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REVIEW

Oligomerization of drug transporters: Forms, functions, and mechanisms

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Abstract Drug transporters are essential players in the transmembrane transport of a wide variety of clinical drugs. The broad substrate spectra and versatile distribution pattern of these membrane proteins infer their pharmacological and clinical significance. With our accumulating knowledge on the three-dimensional structure of drug transporters, their oligomerization status has become a topic of intense study due to the possible functional roles carried out by such kind of post-translational modification (PTM). In-depth studies of oligomeric complexes formed among drug transporters as well as their interactions with other regulatory proteins can help us better understand the regulatory mechanisms of these membrane proteins, provide clues for the development of novel drugs, and improve the therapeutic efficacy. In this review, we describe different oligomerization forms as well as their structural basis of major drug transporters in the ATP-binding cassette and solute carrier superfamilies, summarize our current knowledge on the influence of oligomerization for protein expression level and transport function of these membrane proteins, and discuss the regulatory mechanisms of oligomerization. Finally, we highlight the challenges associated with the current oligomerization studies and propose some thoughts on the pharmaceutical application of this important drug transporter PTM.

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1. Introduction

Drug transporters mediate the transport of clinical drugs across cell membranes, and work in concert with drug-metabolizing enzymes to regulate the absorption, distribution, metabolism, and excretion (ADME) of a wide variety of therapeutic agents. Human drug transporters are members of the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies. ABC transporters directly use the energy generated by ATP hydrolysis to mediate drug efflux from the intracellular to the extracellular environment[1]. Major ABC family members that are involved in drug transport include P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated proteins (MRPs/ABCCs), breast cancer resistance protein (BCRP/ABCG2), and bile salt export pump (BSEP/ABCB11)[2]. On the other hand, most SLC family members are influx transporters that transport drugs into cells by facilitated diffusion or secondary active transport. The SLC transporters that are widely recognized as important players in drug absorption and disposition are organic anion-transporting polypeptides (OATPs/SLCOs), organic anion transporters (OATs/SLC22As), organic cation transporters (OCTs/SLC22As), organic cation and carnitine transporters (OCTNs/SLC22As), peptide transporters (PEPTs/SLC15As), nucleoside transporters [concentrative nucleoside transporters (CNTs/SLC28); equilibrative nucleoside transporters (ENTs/SLC29)], bile salt transporters [Na^+-taurocholate co-transporting polypeptide (NTCP/SLC10A1); apical sodium-dependent bile acid transporter (ABST/SLC10A2)], organic solute transporter α/β (OSTα/β/SLC51 A/B). In addition, multidrug and toxin extrusions proteins, which belong to the SLC47A family, mainly function as efflux transporters[3].

Generally, drug transporters are found to be expressed in the liver, kidney, small intestine, and brain, sharing overlapping substrates. In addition to therapeutic drugs, they also transport toxins, metabolites, nutrients, and endogenous compounds such as bile salts, hormones, and signaling molecules. The diverse expression pattern and substrate specificity infer their important roles in tissue protection, remote sensing and signaling, immunology, and cell homeostasis maintenance[4,5]. Owing to their physiological and pharmacological significance, studies on drug transporters have attracted much attention in recent years. Basic characteristics of these transporters such as their substrates, tissue distribution, membrane localization, and functionality have been summarized in several excellent reviews[6–11].

In the human genome, around 25%−35% of genes encode integral membrane proteins[12,13]. Most integral membrane proteins do not function as individual units but form oligomers. A cursory analysis of the Protein Data Bank (PDB) for transmembrane (TM) proteins revealed that a large fraction of the membrane proteins (~65%) are obligate oligomers[14]. Residing within membrane lipid bilayers, transmembrane proteins have the propensity to form functional oligomers. The lipid bilayer and the topology of membrane proteins restrict and define the orientation of the individual proteins (subunits), eventually facilitating their assembly. The formation of oligomers reduces energetically unfavorable protein-lipid interactions. Additionally, interactions between TM proteins help shield large parts of the protein surface area from the lipid and thus stabilize the protein[15].

Oligomerization is also closely linked to the expression level and function of various proteins. It is involved in the folding, quality control, trafficking, and targeting of the protein complexes[16]. In addition, the formation of oligomers may create new substrate binding sites and transporting pathways, or result in cooperative or allosteric regulation[17]. Integral membrane proteins can be grouped into four types: receptors, channels and transporters, membrane enzymes, and co-factor scaffolding proteins, participating in cell signal transduction, ions and molecules transportation, enzymatic reactions, and orientational confinement, respectively[18]. The acquisition of these functions requires a very diverse array of protein architectures by a possible evolutionary strategy to create larger proteins and complexes from smaller structural units[14].

Although the regulation and function of drug transporters have been subjected to extensive investigations through mutagenesis and pharmaceutical studies over the past decades, our knowledge of the structure and molecular mechanisms of most drug transporters is still limited. Growing evidence indicates that many drug transporters form higher-order oligomers constituted of identical (homo) or different (hetero) monomers, with functional properties distinct from their protomeric components.

In this review, different forms of oligomeric complexes that involve drug transporter are summarized. Since oligomerization is often used more loosely to refer to any type of protein association, information on the protein−protein interaction between drug transporters and other regulatory proteins was also included. The structural basis of oligomerization, as well as its role in regulating the protein level and function of drug transporters, along with its regulatory mechanisms, were described. The possible pharmaceutical implications for the oligomerization of drug transporters were discussed.

