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Multi-target ABC transporter modulators: what next and where to go?

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“the huge knowledge accumulated until today could bring several new promising candidates into the clinic, which increases the chance of a real clinical breakthrough.”

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Cancer & ABC transporters: the background

Several ABC transporters (e.g., ABCB1, ABCB4, ABCC1–5, ABCC10, ABCG2 and ABCG4) are known to contribute to the phenomenon of cancer-related multidrug resistance (MDR) [1]. Expression occurs either inherently (chemo-naïve patient) or is acquired after initial cancer chemotherapy (chemo-treated patient) in certain cancer types (such as bladder, blood, brain, breast, colon, kidney, lung, ovary and pancreas) [1]. ABC transporters recognize a broad range of structurally unrelated antineoplastic agents inside (cancer) cells and translocate these into the exterior using the energy of ATP hydrolysis. The substrate range of the most studied representatives ABCB1 (P-gp), ABCC1 (MRP1) and ABCG2 (BCRP) covers almost the whole range of (small-molecule) antineoplastic agents in use today, for example, anthracyclines (B1, C1), antifolates (B1, C1, G2), camptothecins (B1, C1, G2), epipodophyllotoxins (B1, C1, G2), nitrogen mustards (C1), taxanes (B1), tyrosine kinase inhibitors (TKIs [B1, C1, G2]) or vinca alkaloids (B1, C1) [1–3]. Occurrence of ABCB1, ABCC1 and/or ABCG2 is recognized as a negative marker for the outcome of chemotherapeutic intervention, and relapsed/refractory malignancies are associated with overexpression of either of these transporters [4]. The most recent analyses confirmed that these three transporters are also co-expressed in certain cancers, such as leukemia [5]. However, there is still a lack of understanding regarding the interplay between ABC transporters in general, and a correlation between co-expression of certain representatives and prognosis in cancer has only very recently been addressed in the literature [6].

Inhibitors of ABC transporters: a historic approach

Ever since their discovery, two questions arose with regard to ABC transporters: (i) how do they work?; and (ii) how can their activity be manipulated? Small-molecule ABC transporter modulators play(ed) a huge role in changing transport activity to draw conclusions regarding function of these efflux pumps. Nearly 30 years have passed since the discovery of the very first high-throughput screening- and synthesis-derived inhibitors of ABCB1. Before, inhibitors have only been known from other pharmacological fields (so-called ‘first generation’ inhibitors; for example, verapamil or cyclosporine A) [2,3], exerting additional (negative) pharmacological effects. Pharmaceutical companies like CBA Pharma, Eli Lilly, Immunex, Janssen, GlaxoSmithKline, Mitsui, Pfizer, Sandoz, Vertex or Xenova spent huge effort to generate novel, highly potent and nontoxic ABCB1 inhibitors [2,3]. CP100356, CBT-1, GF120918 (elacridar), LY335979 (zosuquidar), MS-209 (dofequidar), PSC 833 (valsopodar), R101933 (laniquidar), S 9788, VX-710 (biricodar), XR9051 or XR9576 (tariquidar) are the most famous representatives that marked the fundament of modern ABC transporter inhibitors, also regarding ABCC1 and ABCG2. However, only few compounds found the way into clinical oncology (including CBT-1, LY335979, MS-209, PSC 833, VX-710 and XR9576 against ABCB1 [3,7]; MS-209 and VX-710 against ABCC1 [7]; and GF120918 against ABCG2 [8]). Most trials were terminated due to severe side effects, leaving sparse agents still under consideration

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for ongoing clinical evaluation (e.g., CBT-1) [3,4]. No authority-approved inhibitor of ABC transporters exists until today. Hence, what is the problem?

