC transporters (Masereeuw et al., 2000), whereas fluo-cAMP has been described as a specific probe for ABCC4 (Reichel et al., 2007).

The different classes of ABC transporters also overlap regarding their specificity to inhibitors. The inhibitors verapamil and cyclosporin A interact with both ABCB1 and ABCCs (Barrand et al., 1993; Zaman et al., 1994), with median effective concentrations only slightly lower in ABCB1-overexpressing cell lines than in cells showing increased levels of ABCC1 (Holló et al., 1996). Compared to the parent compound, the cyclosporin A derivative PSC-833 shows an improved but not complete specificity towards ABCB1 (Leier et al., 1994). The compound tariquidar has been shown to inhibit both ABCB1 and ABCG2 (Gardner et al., 2009). Inhibitors described as specific for ABCB1 include LY335979 (Shepard et al., 2003) and the hydrophobic peptides reversin 121 and 205 (Sharom et al., 1999). The leukotriene LTD_4 receptor antagonist MK571 is a specific inhibitor of ABCC transporters (Gekeler et al., 1995), whereas fumitremorgin C and Ko134 have been described as selective ABCG2 inhibitors (Allen et al., 2002; Robey et al., 2004).

Different types of teleost tissue preparations have been used for transport measurements. Polarised epithelia can be mounted in Ussing chambers, allowing to establish rates of basolateral-to-apical and apical-to-basolateral substrate translocation under different conditions (Sussman-Turner and Renfro, 1995). In a number of marine teleosts, isolated kidney tubules reseal spontaneously in vitro, so that the uptake of fluorescent substrates from cell culture media and their secretion into the tubular lumen can be observed using confocal microscopy (Miller, 1987). In non-differentiated cells, ABC drug transporter activities can be established as the difference in substrate accumulation between parallel treatments differing in the presence or absence of a suitable inhibitor, reflecting that the activity of ABC drug transporters can limit the accumulation of substrates. In this approach, levels of the fluorescent substrate can be determined either on cell monolayers following extraction (Sturm et al., 2001b), or by flow cytometry using cells in suspension (Kobayashi et al., 2008).
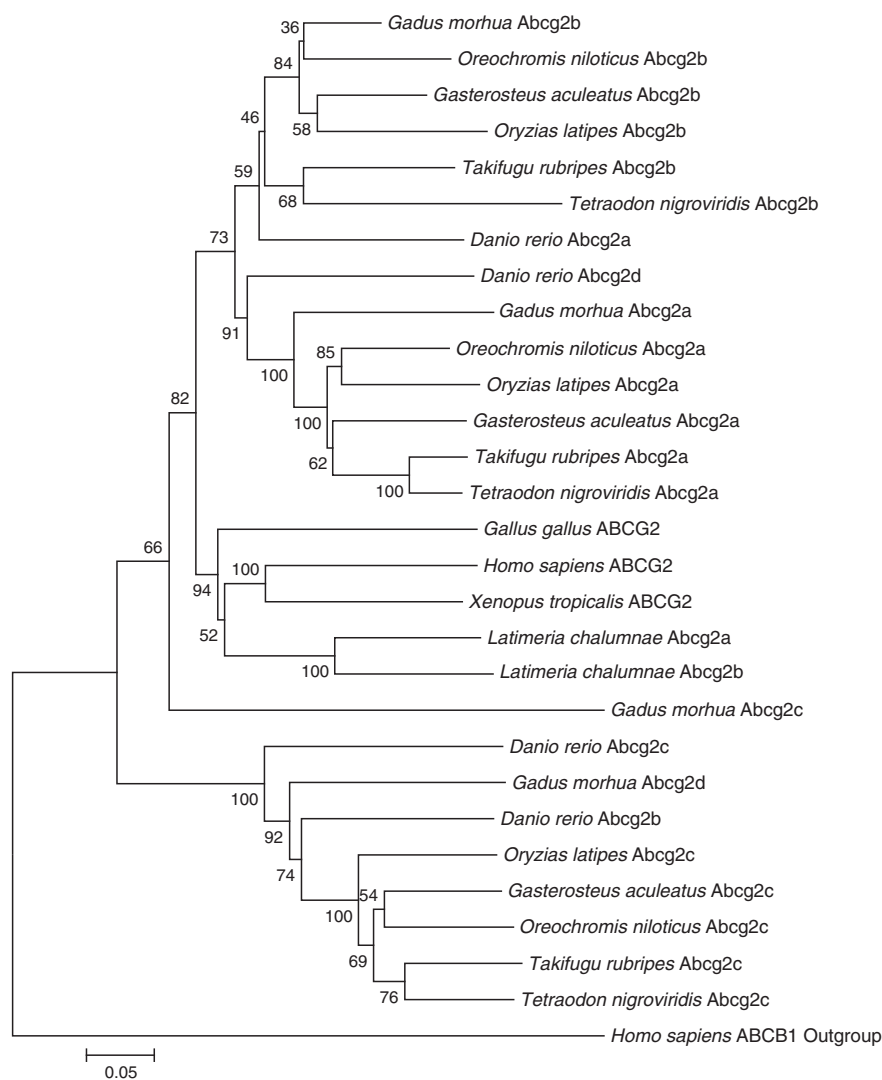
these teleost species (Fig. 5). Results in medaka, stickleback, tilapia, pufferfish and in cod resembled that in green puffer, with *Abcg2a* being the only Abcg2 isoforms showing synteny to ABCG2 (data not shown).

Fig. 4. Phylogenetic analysis of vertebrate ABCG2/Abcg2 transporters. Included in the analysis are transporter amino acid sequences from teleosts (*Danio rerio, Gasterosteus aculeatus, Gadus morhua, Oreochromis niloticus, Oryzias latipes, Takifugu rubripes, Tetraodon nigroviridis*) and other vertebrates. For further details see the legend of Fig. 1.

As reviewed in detail in the subsequent sections, teleost tissues in which the expression and activity of ABC drug transporters has been ascertained include the kidneys, the liver, the intestine and the capillary endothelia forming the blood–brain barrier (Fig. 6). Selected model substrates and inhibitors used in transport activity measurements in teleost tissues are summarised in Table 1. Roles of ABC drug transporters in further teleost tissues are likely to exist but require further investigation.

3.1. Kidney

In teleosts, the kidneys have central roles in the maintenance of the internal electrolyte and acid–base balances (Marshall and Grosell, 2006) and in the excretion of metabolic waste products and chemicals of endogenous or foreign origin (Kleinow et al., 2008). Glomerular ultrafiltration and tubular active secretion contribute to the renal excretion of organic chemicals. Glomerular filtration rates are greatly reduced in euryhaline teleosts adapted to seawater, and some marine teleosts possess nephrons lacking glomeruli (Beyenbach, 2004). Within the nephron, the secretion of organic chemicals occurs mainly in the proximal tubule, which is composed of cuboid epithelial cells rich in mitochondria and possessing a dense layer of microvilli on their apical surfaces. In a number of marine teleosts, nephrons consist mainly of proximal tubules (~90% of nephron length), of which in vitro preparations can be

obtained that allow the study of transport of fluorescent model compounds by confocal microscopy (Miller, 1987).

Historically, different transport systems have been functionally characterised in the kidney tubule for "organic cations" (including bases) and "organic anions" (including acids) (Wright and Dantzler, 2004), with both types of systems involving two transmembrane transport steps, basolateral uptake and apical secretion. Members of the SLC superfamily have important roles in these systems; however, these transporters are beyond the scope of this article (for reviews, please refer to El-Sheikh et al. (2008); Koepsell et al. (2007); Wright and Dantzler (2004)). In addition, ABC transporters mediate the secretion of bulkier organic ions into the renal tubule (Wright and Dantzler, 2004).

The transport of the organic base daunomycin (Mw 528 Da) into proximal tubule lumen was inhibited by verapamil and cyclosporin A in winter flounder (*Pleuronectes americanus*) (Sussman-Turner and Renfro, 1995) and killifish (*Fundulus heteroclitus*) (Miller, 1995), consistent with a mechanism involving Abcb1 (Table 1). Immunostaining of flounder proximal tubule primary cultures with antibody C219 showed a signal located to apical microvilli (Sussman-Turner and Renfro, 1995). At pH 8.25, luminal daunomycin secretion in killifish proximal kidney tubules was resistant to inhibition by tetraethylammonium (TEA; Mw 130), a "type I" organic cation (Mw < 400) and model substrate for
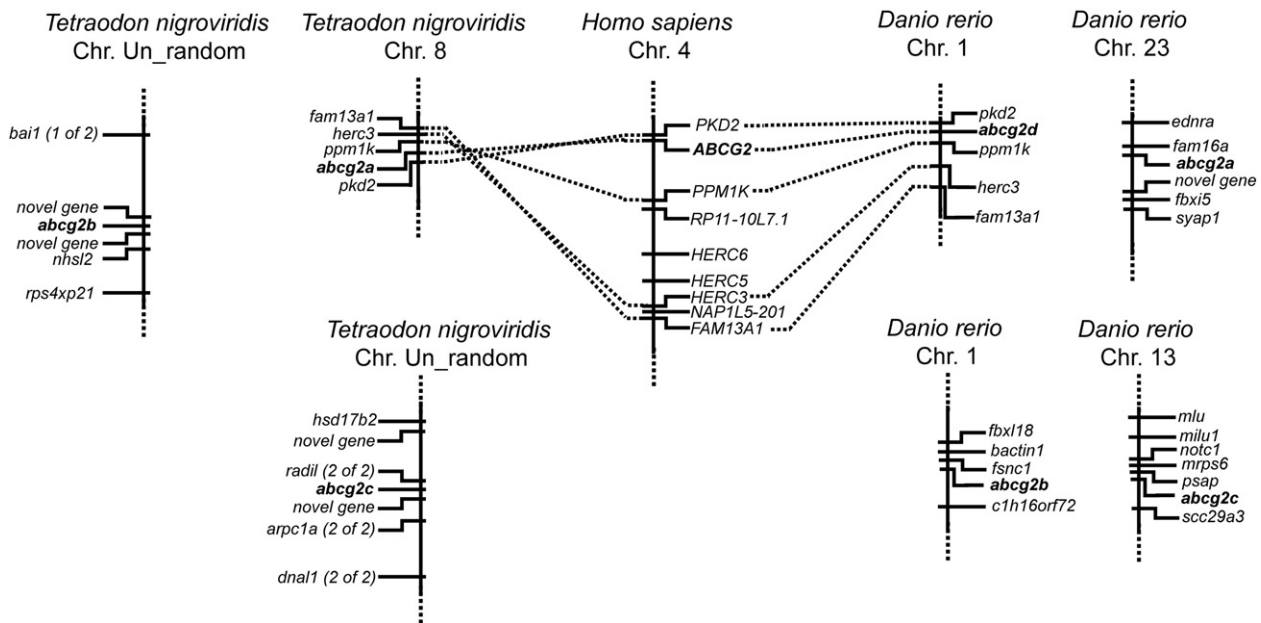
Fig. 5. Conserved synteny of the human (*Homo sapiens*) *ABCG2* region to homologous regions in teleost genomes. Dotted lines illustrate that the human *ABCG2* region is syntenic to green spotted pufferfish *(Tetraodon nigroviridis)* abcg2a and zebrafish *(Danio rerio)* abcg2d.

