Vesicular ATP-binding cassette transporters in human disease: relevant aspects of their organization for future drug development

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“besides ABC transporters themselves, the vesicles in which they reside, the lipid raft microdomains in which they are organized and oligomerized as well as the cytoskeleton are new potential drug targets.”

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ABC transporters in disease: an introduction

ATP-binding cassette (ABC) transporters are membrane-bound proteins that actively translocate endo- and xenobiotic solutes across cellular membranes at the expense of energy by ATP hydrolysis, often against a concentration gradient [1]. In disease, these transporters are often altered in expression and/or functional activity. The multidrug resistance (MDR) phenotype in cancer, for example, occurs due to the overexpression of these transport proteins. Here, ABC transporters confer cross-resistance to structurally and functionally diverse and chemically unrelated antineoplastic agents [1,2]. Virtually every single type of the 49 ABC transporters confers resistance to antineoplastic agents, with many of them having a broad substrate range [2]. Strikingly, certain representatives (e.g., ABCB1, ABCC1 and ABCG2) have an overlapping substrate range [1,2], which enables these transporters to compensate for the functional loss of their siblings after selective inhibition and/or downregulation. On the other hand, some transporters have a differing substrate range, leading to a universal MDR phenotype, covering almost the whole range of first- and second-line anticancer agents when co-expressed. The overlapping substrate range and regulatory compensation of ABC transporters may be one major aspect why clinical studies with selective ABC transporter inhibitors have failed [1].

Another area where ABC transporters have been shown to play an important role is Alzheimer's disease (AD) [3–7]. AD is a neurodegenerative disorder which is characterized by the pathological intracerebral precipitation of extracellular β -amyloid (A β) protein plaques [3]. These neurotoxic A β proteins contribute to a decline of synaptic communication, neuronal atrophy, cognitive impairments and death after long-term accumulation and exposure

to neurons [3]. ABC transporters were discovered as key proteins in the process of production, degradation and clearance of A β proteins [3–7]. Remarkably, the very same representatives that have a bad reputation in cancer-related MDR – ABCB1, ABCC1 and ABCG2 – are associated with (Direct) A β protein export from the brain to the blood stream across the blood–brain barrier (BBB) [1]. However, many other ABC transporters have been linked to the pathogenesis of AD, especially (phospho)lipid and cholesterol transporters (e.g., ABCA1–2, ABCA5, ABCA7, ABCG1–2 and ABCG4) [3]. Similar to cancer-related MDR, the above-mentioned interrelationship between ABC transporter function and compensatory expression applies to AD as well, which has been demonstrated for certain representatives (e.g., ABCB1 or ABCG2) [3]. However, the intricate relationship between the up- and downregulation of ABC transporters in both cancer-related MDR and AD is still poorly understood and potentially underrated.

Both examples, cancer-related MDR and AD, represent the ABC transporter-related dogma of a ‘static’ frontier, where the blood–tumor barrier (BTB) as well as the BBB are commonly believed as their major ‘venue’ of action. However, several reports have already identified ABC transporters as participants in rare lysosomal storage disorders, such as Fabry disease (ABCB11 and ABCC5 [8]) and Nieman–Pick type C disease (ABCG5 and ABCG8 [9,10]) and a huge body of evidence underlines that subcellular(–originating) structures as carriers of ABC transporters may play an essential role in major ABC transporter-related diseases, such as cancer-related MDR or AD.

Subcellular ABC transporters: function beyond logistics

It is crucial to differentiate between subcellular logistics and subcellular function of ABC transporters. There is no doubt that all 49 ABC transporters are native intracellular proteins that are produced, processed and distributed during their synthesis in the endoplasmic reticulum [11], post-translational modification in the Golgi apparatus [11], pooling and/or sorting in the post-/*trans*-Golgi network [12,13], and trafficking, sorting and/or recycling in vesicular bodies [11]. And yet, this is all of logistical nature and the fusion and fission of endosomal and endocytic vesicles with the plasma membrane as well as with one another as part of the above mentioned steps is accompanied by a rather temporary residency of the individual transporter within the respective step – implying no additional function except for its relocation.