The clinical problem: study design

Clinical studies conducted did absolutely not meet the expected results. This is a multi-faceted problem on different levels as cancer-related MDR is a complex issue which mostly cannot be attributed to one single mechanism of resistance (such as the expression of ABC transporters). The involved modes comprise of: (i) decreased influx or reduced uptake of chemotherapeutic agents; (ii) alteration of the cell cycle leading to changed drug target(s); (iii) increased DNA repair; (iv) altered metabolism of the applied drug(s); and (v) increase of energy-dependent efflux [2]. In trials of the 1990's and 2000's, in which patient populations were chosen by tumor type, the presence of a transporter-dominated mode of MDR has not been addressed properly [3]. This can be attributed to the lack of methods to assess ABCB1 in the respective tumor(s). Nowadays, modern biomarkers to image transporters and their function *in vivo* are available [5,9]. In addition, biopsy-derived cells can more easily be screened applying novel fluorescence-based accumulation assays using specific (or broad-spectrum) ABC transporter inhibitors [10]. Western-blotting and PCR complement the fund of methods to determine the participation of ABC transporters in cancer-related MDR. Including the mentioned methods in future study designs, (i) a putative transporter-dominated resistance mechanism could be determined, and (ii) proper patient populations could be selected. Furthermore, a clever randomization should be performed, as run-in phases with antineoplastic agents, or (usually chosen) cross-over designs could have the disadvantage of promoting resistance of cancer cells and therefore *de facto* change the preconditions of the patient populations [4]. Hence, a modern and innovative study design is needed to address all peculiarities of cancer-related MDR.

The clinical problem: compound-related aspects

The first studies against ABCB1 were performed using pharmacological drugs with inherent pharmacological profile (e.g., verapamil) [2,3]. Due to low potency of these drugs, high doses were necessary to obtain the desired effect (ABC transporter inhibition), which went along with severe adverse events (such as heart block [verapamil]). Second- and third-generation small-molecule ABC transporter inhibitors were used at much lower dose, as they were significantly more potent than first-generation drugs such as verapamil. Nevertheless, unforeseen adverse events of potentially life-threatening severity are still one major issue in clinical evaluations, which already led to termination of many trials [3,4]. Adverse events and their severity can only marginally be anticipated by *in vitro* cytotoxicity/cell viability assays and *in vivo* experiments. Hence, it must be pondered: the potential risk of a new therapy against the death in the immediate future of relapsed/refractory cancer patients.

The clinical problem: preclinics

It is recognized that one major impediment of clinical success were (and still are) preclinical models to cast potential candidates for clinical evaluation. These models do not mirror the physiological reality – for example, the expression levels of ABC transporters in used (cancer) cell lines are much different (i.e. higher) than in tumor cells; or one-layer (cancer) cells in a culture flask are completely accessible for adjuvant ABC transporter inhibitors, while cells in a tumor are often not (except for highly vascularized tissue) [3,4]. This makes it difficult to translate *in vitro* results to positive clinical outcomes. On the other hand, many mice xenograft models showed that tumor growth inhibition and shrinkage were achievable by ABC transporter inhibitors in combination with antineoplastic agents. Furthermore, artificial test setups are common and apply for almost all preclinical models in pharmacology. Nevertheless, even with these artificial test setups drugs enter clinical trials and get approval on world health markets. Hence, considering the characteristics of cancer-related MDR in general, such as the different mode(s) of resistance, drug penetration into the tumor(s), ABC transporter polymorphism or expression of multiple ABC transporters [3–6], the development of a targeted therapy in ABC transporter-driven cancer-related MDR is feasible.

The clinical problem: state of the art

Within the last 20 years, TKIs were developed as the newest class of small-molecule anticancer agents. Soon, it was discovered that these compounds are also inhibitors of the three major ABC transporters [8,11]. Several clinical evaluations have been conducted using common antineoplastic agents with adjuvant TKIs, including lapatinib, nilotinib and sunitinib against ABCB1 [8,11,12]; nilotinib against ABCC1 [12]; and gefitinib, lapatinib and sunitinib against ABCG2 [8,11]. However, these trials encountered well-known impediments: (i) there has

been only partial benefit in certain trials (complete remission or enhanced progression-free period), while many patients did not respond; (ii) in some cases the trial had to be aborted due to severe side effects (cytotoxic agent- or TKI-related effects); and (iii) certain TKIs were identified as substrates of ABC transporters, hence the same resistance (promoting) mechanism applied compared with antineoplastic agents. On the other hand, two major achievements have been made: (i) proof was given that MDR reversers can be used (under certain circumstances) against cancers with expression of ABC transporters in general; and (ii) since most TKIs have affinity to more than only one ABC transporter, a multi-target approach became promising. However, one major obstacle is the use of existing data to contribute to the development of novel agents. It is a problem of compound quantification.

