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ABC transporters in anticancer drug transport – Lessons for Therapy, Drug Development and Delivery Systems

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Abstract:
The structural aspects as well as the classification of the ABC superfamily (the largest group of transmembrane proteins) has been highlighted. Over-expression of one or more of these transporters, barring exceptions, can correlate with an increased drug resistance (the multi-drug resistance phenotype). Hence, studying these proteins, using experimental and *in silico* approaches, has tremendous benefit for patient selection as well as stratification into "good" and "poor" drug responders. Further, the need to obtain a better insight into "intrinsic" and "extrinsic" mechanisms of resistance were reiterated upon, based on the relative recruitment of the different signal transduction molecules. The concept of the reversal of the MDR phenotype, has been discussed and extended in the context of combination therapy. This form of therapy involves the use of a cocktail of synthetic and biopharmaceutical drugs as well as nanotechnology-based approaches, for improvements in their pharmacokinetic (PK) and pharmacodynamic (PD) profile. Such strategies have targeted the heterogeneous cancer and cancer stem cells, signaling molecules, marker enzymes as well as the microenvironment for improved efficacy and safety as well as to minimize the chance of relapse.

Keywords: ABC transporter; P-gP glycoprotein; drug efflux; drug uptake; SLC; MDR phenotype; polypharmacy; combination therapy; cancer stem cells; nanoparticle /nanotechnology.

1. Introduction to ATP – binding cassette (ABC) transporters and P-glycoprotein (P-gP)

ABC transporters are the largest family of transmembrane proteins (Scott et al., 2012; Karwar, 2014), which utilize the energy from ATP hydrolysis to translocate many molecules across biological membranes. This translocation may be from within the cell to the endoplasmic reticulum; mitochondria or to the exterior of the cell. In the latter case, the predominantly unidirectional pump may be necessary for the transport to other organs or excretion from the body. Further, the membrane protein has certain non-transport functions, as in translation and DNA repair (Davidson et al., 2008; Goffeau et al., 2004). They have acquired this name, since they have a characteristic ATP-binding domain (NBF-nucleotide binding fold). This domain (found in all ATP-binding proteins) consists of a Walker A and a Walker B motif separated by about 90-120 amino acids, along with a distinctive C-motif upstream of the Walker B site. This protein has two each of the transmembrane domains (typically 12, however, the number of helices can vary) and the NBFs (localized in the cytoplasm and involved in energy transfer for substrate transport). Heterogeneity in the TM domains has been reported and they have been reclassified as Type I importer; Type II importer and ABC exporter (Locher, 2008). These transporters can have 1 TM and 1 NBF (half-transporter) or can be a full transporter (2 TMs and 2 NBFs). In the former case, they can homo or hetero-dimerize to produce a fully functional transporter (Borst and Elferink 2002). These transporters are involved in ferrying endogenous compounds like metabolic products, lipids, sterols across the cellular membranes, apart from exogenous drugs including those for treating cancer (Deeley et al., 2006).