2. Forms of oligomerization

2.1. *Homo-oligomerization*

Homo-oligomerization is commonly found in members of the ABC and SLC families. MRP1 (ABCC1) dimer was first discovered by radiation inactivation of the human erythrocytes[19] and electron microscopy (EM) imaging of the purified transporter protein[20]. Subsequently, Yang et al.[21] examined the oligomeric status of human MRP1 using techniques such as perfluorooctanoic acid (PFO)-polyacrylamide gel electropheresis, non-denaturing polyacrylamide gel electropheresis, gel filtration chromatography, sucrose density gradient sedimentation, chemical cross-linking, and co-immunoprecipitation. It was demonstrated that human MRP1 is a homodimer and that the amino-terminal MSD0L0 (membrane-spanning domain, where L0 is loop 0) region is essential and sufficient for MRP1 homodimerization[21]. Compared to other ABC family members that contain 12 transmembrane helices (TMs), BCRP (ABCG2) only has six TMs. Hence is regarded as a "semi-transporter". It has been reported in quite a few studies that BCRP forms homodimers or higher-order oligomers such as tetramers, octamers, or dodecamers[22–24]. Using single particle imaging technique, Wong et al.[25] demonstrated that tetramers and dimers consist of 90% and 10% of the GFP-tagged BCRP in 293T cells, respectively. However, cryo-electron microscopy (cryo-EM) structure analysis revealed that BCRP is only a dimer[26–28]. In contrast to MRP1 and BCRP, related studies on P-gp (ABCB1) showed controversial results and its exact oligomerization status remains unclear. Using biochemical and biophysical techniques, P-gp was demonstrated to form oligomers at the cell membrane in early studies[29–32]. Sedimentation velocity centrifugation of detergent extracts from hamster and human multidrug resistance (MDR) cell lines also suggested an

oligomeric form of P-gp[33]. Jette et al.[34] reported the possible existence of P-gp dimer in brain capillaries and renal brush border membranes as well. However, other studies failed to find similar evidence of P-gp oligomerization. A molecular complementation analysis suggested the functional unit of P-gp appeared to be a monomer[35]. Electron microscopy and single particle image analysis of both detergent-solubilized and lipid-reconstituted P-gp protein showed a protein size most consistent with a monomeric form[36]. Taylor et al.[37] also demonstrated that P-gp is normally monomeric with biochemical and genetic approaches. A series of cryo-EM structures of human P-gp were reported in recent years, all of which inferred a monomeric P-gp[38–40].

By applying the methods of cross-linking, co-immunoprecipitation, gel chromatography, and biotin labeling, Hong et al.[41] demonstrated that human OAT1 (hOAT1) formed homo-oligomers at the plasma membrane of the OAT1-expressing LLC-PK1 cells. In addition, homo-oligomerization of rat Oat1 (rOat1) can be detected in rat kidney homogenates. Oligomeric forms of rOat1 and rOct1 were also found in an *in vitro* synthetic recombination system[42]. Human OCT2 was also shown to exist as oligomers both in HEK293 cells over-expressing the transporter and in human kidneys[43].

With similar approaches, homo-oligomeric forms of OATP1B1 and OATP1B3 were verified[44,45]. Other OATP family members such as OATP2B1 and OATP5A1 were demonstrated to form homo-oligomers as well[46,47], suggesting that oligomerization may be a common feature of OATP family members.

NTCP and ASBT, two members of the SLC10 family, also exist as homo-oligomers[48,49]. A more recent study investigated the oligomerization of NTCP in Sf9 cells and found that dimer is the predominant form of the transporter in the insect cell system[50]. Further investigation by Noppes et al.[51] revealed that all SLC10 family members form homo-oligomers.

CNTs and ENTs also undergo homo-oligomerization. Based on the knowledge of the crystal structures of *Vibrio cholerae* CNT (vcCNT)[52] and its orthologue from *Neisseria wadsworthii* (CNTNW)[53], Stecula et al.[54] utilized size exclusion profiling, glutaraldehyde cross-linking, and mutagenesis analysis to show the existence of human CNT3 homotrimers. Thereafter, the oligomerization status of CNT3 was confirmed by Zhou and co-workers[55]. CNT3ins, which is a human CNT3 truncated isoform (Δ1−69) and exhibits identical binding affinity with uridine as the full-length CNT3, was shown in cryo-EM images to form a trimeric shamrock-shaped architecture through contacts of the central helices from each protomer[55]. Additionally, the homo-oligomeric forms of ENT1 and ENT2 were also found in a phosphorylation regulation study[56].

It should be noted that though quite a few of the drug transporters were shown to form dimers or even multimers (Table 1), the exact stoichiometry of the oligomeric quaternary structure of most drug transporters remained unclear except for CNT3.

2.2. *Hetero-oligomerization*

Studies have found that proteins that form homo-oligomers may gradually acquire the capability to form hetero-oligomers through evolution. Hetero-oligomerization among proteins can promote the acquisition of multiple functions of proteins[66,67]. This phenomenon also exists in drug transporters (Tables 1 and 2).

Table 1 Formation of oligomeric complexes among drug transporters.

Family	Transporter	Oligomerization partner	Effect on the transporter	Ref.
ABC	MRP1 (ABCC1)	MRP1	N/A	19–21
	BCRP (ABCG2)	BCRP	Formation of functional unit	22–24,26–28,57
	P-gp (ABCB1)	P-gp	N/A	29–34
SLC	OAT1 (SLC22A6)	OAT1	Required for cell surface expression	41,58
	rOat1 (Slc22a6)	rOat1	N/A	41,42,59
	rOat3 (Slc22a8)	rOat3	N/A	60
	OCT1 (SLC22A1)	OATP1B3	No	61
	OCT2 (SLC22A2)	OCT2	N/A	43
	rOct1 (Slc22a1)	rOct1	Required for cell surface expression	42,59
	OATP1B1 (SLCO1B1)	OATP1B1	Formation of functional unit and required for expression	44,62
		OATP1B3	No	61
	OATP1B3 (SLCO1B3)	OATP1B3	N/A	45
		OATP1B1	Increased expression and decreased k_{cat}	61
		NTCP	Increased expression and decreased k_{cat}	61
		OCT1	Decrease expression and increased k_{cat}	61
	OATP2B1 (SLCO2B1)	OATP2B1	N/A	46
	rOatp1a4 (Slco1a4)	rOatp1a1	Required for cell surface expression	63
	NTCP (SLC10A1)	NTCP	Required for internalization	48,64
		SLC10A4	Decreased cell surface expression	48
		SOAT	N/A	48
		OATP1B3	No	61
	ASBT (SLC10A2)	ASBT	Formation of functional unit	49
	CNT3 (SLC28A3)	CNT3	Shortens substrate translocation distance	54,55
	ENT1 (SLC29A1)	ENT1	N/A	56
		ENT2	Decreased transport activity	56
	ENT2 (SLC29A2)	ENT2	N/A	56
		ENT1	Increased transport activity	56
	OSTα (SLC51A)	OSTβ	Formation of functional unit and required for expression	9,65

N/A, not available.