"classical" organic cation transport systems (Miller, 1995). When the pH of the media was decreased to 7.25, however, TEA caused a reduction in cellular and luminal accumulation of daunomycin (Miller, 1995). This finding indicated that at the lower pH "classical" organic cation transport systems became involved in daunomycin transport in addition to the Abcb1-like mechanism, which is in line with the higher proportion of cationic daunomycin expected at pH 7.25 when compared to pH 8.25 (Miller, 1995).

The secretion of the large organic anion fluorescein-methotrexate (Mw 923 Da) to killifish proximal tubular lumen was inhibited by LTC$_4$, cyclosporin A and verapamil, but remained unaffected by glutarate and ouabain, and was largely sodium independent (Masereeuw et al., 1996) (Table 1). Accordingly, basolateral uptake and luminal secretion of fluorescein-methotrexate were by mechanisms distinct from the ouabain-sensitive, sodium-dependent "classical" transport system involving exchange for dicarboxylates that had previously been described for small organic anions such as fluorescein (Mw 130 Da) (Masereeuw et al., 1996). The inhibitory effects of LTC$_4$ and cyclosporin A suggested the luminar secretion of fluorescein-methotrexate by a teleost homologue of ABCC2 (Miller and Pritchard, 1997), an ABC drug transporter known to show apical expression in the mammalian proximal tubule (Table 1). Supporting this interpretation, the luminal membrane of killifish proximal tubule showed immunoreactivity against an antibody

raised against rabbit ABCC2 (Masereeuw et al., 2000). Examination of the transport of other organic ions suggested the presence of different transport systems in killifish tubules. In addition to activity with the "classical" transport system, transport by an ABCC2-like efflux mechanism could be demonstrated for sulforhodamine 101 (Mw 606 Da) (Masereeuw et al., 1996) and lucifer yellow (Mw 444 Da) (Masereeuw et al., 1999) (Table 1). In addition, the presence of a putative teleost ABCC4 homologue in killifish kidney was suggested by the cellular-to-luminar transport of a fluorescent cAMP analogue, which was inhibited by the general MRP inhibitors MK571 and LTC$_4$ and the ABCC4-specific compounds cAMP and adefovir (Reichel et al., 2007) (Table 1).

The teleost proximal tubule system was subsequently applied to identify classes of renal drug efflux transporters interacting with selected pharmaceuticals by studying custom-made fluorescent analogues of the relevant drugs. ABCB1-like transport was demonstrated for fluorescent derivatives of the anthelminth ivermectin and the immunosuppressants cyclosporin A and rapamycin (Schramm et al., 1995; Miller et al., 1997; Fricker et al., 1999) (Table 1). The HIV protease inhibitors ritonavir and saquinavir inhibited ABCB1- and ABCC2-like transport in killifish kidney tubules, and experiments with a fluorescent analogue of saquinavir further suggested that this compound is transported by both transporters (Gutmann et al., 1999). Using a
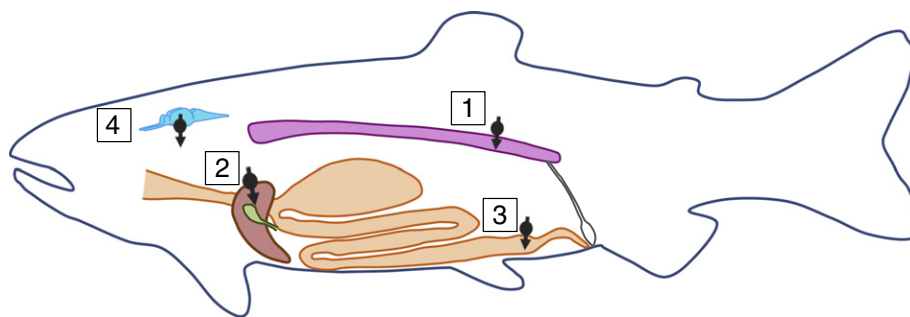
Fig. 6. Expression and activity of ABC drug transporters in teleost tissues. Currently available data support the view that ABC drug transporters are involved in the renal (1) and biliary (2) excretion of organic chemicals and limit the uptake of food-borne chemicals in the gut (3). Moreover, ABC drug transporters in the endothelial capillaries constituting the blood–brain barrier contribute to the low penetration of foreign chemicals into the brain (4). ABC drug transporters could potentially have roles in further teleost tissues including the gill epithelium and the blood–gonadal barriers; however, more research is required to substantiate or refute such roles.

Table 1
Measurement of ABC transporter-related drug efflux activities in teleost tissues. The table provides an overview of systems in which the activity of teleost ABC transporters has been measured, providing details regarding the tissue preparation and species used, the transporter activity recorded and the model substrates and inhibitors applied.

Preparation	Species	Transporter	Substrates	Inhibitors	Study
Isolated renal proximal tubules	Killifish (*Fundulus heteroclitus*)	ABCB1-like	Daunomycin, NBD-cyclosporine A, BODIPY-ivermectin	Verapamil, cyclosporin A, vinblastine, PSC-833	Miller, 1995; Schramm et al., 1995; Fricker et al., 1999
		ABCC2-like	Fluorescein-methotrexate, lucifer yellow	Probenecid, bromosulfophtalein	Masereeuw et al., 1996, 1999
		ABCC4-like	Fluo-cAMP	MK571, LTC$_4$, cAMP, adefovir	Reichel et al., 2007
Isolated hepatocytes	Rainbow trout (*Oncorhynchus mykiss*)	ABCB1-like	Rhodamine 123, calcein-AM, BODIPY-verapamil, doxorubicin	Verapamil, cyclosporin A, vinblastine, reversin 205	Sturm et al., 2001b; Zaja et al., 2008b
Intestine membrane vesicles	Channel catfish (*Ictalurus punctatus*)	ABCB11-like	Dihydrofluorescein diactetate	taurocholate, taurochenodeoxycholate	Zaja et al., 2008b
		ABCC2-like	Rhodamine 123, calcein-AM	MK571, verapamil	Zaja et al., 2008b
		ABCB1-like	[3H] vinblastine	Verapamil	Doi et al., 2001
Isolated brain capillaries	Killifish (*Fundulus heteroclitus*)	ABCB1-like	NBD-cyclosporin A, BODIPY-verapamil	PSC-833	Miller et al., 2002
		ABCC2-like	Fluorescein-methotrexate	LTC$_4$	Miller et al., 2002
Embryo	Zebrafish (*Danio rerio*)	Abcb4 (ABCB1-like)	rhodamine B, calcein-AM, BODIPY- vinblastine	cyclosporin A, PSC-833, MK571	Fischer et al., 2013

fluorescent derivative of the somatostatin analogue octreotide, both ABCB1- and ABCC2-like transport of the compound could be measured in killifish kidney tubules (Gutmann et al., 2000).

3.2. Liver

The teleost liver has major roles in the homeostasis of amino acids, carbohydrates and fatty acids. Moreover, numerous proteins are synthesised in the liver and secreted into blood, for instance albumin-like proteins, fibronectins and vitellogenin (Hinton et al., 2008). In addition, the liver plays a key role in the detoxification of endogenous and foreign compounds, including many organic chemicals having relevance as environmental pollutants (Schlenk et al., 2008). Hepatocytes are in contact with blood perfusing the hepatic parenchyma at their sinusoidal (basolateral) membranes, at which blood-borne chemicals can be taken up. In the hepatocyte, chemicals may undergo phase I and II biotransformation metabolism (Schlenk et al., 2008). At the same time, chemicals or their metabolites may be subject to efflux transport by canalicular ABC pumps, resulting in biliary excretion. The importance of the biliary route of excretion is illustrated by the large range of compounds found in fish bile, which includes industrial chemicals such as chlorophenols (Oikari and Kunnamo-Ojala, 1987), combustion products such as polycyclic aromatic hydrocarbons (Collier and Varanasi, 1991) and agricultural compounds such as pesticides (Lech et al., 1973; Bradbury et al., 1986). Alternatively, chemicals or their metabolites can leave the hepatocyte by being transported back into the blood by sinosoidal ABC transporters, after which they may be subject to renal excretion.

In mammals, the hepatic ABC drug transporters ABCB1, ABCC2 and ABCG2 localise to the canalicular membrane, while ABCC1 and 3 are expressed in the sinusoidal membrane (Chan, 2004). Other canalicular ABC transporters mediate the secretion of bile acids (ABCB11), phospholipids (ABCB4) and cholesterol (ABCG5/ABCG8) into the bile fluid (Hazard and Patel, 2007; Oude Elferink and Paulusma, 2007; Stieger et al., 2007). Although not drug transporters, these ABC proteins contribute to maintaining bile flow and thus facilitate chemical excretion, and for this reason existing studies on these pumps in fish will be considered in this section.

In immunohistochemical investigations of guppy (*Poecilia reticulata*) tissues using antibodies raised against mammalian P-glycoprotein, monoclonal antibodies C219 and JSB-1 specifically stained bile canaliculi (Hemmer et al., 1995). Subsequently, C219 has been widely used to detect hepatic P-glycoprotein in teleosts, in which it stained canalicular structures in immunohistochemical experiments, and detected protein fractions of the expected molecular mass of P-glycoprotein of ~170 kDa in immunoblots (Hemmer et al., 1998; Cooper et al., 1999; Albertus and Laine, 2001; Sturm et al., 2001b; Bard et al., 2002b). However, as the epitope recognised by C219 (VQEALD/VQAALD) is conserved in ABCB4 and ABCB11 (Georges et al., 1990), as well as in Abcb11 from winter flounder (*Pleuronectes americanus*) (Chan, 1992), C219 can be expected to cross-react with ABC transporters other than Abcb1. The bile salt export pump Abcb11 shows high mRNA expression in teleost liver (Loncar et al., 2010), and could therefore contribute to the hepatic signal detected by C219 in teleosts.

Data on the hepatic mRNA expression of ABC drug transporters are available for a number of teleost and elasmobranch species. Tutundjian et al. (2002) cloned a fragment of *abcb1* in turbot (*Scophthalmus maximus*) and demonstrated expression of its mRNA in brain, intestine, kidney and liver by RT-PCR. A cDNA encoding an *abcc2* homologue was cloned in the elasmobranch little skate (*Raja erinacea*), and showed high expression levels in kidney, intestine and liver, where it located to the canalicular membrane (Cai et al., 2003). The group of Smital and co-workers isolated cDNAs of *abcb1* and *abcc2* from rainbow trout liver (Zaja et al., 2008b), and reported mRNA copy numbers per ng of total liver RNA of 8.61×10^2 for *abcb1*, 1.29×10^3 for *abcc2*, 2.55×10^1 for *abcc3* and 2.78×10^2 for *abcg2* (Loncar et al., 2010). Expression of

abcc1, *abcc4* and *abcc5* was also analysed, but found to be low in liver (<5 copies/ng of total RNA) (Loncar et al., 2010). The hepatic mRNA expression of *abcb1*, different *abcc* paralogs, and *abcg2* has been confirmed in a number of further teleost species in the context of ecotoxicological studies (Bresolin et al., 2005; Paetzold et al., 2009; Zucchi et al., 2010; Costa et al., 2012; Diaz de Cerio et al., 2012), which will be discussed in detail below.