These transporters (highly conserved throughout evolution) have been classified into seven mammalian subfamilies (ABCA; ABCB; ABCC; ABCD; ABCE; ABCF; ABCG) based on the structure of the gene (49 genes in humans); sequence homology in the TM and NBF domains; as well as in the organization of the domains. ABCA1 subfamily (12 transporters) has been further classified into 2 groups. Seven proteins form part of 1 group, while the other group has five proteins. The former category of proteins has their genes dispersed in different chromosomes, while in the case of the latter; they are all clustered together on one chromosome (17q24). The ABC – transporters P-glycoprotein (ABCB1) is one of the most clinically relevant protein and was the first to be characterized for its ability to confer an **M**ulti **D**rug **R**esistance (MDR) phenotype in cancer cells. Members of this subfamily are four full transporters and seven-half transporters and are mainly localized in the brain and in the liver (Dean et al., 2001). This transporter is able to efflux structurally diverse category of hydrophobic drugs (broad spectrum) including colchicine, adriamycin, vinblastine and VP16. The intriguing and puzzling finding of cross-resistance to a multitude of different classes of anti-cancer drugs (with notable exceptions being platinum compounds, nucleoside analogs, or alkylating agents) was resolved, in major part, by the discovery of the aforesaid flexible, 170 kDa P-glycoprotein (P-gP) in the 1970s (Juliano and Ling, 1976; Juranka et al., 1989). Corroborative evidence for the flexibility of this protein has been demonstrated in mice and humans using a combination of chemical, biochemical, biophysical, genetic and molecular modeling approaches. Further, the protein has been shown to have a common drug-binding pocket with partially overlapping sites for substrate binding (Chufan et al., 2015). However, results obtained were based on X-ray crystallography data from mice. Currently, further research has been hampered by the lack of X-ray crystallography data on the human P-gP necessitating computational approaches with the homology-modeled human glycoprotein (Cleave SS et al. 2013). It is hoped that such an approach may provide more insights into their possible differential affinity of binding (anti-cancer drugs) with the inward (closed conformation) and the outward (open conformation) forms of the protein and hence may provide a better understanding of drug resistance. While the ABCC subfamily has 12 transporters, the proteins relevant to drugs are ABCC1, ABCC2 and ABCC3. ABCC1 has a similar "drug-efflux profile" when compared with that of ABCB1. However, these proteins are involved mainly in removing drugs conjugated to glutathione and other organic anions Four genes make up the ABCD subfamily in humans. They are not directly involved in drug transport. They (half-transporters) have been reported to be involved in fatty acid transport and oxidation in the peroxisome. Both ABCE and ABCF subfamilies have no TM region and hence are not involved in anti-cancer drug transport, despite possessing ATP-binding domains. Members of the last family (ABCG) have six "reverse" half-transporters. These proteins have a NBF at the amino terminus and a TM domain at the carboxyl terminus. Of all the genes in this family, drug resistance has been attributed to ABCG2 (ABCP, MXR1, BCRP). Gene amplification or chromosomal translocation may be the major mechanisms responsible for the observed resistance to chemotherapeutic drugs in the anthracycline family (Dean et al. 2001). Apart from the family of ABC transporters, there are some proteins that are related, at least in part,

structurally to this family. They belong to the multidrug resistance associated protein family (MRP) and consists of nine members (Kruh and Belinsky 2003). This aspect (topic for another review) should also be seen from the oft repeated paradigm, in terms of redundancy of toxicant efflux mechanism (back-up) being used for the fortuitous removal of anti-cancer drugs.

2. Chemotherapy –Targeting Multi Drug resistance (MDR) in cancer

Drug resistance is the prime cause of death in cancer and 30-80% of the cancer treatments fail due to resistance to cytotoxic drugs (Velingkar and Dandekar, 2010). The expression of ABC transporters increases with the exposure to a wide spectrum of anti-cancer drugs which are synthetic or natural in origin. These drugs fit into one among the several classes and include mitotic spindle disruptors (vinca alkaloids -vinorelbine, vincristine, vinblastine); DNA intercalators that inhibit the progression of topoisomerase II complexes as well as cytotoxic free radical generators (anthracyclines –doxorubicin; daunorubicin; epirubicin); microtubule-disrupting diterpenes (taxanes -Paclitaxel, Docetaxel); cytotoxic topo-II inhibitors-epipodophyllotoxins (etoposide, teniposide); Topoisomerase I inhibitor (topotecan); polypeptide antibiotics which inhibit DNA transcription (dactinomycin); and aziridine-containing, natural DNA crosslinker-mitomycin C and, barring exceptions (platinum compounds, nucleoside analogs as well as alkylating agents) are known to be effluxed by these transporters, and they have a broad substrate specificity. This is the case, despite the differences in the respective mechanisms of action of the aforesaid different types of drugs. (Krishna and Mayer, 2000). Increased expression of P-gP has been correlated with poor prognosis. This aspect has been reported for both solid tumors as well as for leukemias. For e.g., In a study involving 50 patients with locally advanced breast cancer, NeoAdjuvant ChemoTherapy (NACT) and immuno-histochemical findings provided evidence for an inverse correlation between P-gP expression and drug response. In other words, the patients who were positive for P-gP expression before NACT, were poor responders (Singh et al., 2005). Calcein efflux assay (quantitative, standardized, inexpensive screening test for the detection of P-glycoprotein as well as multidrug resistance-associated protein activities) indicated that there was a 69% chance of the therapy failing in patients with MDR positivity. Further, there was a 72% chance of a positive response in patients testing negative for the MDR phenotype (Karászi et al., 2001).