Table 2 Protein−protein interaction (hetero-oligomerization) of drug transporters and regulatory proteins.

Family	Transporter	Interaction partner	Effects on transporter	Ref.
ABC	P-gp (ABCB1)	Pim-1	Phosphorylation, glycosylation, and cell surface expression, increased stability	72
		PKC$\alpha/\beta/\gamma/\varepsilon/\varphi$	Phosphorylation	73
		Receptor for activated C kinase 1 (Rack1)	Promotes the interaction with Anxa2	74
			Promotes the interaction with Src	75
		Src tyrosine kinase	Reduced association with Cav1, and increased transport activity;	75
			Affect Anxa2 phosphorylation	76
		Caveolin-1 (Cav1)	Decreased transport activity	75,77–79
		Caveolin-2 (Cav2)	N/A	77
		Annexin A2 (Anxa2)	N/A	74,80
		Ezrin	Required for the association between ABCB1 and actin	81
			Required for localization to lipid rafts and transport activity	82
		Radixin	Required for the association between ABCB1 and actin	81
			Increased expression	83
		Moesin	Required for the association between ABCB1 and actin	81
		Actin	Required for polarized membrane localization and transport activity	81
		Tubulin	N/A	84
		CD44	Required for expression	85
		CD147	Required for expression	86
		Rab4	Increased localization in cytosolic endosome, and decreased cell surface expression	87
		Rab14	N/A	87
		Nedd4-1	Decreased cell surface expression	88
		Ubiquitin	Increased degradation	89
		RING finger protein 2 (RNF2)	Increased degradation	90
		Bap29varP	Trapped in the ER and intracellular vesicles	91
		FBXO15	Enhanced ubiquitination, and increased degradation	92
		Ube2r1	Enhanced ubiquitination, and increased degradation	92
		FBXO21	Enhanced ubiquitination, and increased degradation	93
		Hsc70	Required for protein folding	94,95
		Calnexin	Required for glycoprotein folding	95,96
	MRP1 (ABCC1)	ATP Synthase α	N/A	97
		Tubulin	N/A	98
		Glycogen synthase kinase 3$\alpha\beta$ (GSK3$\alpha\beta$)	Increased protein stability	99
	MRP2 (ABCC2)	NHERF1	N/A	100
		NHERF3 (PDZK1)	Required for apical membrane localization	101,102
		NHERF4	N/A	100
		Ezrin	Required for apical membrane localization in Caco2 cells;	103
			Promotes internalization in human obstructive cholestasis cells	104
		Radixin	Required for apical membrane localization in Caco2 cells	103
		Clathrin	Required for endocytosis	105
		Adaptor protein 2 (AP2)	Required for endocytosis	105
	MRP4 (ABCC4)	NHERF1	Promotes internalization in HeLa cells;	106 107
			Required for apical membrane localization in MDCKI cells and LLC-PK1 cells	
		NHERF2	N/A	108

(*continued on next page*)

Table 2 (*continued*)

Family	Transporter	Interaction partner	Effects on transporter	Ref.
		NHERF3 (PDZK1)	Promotes the interaction with CFTR; Increased protein stability, and reduced internalization	108, 109
		Sorting nexin 27 (SNX27)	Promotes internalization	110
		MPP1	Increased membrane stability	111
	BCRP (ABCG2)	Pim-1	Phosphorylation, oligomerization, and function	112
		JAK3	Required for phosphorylation and its interaction with β-catenin	113
		β-Catenin	Required for cell surface expression	113
SLC	OAT1 (SLC22A6)	SGK2	Increased protein stability and transport activity	114
		Nedd4-1	Enhanced ubiquitination, and decreased cell surface expression	115
		Nedd4-2	Enhanced ubiquitination, and decreased expression	116
	OAT3 (SLC22A8)	Nedd4-2	Enhanced ubiquitination, and decreased cell surface expression	117
		PKCζ	Activation of PKCζ increased OAT3 transporter activity	118
		Myosin	Required for localization to lipid rafts and transport activity	119
		β-Actin	Required for localization to lipid rafts and transport activity	119
		Caveolin-1 (Cav1)	Required for localization to lipid rafts and transport activity	119
	OAT4 (SLC22A11)	Nedd4-2	Enhanced ubiquitination, and decreased cell surface expression	120
		NHERF1	Required for cell surface expression	121
		NHERF3 (PDZK1)	Required for cell surface expression	121
		Caveolin-1	Increased transport activity	122
	OCT1 (SLC22A1)	LAPTM4A	N/A	123
	OCT2 (SLC22A2)	Yes-1	Phosphorylation and function	124
		LAPTM4A	Induced endocytotic degradation	123
		CD63	Recycling from endosomes to the basolateral membrane	125
	OCT3 (SLC22A3)	PDZK2	N/A	126
		LAPTM4A	N/A	123
	OCTN1 (SLC22A4)	NHERF3 (PDZK1)	N/A	127
	OCTN2 (SLC22A5)	NHERF3 (PDZK1)	Increased transport activity	127
		PDZK2	Increased membrane stability	128
	OATP1A2 (SLCO1A2)	NHERF1	Increased protein stability, and reduced internalization	129
		NHERF3 (PDZK1)	Increased protein stability, and reduced internalization	129
	OATP1B1 (SLCO1B1)	Ubiquitin	Enhanced ubiquitination	130
		NHERF3 (PDZK1)	Required for cell surface expression	131
	OATP1B3 (SLCO1B3)	Ubiquitin	Enhanced ubiquitination	130
	OATP2B1 (SLCO2B1)	NHERF3 (PDZK1)	Required for cell surface expression	132
	NTCP (SLC10A1)	EGFR	Required for NTCP oligomerization during HBV infection	133
	ASBT (SLC10A2)	Ubiquitin	Required for proteasomal degradation	134

N/A, not available.