The transport activity of hepatic ABC drug transporters in teleosts has been investigated by assessing the effects of selective inhibitors on the accumulation, efflux or transport of fluorescent or radiolabelled model substrates, employing experimental models including whole animals, livers perfused in situ, primary cultures of isolated hepatocytes and membrane vesicles. The waterborne exposure of common carp (*Cyprinus carpio*) to 3 mM rhodamine B resulted in a time-dependent accumulation of the dye in liver and bile fluid over 3 hours (Smital and Sauerborn, 2002). When rhodamine B was combined with the ABCB1 inhibitors verapamil or cyclosporin A this resulted in elevated hepatic and biliary dye accumulation compared to fish exposed to the fluorescent probe alone (Smital and Sauerborn, 2002). In channel catfish (*Ictalurus punctatus*) in situ liver perfusions, transport of rhodamine 123 from perfusate to bile was significantly decreased in the presence of verapamil (Kleinow et al., 2004). Verapamil also inhibited perfusate-to-bile transport of estradiol, which in turn competed with rhodamine 123 transport (Kleinow et al., 2004). Similarly, verapamil enhanced the accumulation of doxorubicin by killifish (*Fundulus heteroclitus*) hepatocytes (Albertus and Laine, 2001), and verapamil, cyclosporin A, doxorubicin and vanadate increased the accumulation of rhodamine 123 by rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Sturm et al., 2001b) (Table 1). While the above findings were interpreted as evidence for the presence of ABCB1-like transporters in the teleost liver, a later study employing further inhibitors (Abcb1: reversin 205, Abcc: MK571) demonstrated that both ABCB1- and ABCC-like transporters are involved in the transport of rhodamine 123 in trout hepatocytes (Zaja et al., 2008b) (Table 1). Two further studies with trout hepatocytes used the inhibitor tariquidar in combination with rhodamine 123 or doxorubicin to measure transport activity attributed to Abcb1 (Bains and Kennedy, 2005; Hildebrand et al., 2009). While the ABCB1 inhibitor tariquidar does not inhibit ABCCs, it can interact with ABCG2 (Robey et al., 2004). In the elasmobranch little skate, liver membrane vesicles were used to investigate ABCC-like transport activity (Rebbeor et al., 2000). The uptake of tritiated *S*-dinitrophenyl-glutathione and glutathione showed an ATP-dependent component that was inhibited by ABCC substrates and GSH (Rebbeor et al., 2000).

Relatively little is known on fish ABC transporters involved in the secretion of bile constituents. Conjugated bile salts are found in the bile of all vertebrates, but different vertebrate groups show distinct differences regarding the subclass composition of bile salts present in bile fluid (Moschetta et al., 2005). The *abcb11* gene, which encodes the hepatic bile salt export pump (BSEP), was originally isolated in a teleost (Chan, 1992; Childs et al., 1995; Gerloff et al., 1998), suggesting a conservation of *abcb11* across the vertebrates. In the elasmobranch little skate (*Raja erinacea*), an antiserum raised against rat ABCB11 reacted with proteins of an apparent mass of 210 kDa in immunoblots and resulted in the immunostaining of bile canaliculi (Ballatori et al., 2000). Subsequently, little skate *abcb11* was cloned and shown to transport taurocholate upon transfection into Sf9 cells (Cai et al., 2001). In rainbow trout, *abcb11* was highly expressed in liver (Loncar et al., 2010), and BSEP-like activity was detectable in cultured hepatocytes (Zaja et al., 2008b) (Table 1). Cholesterol is found in bile fluid in different vertebrate groups, however it can greatly vary in concentration (Moschetta et al., 2005). While the locus encoding the mammalian hepatic cholesterol transporters ABCG5 and ABCG8 is highly conserved between mammals and teleosts (Hazard and Patel, 2007), no direct evidence is available regarding the hepatic expression and functional activity of Abcg5 and Abcg8 in fish. Phospholipids, which are absent from the bile of elasmobranchs and reptiles, are lacking or show very low

concentrations in the bile of teleosts (Goto et al., 2003), and are found at widely variable levels in the bile of different mammalian species (Oude Elferink et al., 2004; Moschetta et al., 2005). Apparently there is no active translocation of phospholipids into teleost bile indicating the absence of functional homologues to mammalian ABCB4 in teleosts. On the basis of available genomic data, some studies have suggested that teleosts lack an *ABCB4* orthologue (Annilo et al., 2006; Moitra et al., 2011; Liu et al., 2013), while others came to the conclusion that teleosts possess an *ABCB4* orthologue resembling mammalian *ABCB1* in function (Fischer et al., 2013).

3.3. Intestine

The gastrointestinal tract of fish has multiple functions, which include digestion and nutrient absorption, osmoregulation and maintenance of the acid–base balance, and barrier and immune functions (Marshall and Grosell, 2006; Bakke et al., 2011; Cain and Swan, 2011). As in other animals, the intestine is a potential site of uptake of chemical compounds other than nutrients from food (Kleinow et al., 2008), which include anthropogenic contaminants as well as toxic chemicals from natural sources. As an adaptation limiting potential adverse effects of food-borne toxicants, biochemical defence systems, including biotransformation enzymes and drug transporters, are expressed in the intestinal epithelium (Chan, 2004; Schlenk et al., 2008). In mammals, the intestine is a major site of expression of ABC drug transporters, some of which limit the oral absorption of drugs that are transport substrates (reviewed by Oostendorp et al., 2009). Relatively little is known about the expression and activity of ABC transporters in the gastrointestinal system of fish. The immunohistochemical staining of lumenal surfaces of the intestinal epithelium in guppy (*Poecilia reticulata*) suggested the expression of ABCB1-like proteins in the teleost gut (Hemmer et al., 1995). In channel catfish (*Ictalurus punctatus*), reactivity with mAB C219 in immunoblots was lower in the intestine than in the liver (Doi et al., 2001), whereas immunohistochemistry revealed a more pronounced expression of reactive protein(s) in the distal than the proximal regions of the gut (Kleinow et al., 2000). [^{3}H] vinblastine uptake of membrane vesicles prepared from catfish intestinal mucosa was stimulated by ATP and inhibited by verapamil, consistent with the presence of an ABCB1-like transporter (Doi et al., 2001) (Table 1). Among different rainbow trout (*Oncorhynchus mykiss*) ABC drug transporters, *abcb1*, *abcc2*, *abcc3* and *abcg2* showed major mRNA expression in the intestine, whereas *abcc1*, *abcc4* and *abcc5* mRNAs were present at low abundances (<15 copies per ng of total RNA) (Loncar et al., 2010). Of these transporters, *abcb1*, *abcc2*, and *abcg2* showed higher levels of mRNA expression in the distal than the proximal intestine (Loncar et al., 2010).

3.4. Blood–brain barrier and blood–cerebrospinal fluid barrier

To ensure optimal functioning of the vertebrate central nervous system (CNS), the chemical composition of its extracellular fluids is under tight physiological control and the movement of molecules between blood and CNS is restrained at anatomical interfaces such as the blood–brain barrier and the blood–cerebrospinal fluid barrier (Redzic, 2011). The blood–brain barrier is comprised of the endothelia of the brain capillaries and separates blood from brain interstitial fluid (Cserr and Bundgaard, 1984). Its function is to protect the brain from potentially neurotoxic compounds and prevent its exposure to the physiological fluctuations in concentrations of plasma solutes while at the same time allowing for the exchange of ions, nutrients, metabolic waste products and signalling molecules between blood and brain interstitial fluid (Redzic, 2011). The endothelial cells forming the blood–brain barrier have low pinocytotic activity and possess tight junctions interconnecting adjacent cells, which foreclose the paracellular passage of molecules (Ueno, 2009; Redzic, 2011). In consequence, the capillary brain endothelium shows a high transendothelial electrical resistance

in the range of 1500 W cm^2, and is in this respect reminiscent of tight epithelia (Crone and Christensen, 1981). Besides constituting an anatomical barrier, brain endothelial cells actively mediate the transport of ions, nutrients, neurotransmitters and metabolic waste products across the endothelium through a multitude of transporters expressed at the apical (blood side) and/or basolateral (brain side) cell membranes (Miller, 2010; Redzic, 2011). In mammals, ABC drug transporters showing an apical expression at the blood–brain barrier comprise ABCB1, ABCC2, ABCC4, ABCC5 and ABCG2 (Ueno, 2009; Redzic, 2011). ABCC1 is present in brain endothelial cells but its subcellular localisation is still a matter of debate (Dallas et al., 2006; Redzic, 2011).

Early studies have characterised the teleost blood–brain barrier as tight based on its impermeability for dyes, inulin, iodine and horseradish peroxidase (HRP); however, conflicting results suggesting a less restrictive barrier function were obtained with epinephrine and thiocyanate (Cserr and Bundgaard, 1984). Recent studies confirmed the presence of a fully functional blood–brain barrier in zebrafish using HRP, a biotinylation agent and an ectopically expressed recombinant protein as markers and demonstrated the presence of claudin-5 and other tight-junction proteins in the microvessels (Jeong et al., 2008; Xie et al., 2010). A role of ABC transporters in the teleost blood–brain barrier is suggested by the observation that inhibitors of ABC drug transporters increase the retention of rhodamine 123 in the zebrafish brain (Park et al., 2012).

The high therapeutic margin of the anti-parasitic drug ivermectin in mammals is partly explained by its selectivity for ecdysozoan GABA- and glutamate-gated ion channels (Lynagh and Lynch, 2012), and partly due to the fact that ABCB1 activity in the brain capillary endothelia limits the brain penetration of ivermectin, preventing interaction with related vertebrate ion channels expressed in the central nervous system (Schinkel et al., 1994). Relatively high brain levels of ivermectin were measured in teleost fish following parenteral administration (Høy et al., 1990; Katharios et al., 2004). While this suggests a limited efficacy of the teleost blood–brain barrier towards ivermectin, co-administration of ivermectin with the ABCB1 inhibitor cyclosporin A resulted in increased adverse behavioural effects compared to a treatment with ivermectin alone (Bard and Gadbois, 2007), suggesting that ABC pumps of the teleost blood–brain barrier provide a partial protection against ivermectin neurotoxicity.

Further evidence for roles of ABC transporters in the teleost blood–brain barrier is provided by in vitro studies. Isolated killifish brain capillaries accumulated ABC transporter substrates from incubation media and secreted the compounds into the capillary lumen in a concentrative fashion (Miller et al., 2002). The transport of fluorescent derivatives of cyclosporin A and verapamil was inhibited by PSC-833 but not LTC$_4$, which is consistent with an Abcb1-dependent luminal transport mechanism (Miller et al., 2002) (Table 1). In contrast, luminal accumulation of fluorescein-methotrexate and sulforhodamine 101 was inhibited by LTC$_4$ but not PSC-833, suggesting the involvement of Abcc transporters (Miller et al., 2002) (Table 1). Similar luminal accumulation of fluorescent derivatives of cyclosporin A, verapamil and methotrexate was demonstrated in isolated brain capillaries of spiny dogfish shark (*Squalus acanthias*) (Miller et al., 2002). Immunostaining using mAB C219 and an antibody directed against rabbit ABCC2 localised ABCB1-like and ABCC2-like proteins to the luminal surface of killifish brain capillaries (Miller et al., 2002). In accordance with suggested roles in the blood–brain barrier, ABC transporters showing transcript expression in the rainbow trout brain were, in the order of decreasing abundance expressed as copy numbers per ng of total RNA, *abcb1* (1.38×10^3/ng), *abcc2* (1.38×10^2/ng), *abcg2* (1.02×10^2/ng), *abcc3* (8.4×10/ng), *abcc5* (2.65/ng), *abcc4* (1.68/ng) and *abcc1* (0.72/ng) (Loncar et al., 2010).