Increased Expression of P-gP has been correlated with the increasing levels of acquired drug resistance and the development of the MDR phenotype. This form of resistance is known as having been "acquired" and may be due to copy number changes and/or increases in gene expression and has been demonstrated in laboratory-based experiments and clinical studies (Grogan et al., 1993). Further, others have reported a possible intercellular transfer of the P-gp as well as the "acquired resistant" MDR phenotype to the PgP negative cells (Levchenko et al., 2005). In other cases, the endogenously-high, intrinsic tissue-specific expression of P-gP (for e.g., epithelial cells of the colon, kidney, adrenal, pancreas, and liver) accounts for the initially high efflux, and hence faster development of resistance to such anti-cancer drugs. This resistance is translated into a decrease in the

efficacy and the possible dose-dependent increase in toxicity (Fardel et al., 1996). Further, intrinsic resistance may be related to the acquisition of genetic mutations in the genes encoding for this transporter protein (Szakács et al., 2006). These findings have provided an impetus for mechanistic studies to be performed to document the differences as well as common signal transduction pathways that can provide a better molecular profile of drug resistance. In this regard, for paclitaxel, NF-kB as well as pregnane X receptor (PXR)-mediated pathways were involved in P-gP-mediated drug resistance. In the case of doxorubicin, it has been reported that NF-κb pathway was primarily involved. Hence, different strategies may need to be adopted for P-gP-mediated resistance to different anti-cancer drugs (Xu et al., 2014) in the context of target identification/validation. Further, several polymorphisms in the MDR1 gene have been identified and such genetic variants can contribute to alterations in the drug efflux capabilities. For e.g., a case-control study showed that the CC/TT genotype (1236 codon in exon 12) in the MDR1 gene conferred the "poor responder status", in a Saudi Arabian population of breast cancer patients, to the standard chemotherapeutic regimen (Alsaif et al., 2013). In certain cases, even synonymous changes can alter the conformation and hence the substrate and inhibitor binding sites despite their being no change in the coding sequences (Kimchi-Sarfaty et al., 2007).

3. Strategies on the reversal of MDR in cancer

Research is ongoing globally to decrease toxicity and improve the efficacy of P-gP inhibitors (improved PK/PD profile). These efforts include micronization-based strategies of existing drugs; improvements in the physico-chemical properties (hydrophobilicty vs hydrophilicity and nanotechnology-based based polypharmacy (combination therapy) methods (Liu et al., 2014). This mode of therapy has also been demonstrated model systems. For e.g., upregulation of proteins including MDR1-mediated resistance to paclitaxel has been reported in certain cell lines. As a possible strategy to circumvent this problem, resveratrol (a flavanoid from a natural source) has been used in combination with paclitaxel (Sprouse and Herbert, 2014). Also, repurposing of existing drugs can also been done, especially done after an improved understanding has been obtained of the factors contributing to the intrinsic and extrinsic resistance to existing anti-cancer drugs. For e.g., certain micro-RNAs have been known to contribute to drug resistance in non-small cell lung cancer. A better understanding of these pathways can provide a more-targeted approach to resolving this age-old problem of MDR (Tibaldi et al., 2015). The current research efforts are also geared towards the development of nano-particle-based approaches for inhibiting or circumventing these efflux mechanisms. Such efforts would complement the existing strategies of using compounds that are known to bypass P-gP (Platinum compounds, nucleoside analogs and alkylating agents) or to develop modified versions of the existing drugs, such that they are not substrates of this transporter protein (Yoshikawa et al., 2004; Nobili et al., 2011). While this topic has been reviewed by others, a brief summary here, especially in the context of drug efflux and the MDR phenotype would enable the reader to obtain a comprehensive understanding of the current developments in the field. These include the development of polymers, lipids,