2.2.1. Hetero-oligomers formed among drug transporters

Using proximity ligation assay, Zhang et al.[45] detected co-localization of OATP1B3 with OATP1B1 or NTCP in HEK293 cells over-expressing the corresponding transporters, suggesting the formation of hetero-oligomers between these proteins. The association of OATP1B3 and NTCP was also demonstrated in frozen human liver tissue. A follow-up investigation showed that a hetero-oligomerization occurred between OATP1B3 and OCT1 as well[61]. When exploring the membrane targeting mechanism of rOatp1a4 in hepatocytes, Wang et al.[63] found the direct hetero-oligomerization between rOatp1a4 and rOatp1a1. NTCP was found to form hetero-oligomer with family members SLC10A4 and sodium-dependent organic anion transporter (SOAT/SLC10A6) in U2OS cells. However, given the different

tissue distribution of NTCP with these SLC10A members, *i.e.*, NTCP is specifically expressed in the liver; while SLC10A4 is mainly detected in the brain, eyeball, adrenal gland, and small intestine, the true presence of such an association in the human body needs to be further validated[48]. When triggered by protein kinase C (PKC) activation, ENT1 and ENT2 on the cell surface can form ENT1−ENT2 hetero-oligomer to participate in functional regulation[56].

OSTα/β is a hetero-multimer that is composed of two completely different units. OSTα that is encoded by *SLC51A* is a protein that contains 340 amino acid residues and is predicted to have 7-TM domains; whereas OSTβ is encoded by *SLC51B* and has a length of 128 amino acids with a putative single-TM domain[65]. Activation of farnesoid X receptor (FXR) induced the synthesis of more copies of Ostβ than Ostα, implicating a large transporter oligomeric complex may utilize several copies of Ostβ for each Ostα subunit[68−70].

2.2.2. *Hetero-oligomers formed between drug transporters and regulatory proteins*

Regulation of drug transporters requires the accessory of interactions with enzymes, scaffold proteins, molecular chaperones, and other proteins. Although oligomerization often refers to the stable and physical contact of proteins with similar structures, it was also proposed that such a phrase can be used to indicate the association of different types of proteins[71]. We hence also categorized the interactions between drug transporters and regulatory proteins as hetero-oligomerization as listed in Table 2.

Polarized expression of many drug transporters in cells depends on direct interaction with scaffold proteins such as the postsynaptic density protein-95, *Drosophila* disc large tumor suppressor, and zonula occludens-1 (PDZ) and ezrin-radixin-moesin (ERM) proteins. ABC transporters such as MRP2 and MRP4, as well as SLC transporters including OAT4, OCT3, OATP1A2, OATP1B1, OATP1C1, OATP2B1, OATP3A1, OATP4A1, OCTN1/2, and PEPT1/2 were shown to interact with PDZ proteins[131,135,136]. P-gp and MRP2 were found to interact with ERM proteins[137].

Recent publications have demonstrated that many drug transporters are localized within lipid rafts on the cell membrane and interact with lipid raft-related proteins. For example, P-gp was shown to interact with caveolin-1 and caveolin-2[77]. The interaction of OAT3 with caveolin-1, β-actin, and myosin is required for its localization at the lipid rafts and transport activity[119].

Oligomerization of drug transporters with certain proteins was shown to affect their cell surface expression and/or trafficking. For example, human OCT2 was shown to be associated with the lysosomal-associated protein transmembrane 4 alpha (LAPTM4A). The over-expression of LAPTM4A reduced the total and cell surface level of the transporter, possibly by regulating its endocytotic recruitment[123]. The cellular location of OCT2 was also found to be regulated by CD63, a ubiquitously expressed member of the tetraspanin superfamily. Investigation with polarized Madin−Darby kidney canine kidney (MDCK) cells and CD63-knock-out mice suggested that CD63 may play a role in OCT2 recycling from the endosomes to the basolateral membrane of polarized epithelia[125].

Kinases and ubiquitin ligases were demonstrated to directly associate with their substrate proteins. For example, P-gp and BCRP contain the consensus sequence of the serine/threonine kinase proviral integration site for Moloney murine leukemia virus 1 (Pim-1), which regulates the phosphorylation of these transporters *via* protein−protein interaction[72,112]. Janus kinase 3 (JAK3) directly phosphorylates BCRP, promoting its interactions with β-catenin for maintaining the expression, surface localization, intestinal drug efflux, and barrier function of the transporter protein[113]. E3 ubiquitin ligase neural precursor cell-expressed developmentally down-regulated 4-2 (Nedd4-2) was found to interact with OAT family members OAT1, OAT3, and OAT4, affecting their ubiquitination status and protein stability[116,117,120].

A more detailed list of the hetero-oligomeric complexes formed between drug transporters and regulatory proteins was summarized in Table 2.

3. Structural basis of oligomerization

Drug transporters usually consist of 12−14 hydrophobic transmembrane helices, connected by hydrophilic intracellular and extracellular loops, with N- and C-terminal regions of diverse lengths that are mostly hydrophilic and cytoplasm facing. These regions that show versatile compositional characteristics provide the structural basis for drug transporter oligomerization (Fig. 1).

3.1. *Transmembrane helices*

Transmembrane helices are important structural features of drug transporters, some of which are involved in the formation of translocation pathways; while others are embedded in the lipid bilayer to stabilize the protein conformation. These peripheral transmembrane regions may also act as interactive interfaces for the formation of oligomers (Fig. 1). Conserved sequences related to the oligomerization of transporters include motifs such as GXXXG, leucine heptad, polar-XX-polar, and aromatic-XX-aromatic, which promote the interaction between transmembrane regions through different mechanisms[138].

The GXXXG motif is a well-recognized helix−helix packing domain that is frequently tested in the oligomerization studies of drug transporters. Due to its small side chain structure, glycine in the GXXXG motifs permits the transmembrane regions to be closely associated with each other, allowing the formation of oligomeric complexes[139]. Proposed GXXXG motifs were tested with site-directed mutagenesis in BCRP-TM1[140], OAT1-TM2/5[141], OATP1B1-TM8[44], and NTCP-TM2/7[142]. The corresponding mutations were demonstrated to affect the folding, trafficking, and expression level of the membrane proteins. However, whether these effects indeed result from the disruption of oligomerization is unclear, as GXXXG motifs not only mediate protein oligomerization but also play a role in the correct folding of proteins[138]. Cryo-EM analysis in recent years also pointed out that GXXXG motifs previously proposed to be involved in the oligomerization of drug transporters may not be situated at the right interface for the formation of oligomeric complexes. For example, the respective residues of the two GXXXG motifs within the TM1 of BCRP were found to be opposed to each other in a recently reported cryo-EM structure of the transporter, implicating that these motifs may not contribute to the dimerization process[26].