The quantification problem: data deviation

Today there is a unique chance to re-evaluate the data available and re-consider clinical evaluation of certain representatives [13–15]. Compared with the 1990's, huge information has accumulated regarding ABC transporter inhibitors – selective or nonselective – especially concerning ABCB1, ABCC1 and ABCG2. This information deviates of course on a natural basis, as different work groups use different assays – dependency on (i) detection method (such as radioactivity vs fluorescence); (ii) tracer (substrate of ABC transporters; for example, doxorubicin vs. calcein AM); or (iii) ABC transporter-carrying host (such as reconstituted/enriched inside–out membrane vesicles vs selected/transfected living cells). Nevertheless, a computational virtual screening approach taking different (naturally deviating) data on ABCC1 inhibitors into account led most recently to the discovery of several specific, dual and broad-spectrum ABC transporter inhibitors [16]. Hence, data deviation has a certain impact on such novel methods, but this impact has limits and does not necessarily compromise the experimental outcome. Instead, this method gave proof that the right quantification measure will lead to novel agents. On the other hand, data can vary, sometimes over a wide range, which makes specific compound-related measures, such as inhibitory power or activating nature, also assay specific. Hence, characteristic measures of compound evaluation such as the IC_{50} are at best not only determined and evaluated in one single assay, but in different assays that (i) either use different detection methods, or (ii) at least different tracers for cross-over validation. In addition, new measures are needed to further characterize compounds properly.

The quantification problem: finding new measures

In the 1970s to 1990s, single-point measurements in MDR reversal assays were very common, providing certain measures (e.g., fold reversal, resistance factors or percentage reversal). One disadvantage is that these values are rather incomparable to each other, and can barely be used for ongoing evaluation without accepting huge (mathematical) errors. However, within the last 20 years, the same MDR reversal assays were conducted extensively with multiple-point measurements to translate the ABC transporter-inhibiting feature of a compound (IC_{50}) to its capability to reverse MDR. This much more detailed analysis of potent ABC transporter inhibitors became more important as a new measure emerged that was introduced within the last 2 years: the half-maximal reversal concentration (EC_{50}) [16–19]. The EC_{50} gives the concentration of an inhibitor which is needed to half-maximally sensitize ABC transporter-expressing (cancer) cells, and can be interpreted as the IC_{50} equivalent of MDR reversal assays. This measure can be deduced from an inverse-sigmoid curve that results when plotting the resistance factors of the (cancer) cells against the used inhibitor concentration. Principally, the data to generate this measure exist for many compounds (such as the TKIs pelitinib, sunitinib or vatalanib) [20–22], but the final step from rows of (raw) data to the final comparable measure (EC_{50}) has mostly not been taken except for very few representatives [16–18]. IC_{50} and EC_{50} values can be used to validate each other. The first value discriminates between potent and nonpotent, the second value discriminates the (usually) potent compound between effective and noneffective against (cancer) cells. Correlating both gives answer to the questions: (i) is the initial surrogate of compound evaluation (e.g., fluorescence intensity) an adequate measure for efficacy (prediction) in MDR reversal assays?; and (ii) do structure–activity relationships (SAR) obtained in the inhibition assays reflect also in MDR reversal assays? This validation is barely reported in the literature (pyrrolopyrimidines against ABCC1 in daunorubicin assay and reversal of MDR against daunorubicin) [23]. A third up to now never reported measure is time-dependency of MDR reversal *in vitro*. Besides the proof that a potent compound (IC_{50}) reversed concentration-dependently MDR in (cancer) cells (EC_{50}), it should also be highlighted whether a low, nontoxic concentration ($\leq EC_{50}$) of this compound in combination with an antineoplastic agent time-dependently kills (cancer) cells more efficiently compared with the antineoplastic agent alone. Finally, the capability of the compounds to either up- or down-regulate the evaluated ABC transporters (e.g., by western blot and/or PCR) should be assessed. These mentioned measures: (i) IC_{50} , (ii) concentration-

(EC₅₀), and (iii) time-dependent MDR reversal in combination with a cross-correlation of these values and consideration of 'side effects' of the evaluated compounds like transporter induction will help significantly: (i) to feed new computational approaches (such as similarity search and/or pharmacophore modeling) with purpose to obtain new scaffolds for reversal of ABC transporter-mediated MDR applying *in silico* screenings, and (ii) to assist in the decision process to go on with *in vivo* evaluations or even with clinical studies in cancer patients.