Nevertheless, many ABC transporters have (sometimes in addition to their lamellar localization and function) an intracellular purpose and target *ab initio* (multi)vesicular bodies and organelles inside the cell as a real subcellular destination rather than an intermediate ‘stopover’. These destinations include [early/late (acidic)] endo- or lysosomes (e.g., ABCA4 [14], ABCA5 [15], ABCB1 [11], ABCB6 [16], ABCB9 [17], ABCC4–5 [18,19], ABCD4 [20], ABCG1 [21,22] or ABCG4 [22]), proteasomes (e.g., ABCB9 [17]), mitochondria (e.g., ABCB7–8 [16], ABCB10 [16], ABCC1 [23] or ABCC6 [24]), peroxisomes (e.g., ABCD1–3 [20]) or phago(lyso)somes (ABCA4 [14] or ABCF1 [25]). Beyond their regular (sub)cellular function, it has also been established that (re)localization of ABC transporters into intracellular vesicles may cause, or at least contribute to, human diseases. Regarding cancer-related MDR, several ABC transporters were proven to sequester antineoplastic agents into (early/late) endo- or lysosomes, for example, ABCA2–3 [11,26,27], ABCB1 [11,28–30], ABCC1–2 [11,29–31] or ABCG2 [11,29–31]. These transporters were shown to be recruited from the cell membrane surface *via* internalization into (early/late) endo- or lysosomes, which was demonstrated in colocalization studies with these ABC transporters and endo- or lysosomal markers, such as early endosomal antigen 1 (EEA1) [11], lysosomal-associated membrane protein 1 (LAMP1) [27], LAMP2 [11,27–29], LysoTracker Blue[®] [29], LysoTracker Green[®] [11], LysoTracker Red[®] [11,27], cathepsin D [11,28,30] or synaptotagmin VII [30]. This is in agreement with colocalizations of other endo- or lysosomal markers, such as caveolin 1 [14], CD63 [12], Nieman–Pick type C [21], RhoB-GFP [22], Rab4 [19], Rab5 [14,19,21], Rab9 [19], Rab11 [19,21], ribophorin-2 [32], surfactant protein C [12] or syntaxin 7 [15], with other ABC transporters (e.g., ABCA1 [12], ABCA4–5 [14,15], ABCC5 [19], ABCC8 [32], ABCG1 [21,22] or ABCG4 [22]).

The often stress-induced [11,28,33] sequestration of cytotoxins into (intracellular or -luminal) vesicles serves two main objectives: containment, where the respective antineoplastic agent is safely contained away from its cellular target, thereby preventing its harm to cellular functions, which ultimately results in (cancer) cell survival; and disposal, where vesicles containing cytotoxic agents are processed to the cell surface to form either (multivesicular) lamellar [27] or barrier bodies [11,29], or may be segregated to constitute extracellular vesicles [31,34] before phagocytic processing including degradation of the contained harmful toxins [29]. Extracellular vesicles such as exosomes have also been associated with other ABC transporters that are not directly linked to vesicle-mediated MDR (e.g., ABCB6 [35]). Interestingly, ABC transporters themselves may also serve as cargo in extracellular vesicles. This

was shown to lead to a donor-to-recipient passing of transport proteins which resulted in an inherited MDR of previously sensitive cells (e.g., ABCB1 [29] or ABCC1 [36]).

Concerning AD, the safe containment of A β proteins in lysosomal compartments and subsequent degradation and/or phagocytic (microglial) processing is vital for cerebral A β protein clearance [3,37–39]. Indeed, a disorder in lyso- and autophagosome formation and processing in AD has been described [40,41]. Although there is no direct link between vesicular ABC transporters and lysosomal A β storage, it must be considered that several ABC transporters connected to (vesicular) lipid raft composition, organization of (organellar) membrane bilayer microdomains and (cerebral) cholesterol homeostasis, such as ABCA1–2, ABCA5, ABCA7, ABCG1–2 and ABCG4, are highly associated with the emergence and progression of AD [3]. Interestingly, most of these ABC transporters were reported to reside in endo- or lysosomal compartments [11,12,15,21,22,26,30,31], and certain ABC transporters were generally associated with phago- and endocytosis as well as autophagy, such as ABCB1 [28,29] or ABCF1 [25]. This implies an important role of ABC transporters in the degradation and recycling of (sub)cellular components and (macro)molecules. Hence, considering the contribution of ABC transporters to the formation, trafficking and endo- (as well as exo-)cytosis of intra- (and extra-)cellular vesicles, as well as the formation and establishing of lipid raft microdomains as (immune) response against A β protein excess (so-called ‘inflammarafts’) [42], a contribution to the emergence and development of AD by dysfunctional and/or defective vesicular ABC transporters seems likely.