and/or surfactants that can inhibit efflux transporters. For e.g., Surfactants (amphiphilic molecules comprising both hydrophilic and hydrophobic groups) can assist drug delivery by increasing the fluidity of the cell membrane (for e.g., Cremophor® EL), and the mitochondrial membrane (Pluronics). This, in turn, may have contributed to the change in mitochondrial polarity as well as a concentration-dependent decrease in ATP levels. Polymer (poly(alkyl cyanoacrylate) can modify the charge properties of a chemotherapeutics, thereby improving its uptake and transport. Receptor-mediated endocytosis, unlike diffusion, of the nanoparticle-encapsulated drug and its subsequent trafficking can occur via endocytic vesicles. This mechanism can position a higher concentration of the drug closer to the site of action. While more corroborative evidence is necessary, evidence (for an alteration in the intracelullar distribution due to a different mode of uptake) is available for nanoparticle-drug conjugates (for e.g., N-(2-hydroxypropyl) methacrylamide (HPMA) – adriamycin conjugate). Since late endosomes have a lower pH, this different, physiological property can be exploited to trigger drug release at that site. This aspect can be combined by the use of conjugates of different pore sizes (mesoporous silica) to further regulate/fine-tune drug release. Use of the aforesaid combination therapy approach has its limitations in terms of the PK of the efflux inhibitor altering the PK of the chemotherapeutic drug. It is also important to avoid side-effects (toxicities due to the efflux inhibitors or the anti-cancer drug inhibiting the physiological functions of P-gP in normal cells). To address this problem, nanoparticles encapsulating the aforesaid combination of an efflux inhibitor and a chemotherapeutic drug can be used. For e.g., drug cytotoxicity can be induced in drug-resistant cell lines by loading nanoparticles with the drug (doxorubicin) and a P-gP inhibitor (tariquidar). As a logical extension of this concept to possible overcome drug resistance, drugs (for e.g., paclitaxel) were encapsulated with an siRNA to silence the MDR1 transcript. Further, this nano-conjugate was conjugated with biotin, thereby converting it into an active drug transport vehicle (Kirtane et al., 2013). Alternatively, substrates for the uptake transporter (SLC - solute carrier family) can be used to improve PK and avoid MDR of conventional drugs (Huang et al., 2006). Finally, natural products (flavonoids and their synthesized synthetic derivatives as efflux inhibitors) can be used in combination with chemotherapeutic drugs to reduce toxicity and improve efficacy (Conseil et al., 1998; Zhang et al., 2004). Also, kaempferol has been shown to be an ABCG2 inhibitor and contributes to the increased intracellular accumulation of quercetin, thereby providing a mechanistic rationale for the use of this combination therapy to improve bioavailability (An et al., 2011). The recurrence of tumors, despite therapy (singly and/or in combination), has been attributed, at least in part, to the presence of the relatively resistant cancer stem cells (CSCs) or side population (SP) cells that share features with CSCs. Such cells have been shown to have an increased expression of the P-glycoprotein as well as the other proteins of the MDR family (MRP). Increased expression of multiple members of the family is significant and should be borne in mind, since it is known that drugs that bypass p-GP (for e.g., nucleotide analogs) can possibly be effluxed by another transmembrane protein in the same family (for e.g., MRP2 (ABCC2) (Hoffmann et al.2004) and may also be overexpressed in a

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subset of recalcitrant, relatively resistant tumor cells (CSCs).