The main function of the choroid plexus (CP) epithelium is the secretion of cerebrospinal fluid (Cserr and Bundgaard, 1984). It consists of mitochondria-rich cuboid cells that are interconnected by tight junctions and possess microvilli on their apical sides facing the cerebrospinal

fluid. The endothelial cells of the capillary network underlying the CP epithelium on the basolateral side are fenestrated, so that the CP epithelium constitutes the site of the blood–cerebrospinal fluid barrier (Cserr and Bundgaard, 1984; Redzic, 2011). TEER estimates obtained on bullfrog CP explants mounted in Ussing chambers were in the range of 150 W cm^2, which is similar to values obtained in leaky epithelia (Saito and Wright, 1984). In mammals, ABC transporters expressed in the CP epithelium include ABCC1 and ABCC4, which adopt a basolateral localisation, ABCB1 and ABCG2, which are expressed apically, and ABCC5, the localisation of which remains to be elucidated (Redzic, 2011). While ABCC1 is the most abundant of ABC transporter in the CP epithelial cells, ABCB1 levels in the CP epithelium are low (Redzic, 2011).

No studies on the function of the CP exist in teleosts. However, the CP epithelium in the elasmobranch spiny dogfish shark (*Squalus acanthias*) is anatomically easily accessible and explants of the CP in this species have been used for in vitro transport studies using confocal microscopy (Baehr et al., 2006; Reichel et al., 2008). Fluorescein-methotrexate and sulforhodamine 101 were accumulated by CP cells from incubation media, provided at the cerebrospinal fluid side, and actively secreted into the subepithelial/vascular space (Baehr et al., 2006; Reichel et al., 2008). Both cellular accumulation and vascular secretion of the dyes were sensitive to inhibition by the ABCC inhibitors MK571 and LTC$_4$ (Baehr et al., 2006; Reichel et al., 2008).

3.5. Other tissues

In teleosts, the gill is a main site of gas exchange, osmoregulation and excretion of nitrogenous waste products (Evans et al., 2005). Due to the large surface of the gill epithelium, as well as the effective respiratory ventilation of water and circulation of blood, the gills represent a dominant site both for the absorption and the elimination of xenobiotics (Kleinow et al., 2008). Relatively little is known about the expression of ABC drug transporters in the fish gill. A study in guppy using mAB C219, directed against mammalian ABCB1 but also known to crossreact with other ABC transporters, reported a strong staining reaction in gill chondrocytes and an absence of signal in filaments and lamellae (Hemmer et al., 1995). In contrast, in gill tissue from killifish (*Fundulus heteroclitus*) or high cockscomb blenny (*Anoplarchus purpurescens*) no immunoreactive bands were visible in Western blots with C219 (Bard et al., 2002a,b). In a study with rainbow trout, ABC drug transporters showing major mRNA expression in gills were *abcc2* and *abcc3* when data were reported as copy numbers per mass unit of total RNA, whereas mRNAs encoding Abcc3, Abcg2 and to a lesser extent also Abcc5 were the most abundant ABC transcripts based on quantification relative to the reference gene *ef1a* (Loncar et al., 2010). No data are available on the activity of ABC transporters in the fish gill.

The rectal gland is a NaCl-secreting organ of marine elasmobranchs (Marshall and Grosell, 2006). In isolated rectal gland tubules from dogfish shark (*Squalus acanthias*), sulforhodamine 101 was taken up from incubation medium and secreted into tubule lumen, suggesting additional roles of the rectal gland in xenobiotic excretion (Miller et al., 1998a). Luminal secretion of sulforhodamine 101 in shark rectal gland tubules was saturable, concentrative and sensitive to inhibition by cyclosporin A and LTC$_4$, but not verapamil, p-aminohippurate or TEA, suggesting an involvement of Abcc pumps in the transport mechanism (Miller et al., 1998a).

In mammals, a number of ABC drug transporters are expressed in the blood–testis barrier and in the placenta (Leslie et al., 2005). Little is known about blood–gonadal tissue barriers in fish. A number of studies have found marked mRNA expression of ABC transporters in gonads of fish, which could be related to roles of the pumps in blood–tissue barriers. In rainbow trout, transcripts of *abcb1, abcc2–4* and *abcg2* were abundant in the ovaries (Loncar et al., 2010), whereas in zebrafish *abcc1* mRNA showed a marked expression in both male and female

gonads and *abcc5* mRNA was highly expressed in testis (Long et al., 2011a,b).

Evidence for the presence and activity of ABC drug transporters in the teleost epidermis was provided in investigations using a rainbow trout skin primary culture system (Shúilleabháin et al., 2005). In epidermal cell cultures, the efflux of rhodamine 123 was inhibited by verapamil. Moreover, a fraction of the cells in epidermal primary cultures displayed positive immunoreactivity with the mAB C219 (Shúilleabháin et al., 2005). Exposure of cultured trout epidermal cells to sediment elutriates from a polluted field site resulted in an increase of the number of intensely JSB1 positive cells and enhanced rhodamine 123 efflux activity (Shúilleabháin et al., 2005).

Mammalian stem cells show an increased expression and activity of ABC efflux transporters, which are used as markers of stem cell identification (Bunting, 2002). Side population cells from the hematopoietic tissue of zebrafish kidney were characterised by an increased Hoechst 33342 dye efflux activity and showed marked *abcg2a* expression (Kobayashi et al., 2008). Moreover, zebrafish kidney side population cells were enriched in hematopoietic stem cells (Kobayashi et al., 2008).

3.6. Fish embryos

Most teleosts are oviparous, with the complete embryonic development taking place outside the maternal organism. The developing "orphan" embryo thus is directly exposed to potentially adverse environmental conditions that could affect development and it is a common perception that this ontogenetic life phase is therefore particularly sensitive (McKim, 1985; Oberemm, 2000). Correlations of toxicity data for fish embryo and adult fish for various chemicals, however, show that sensitivities of the different life stages are generally highly comparable (Belanger et al., 2013), contradicting the notion of increased vulnerability of teleost embryos to environmental stressors as compared to adult stages. Indeed, despite the lack of differentiated organs "orphan" embryos across aquatic animal taxa employ various cellular defence mechanisms enabling them to deal with a range of stressors in development including changes in temperature, hypoxia, pathogens, UV radiation, free radicals, and toxicants (Hamdoun and Epel, 2007). One important component of the suite of defensive mechanisms are MDR transporters (Hamdoun and Epel, 2007), which are also expressed and active in teleost embryos. It was recently shown that the P-glycoprotein Abcb4 acts as protective barrier against the uptake of toxic compounds dissolved in the water by the zebrafish embryo (Fischer et al., 2013). Fish embryos where expression of functional Abcb4 was disrupted by morpholino knockdown or by co-exposure to ABC transporter inhibitors, such as cyclosporin A or PSC-833, showed increased uptake of fluorescent ABC transporter substrates rhodamine B and calcein-AM (Table 1) and their sensitivity to the toxic impact of the ABC transporter substrate vinblastine was increased (Fischer et al., 2013). By effluxing incoming chemicals Abcb4 keeps cellular levels of those compounds in embryo tissues and cells low and forms a multidrug-resistance type environment–tissue barrier, in analogy to endogenous blood–tissue barriers. *Abcb4* transcripts are found in the early embryo before de novo transcription starts, which indicates that they are maternally transferred to the embryo, and efflux activity can be observed already in the very early embryo one hour after fertilisation (Fischer et al., 2013). Similarly, transcripts of the MRPs *abcc1* (Long et al., 2011a) and *abcc5* (Long et al., 2011b) are maternally transferred to the zebrafish egg, whereas transcripts of *abcc2* occur not before 72 h postfertilisation, indicating that this transporter has no relevant function in the early embryo (Long et al., 2011d). The authors related a function of all three transporters Abcc1, Abcc2 and Abcc5 with heavy metal detoxification in zebrafish embryos and larvae (Long et al., 2011a,c,d), thus associating them to the suite of molecular detoxification systems in zebrafish early life stages. Constitutive transcript expression levels of ABC transporters were also determined in early life stages of Nile tilapia (*Oreochromis niloticus*). As in zebrafish, transcripts of P-glycoprotein

abcb1b and of *abcc1* were found in early developmental stages directly after fertilisation of the egg, whereas *abcc2*, together with *abcg2a* and *abcb11*, occurred in later stages (from pharyngula stage on) (Costa et al., 2012). The function of Abcb11 as bile salt export pump (BESP) in liver has been shown to be highly conserved across vertebrate taxa (Ballatori et al., 2000; Cai et al., 2001) and accordingly high *abcb11* transcript levels were found in teleost liver (Loncar et al., 2010); the occurence of *abcb11* transcripts in teleost embryos (Costa et al., 2012) may thus be associated with the appearance of the embryonic liver.

An interesting question regards whether ABC transporters have specific functions in the developing embryo that are distinct from the function in adult tissues. Indeed, a function essential for development of the zebrafish embryo was found for Abcc6a. The exact function of the mammalian orthologue ABCC6 is not clear, but null mutations of *ABCC6* are associated with the inherited disorder pseudoxanthoma elasticum (PXE), which is characterised by dystrophic mineralisation and fragmentation of soft connective tissues (Pfendner et al., 2007). In zebrafish embryos, expression of *abcc6a* is located in the Kupffer's vesicles and in the tail buds; upon knockdown of functional Abcc6a protein expression embryos at 1 day after fertilisation showed shortening of the body, delay of the development of the head, decreased tail length, and curving of the caudal part and older embryos developed severe heart edema and died at 8 days after fertilisation (Li et al., 2010). The Abcc6 knockdown effect in zebrafish embryos was rescued by co-injection of mouse *Abcc6* mRNA (Li et al., 2010), which indicates that zebrafish Abcc6a and mouse ABCC6 have similar functional properties, but probably have different functional physiological roles. The knockdown of the Abcb4 multidrug transporter and of Abcb5 did not result in visible developmental effects (Fischer et al., 2013) providing no evidence for developmental roles of these transporters.

4. Substrates and inhibitors of teleost ABC transporters

While chemicals have been classified as transport substrates or inhibitors of specific ABC drug transporters, these two categories are not mutually exclusive, as many substrates inhibit the transport of other compounds, while some compounds classified as inhibitors are themselves transported. Chemical interaction has been studied in considerable depth for the mammalian drug transporter ABCB1, and to a lesser extent for other drug transporters. These research efforts have had two main drivers. Firstly, in order to overcome ABC transporter-related MDR of cancers (Gottesman et al., 2002), studies have searched to identify non-cytotoxic inhibitors of ABCB1 and other MDR pumps, which are called reversal agents, resistance modifiers or chemosensitisers (Ford and Hait, 1993; Choi, 2005). Secondly, ABCB1 and possibly other drug transporters can limit oral drug absorption and drug penetration into sanctuary sites of the body. In consequence, ABC drug transporters can provide obstacles for drug delivery and be the basis for drug–drug interactions, so that for drugs other than reversal agents chemical interaction with ABC pumps is generally an undesired trait (Calcagno et al., 2007; Szakács et al., 2008).

Mammalian ABC drug transporters accept a wide range of structurally and functionally unrelated pharmaceuticals as transport substrates and are inhibited by a similarly wide range of drugs (Ford and Hait, 1990; Ambudkar et al., 1999; Choi, 2005; Deeley et al., 2006; Krishnamurthy and Schuetz, 2006; Calcagno et al., 2007). Interaction of mammalian ABC drug transporters has further been demonstrated for environmentally relevant compounds including surfactants and pesticides (Siegsmund et al., 1994; Bain and LeBlanc, 1996; Lanning et al., 1996; Bain et al., 1997; Loo and Clarke, 1998; Oosterhuis et al., 2008).