Another prominent motif that was often investigated in oligomerization studies is the leucine heptad with a basic composition of L-XXXXX-L, in which leucine can be replaced by isoleucine/valine. The hydrophobic property of leucine heptad promotes the formation of oligomers among proteins[143]. When three short-form leucine heptad repeats in OATP1B1 TMs were investigated, it was demonstrated that the disruption of leucine heptad repeats within

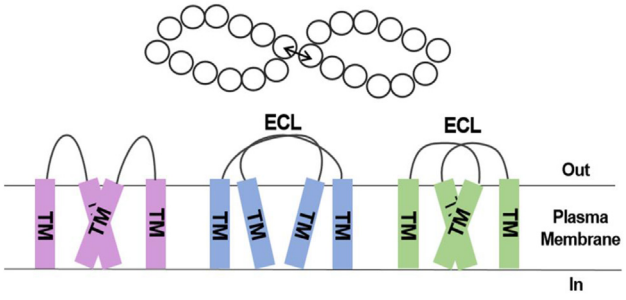

Figure 1 Structural basis for drug transporter oligomerization. Upper panel, the arrangement of drug transporter TMs viewed from the cytoplasmic side, the oligomerization interface is indicated with an arrow. Lower panel, models for the TM−TM, ECL−ECL, or TM−TM + ECL−ECL contacts, with rectangles representing transmembrane segments (TM) and curved lines standing for extracellular loops (ECLs).

TM3 significantly decreased the uptake function and oligomerization of OATP1B1. Sequence comparison revealed that almost all the OATP1 and OATP2 subfamily members contain identical leucine heptad at TM3, suggesting that it may serve a similar role in the oligomerization of different OATPs[62].

It is worth noting that some transmembrane helices may not contain the above-mentioned oligomerization-related motifs, yet are still important for the formation of oligomeric complexes among drug transporters. For example, TM6 of OAT1[58], TM5 of MRP1[144], and TM5/6 of BCRP[145], are all demonstrated to be important for the oligomerization process. Whether novel oligomerization-related motifs or unknown mechanisms are involved needs further investigation.

3.2. *Extracellular loop*

In transmembrane proteins, the intracellular and extracellular loops that connect the domains embedded within lipid bilayers are important for their stability and substrate binding. Reports have shown that extracellular loops (ECL) may be involved in the oligomerization of drug transporters as well. The ECL3 and TM5 jointly mediate the oligomerization of MRP1. It was proposed that it is the hydrophobicity of TM5 and the length of ECL3, but not the specific amino acid sequences in these regions that contribute to the dimerization of the transporter[143].

Oligomerization mediated by the ECLs may rely on the formation of inter- or intra-molecular disulfide bonds. ECL3 of BCRP, along with TM5 and TM6, constitute an oligomerization interface for the transporter[145]. Cys603 localized in the ECL3 is involved in the formation of a symmetrical intermolecular disulfide bond between BCRP monomers[146]. Such an intermolecular disulfide bond was confirmed in the cryo-EM structure of BCRP[26]. The dimers or higher oligomeric forms of OCT2 were also demonstrated to be formed by covalent disulfide bonds between monomeric subunits as well. When the first (Cys51) and the last cysteines (Cys143) of the large ECL1 of OCT2 were simultaneously mutated, oligomerization of the transporter was completely abolished[43]. Intramolecular disulfide bonds were also found to promote the formation of oligomers. The large extracellular loops of rOct1 or rOat1 are pivotal for oligomerization. For rOct1, the tertiary structure of the large extracellular loop is stabilized by intramolecular disulfide bonds. Replacement of the cysteine residues in the large ECL of rOct1 with serines or the disruption of disulfide bonds with dithiothreitol (DTT) prevented the oligomerization of the transporter[59].

3.3. *Amino and carboxyl termini*

The amino and carboxyl termini are important for the subcellular localization, trafficking, substrate binding specificity, protein−lipid interactions, oligomerization, and signal transduction of transporters[147]. Although reports concerning the involvement of these structural features in the homo-oligomerization of drug transporters are lacking, they were shown to be essential for the interaction of drug transporters with PDZ and ERM proteins, which serve important roles for the proper targeting and trafficking of drug transporters[136,137].

4. Functional roles of oligomerization

4.1. *Oligomerization and protein level*

The expression level of drug transporters is in a dynamic equilibrium among the synthesis, membrane targeting, internalization, recycling, and degradation processes. In addition to molecular chaperones and specific enzymes, oligomerization also plays an important role in maintaining the proper amount and localization of these membrane proteins (Fig. 2).

When the oligomerization status of OATP1B1 is disrupted, a reduction in protein level was observed[44,62]. Inhibition of OAT1 oligomerization disturbed its targeting to the cell membrane[58]. In view of the fact that protein oligomerization may be involved in quality control and that only correctly folded proteins or those that form oligomers can go through endoplasmic reticulum (ER)-exit[148], the formation of oligomers may help drug transporters to meet the stringent quality control within the ER. Hetero-oligomerization between drug transporters may also lead to the change of protein level. The association of OATP1B3 with OCT1 resulted in a reduced level of OATP1B3; while its interaction with OATP1B1 or NTCP increased the amount of the transporter protein. However, the underlying regulatory mechanism of such a phenomenon remains unknown[61].

After biosynthesis and exit from the ER, properly targeting to the plasma membrane is also a crucial step for the functionality of membrane proteins. Drug transporters may reach their target sites with the help of hetero-oligomerization. The formation of OSTα/β hetero-oligomers enables the complete transporter to be localized to the cell surface membrane and enhances the stability of the proteins. Truncation of OSTβ or the N-terminus of OSTα resulted in a reduced plasma membrane level of OSTα/β[9]. Rat Oatp1a1 (rOatp1a1) relies on PDZ protein for its localization on the cellular membrane; while rat Oatp1a4, lacking the PDZ binding domain, forms hetero-oligomers with rOatp1a1 to promote protein maturation and its proper localization at the liver cell membrane[63]. NTCP forms hetero-oligomeric complexes with SLC10A4 and SLC10A6 in U2OS cells. The co-expression of SLC10A4 retained NTCP within the cell, resulting in a decreased cell surface level of NTCP and reduced taurocholate transport activity[48].