This gains importance as lipid raft formation and membrane fluidity are major contributors to the formation of (functional) oligomeric ABC transporter structures. This does not only account for the so-called ‘half-transporters’, such as ABCB2–3 [3,17,35], ABCB6–10 [3,17,35] and ABCC12–13, as well as the D and G subclass ABC transporters [2,9,20–22], for which a homo- (or hetero- [20]) dimerization is proposed. But also so-called ‘full-transporters’ were reported to dimerize (e.g., ABCB1 [43], ABCC1 [44] or ABCC9 [45]), and the ‘half-transporter’ ABCG2 was reported to agglomerate to a tetramer [46], which equals a ‘full-transporter’-dimer. Hence, oligomerization to form ABC transporter units is vital for their function and highly dependent on membrane fluidity.

Implications for drug design & development: multitarget approaches to uncover black spots

The discovery of new small molecules to affect underexplored transporters can be challenging. For example, the A subclass ABC transporters comprise of representatives highly associated with AD [3]. Especially dysfunction of ABCA1 and ABCA7 are highly connected to the development and progression of AD [3]. In fact, ABCA7 was identified as the second loci showing highest odds ratio for increased AD risk (1.23), just after APOE [47]. However, there are only very few directly interacting small-molecule modulators of these transporters [48,49]. Considering the existence of transporters with no knowledge about their structure, function or interfering small molecules, one is confronted with the question: How can a potent and selective modulator be developed to intervene with the function of such an ABC transporter?

Fortunately, there are several options to answer this question. The first option is the use of structure-based information: recently, the cryo-electron microscopy (cryo-EM) structures of ABCA1 [50,51] and ABCG2 [52] have been reported, which may be utilized to gain (virtual) information of other ABC transporters of the same subgroup by, for example, homology models. From this structural data, conclusions can be drawn for future drug design and development of small-molecule modulators. With scientific progress, more structural data of ABC transporters will be reported and available for computational analysis, and hence, the (re)evaluation of (existing) computational models. This will facilitate for more accurate extrapolation and prediction of structural information to unexplored ABC transporters. The second option is the use of ligand-based information: biological data of ABC transporter modulators can be used and transferred to transporter subgroup relatives – or even for their counterparts in other subgroups [53]. This may seem counterintuitive, as the sequence identities (and similarities) of nonclosely related transporters of different subgroups are rather low. However, it has already been shown for broad-spectrum ABCB1, ABCC1 and ABCG2 inhibitors [54–57] and reversers of ABCB1-, ABCC1- and ABCG2-mediated MDR [54–56], that a cross-family modulation of ABC transporters is possible with one single molecule. This is a striking fact, as the sequence identity (similarity) of these three MDR-conferring transporters is rather low according to sequence analyses [ABCB1, ABCC1: 19.2% (34.8%); ABCB1, ABCG2: 13.6% (23.2%); ABCC1, ABCG2: 9.2% (15.6%)]. Consequently, it can be assumed that common amino acid sequences and structural motifs within the transporters exist beyond the highly conserved nucleotide-binding domains (NBDs). These sequences and motifs may represent a common structural or functional similarity and could be harnessed for drug design and development [53]. This also raises the question if the phylogenetic categorization of ABC transporters is still contemporary considering their functional similarities.

Technically, multitarget modulation of ABC transporters has until now only been described for inhibitors [54–57], which potentially qualifies them for application in cancer-related MDR [1]. However, in AD, these inhibitors may serve as template structures for ABC transporter activation as a potential treatment option against AD development and/or progression, since certain compound classes were shown to comprise of both inhibitors and activators [58,59]. Hence, considering all data available on (multitarget) modulators of ABC transporters [1], analysis of the biological data and its correlation to functional substructures of the respective molecule(s), as well as identification of molecular components as indicators for broad-spectrum interaction [53], novel nonselective (promiscuous) activators might be found. This strategy can be supported by computer-based approaches, such as substructure or similarity search, pharmacophore modeling or molecular docking, which already led to the discovery of novel pan-ABC transporter inhibitors [55,57]. Nonselective (promiscuous) activators may be further optimized for selectivity regarding the desired transporter by organic synthesis. On the other hand, selectivity might not even be necessary, as a simultaneous attack on multiple (sub)cellular targets allows for interruption or potentiation of several pathways, and hence, may offer better treatment options [1].