Substrates and inhibitors of ABC transporters can be identified through vectorial transport studies in cell monolayer systems supporting polarised expression of the ABC transporter of interest (Kim et al., 1998; Polli et al., 1999). However, fish cell lines suitable for this type of approach await being identified. Moreover, transport studies using assay monolayer systems are labour intensive to

perform and require analytical quantification of the drugs studied, constraining the usefulness of this methodology for chemical screening.

Methodologies to identify chemicals interacting with ABC transporters at comparatively high sample throughput include cytotoxicity and/or dye accumulation assays in drug-resistant cell lines overexpressing specific ABC drug transporters. Such resistant cell lines can be generated by subjecting non-resistant cell lines to a step-wise selection with increasing levels of cytostatic drugs (Riordan and Ling, 1985) or by transfection of suitable cell lines with the transporter in question (Ueda et al., 1987). Cell lines overexpressing specific ABC drug transporters show a decreased cellular accumulation and toxicity of substrates of the relevant pump. Accordingly, transporter substrates can be identified in cytotoxicity assays as compounds showing a decreased toxicity in the resistant when compared to the parental cell line (Szakács et al., 2008). In contrast, in dye accumulation assays with drug-resistant cell lines, both substrates and inhibitors increase the cellular accumulation of fluorescent dyes that are substrates of the studied transporter (Homolya et al., 1993; Holló et al., 1994).

Another approach to investigate chemical interaction with ABC pumps at high sample throughput is based on the measurement of transporter ATPase activity in cell membrane fractions obtained from cell lines overexpressing specific transporters (Ambudkar et al., 1992; Doige et al., 1992) or generated in recombinant baculovirus/insect cell expression systems (Sarkadi et al., 1992; Germann, 1998). In these experimental systems, ABCB1 (P-glycoprotein) shows basal ATPase activity in the absence of added substrates of the transporter, which is dependent on the presence of cholesterol in the cell membrane (Garrigues et al., 2002). Chemicals interacting with ABCB1 typically stimulate basal transporter ATPase activity in a concentration-dependent way. For some compounds, ATPase activities follow a biphasic concentration-effect profile, in which increasing levels of the compound provoke increasing ATPase activation up to an optimal concentration beyond which inhibition occurs (Sarkadi et al., 1992; Litman et al., 1997). Other chemicals cause inhibition of basal ABCB1 ATPase activity only, with no apparent stimulation at low levels (Litman et al., 1997).

The study of chemical interaction with ABC transporters by different methodologies can at times lead to conflicting results, particularly with systems in which transport is not measured directly (Szakács et al., 2008). The transport of chemicals by ABC drug transporters is a complex process influenced by various factors, including access and affinity to the transporter's drug binding site(s) and the ability of the compound to induce ATPase hydrolysis and concomitant conformation changes of the protein effecting transmembrane translocation of the chemical. The recently achieved X-ray structure of ABCB1 in the apo and drug-bound state (Aller et al., 2009) has revealed that ABCB1 possesses a large internal cavity which accommodates distinct drug-binding sites and has portals allowing the entry of substrates from both the cytoplasm and the inner leaflet of the cell membrane. In addition to direct chemical interaction with ABCB1, the chemical's permeability for the cellular membrane is an important factor in determining whether apparent transmembrane transport will occur (Stein, 1997). Compounds effectively extruded by ABC efflux pumps, such as the rhodamine 123 and doxorubicin, typically show low to moderate rates of passive membrane permeation (Eytan et al., 1996b; Von Richter et al., 2009). In contrast, reversal agents that are not transported themselves, such as quinidine and verapamil, typically show high membrane permeability (Eytan et al., 1996b; Von Richter et al., 2009).

The following sections summarise studies providing evidence for the interaction of chemicals with teleost ABC drug transporters based on studies employing tissue, cellular and subcellular models. Subsequently, the metabolic costs of chemical interaction with ABC transporters are addressed.

4.1. Results for primary cell culture systems

As reviewed in detail in Section 3, the measurement of the activity of ABC drug transporters in cell or tissue cultures involves the demonstration of effects of specific inhibitors on the accumulation or efflux of model substrates. Model substrates and inhibitors include a number of pharmaceuticals, such as the anthracyclins daunorubicin and doxorubicin, the calcium channel blocker verapamil and the anti-retroviral drug adefovir (Table 1). Studies with isolated kidney tubules have further demonstrated the interaction of renal teleost Abcb1 and/or Abcc transporters with a range of drugs, including the immunosuppressants cyclosporin A and rapamycin (Schramm et al., 1995; Miller et al., 1997), the anthelmintic ivermectin (Fricker et al., 1999), the HIV protease inhibitors ritonavir and saquinavir (Gutmann et al., 1999), and the somatostatin analogue octreotide (see Section 3.1 for details). These data suggest that teleost ABC drug transporters resemble their mammalian counterparts in that they interact with a wide range of chemicals.

A few studies have used primary cell culture systems or organ perfusions to investigate the interaction of teleost ABC transporters with environmentally relevant chemicals. The industrial chemical bisphenol A, which is present in many plastics and the coating of food cans, exerted differential effects on xenobiotic transport in killifish kidney tubules, inhibiting ABCC-like luminal secretion of sulforhodamine but stimulating the efflux of mitoxantrone (Nickel et al., 2013). The fungicide prochloraz and, less effectively, the xenoestrogen nonylphenol ethoxylate inhibited efflux of rhodamine 123 from rainbow trout hepatocytes (Sturm et al., 2001a). Similarly, the surfactant linear alkylbenzene sulfonate decreased rhodamine 123 transport into bile in perfused catfish liver (Tan et al., 2010).

4.2. Results for cell lines

Step-wise selection of the topminnow (*Poeciliopsis lucida*) hepatoma cell line PLHC-1 with increasing levels of doxorubicin was used to generate the subline PLHC-1/dox that shows a 45-fold reduction in doxorubicin sensitivity compared to the parental cell line (Zaja et al., 2008a). Using relative quantification by RT-qPCR, *abcb1* expression in PLHC-1/dox compared to PLHC-1 was found to be 42-fold increased when normalised to β-actin (Zaja et al., 2008a) and 160-fold increased when normalised to 18S RNA (Zaja et al., 2011), with Abcb1 overexpression in the selected cell line further being apparent from immunoblot analyses (Zaja et al., 2008a). In addition, PLHC-1/dox cells showed small increases (≤4.4-fold) in transcript levels of *abcc1, abcc2, abcc4* and *abcc10,* whereas *abcg2* mRNA levels were decreased (Zaja et al., 2011).

Compared to PLHC-1, cytotoxicity in PLHC-1/dox cells was decreased not only for doxorubicin, but also for other cytostatic drugs known to be substrates of human ABCB1, including the anthracyclines daunorubicin, the Vinca alkaloids vinblastine and vincristine, and the topoisomerase inhibitor etoposide (Zaja et al., 2008a) (Table 2). However, the resistance spectrum of PLHC-1/dox did not extend to the MRP substrates methotrexate and cisplatin (Zaja et al., 2008a) (Table 2). A number of pharmaceuticals known to be substrates and/or inhibitors of human ABCB1, including the calcium channel blockers verapamil, nicardipine and diltiazem and the alkaloids quinidine, reserpine and colchicine also interacted with *P. lucida* Abcb1, as indicated by positive effects on dye accumulation in PLHC-1/dox cells (Zaja et al., 2011) (Table 2). Dye accumulation in the Abcb1-overexpressing cell line was further increased by model inhibitors (Table 2), of which cyclosporin A, PSC-833 and reversin 205 are considered to be specific for ABCB1, whereas MK571 and Ko143 are in mammalian systems selective for MRPs and ABCG2, respectively (Zaja et al., 2011). As would be expected, some of the model inhibitors further had activity as reversal agents capable of abrogating the doxorubicin resistance of PLHC-1/dox cells (Zaja et al., 2008a) (Table 2).

PLHC-1/dox cells were further used to investigate the interaction of environmental pollutants with *P. lucida* Abcb1. Environmental

Table 2

Interactions of chemicals with teleostean ABC efflux transporters. Different types of cytotoxicity assays, as well as fluorescent dye accumulation and ATPase activity assays, have been used to reveal chemical interaction with teleost ABC transporters. In these assays, chemicals can interact with ABC transporters as substrates (S) and/or inhibitors (I), or fail to show such interaction (non-interacting compounds, N). Teleost transporters studied to date include *Poeciliopsis lucida* Abcb1, which is overexpressed in the cell line PLHC-1/dox (Caminada et al., 2008; Zaja et al., 2008a, 2011), and *Danio rerio* Abcb4, which has been expressed in baculovirus-transfected insect cells (Fischer et al., 2013). Compounds showing decreased cytotoxicity in PLHC-1/dox as compared to the parental cell line PLHC-1 are putative transport substrates of *P. lucida* Abcb1, as are chemicals the cytotoxicity of which is potentiated by the ABCB1/Abcb1 inhibitor cyclosporin A. Chemicals reversing the doxorubicin resistance of PLHC-1/dox or enhancing the accumulation of the fluorescent ABCB1/Abcb1 substrate calcein-AM are regarded inhibitors of *P. lucida* Abcb1 transport activity. In ATPase assays, substrates have been defined as chemicals stimulating ATPase activity, while inhibitors have been defined as substances decreasing basal ATPase activity in a study with *P. lucida* Abcb1 (Zaja et al., 2011) and as compounds decreasing verapamil-stimulated activities in a study with *D. rerio* Abcb4 (Fischer et al., 2013). Please see text for further explanations.

Substance	*Poeciliopsis lucida* Abcb1			*Danio rerio* Abcb4
	Cytotoxicity assays	Calcein-AM assay	ATPase assay[1]	ATPase assay[2]
Cytostatic drugs				
Doxorubicin	S[3]	N[4]	N	I[5]
Daunorubicin	S[3]	n.d.	n.d.	n.d.
Vinblastine	S[3]	N[4]	N	I, S[5]
Vincristine	S[3]	n.d.	n.d.	I[5], S[5]
Etoposide	S[3]	N[4]	N	n.d.
Mitoxantrone	n.d.	N[4]	I	n.d.
5-Fluorouracil	n.d.	N[4]	I	n.d.
Methotrexate	N[3]	n.d.	N	n.d.
Cisplatin	N[3]	n.d.	N	n.d.
Calcium channel blockers				
Verapamil	I[6]	I[4]	S	S
Nicardipine	n.d.	I[4]	S	n.d.
Diltiazem	n.d.	I[4]	S	n.d.
Alkaloids				
Quinidin	n.d.	I[4]	S	n.d.
Reserpine	n.d.	I[4]	I	n.d.
Colchicine	S[3]	I[4]	S	n.d.
Fluorescent model substrates				
Rhodamine 123	n.d.	n.d.	S	n.d.
Rhodamine B	n.d.	n.d.	n.d.	S[5]
Calcein-AM	n.d.	n.d.	n.d.	I, S
Hoechst 3334	n.d.	I[4]	I	n.d.
Model inhibitors				
Cyclosporin A	I[6]	I[4]	I	I, S
PSC-833	I[6]	I[4]	I	I, S
MK571	N[6]	I[4]	I	I
Reversine 205	n.d.	I[4]	S	n.d.
Ko143	n.d.	I[4]	N	n.d.
Environmental pharmaceuticals				
Pravastatin	n.d.	N[4]	S	n.d.
Atorvastatin	S[7]	I[4]	I	n.d.
Sildenafil	I[6]	I[4]	S	n.d.
Propranolol	S[7]	I[4]	S	n.d.
Tamoxifen	I[6]	I[4]	S	n.d.
Fenofibrate	I[6]	n.d.	n.d.	n.d.
Acebututolol	S[7]	n.d.	n.d.	n.d.
Pesticides				
Endosulfan	n.d.	I[4]	I	n.d.
Diazinon	n.d.	I[4]	S	n.d.
Fenoxycarb	n.d.	I[4]	N	n.d.
Chlorpyrifos	n.d.	N[4]	S	n.d.
Malathion	n.d.	I[4]	N	n.d.
Fosalon	n.d.	I[4]	S	n.d.
Dichlorodiphenyl-dichloroethylene	n.d.	I[4]	I	n.d.
Polycyclic musks				
Galaxolide	n.d.	n.d.	n.d.	I, S
Tonalide	n.d.	n.d.	n.d.	I[5], S
Polycyclic aromatic hydrocarbons				
Benzo[*a*]pyrene	n.d.	N[4]	N	n.d.
Phenanthrene	n.d.	n.d.	n.d.	I, S[5]
Toxic metals				
As$_2$O$_3$	n.d.	N[4]	S	n.d.

n.d.: not determined.