4. MDR - CSCs -Strategies for Targeting

CSCs are considered to be the major reservoir for cancer relapse as well as the originator of metastatic cell growth. Their relative resistance, following a chemotherapeutic drug regimen (part of the "acquired resistance" phenotype), and their important contribution to the MDR phenotype, has been attributed to the increased expression of drug efflux transporters, apart from the contributions made by alterations in DNA repair capabilities and apoptosis (Trumpp and Wiestler 2008). These aberrations have been reported at the membrane, cytosol as well as in at the nuclear levels (Stavrovskaya, 2000). This may complement the development of selection-pressure-induced (could be related to the hypoxic niche/microenvironment) "intrinsic resistance" (Pan et al., 2006). While drug-efflux-mediated evasion of apoptosis is an important mechanism, other hallmarks of cancer (independence-from the need for growth-stimulatory signals - for e.g., constitutive activation of the EGF/EGFR proliferation pathway; refractoriness to growth–inhibitory signals; increased angiogenesis-mediated supply of nutrients to the growing tumor; invasion and metastasis as well as enabling replicative immortality) have to be considered for developing/refining the drug cocktail for CSCs. Also, the emerging hallmarks (reprogramming of the metabolic pathways and the evolution of the metabostemness phenotype (Menendez and Alarcón 2014; Menendez et al., 2014) as well as the immune evasion mechanisms developed) (Hanahan and Weinberg 2011) will contribute to drug resistance as well as the outgrowths from CSCs. Further, there is evidence in the literature, that in certain cases, the evolution of tumors may not follow a hierarchical CSC model (each cell may have an equal chance of becoming tumorigenic) with stochastic genetic/epigenetic changes resulting in phenotypically similar cells. In other cases, there can be diverse cells in terms of their phenotype and these cells can undergo reversible changes, necessitating the need to accurately identify and possibly quantify CSCs as well as their subsets. However, it is widely accepted that both models need not be mutually exclusive (clonal evolution may be important for both CSCs and non-CSCs) and an effective therapeutic strategy would have to target the CSCs, non-CSCs (large numbers of tumorigenic cells) as well as the microenvironment harboring both these categories of aberrant cells (Suresh 2015; Vermeulen et al., 2012). Some of these stem cells, known as side population cells, have been identified, due to their differentially high efflux of Hoechst dye and Rhodamine (substrates of both ABCB1 and ABCG2). These cells are relatively quiescent; resistant to chemotherapeutic agents, apart from being anti-apoptotic (Zhao et al., 2013). This heterogeneity (temporal and/or spatial) notwithstanding, the approach to target CSCs, adopted by many, warrants a conceptual understanding of the approaches taken to target this important set/subset of cancer cells, with classical examples of each strategy. Further, it can be expected that any drug cocktail developed to target these relatively resistant, rare cells should be capable of eliminating the more sensitive larger numbers of non-CSC tumorigenic cells. Apart from the aforesaid combination therapy targeting the efflux inhibitors in cancer cells, inhibiting one of the CSC enzyme (aldehyde dehydrogenase) using drugs (for e.g., all –trans-

retinoic acid), is associated with chemosensitization of such cells to certain chemotherapeutic drugs. This observed chemo-sensitization was associated with a concomitant reduction in the levels and activity of the two isozymes of ALDH (ALDH1 and ALDH2) (Arrieta et al., 2010). Apart from such approaches, drugs that target the niche (housing the CSCs) as well as those that act on key signaling molecules (for e.g, Hedge Hog; Notch; Wnt etc) are some of the most important, currently used strategies to combat MDR using a cocktail of conventional and/or biopharmaceutical strategies (siRNA) (Di and Zhao 2015). Further, it was shown that colo-rectal cancer cells with Kras mutations that were resistant to conventional EGFR therapy could be treated with Notch antagonists (Fischer et al., 2011). These antagonist could potentially decrease self-renewal and blood vessel formation, and hence help in the management of patients not suited for the conventional anti-EGFR therapy. Hence, apart from chemo-resistance reverting strategies, approaches that targets/inhibits key self-renewal signaling molecules can be used, as long as the delivery payload is selective, efficacious and safe (Iyer et al., 2013).

5. Conclusions

As mentioned by Kawar S., the phenomenon of drug resistance or the MDR phenotype can attributed, in major part, to be due to the drug efflux transporter pumps. Hence, this calls for an improved understanding of the structural and functional aspects (using experimental and *in silico* approaches) of the normal and polymorphic variants. Further, the conventional polypharmacy approach can be modified using nanotechnology-based strategies for improving the PK/PD profile of the chemotherapeutic drug and hence, the dosage regimen. These strategies can be used to target CSC – reservoirs of relapsing cancers and the originator of metastatic cancer growth.

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