Vesicular ABC transporters: implications for pharmacokinetics

Promiscuity of molecules could be the first step in the development of novel, highly potent and selective modulators of ABC transporters that were previously unable to be targeted by small molecules. However, when it comes to target localization, diseases like cancer-related MDR or AD have an additional pharmacokinetic dimension, where the distribution and targeting of small-molecule agents to their (sub)cellular target(s) can be a huge challenge. Nevertheless, targeting of subcellular ABC transporters has already successfully been demonstrated [11,28]. Several studies have shown that designing ABCB1 substrates that are lysosomotropic (prone to lysosomal trapping) can induce lysosomal membrane permeabilization (LMP) and cancer cell death by generation of reactive oxygen species (ROS) inside the lysosomes [11,28]. In this instance, the small molecule can use the ABC transporter to its advantage to increase its concentration within lysosomes to exert its lethal effect on cancer cells. Considering that nearly all ABC transporters contribute to the MDR phenotype [2] and many representatives are functionally active in intracellular vesicular systems [11,26–28,30,31], the above described targeting strategy [11,28] may serve as template to be utilized in conjunction with other ABC transporters.

Again, the situation in AD is more complex, as substrates of ABC transporters may either already fail to cross the BBB or fail to be retained in the brain. In this case, these agents will never reach their intended subcellular target(s). Such promiscuous or selective substrates would be of great use as AD diagnostics [e.g., positron emission tomography (PET) tracers] to track ABC transporter function at the BBB itself [60] (e.g., ABCB1 [61,62] or ABCC1 [63,64]). However, for future drug design of AD therapeutics to affect ABC transporters participating in (subcellular) vesicle membrane formation and composition, these compounds should rather not be substrates but allosteric modulators. As modulation would most likely be conferred through the inter-membrane space, the compounds should be rather lipophilic. This would also be of use when focusing on lipid raft microdomains as potential drug targets [42].

Besides, the oligomerization of ‘half-’ and ‘full-transporters’ [43–46] generates new interaction sites, therefore target options for interfering drugs. These sites contain certain motifs and/or domains for vesicular targeting and/or anchorage of ABC transporters (e.g., A subclass ABC transporters [12], ABCB6 [65], ABCD1–3 [20,65] or ABCG1 [21]). Finally, it must be noted that the lipid raft microdomain organization, but also the movement of intracellular ABC transporter-containing vesicles, is orchestrated by the cytoskeleton [11], which for its part was demonstrated to be influenced by ABC transporter function (e.g., ABCA1 [42]). In summary, besides ABC transporters themselves, the vesicles in which they reside, the lipid raft microdomains in which they are organized and oligomerized as well as the cytoskeleton are new potential drug targets.

Pan-targets & pan-agents: concluding remarks

Awareness of the target and its localization is key for future drug development, which applies to cancer-related MDR, AD and other diseases with ABC transporter participation (e.g., atherosclerosis). Within the last 40 years, a comprehensive knowledge regarding ABC transporters, their basic functional aspects, as well as interfering small molecules has accumulated [59,66]. However, we still have a limited understanding of ABC transporters, their functional roles, localization sites and specific involvement in disease development and progression. Many ABC transporters have barely been studied, especially in the A and G subfamily. For future target identification and drug design, one must be aware that certain aspects of particular ABC transporters, such as function and localization, may

also apply for their subgroup relatives – or even for their non-subgroup counterparts. For example, the sequestration of anticancer drugs into lysosomes has been shown for certain ABC transporters of different subgroups [11,26–28,30,31]. However, the capability of a cell to develop cross-resistance might not be limited to the expression of these particular ABC transporters but could rather be transferred to other understudied ABC transport proteins as well. These may act solely or in association with other transporters, either functionally [1,2] or via a regulatory interrelationship [1], as already proven for certain representatives [1,3]. Moreover, ABC transporters may have a critical contribution to lipid raft microdomain composition and organization, which might not only be limited to (phospho)lipid and cholesterol transporters, which offers a new perspective on target identification in AD as well. Functional aspects that were formerly perceived as exclusive to a small group of ABC transporters may be eligible for many other ABC transporters as well, especially regarding their localization and function in subcellular organelles as well as membrane vesicles. From this, a general intracellular function of these ABC transporters could be concluded, questioning the widely accepted legitimacy of the phylogenetic categorization of ABC transporters. In all, this seems to be a major challenge for the development and application of novel interacting agents. On the other hand, this complementary information provides a unique opportunity to obtain small molecules as novel and innovative interactors of these understudied targets, to uncover their impact in human diseases and reveal critical pathological aspects. Appreciation of these aspects will truly guide future drug discovery in ABC transporters-mediated diseases.

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