The internalization of transporter triggered by chemicals and enzymes may be associated with the change of oligomerization status as well[149,150]. NTCP-mediated hepatitis B (HBV) infection provides an example of the association between oligomerization

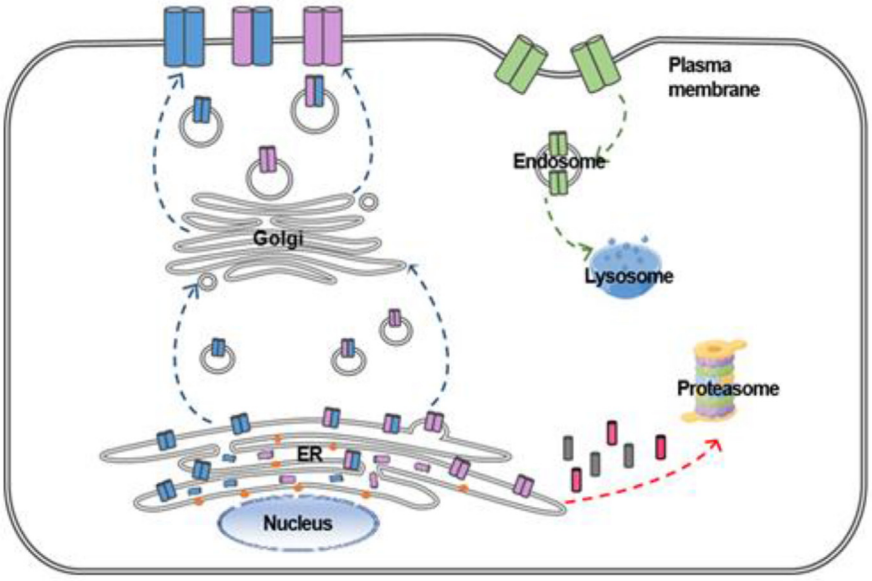

Figure 2 Role of oligomerization in drug transporter expression level and trafficking. The oligomerization and quality control (QC) pathways are interconnected and work in concert. Export from the ER requires specific signals in the amino acid sequence or post-translational modifications such as oligomerization. These mechanisms assist the transporters to pass the stringent quality control in the ER and continue for the following processes, eventually targeting to the cell membrane (blue route); while those being disrupted may be translocated to the proteasome for degradation (red route). Oligomerization may also be involved in the internalization and recycling of the drug transporters (green route).

and internalization. While NTCP oligomerization was abrogated, it is accompanied by an inhibition of NTCP internalization and ultimately impeded viral infection[64].

4.2. *Oligomerization and protein function*

The proper function of drug transporters not only relies on the protein level but also on the correct and complete conformation of the protein structure. Studies have shown that oligomerization may be involved in the exerting of various functions of drug transporters as well.

4.2.1. *Formation of functional unit*

Many membrane proteins seem to be functionally competent as monomers, despite their participation in oligomer assembly. However, reports have demonstrated that drug transporters may need to form oligomers for their proper functions. Unlike other members of the ABC family, the BCRP protomer only possesses one nucleotide binding domain (NBD) and one transmembrane domain (TMD), which oblige the efflux transporter to at least form a dimer to gain full functionality[57]. Such a functional dimerization is confirmed by the cryo-EM structure analysis[26–28].

The functional unit of transporters was identified mainly through co-expression of wide-type and lost-of-function protomer due to the lack of high-resolution structures of most of the drug transporters. The lost-of-function protein retains normal interaction ability and protein expression, hence oligomerization still occurs. When a functional dominant-positive/negative effect is observed, it is likely the functional units are oligomers. Numerous studies have shown that OATP1B1 has two binding sites for estrone-3-sulfate (ES)[151–153]. During the investigation of OATP1B1 oligomerization, it was found that this transporter may function as monomers at the low-affinity site; while oligomeric structures are needed for carrying out the uptake function at the high-affinity site. It was proposed that the formation of oligomers may create new binding sites for the transporter[44]. With a similar co-expression approach, it was found that the non-functional Cysless ASBT exhibited a dominant-negative effect on the wide-type ASBT, suggesting that ASBT exists as an active dimer and/or oligomer[49]. Both Ostα and Ostβ subunits are essential components of the taurocholate transport system. Ostα constitutes the substrate translocation subunit; while the interaction of Ostβ with Ostα is essential for membrane expression and function of the Ostα/β heteromeric protein[9].

4.2.2. *Regulation of transport function*

Aside from participating in the formation of functional units, oligomerization can also regulate the function of transporters. For example, though the translocation path of hCNT3 is confined to the monomers, the helices in the trimerization and scaffold domains in the three protomers form a large aqueous basin within the membrane that significantly shortens the substrate translocation distance from approximately 40 to 25 Å, modulating the transport function through allostery[54].

Hetero-oligomerization may also affect the functional characteristics of drug transporters. In addition to altering the protein level, the co-expression of NTCP, OCT1, or OATP1B1 significantly changed the kinetic parameters of OATP1B3. OCT1 increased the relative turnover number (k_{cat}) of OATP1B3 from 166 $\pm$ 12.4 to 253 $\pm$ 26.2 pmol/mg protein/min; while both OATP1B1 and NTCP exhibited a suppressive effect (from 510 $\pm$ 36.7 to 164 $\pm$ 10.3 and 224 $\pm$ 27.2 pmol/mg protein/min, respectively). It was proposed that OATP1B1/NTCP/OCT1 and OATP1B3 may co-localize in a microdomain at the cell surface membrane. Such kind of transporter-dependent modifications could be overlooked in cellular systems that over-expressing only

a single transporter, and its effect on the pharmacokinetics of drugs may therefore under-/over-estimated[61].

5. Modification of oligomerization

As improper oligomerization may at times lead to structural and/or functional consequences, it needs to be tightly regulated. Post-translational modifications (PTM) such as phosphorylation can interact and regulate oligomerization. Moreover, oligomerization can be modified by various peptides and small molecules, implicating a novel strategy for drug design.