[1] Inhibition effects were determined on basal ATPase activities (Zaja et al., 2011).

[2] Inhibition effects were determined on verapamil-stimulated ATPase activities (Fischer et al., 2013).

[3] S: The cytotoxicity in PLHC-1/dox was decreased as compared to that in PLHC-1; N: The cytotoxicity was similar in both cell lines (Zaja et al., 2008a).

[4] Zaja et al. (2011).

[5] Effects were statistically not significant (Fischer et al., 2013).

[6] I: The compound enhanced the cytotoxicity of doxorubicin in PLHC-1/dox; N: The compound had no effects on doxorubicin cytotoxicity (Caminada et al., 2008; Zaja et al., 2008a).

[7] The cytotoxic effects of the compound were enhanced by the ABCB1/Abcb1 inhibitor cyclosporin A (Caminada et al., 2008).

pharmaceuticals showed positive effects in cellular dye accumulation assays in PLHC-1/dox cells and/or acted as reversal agents (Caminada et al., 2008) (Table 2). Another experimental approach to reveal chemical interaction with *P. lucida* Abcb1 involved testing whether the toxicity in PLHC-1/dox cells was enhanced by the co-treatment of cells with the ABCB1 inhibitor cyclosporin A. Again, several environmental pharmaceutics showed positive results, suggesting they represent substrates of *P. lucida* Abcb1 (Caminada et al., 2008) (Table 2). Effects on dye accumulation by PLHC-1/dox cells were further demonstrated for a range of pesticides, suggesting they might be substrates and/or inhibitors of *P. lucida* Abcb1 (Zaja et al., 2011) (Table 2). Similarly, inhibitory activity on Abcb1-related transport in PLHC-1/dox was demonstrated for a range of waste water components (Smital et al., 2011).

In zebrafish, cadmium selection of the fibroblast-like cell line ZF4 was used to generate the cadmium-resistant line ZF4-Cd (Long et al., 2011c). ZF4-Cd cells are cross-resistant to cadmium, mercury, arsenate and arsenite and show an enhanced mRNA expression of *abcc2*, *abcc4* and *mt2* genes (Long et al., 2011c). Moreover, ZF4-Cd cells possess elevated levels of glutathione (Long et al., 2011c).

A number of permanent fish cell lines have been shown to constitutively express ABC transporters, suggesting it may be feasible to create further cellular models overexpressing ABC pumps. Lines characterised regarding their complement of ABC transporters include the SAE *Squalus acanthias* shark embryo-derived cell line (Kobayashi et al., 2007), seven rainbow trout cell lines (Fischer et al., 2011) and the topminnow (*Poeciliopsis lucida*) hepatoma cell line PLHC-1 (Zaja et al., 2007). Relative mRNA levels of ABC transporters were similar among rainbow trout cell lines RTL-W1, R1, RTH-149, RTgill-W1, RTG-2, RTgutGC and RTbrain, with mRNA levels *abcc1-3* and *abcc5* exceeding those of Abcb and Abcg subfamily drug transporters by 80- to over 1000-fold (Fischer et al., 2011).

4.3. Results with ATPase assays

In teleosts, ATPase assays have been performed with *P. lucida* Abcb1 and *D. rerio* Abcb4, respectively, testing a range of compounds that comprise known substrates and inhibitors of mammalian ABC transporters and environmentally relevant chemicals (Table 2) (Zaja et al., 2011; Fischer et al., 2013). Recently, ATPase assays were also used in effect directed analysis to detect compounds interacting with *P. lucida* Abcb1 in environmental samples (Zaja et al., 2013). For ATPase assays, cell membranes enriched in *P. lucida* Abcb1 were prepared from the PLHC-1/dox cell line overexpressing the transporter (Zaja et al., 2011), whereas membrane fractions containing *D. rerio* Abcb4 were obtained following baculovirus/insect cell expression (Fischer et al., 2013).

Chemicals that stimulated the ATPase activity of *P. lucida* Abcb1 were categorised as substrates of the transporter, while inhibitors of the basal ATPase activity of *P. lucida* Abcb1 were classified as transporter inhibitors (Zaja et al., 2011) (Table 2). In mammalian systems, chemicals stimulating the ATPase activity of ABCB1 are known to comprise substances that are substrates in cellular transport assays as well as compounds that are inhibitors of transport activity in cellular assays (Sarkadi et al., 1992; Litman et al., 1997). Conversely, inhibitors of ABCB1 ATPase typically constitute inhibitors of transport activity in cellular assays (Litman et al., 1997; Von Richter et al., 2009). Taking this into account, the results from *P. lucida* Abcb1 ATPase assays are in accordance with earlier findings from cytotoxicity and dye accumulation assays in PLHC-1/dox cells for many compounds, including calcium channel blockers and alkaloids, as well as most of the model inhibitors, pesticides and environmental pharmaceutics (Table 2).

However, diverging results between assays were observed for the cytostatic drugs etoposide and doxorubicin, the latter of which was used in generating the resistant PLHC-1/dox cell line (Table 2). The lack of effects of these chemicals on Abcb1 ATPase activity (Table 2) may seem surprising, considering that Abcb1 overexpression has been suggested as the main molecular factor behind the resistance of PLHC-

1/dox cells to these cytotoxic compounds, which implies their cellular efflux by this transporter (Zaja et al., 2008a, 2011). However, only small stimulating effects on the ATPase activity of human ABCB1 have been reported for a number of confirmed ABCB1 substrates, which include etoposide and doxorubicin (Polli et al., 2001). As ABCB1 displays a high basal ATPase activity in the absence of added substrate, it has been hypothesised that substrates stimulating ATPase activity only by a small degree may go unnoticed due to the high basal ATPase activity of ABCB1 (Eytan et al., 1996a).

Following expression of *D. rerio* Abcb4 in the baculovirus/insect cell system, a range of chemicals were examined for effects on ATPase activity of the transporter (Fischer et al., 2013) (Table 2). The ABCB1 reversal agent verapamil and the ABCB1 substrate rhodamine 123 stimulated basal ATPase activity of *D. rerio* Abcb4, and were thus classified as substrates of the transporter (Fischer et al., 2013) (Table 2). The ABCB1 substrate doxorubicin and the ABCC/Abcc inhibitor MK571 inhibited verapamil-stimulated ATPase activities of *D. rerio* Abcb4 and were therefore categorised as inhibitors of the transporter (Fischer et al., 2013) (Table 2). The remaining compounds tested qualified both as substrates and inhibitors according to the above criteria and included the Vinca alkaloids vinblastine and vincristine, the model compounds calcein-AM, cyclosporin A and PSC-833, and the environmental pollutants phenanthrene, galaxolide and tonalide (Table 2) (Fischer et al., 2013). (Table 2).

The teleostean Abcb1 and Abcb4 proteins and human ABCB1 appear to be largely corresponding with regard to function and substrate spectra, but ATPase assays reveal subtle differences among transporter specificities. While the ABCB1 reversal agent cyclosporin A inhibits ATPase activity of ABCB1 (Von Richter et al., 2009) and *P. lucida* Abcb1 (Table 2) (Zaja et al., 2011), it provoked a marked stimulation of *D. rerio* Abcb4 ATPase activity (Table 2) (Fischer et al., 2013). Furthermore, the ABCB1 substrate vinblastine stimulated ATPase activities of human ABCB1 (Sarkadi et al., 1992) and *D. rerio* Abcb4 (Fischer et al., 2013) but had no effects on *P. lucida* Abcb1 ATPase activity (Zaja et al., 2011) (Table 2).

4.4. Metabolic costs of chemical interaction with ABC transporters

ABC transporters mediate the translocation of their substrates across membranes by an ATP-dependent mechanism. The energetic costs of transport of rhodamine 123 and doxorubicin were estimated in two studies with isolated cultured rainbow trout hepatocytes (Bains and Kennedy, 2005; Hildebrand et al., 2009). Exposure of hepatocytes to 5 and 10 mM rhodamine 123 increased respiration by 18.5% and 25.7% over basal rates, respectively (Bains and Kennedy, 2005). The altered respiration rates were not due to direct effects of rhodamine 123 on mitochondria. Co-treatment of hepatocytes with the ABCB1 inhibitor tariquidar inhibited the cellular efflux of rhodamine 123 and caused respiration rates to return to basal levels (Bains and Kennedy, 2005). When doxorubicin-treated hepatocytes were allowed to efflux the anthracycline for 3 h, this resulted in an up to 25% decrease of cellular ATP levels compared to parallel incubations of untreated cells (Hildebrand et al., 2009). In contrast, following the incubation of doxorubicin-treated hepatocytes in the presence of tariquidar, a decreased efflux of doxorubicin was observed and cellular ATP levels remained unchanged (Hildebrand et al., 2009). While these data suggest that ABC drug transporter activity is associated with significant energy costs, exposure of rainbow trout to restricted feeding intake or fasting for up to 9 weeks did not result in significant changes in hepatic rates of rhodamine 123 transport (Gourley and Kennedy, 2009).

5. Regulation of expression of teleost ABC drug transporters

Genomic and non-genomic mechanisms contribute to the regulation of ABC transporter activity in different tissues. Genomic mechanisms of regulation include genetic and epi-genetic effects, the modulation of

transcription rates, effects on mRNA stability and translational silencing by miRNAs (Masereeuw and Russel, 2012). Mechanisms of non-genomic regulation comprise the insertion of transporters into and their retrieval from the cell membrane, as well as posttranslational modifications such as phosphorylation and glycosylation and protein–protein interactions (Masereeuw and Russel, 2012). Comprehensive reviews are available on the regulation of mammalian ABC transporters in tumour cells (Scotto, 2003; Chen and Sikic, 2012; Chen et al., 2012) and in normal tissues including liver (Kipp and Arias, 2002; Chan, 2004; Roma, 2008), intestine (Estudante et al., 2013), kidney (Masereeuw and Russel, 2012), testis (Mruk et al., 2011) and brain (Miller, 2010; Chan et al., 2013).