The quantification problem: finding the right target(s)

In earlier clinical studies transporters other than ABCB1 have not been assessed properly [3]. The same principle holds true in ABC transporter inhibitor research: while highlighting one specific transporter, testing – or at least screening – of others was (and still is) often omitted, which needs to be changed. Instead, all compounds must routinely be screened against other transporters as well – at least against the three major ABC transporters ABCB1, ABCC1 and ABCG2. Determination of IC₅₀ values – even if in high single-digit or low double-digit micromolar concentration range – is important to determine critical features needed for selective or broad-spectrum ABC transporter inhibition. Until now, approximately 800 compounds have been evaluated against ABCB1, ABCC1 and ABCG2 with definite IC₅₀ values, only one tenth has been revealed as triple ABCB1, ABCC1 and ABCG2 inhibitors [16]. Less than 30 exerted their inhibitory power against all three transporters below 10 μM, only approximately ten representatives had activities below 5 μM. Nevertheless, multi-target ABC transporter inhibition has emerged as a new approach in ABC transporter science, which has increasingly been recognized within the past couple of years [5,24,25].

Multi-target inhibitors of ABC transporters: a new approach in ABC transporter-driven MDR

Simultaneously targeting several transporters gives benefit due to several reasons: (i) it was recently proven that the three major ABC transporters (ABCB1, ABCC1 and ABCG2) might be co-expressed in one cancer cell line (inherent co-expression) [5,6]. Since these have differing substrate preferences, a co-expression extends their substrate (= antineoplastic agent) range, which increases resistance against chemotherapeutics in general; and (ii) it was also reported that downregulation or selective inhibition of one transporter might be compensated by upregulation of another (triggered upregulation) [26–28], which can be attributed to their (partially) overlapping substrate range. Considering that certain cancers have the ability to regulate the presence of ABC transporters and therefore change or maintain their resistance profile by supplementary or compensatory ABC transporter (over)expression, the next logical step is to counteract these effects by designing an inhibitor that affects all putatively upregulated transporters. This includes the three major ABC transporters mostly associated with cancer-related MDR (ABCB1, ABCC1 and ABCG2). In ongoing research, other MDR-relevant transporters have also to be taken into account as well (e.g., ABCB4 and ABCC2-5) to extend the interaction profile of multi-target inhibitors. Until now, only approximately 80 triple ABCB1/ABCC1/ABCG2 inhibitors with definite IC₅₀ values exist, and only five compounds were shown to reverse ABCB1-, ABCC1- and ABCG2-mediated MDR (e.g., the TKIs pelitinib, sunitinib and vatalanib) [16,18,20–22]. Consideration of both critical measures (IC₅₀ and EC₅₀) in a new double-tracked computational approach with recourse on the huge knowledge accumulated until today could bring several new promising candidates into the clinic, which increases the chance of a real clinical breakthrough.

Multi-target activators: a new field in Alzheimer's disease

Within the last 20 years, it was discovered that ABC transporters play a crucial role in Aβ clearance at the blood–brain barrier [29]. Strikingly, the very same three representatives in context of cancer-related MDR – ABCB1, ABCC1 and ABCG2 – are up to now the only ones associated with direct Aβ protein transport [30–32]. The most modern concept of ABC transporter science is the discovery of new activators of these efflux pumps to lessen Aβ burden in Alzheimer's disease-affected brains. However, the ability to activate ABC transporters was demonstrated for only few compounds [33]. As certain modulators exert their effects on ABC transporters often in a biphasic mode (activation at low concentrations and inhibition at higher concentrations) [33], a full-blown compound characterization in broad concentration range is needed. With computational approaches to classify potent and less potent activators – starting from the few known representatives that exert activation – a bulk of compounds could be generated to elucidate the mode of action and SAR of activators for ABC transporters in general.

Conclusion: where to go from here?

Multi-target ABC transporter modulators are of high (clinical) importance, not only due to their role in illnesses with high incidence in aging society (such as cancer-related MDR or Alzheimer's disease), or due to their huge impact on pharmacokinetics of drugs. With computational approaches, the target preferences of these broad-spectrum modulators with respect to the evaluated ABC transporters can be elucidated. This provides the necessary information needed for the next steps: (i) generation of even more novel chemical compounds as multi-target ABC transporter inhibitors (or activators) which will putatively enter clinical evaluations against cancerous (or neurodegenerative) diseases; and (ii) providing highly specific modulators of ABC transporters which could serve as diagnostic tools (e.g., in basic ABC transporter research and assessment) and could be used in a targeted therapy against cancer or Alzheimer's disease. We are just at the beginning of future compound evaluation and comparison concerning ABC transporters in general, which will found the backbone of future ABC transporter modulators in particular and future medicinal chemistry in general.

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