5.1. Regulation of oligomerization through phosphorylation

Phosphorylation, which alters the conformation and activity of proteins by adding phosphate groups to the side chain of Ser/Thr/Tyr, is by far the most well-studied post-translational modification. Pim-1 kinase is a tumor-associated serine/tyrosine kinase that regulates a variety of proteins including cell cycle regulators, pro-apoptotic proteins, and transcription factors. Xie et al.[112] found that BCRP is a substrate of Pim-1, and phosphorylation of the transporter by Pim-1 promoted its oligomerization, cell surface localization, and transport function. Overexpression of the BCRP dephosphorylated mutant T362A or knockdown of Pim-1 reduced the association among BCRP proteins, suggesting that the phosphorylation of BCRP at Thr362 by Pim-1 is necessary for the oligomerization and function of the transporter.

Grañe-Boladeras et al.[56] demonstrated that the regulation of kinases led to a dynamic transition between homo- and hetero-oligomerization of ENT1 and ENT2. ENT1 forms homologous oligomers on the cell surface membrane under normal conditions; while ENT2 is continuously phosphorylated by casein kinase 2 (CK2) and localized in the submembrane region. When PKC induced the activation of protein phosphatase 1 (PP1), the phosphatase dephosphorylates ENT2, which in turn transfers to the plasma membrane. The relocation of ENT2 breaks down both the homo-oligomers of ENT1 and ENT2, promoting the formation of ENT1−ENT2 hetero-oligomers, and altering the function of both transporters[56].

The phosphorylation status of drug transporters may also affect their interaction with regulatory proteins. The interaction with β-catenin is crucial for the expression and cell surface localization of BCRP. In human and mouse obesity, the loss of JAK3-mediated tyrosine phosphorylation of BCRP disrupts the hetero-oligomerization between BCRP and β-catenin, resulting in a significant reduction of intestinal BCRP expression and compromising the colonic drug efflux and barrier functions[113].

The C-terminal PDZ binding domain of MRP2 contains a PKC consensus phosphorylation site-Ser1542. In Sf9 cells, the phospho-mimicking mutant S1542E showed a stronger preference to interact with Na^+/H^+ exchanger regulatory factor 1 (NHERF1) and NHERF4 than the wide-type MRP2; while the dephosphorylated mimicking mutants S1542A exhibited decreased interaction with NHERF1[100]. Phosphorylation sites (Ser634 and Ser635) are present within the C-terminal PDZ binding domain of rOatp1a1 as well. Choi et al.[154] found that phospho-mimicking mutant oatp1a1EE was significantly more prone to bind to PDZK1 than the dephosphorylated mimicking mutant oatp1a1AA. MRP1 was shown to interact with ATP synthase α or tubulin, and the interactions were modulated by the phosphorylation status of the linker domain of MRP1[97,98].

5.2. Modification of oligomerization with peptides and small molecules

Drug transporters are promising targets for drug development. Recent studies have shown that small peptides and chemical compounds can modulate the oligomerization status of drug transporters. The introduction of a short peptide with the homologous sequence corresponding to the interface of the protein oligomer can act as a competitor and interfere with protein oligomerization and function. These short peptides are tools to investigate the protein oligomerization, because such effects are highly selective−one homologous sequence only inhibited its "parental and cognate" proteins. Additionally, utilization of these short peptides avoids the unexpected mutation-derived conformational change of the protein. For example, the TM6 peptide of OAT1 was demonstrated to serve as a potent inhibitor for the formation of oligomeric complexes. Overexpression of TM6 peptides perturbed the oligomerization of OAT1 and subsequently affected its cell-surface expression and transport function[58]. In OATP1B1, the fragmental expression of TM3 interfered with the self-association of OATP1B1 and the formation of hetero-oligomers between OATP1B1 and OATP1B3[62].

The potent and selective inhibitory effects of short peptides implicate their potential application in clinical therapy. The amino-terminal MSD0L0 region mediates the dimerization of MRP1. When co-expressed with wild-type MRP1, MSD0L0 exhibited a dominant-negative effect and inhibited the transport of cysteinyl leukotriene C4 (LTC_4) by MRP1. It is likely that the presence of MSD0L0 disrupted the formation of MRP1 dimers and subsequently suppressed its function[21]. Further investigation minimized the dimerization interface region to TM5 and ECL3 within MSD0L0. It was proposed that these peptides can be developed into drugs to sensitize MRP1-induced multidrug resistance in cancer chemotherapy[144]. Similarly, co-expression of the TM5-ECL3-TM6 domain of BCRP, which constitutes the oligomerization interface of the transporter, significantly inhibited drug efflux, reducing the cell resistance to anticancer drugs mitoxantrone and VP-16[145]. By competitively inhibiting the interaction between MRP2 and NHERF1, synthetic TAT-PDZ1 peptides containing the trans-activator of transcription (TAT) peptide and MRP2-PDZ1 core-binding motif decreased the MRP2 activity in HepG2 cells[155]. Two peptides, consisting of aa 221−240 and aa 271−290 of NTCP, were shown to block NTCP oligomerization, impeding viral internalization and infection[64]. Studies also demonstrated that the peptide consisted of aa 131−150 of NTCP (a region that includes Gly144 and Gly148 that are responsible for the interaction of NTCP with epidermal growth factor receptor) interfered with the interaction between NTCP and EGFR[136]. Since NTCP oligomerization occurs downstream of the NTCP−EGFR interaction during HBV internalization, the aa 131−150 peptide fragment of NTCP may be utilized as a competitive blocker for NTCP oligomerization and HBV internalization[156].

Oligomerization of NTCP can be blocked by small molecules as well. The oligomerization of NTCP occurs after the transporter interacts with the myristoylated N-terminal PreS1 domain of the large surface protein of HBV, and is important for the internalization and entry of the virus into the cells. Troglitazone, a compound that contains the thiazolidinedione moiety, was shown to inhibit the oligomerization of NTCP and dramatically interfere with the internalization and infection of HBV[64]. Troglitazone was

found to directly bind to NTCP, non-competitively inhibiting the uptake function and oligomerization of the transporter *via* an allosteric effect[156].