5.1. Regulation of ABC transporters in cancer cells

MDR in cancer often is based on the enhanced expression of ABCB1 and other ABC drug transporters in tumour cells (Gottesman et al., 2002). A number of genetic and epi-genetic changes commonly observed in MDR tumour cells are believed to contribute to enhanced ABC drug transporter expression (reviewed by Chen and Sikic, 2012). Genetic changes include gene rearrangements leading to a juxtaposition of other active promoters to the *ABCB1* promoter, thus effecting its de-repression (Chen and Sikic, 2012). Moreover, enhanced expression of ABCB1 following cancerous cellular transformation has been found to be linked to mutations in oncogenes and tumour suppressor genes such as p53 (Chen and Sikic, 2012). Among epi-genetic changes affecting ABC drug transporter expression in cancers, de-methylation and complex changes in acetylation status have been suggested to contribute to the de-repression of the *ABCB1* promoter (Chen and Sikic, 2012).

In a number of marine and freshwater fish, an increased incidence of different hepatic lesions including neoplasms has been reported from polluted habitats (Black and Baumann, 1991; Myers et al., 1991; Vethaak and Jol, 1996; Vethaak and Wester, 1996). Epidemiological analyses of available data strongly support a role of environmental contaminants, particularly PAHs, in the aetiology of these pathologies (Rotchell et al., 2008). Biochemical changes associated with hepatocellular carcinogenesis have been studied in European flounder (*Platichthys flesus*) from polluted sites (Köhler et al., 1998; Koehler et al., 2004). Compared to healthy liver tissue, preneoplastic basophilic foci, as well as hepatic adenomas and carcinomas showed an increase in immunoreactivity with mAB C219, which was interpreted as an up-regulation of Abcb1 (Koehler et al., 2004). In contrast, C219 reactivity was unchanged in early eosinophilic preneoplastic foci (Koehler et al., 2004). Similarly, in killifish from a creosote-contaminated environment the immunochemical C219 signal was increased in hepatocellular carcinomas, but not in early proliferative hepatic lesions (Cooper et al., 1999). In addition, flounder hepatocellular carcinomas showed an increase in glucose-6-phosphate dehydrogenase activity and glutathion-S-transferase (GST)-A expression, and a decrease in CYP1A1 levels compared to healthy hepatic tissue (Koehler et al., 2004). Differences in gene expression between normal liver tissue and hepatic adenomas and carcinomas were investigated using microarray technology in dab (*Limanda limanda*) (Small et al., 2010). Fifty genes that best characterised the tumour type and sex of the host included CYP1A and other biotransformation enzymes, but no ABC transporters (Small et al., 2010). Compared to hepatic tissue from non-cancer bearing dab, there was a significant decrease in global DNA methylation in hepatocellular carcinomas and surrounding non-cancerous liver tissue (Mirbahai et al., 2011).

5.2. Regulation of ABC transporters as part of a general cellular stress response

In mammalian tissues, the expression of ABCB1 can be upregulated in response to cellular stress signals including heat shock, injury, inflammation, hypoxia, and UV and X radiation exposure (Scotto, 2003). The effects of heat shock on renal drug transport were studied in primary cultures of winter flounder (*Pleuronectes americanus*) renal proximal tubule cells (Sussman-Turner and Renfro, 1995). As reviewed above, peritubular to luminal daunorubicin transport in this system involved Abcb1-like transporters. Mild heat shock (5 °C elevation for 6–8 h followed by incubation at normal temperature) stimulated transepithelial transport of the ABCB1 substrate daunorubicin, with effects being protein synthesis dependent (Sussman-Turner and Renfro, 1995).

5.3. Roles of transcription factors in the regulation of ABC transporters by chemicals

Different nuclear receptors are involved in the transcriptional regulation of mammalian ABC drug transporters by endogenous and foreign chemicals (Chen et al., 2012). The pregnane xenobiotic receptor (PXR) was isolated for its role as a factor mediating the chemical regulation of isozymes of the CYP3A subfamily (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998). PXR is further involved in the regulation of other biotransformation enzymes (Blumberg et al., 1998; Xie et al., 2003) as well as ABC transporters including ABCB1 and ABCC2 (Dussault et al., 2001; Geick et al., 2001; Synold et al., 2001; Kast et al., 2002). While ligands of PXR comprise steroid hormones, bile acids and a wide range of organic chemicals, pronounced species differences in ligand selectivity exist (Jones et al., 2000; Ekins et al., 2008). The constitutive androstane receptor (CAR) shows sequence homology to PXR and overlaps with PXR in ligand spectrum and range of target genes (Waxman, 1999; Moore et al., 2000; Maglich et al., 2002). Teleosts possess one receptor showing sequence homology to both PXR and CAR (Maglich, 2003; Handschin et al., 2004), which has been named PXR because functionally it resembles PXR rather than CAR (Moore et al., 2002). The zebrafish PXR has a more restrained ligand spectrum than its mammalian counterparts, accepting only a subset of xenobiotic and steroid agonists of mammalian PXRs (Moore et al., 2002; Ekins et al., 2008; Fidler et al., 2012). Among different bile salts tested, only the main zebrafish bile salt cyprinol sulphate activated zebrafish PXR (Moore et al., 2002; Krasowski et al., 2005). In contrast, in green spotted puffer, which shows a more diverse physiological bile salt profile, a variety of bile salts were agonists of PXR (Krasowski et al., 2011). In zebrafish hepatocyte cultures, PXR activators induced enzyme activities that are in mammals specific for CYP3As and CYP2Cs (Reschly et al., 2007). Moreover, cyprinol sulphate treatment increased the abundance of hepatic *abcb5* transcripts (Reschly et al., 2007). In another study, treatment of zebrafish with the PXR ligand pregnenolone 16α-carbonitrile provoked increases (1.6- to 1.9-fold) in the mRNAs levels of PXR, *abcb1* (now called *abcb4*) and CYP3A (Bresolin et al., 2005). The insecticide chlorpyrifos is a known PXR ligand in zebrafish and human (Ekins et al., 2008). Exposure of killifish to the metabolically activated oxon form of chlorpyrifos induced hepatic immunoreactivity to the Abcb1 mAB C218 (Albertus and Laine, 2001). Taken together, the above data suggest that teleost PXRs are involved in the regulation of detoxification pathways, which seems to include roles in the chemical induction of certain ABC transporters.

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor of the bHLH-PAS (basic Helix-Loop-Helix Per-ARNT-Sim) family of transcriptional regulators (Hahn, 1998). AhR binds a broad spectrum of planar aromatic organic chemicals and acts as a key transcriptional regulator of a battery of genes that in mammals include the important phase I biotransformation enzymes CYP1A1 and CYP1A2, as well as phase II enzymes (Nebert et al., 2000). Early studies in rodents have demonstrated induction of hepatic ABCB1 by carcinogens that are known AhR agonists, such as 2,3,7,8-tetrachlorodibenzodioxin, 2-acetylaminofluorene and 3-methylcholanthrene (Burt and Thorgeirsson, 1988; Gant et al., 1991; Fardel et al., 1996). However, subsequent investigations revealed that the mechanism of ABCB1 induction by these compounds does not involve the AhR (Teeter et al., 1991; Deng et al., 2001).

In contrast, a role of AhR in the regulation of *ABCG2* has been suggested based on results obtained in the human colon adenocarcinoma cell line Caco-2 (Ebert et al., 2005).

In teleost fish, experimental exposures to AhR agonists had variable effects on the expression of ABC drug transporters. In killifish, injection with 3-methylcholanthrene provoked the expected induction of CYP1A in liver, but had no effects on hepatic levels of ABCB1-like proteins, determined in immunoblots with mABC C219 (Cooper et al., 1999; Bard et al., 2002a). Similarly, injection of cockscomb blennies with β-naphtoflavone did not alter hepatic expression of ABCB1-like proteins (Bard et al., 2002b). The dietary treatment of catfish with AhR agonists (b-naphtoflavone, 3,4,3′,4′-tetrachlorobiphenol and benzo[*a*]pyrene) failed to cause significant expression changes of ABCB1-like proteins in the intestinal mucosa, measured using mAB C219 (Doi et al., 2001). In another study, a significant increase in C219 immunostaining in the mucosa of the distal intestine was observed in catfish treated orally with vinblastine or b-naphthoflavone (Kleinow et al., 2000). Subchronic (14 days) waterborne exposure of Nile tilapia (*Oreochromis niloticus*) to the AhR agonist benzo[*a*]pyrene increased the mRNA levels of *abcc2* in gills and of *abcg2* in liver and proximal intestine, but had no effects on *abcb1* and *abcc1* transcript abundance (Costa et al., 2012).

Peroxisome proliferator-activated receptors (PPARs) bind fatty acids and their metabolites and participate in the regulation of genes involved in energy metabolism (Feige et al., 2006). A role of PPARa in regulating ABCB4 expression has been demonstrated in mice (Kok et al., 2003), while the regulation of ABCG2 by PPARg was shown in myeloid cells (Szatmari et al., 2006). The farnesol X receptor (FXR) is a bile acid receptor mainly expressed in the liver and intestine and regulates bile acid synthesis and transport (Kalaany and Mangelsdorf, 2006). Roles of FXR have been demonstrated in the regulation of *ABCB11* and *ABCC2* (Ananthanarayanan et al., 2001; Kast et al., 2002). Teleost PPARs and FXRs have been characterised (Leaver et al., 2005; Reschly et al., 2008; Krasowski et al., 2011) but no data are available regarding their potential roles the regulation of ABC transporters.

5.4. Hormonal effects on ABC transporters

The glucocorticoid receptor (GR) is a nuclear receptor that binds natural and synthetic glucocorticoids with high affinity and mediates genomic and non-genomic effects of these steroids (Bamberger et al., 1996). In addition to classical "glucocorticoid" roles related to metabolism, immune function, development and response to stressors (Yudt and Cidlowski, 2002; Charmandari et al., 2005), the GR has central roles in the osmoregulation of teleost fish (Bury and Sturm, 2007; Kiilerich et al., 2011). The effects of glucocorticoids on Abcc2-mediated transport were investigated in killifish kidney tubules (Prevoo et al., 2011). The rapid stimulation of Abcc2-dependent transport by the natural hormone cortisol and the synthetic glucocorticoids dexamethasone was inhibited by the GR antagonist RU486 but was insensitive to cycloheximide and actinomycin D, suggesting that dexamethasone effects were mediated by GR in a non-genomic fashion (Prevoo et al., 2011). The mechanism of dexamethasone activation of renal Abcc2 activity involved the tyrosine receptor kinase c-MET and MEK1/2 (mitogen-activated protein kinase/extracellular signal regulated kinase kinase) (Prevoo et al., 2011).

The vasoconstrictive peptide hormone endothelin (ET) reduced the luminal secretion of ABCB1 and ABCC substrates by killifish proximal tubules (Masereeuw et al., 2000). These effects of ET were mediated through B-type ET receptor and involved protein kinase C (PKC) (Masereeuw et al., 2000). Similar inhibitory effects of ET involving PKC were shown on ABCC2-like transport in shark rectal gland (Miller and Masereeuw, 2002). A role of PKC in Abcb1-dependent renal transport in killifish had previously been shown (Miller et al., 1998b). Different nephrotoxicants decreased Abcc2-dependent transport activity by the ET -dependent pathway, most probably by causing an opening of

calcium channels that resulted in an increase of intracellular Ca^{2+} levels which in turn triggered ET secretion (Terlouw et al., 2001). Subsequent studies demonstrated the involvement of nitric oxide synthase and guanylyl cyclase signalling in regulation of Abcc2-activity by ET (Notenboom et al., 2002, 2004). Another study investigated the effects of calciotropic hormones on Abcc2-dependent transport in teleost kidney (Wever et al., 2007). Parathyroid hormone (PTH) is a tetrapod hypercalcemic hormone that has recently been shown to exist in teleost fish, where its roles are unknown. In teleosts, the similar PTH-related protein (PTHrP) functions as hypercalcemic hormone, stimulating calcium uptake from water, whereas stanniocalcin (STC) blocks calcium uptake. PTH and PTHrP caused a similar partial inhibition of Abcc2-activity as ET, and showed additive effects when given together with ET (Wever et al., 2007). STC reversed the effects of PTHrP, but had no effect when given alone (Wever et al., 2007).