In the study of Pitre et al.[111], the researchers found that the drug resistance of hematopoietic progenitor cells depended on the formation of MRP4-membrane-palmitoylated protein 1 (MPP1) oligomeric complexes. MPP1 was bound to MRP4 through the PDZ binding domain, and the formation of the protein complex promoted the localization and maintenance of MRP4 on the cell membrane, thereby enhancing the drug resistance of cells. The small molecule drug antimycin A disrupted the formation of MRP4−MPP1 protein oligomers, thereby reversing the drug resistance that occurred in acute myeloid leukemia (AML) cell lines and the patients' primary AML cells[111].

6. Discussions

As an important kind of PTM, oligomerization plays essential roles in maintaining the quaternary structures of drug transporters and at times serves regulatory roles for the expression level and function of these membrane proteins. Studies combining molecular biology and biochemistry methods along with cyro-EM techniques have revealed a more detailed picture of the oligomerization feature of various drug transporters. However, though high-resolution structures of human drug transporters such as P-gp, BCRP, NTCP, ENT1, and CNT3 have been reported, only the oligomeric structure of BCRP and CNT3 was captured[26,39,55,157,158]. The isolation methods for these membrane proteins varied, some of which may lead to the formation of artificial oligomers or disruption of the oligomeric complexes. Further optimization of protein extraction techniques and the study of these proteins within the context of a living cell will more truthfully represent the structural arrangement of drug transporters under physiological conditions. In particular, since drug transporters are diversely distributed in different tissues and/or organs, investigations using cellular systems with a more native membrane environment are warranted. It should be noted that hetero-oligomerization among drug transporters may result in transporter-dependent modifications, which in turn affects the transport activity. Therefore, cell lines that are generated to overexpress a single transporter may not fully capture the protein−protein interactions that occur in the native condition *in vivo*. When the interactions of transporters and drug molecules were examined in such systems, transport parameters such as k_{cat} or K_m would be under- or over-estimated[159]. A more comprehensive characterization of the oligomerization and functional consequences of different drug transporters is essential for providing reliable information on physiologically based pharmacokinetic studies.

Recent studies implicated that the protein−lipid interaction is another layer of post-translational regulatory mechanism for drug transporters. BCRP, OAT3, rOctn2, NTCP, OATP1B1, and OATP1B3 have been shown or proposed to be localized within the lipid rafts[61,119,160,161]. Further, it was demonstrated that cholesterol affected the trafficking and activity of drug transporters such as rNtcp, ABST, BCRP, OCT2, OCTN2, NTCP, and OCT1[162−167]. Membrane lipid bilayers provide the native environment for integral membrane transporters that potentiate their oligomerization. The role of membrane lipids in the formation of oligomers is an emerging research area for transporter studies[168]. However, little information relating to this issue is available so far.

Accumulating evidence suggests that specific structural features are required for the transporters to form oligomeric complexes. Therefore, the identification of essential oligomerization interface not only helps to expand our knowledge on how these oligomers are formed and organized, but also provides precious clues for drug development and clinical therapy. Since in many cases, the oligomerization of drug transporters exhibits functional consequences, the modification of such a process may offer a promising therapeutic strategy for the treatment of diseases related to oligomeric proteins. The oligomerization interfaces can be modulated by "proteomimics" molecules such as peptides, peptidomimetics, or small organic molecules[169], which by directly binding to the critical motifs at the interface, can competitively block the interaction between the oligomeric units[170]. Based on the interface structure, these "proteomimics" molecules have higher specificity, efficacy, and safety over traditional inhibitors, due to their larger surface area and greater chiral and structural complexity. Moreover, the overall research and development costs of peptide drugs will likely be lower than those of small-molecule counterparts due to their intrinsic synthetic feasibility and lower off-target rate. However, some important challenges remain. Firstly, information on the oligomerization interface for most drug transporters is limited. Additionally, compared to inhibiting the active or binding sites of a transporter, the difficulty in designing small organic molecules that inhibit transporter oligomerization is related to the size of the surface that should be covered by the molecule. Moreover, peptides often suffer from membrane impermeability, poor stability *in vivo*, and rapid renal clearance, thus further progress in techniques is required to improve the administration, stability, and delivery of these peptides. The synthetic peptides and small molecules that potently and selectively inhibit BCRP, MRPs, and NTCP function highlighted the potential of targeting oligomerization in clinical applications[21,64,145,155,156]. Additionally, PDZ domains, which are the largest class of protein−protein interaction modules[171], have been suggested as promising drug targets in neurological disorders, cancer, viral infections, and cystic fibrosis[172]. For example, FSC231 is a therapeutic compound that is under development for the treatment of neuropathic pain and stroke. The chemical blocks the interaction between dopamine transporter (DAT) and protein interacting with C kinase 1 (PICK1) by binding to the PDZ domain of the latter[173]. As PDZ proteins also interact with drug transporters and regulate their membrane targeting and transport activity[135,136], a similar strategy may be utilized for PDZ ligand-containing proteins such as MRP2 and MRP4 to overcome drug resistance during cancer chemotherapy. The study performed by Kawase et al.[155] demonstrated that the synthetic TAT-PDZ1 peptide, which competitively modulates the interaction between MRP and NHERF1, significantly decreased the activity of MRP2 in HepG2 cells. These encouraging developments suggest the drug transporter oligomerization itself could be a novel drug target though much work remains to be done.

Compared to other kinds of transporters, such as those of the neurotransmitter sodium symporter (NSS/SLC6) family[174], nucleobase-ascorbate transporter (NAT/SLC23) family[168,175,176], and sugars will eventually be exported transporter (SWEET/SLC50) family[177], the oligomerization of drug transporters still lacks systematic research. Some important questions remain to be answered to fully appreciate the important roles played by the PTM. Whether the oligomerization of drug transporter varies among individuals in healthy or pathological states? At which

step(s) during the synthesis and processing of drug transporters does the oligomerization take place? What is the purpose of some drug transporters to form oligomers when their functional unit is a monomer? How is the hetero-oligomerization among drug transporters regulated? The information is essential for the comprehensive understanding of the molecular and cellular mechanisms in the drug transport process, which may provide invaluable targets for future drug design and/or improvement of the bioavailability of clinical therapeutics. In-depth investigations will be needed to clarify the role of oligomerization and the mechanism whereby this process is regulated.

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Author contributions

Chunxu Ni: Writing-Original Draft; Mei Hong: Conceptualization, Writing-Review & Editing, Supervision, Funding acquisition.

Conflicts of interest

The authors declare no conflict of interest.

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