5.5. Chemical effects on ABC transporter expression in fish

A limited number of studies have investigated the inducibility of ABC drug transporters in fish by organic chemicals. Results obtained with PXR and AhR agonists have been reviewed above. The effects of the feed adulterants melamine and cyanuric acid on renal ABC transporter transcript levels were studied in a trial with rainbow trout (*Oncorhynchus mykiss*) (Benedetto et al., 2011). While the study does not provide statistical analyses, an apparent stimulation of *abcc2* mRNA levels was observed when both compounds were combined (Benedetto et al., 2011). The anti-parasitic compound emamectin benzoate (EMB), known to be an ABCB1 substrate (Igboeli et al., 2012), was investigated regarding its effects on ABC transporters in rainbow trout following a standard 7-day administration of medicated feed (Cárcamo et al., 2011). *Abcc1* transcripts were up-regulated in all tissues investigated (liver, muscle, gill, kidney, intestine), with effects being most pronounced in the intestine (Cárcamo et al., 2011). In addition, a slight increase in hepatic and a small decrease in intestinal *abcb1* transcript abundances was observed (Cárcamo et al., 2011). Waterborne exposure of juvenile rainbow trout to the cholesterol-lowering drug atorvastatin provoked the up-regulation of *abcb1* and *abcc1* transcripts in gill tissue (Ellesat et al., 2012). The effects of heavy fuel oil and perfluorooctane sulfonate (PFOS) on transcript levels of different ABC transporters (*abcb1, 11, abcc2, 3, abcg2*) were studied in thicklip grey mullet (*Chelon labrosus*) using a semi-quantitative RT-PCR method (Diaz de Cerio et al., 2012). The authors observed moderate increases of *abcb1, abcb11* and *abcg2* mRNA levels in liver and of *abcb11* mRNA levels in brain following exposure to PFOS, as well as complex changes in transporter expression in oil and combined oil and PFOS treatments (Diaz de Cerio et al., 2012).

A number of studies provide evidence for a regulation of teleost ABC drug efflux transporters by heavy metals. Exposure of killifish to sodium arsenite for 4–14 days increased Abcc2 expression and activity in renal proximal tubules, but did not affect *abcc2* mRNA levels (Miller et al., 2007). Pre-exposure of fish to sodium arsenite provided protection against adverse effects of the metal on mitochondrial function in renal tubules (Miller et al., 2007). Cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As) stimulated the expression of *abcc1* and *abcc5* transcripts in the zebrafish cell line ZF4 (Long et al., 2011a,b). Overexpression of Abcc1 in zebrafish embryos reduced the toxicity of Cd, Hg and As (Long et al., 2011a). In contrast, *abcc5* overexpression had protective effects only with respect to Cd toxicity, but did not affect adverse effects of Hg or As (Long et al., 2011b). Exposure of zebrafish to Hg and Pb stimulated *abcc2* transcription in intestine, liver and kidney (Long et al., 2011d). Overexpression of *abcc2* in ZF4 cells and embryos decreased the cellular accumulation of Cd, Hg and Pb (Long et al., 2011d). The interaction of toxic metals (Cd, Cr, Hg, As) with ABC drug transporters was studied in the fish cell lines PLHC-1 (wild type) and PLHC-1/dox (see above) (Della Torre et al., 2012). All metals upregulated mRNA expression of *abcc2, abcc3* and *abcc4*, and inhibited Abcb1- and Abcc-like transport activities (Della Torre et al., 2012). Prolonged exposure to

Hg caused increased Abcc-like transport activity but did not affect mRNA levels of any of the transporters studied (Della Torre et al., 2012). *Abcb10*, a mitochondrial ABC transporter associated with the cellular response to oxidative stress, was significantly upregulated in muscle tissue of zebrafish kept for 5 days in tanks with sediments spiked with Cu and Cd. *Abcb10* transcript levels were also increased in brain, gill and intestinal tissues, albeit to a lesser extent (Sabri et al., 2012).

5.6. Altered ABC transporter expression in fish from polluted habitats

A number of studies have found altered expression of ABC drug transporters in fish from polluted habitats. Using immunodetection with the mAB C219, an elevated hepatic expression of ABCB1-like proteins has been reported in killifish at a creosote-contaminated site when compared to a control site (Cooper et al., 1999). In another study, killifish from New Bedford Harbor (MA, USA), a habitat contaminated with planar halogenated aromatic hydrocarbons, showed decreased hepatic and increased intestinal levels of ABCB1-like proteins when compared to fish from a reference site (Bard et al., 2002a). However, when fish were kept in uncontaminated water in the laboratory for 11 weeks, hepatic levels of ABCB1-like proteins decreased in both populations, whereas intestinal levels decreased only in fish from New Bedford Harbor (Bard et al., 2002a). In another study with the intertidal species cockscomb blenny, hepatic levels of ABCB1-like proteins were elevated at sites affected by pulp mill effluents, but there was no correlation between transporter expression and the distance to the pollution source (Bard et al., 2002b). ABCB1-like protein levels in liver decreased after maintenance of fish under controlled laboratory conditions, but attempts to induce hepatic transporters by exposure of blennies to contaminated sediment or prey items from the field were unsuccessful (Bard et al., 2002b). As mentioned above, experimental treatment of killifish with AhR agonists was without effect on ABCB1-like expression in both killifish and cockscomb blennies (Cooper et al., 1999; Bard et al., 2002a,b). Another study investigated ABC transporter expression in killifish from the Sydney Tar Ponds, Nova Scotia, Canada, which are highly contaminated with PAHs, PCBs and heavy metals (Paetzold et al., 2009). Compared to reference site animals, Sydney Tar Pond killifish showed increased hepatic transcript levels of *abcc2, abcg2, cyp1a1* and *gst-mu*, whereas *abcb1* and *abcb11* mRNA levels were unchanged (Paetzold et al., 2009). Together, the above studies demonstrate that environmental pollutants can affect ABC transporter expression in fish; however, at present, the exact identity of chemical inducers remains unclear. Complicating the elucidation of possible links between pollutant exposure and the expression of ABC pumps in the tissues of feral fish, the possibility exists that chemicals of non-anthropogenic origin may have modulated ABC transporter expression in fish, as exemplified by the induction of *abcb1* mRNA levels and ABCB1-like protein in the gills and liver of the teleost *Jenynsia multidentata* (Amé et al., 2009).

6. Summary and conclusions

In the last years, research on ABC drug transporters in teleost fish has substantially advanced. Progress in the field has been significantly facilitated by the completion of different teleost genome projects, through which ABC transporter sequences have become available and which have enabled the annotation of the ABC gene family in zebrafish and catfish; in addition, partially annotated ABC gene sequences of further teleost species are publically available at the Ensembl Genome Browser (www.ensembl.org). The comparison of the ABC family between genomes of teleosts and tetrapods revealed a number of clear orthologous relationships that are consistent with the hypothesis of conserved function. However, the presence of lineage-specific gene duplications, particularly in teleosts, complicates the picture in some subfamilies and further research is needed to elucidate the functional significance of the duplicated ABC transporter genes in teleosts.

A number of studies have investigated the effects of exposure to chemicals on mRNA levels of ABC transporters in teleost tissues using quantitative RT-qPCR. While these works provide valuable insights into the regulation of ABC drug transporters in teleosts, it needs to be stressed that the relationship between mRNA abundance, transporter protein expression and transporter activity is not necessarily straightforward, reflecting the complexity of the different modes of genomic and non-genomic regulation of ABC proteins. In other words, the challenge is to link mRNA levels to protein expression and transport activity. While some progress has been achieved regarding transport assays of improved specificity, the very limited availability of antibodies allowing to detect specific ABC transporters in teleosts still represents a significant obstacle in understanding the regulation of ABC drug pumps in fish.

About two decades ago, results obtained by transport activity measurements and immunochemical methods revealed that the tissue distribution of the ABC drug transporter P-glycoprotein/Abcb1 in teleosts resembles that in mammals, suggesting similar functional properties and physiological roles of ABC pumps in both vertebrate groups. Since then, ABC protein-dependent transport activities have been characterised in a number of organ culture models from teleosts, employing a range of fluorescent probes and transporter inhibitors of known specificity in mammals. In particular, isolated kidney tubules of certain marine teleosts retain epithelial polarity and transport activities during short-term culture, and have proven a very useful model for the investigation of renal drug transport in teleosts.

However, in vitro models are not available for all teleost tissues of interest, which currently restricts feasible approaches to study ABC transporter function in situ. E.g., very little is known on ABC transporter-mediated processes at teleost branchial and intestinal epithelia. Given the high importance of both the gills and the gut as surfaces forming external body boundaries in fish, studies elucidating the role of ABC transporters in chemical uptake and elimination at these sites are urgently needed to improve current models of chemical fate in fish. The activity of hepatic ABC drug transporters has been studied in a number of teleosts, usually employing isolated cultured hepatocytes. However, the common cell monolayer system of hepatocyte primary culture is not ideally suited for transport studies, as cellular polarity is lost during cell isolation and usually does not re-establish in culture. A number of alternative primary culture systems has been proposed but these experimental model systems remain as yet unexplored regarding their suitability for studies of ABC transporters.

While isolated or cultured tissues are experimental systems depicting the in vivo situation with a certain degree of realism, such primary culture models usually contain a range of drug transporters, which limits their usefulness in studies aiming at the characterisation of specific ABC transporters. Systems allowing functional studies of individual ABC drug transporters have recently become available in teleosts and include drug-selected cell lines overexpressing specific ABC drug transporters, as well as insect cell/baculovirus systems suitable for the recombinant expression of ABC pumps. Another approach to address the functions of specific ABC transporters is provided by reverse genetic methodologies such as gene knockdown studies in zebrafish embryos.

Considering that certain fish species, such as zebrafish, are increasingly used as vertebrate model systems in biomedical and ecotoxicological research, it is of utmost importance to understand the biochemical and cellular factors driving the toxicokinetic and toxicodynamic properties of chemicals. While the relevance of active transport mediated by ABC transporters is well accepted in this regard in pharmacology, ABC proteins have so far attracted only limited attention in aquatic toxicology. It is hoped that the overview of the current state of ABC transporter-related research in teleost fish provided in this article will stimulate research further elucidating organ-specific roles of these transporters as factors affecting the fate and biological effects of chemicals in fish.

In summary, while many details regarding the physiological function and ecotoxicological relevance of ABC transporters await being clarified in teleosts, there has been significant progress in characterising

the complement of teleostean ABC pumps. Moreover, methodologies have become available that allow addressing both fundamental questions of transporter regulation and function in teleosts, and applied aspects such as the interaction of ABC pumps with pharmacologically and ecotoxicologically relevant chemicals. The aspects of chemical transporter interaction need to be taken into account in studies addressing toxicokinetic and toxicodynamic processes of chemicals in fish.

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