Revisiting the role of efflux pumps in multidrug-resistant cancer

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Abstract

Most patients who die of cancer have disseminated disease that has become resistant to multiple therapeutic modalities. Ample evidence suggests that the expression of ATP- binding cassette (ABC) transporters, especially the multidrug resistance protein 1 (MDR1, also known as P-glycoprotein or P-gp), which is encoded by ABC subfamily B member 1 (*ABCB1*), can confer resistance to cytotoxic and targeted chemotherapy. However, the development of MDR1 as a therapeutic target has been unsuccessful. At the time of its discovery, appropriate tools for the characterization and clinical development of MDR1 as a therapeutic target were lacking. Thirty years after the initial cloning and characterization of MDR1 and the implication of two additional ABC transporters, the multidrug resistance associated protein 1 (MRP1; encoded by *ABCC1*)), and ABCG2, in multidrug resistance, interest in investigating these transporters as therapeutic targets has waned. However, with the emergence of new data and advanced techniques, we propose to re- evaluate whether these transporters play a clinical role in multidrug resistance. With this Opinion article, we present recent evidence indicating that it is time to revisit the investigation into the role of ABC transporters in efficient drug delivery in various cancer types and at the blood–brain barrier.

Introduction

Despite heroic efforts to develop new anti-cancer drugs and biological therapies, and to catalog and study hundreds of potential mechanisms of resistance to these treatment modalities[1], most patients with metastatic cancer will die from multidrug-resistant disease. Development of multidrug resistance (MDR)—the resistance to multiple, structurally unrelated compounds—is a frequent problem in the treatment of cancer and should be distinguished from resistance to anti-cancer drugs with precise targets and immune therapies which are not examples of MDR. There are several extensive reviews detailing the history and development of this field[2–5].

Acquired MDR has been intensively studied and the basic science is well-established. Over 50 years ago, Goldstein and colleagues described a HeLa subline with actinomycin D resistance following intermittent exposure[6]. Some 10 years later, June Biedler selected Chinese hamster lung and fibroblast cells in actinomycin D and found the selected cells resistant to the selecting agent, as well as to vinblastine, vincristine, and daunomycin[7]. Keld Dano was the first to demonstrate that daunomycin was actively transported out of multidrug-resistant mouse Ehrlich ascites cells and postulated that this must be due to a membrane transporter[8]. This transporter, P-glycoprotein (P-gp), later found to be an ATP binding cassette (ABC) transporter, was identified in drug-resistant Chinese hamster ovary cells in 1976 by Victor Ling[9]. Gros and colleagues were the first to report a drug resistance gene cloned from multidrug-resistant Chinese hamster cells[10] and the gene was found to confer resistance when transfected into sensitive cells[11]. The human gene—termed *MDR1* (and later renamed *ABCB1*)—was reported soon after in 1986[12,13], thus launching the study of ABC transporters, with a family of 48 human membrane transporters involved in diverse physiologic processes[14].

The second member of the ABC transporter family, MRP (subsequently renamed ABCC1), was reported by Cole and colleagues in 1992[15] and was found to mediate resistance to doxorubicin, etoposide, and vincristine among others[16], but evidence of ubiquitous expression has meant it is unlikely to be a suitable target for anti-cancer therapy. The third ABC transporter identified, breast cancer resistance protein (BCRP and MXR; subsequently renamed ABCG2), was reported by three different groups within the span of a few months[17–19]. These additions increased interest in the study of ABC transporters, but added complexity to the definition of MDR. The substrates and key roles for most of these proteins have been identified—but the extent to which the transporters play a role in clinical MDR has never been clarified. Despite the clinical failure of P-gp inhibitors, recent evidence suggests that expression of ABC transporters does play a role in clinical drug resistance in some settings. We will argue that a contemporary understanding of target biology and biomarker development could identify settings where transporters involved in MDR could be considered important therapeutic targets.

ABC transporters: Structure and function

The 48 human ABC transporter genes are classified into seven subfamilies (termed ABCA through ABCG)[20,21]. For the sake of clarity, we will subsequently refer to transporter proteins by their subfamily names rather than by their common names. Structurally, ABC transporters are typified by a characteristic four-domain architecture consisting of two cytoplasmic nucleotide-binding domains (NBDs) that bind and hydrolyze ATP and two transmembrane domains (TMDs) that recognize and transport substrates. While the structure and function of NBDs are similar throughout families, TMDs are highly heterogeneous, allowing transporters to recognize diverse substrates and use the energy from ATP hydrolysis to translocate molecules across membranes, irrespective of the prevailing concentration gradient[22]. Recent work suggests that while the energy from ATP can help translocate engaged substrates, basal ATP hydrolysis drives a continuously changing conformation that may facilitate ABCB1 binding and transport of a wide range of substrates[23]. High resolution structures of ABCC1[24] and ABCG2[25] determined using cryo-

electron microscopy have helped clarify the drug-binding domains, but have left the precise translocation mechanism unresolved.

ABC transporters regulate cellular levels of hormones, lipids, ions, xenobiotics and other small molecules by transporting molecules across cell membranes and serve a range of physiological roles, including intracellular regulation of organelles such as the mitochondrion[26], lysosome[27], endoplasmic reticulum[28] and Golgi apparatus[29]. Loss of function of the transporter via germline mutation is associated with a number of heritable diseases, including cystic fibrosis (ABCC7, CFTR), pseudoxanthoma elasticum (ABCC6), Stargardt macular degeneration (ABCA4), Tangier disease (ABCA1), sitosterolemia (ABCG5 and ABCG8), and harlequin ichthyosis (ABCA12)[30]. These disorders present significant molecular biological and clinical challenges, as they require the development of small molecules (or gene therapy) that encourage normalization of a mutant transporter *via* strategies such as ribosomal read-through[31], message stabilization, correction of folding defects[32], correction of trafficking defects[33,34], allosteric activators of activity[35], modulation of protein-protein interactions[36], control of post-translational regulation, regulation of protein degradation pathways[37,38], or induction of compensatory mechanisms[39]. Prospects are improved somewhat given the notion that restoration of activity as little as 5% that of wild-type basal activity can be sufficient to partially ameliorate disorder phenotypes[40,41].

It should be noted that among the 48 ABC transporters, some have very precise substrate specificity, while others have a broad range of substrates. It is the transporters with broad substrate specificity profiles that have the potential to transport anticancer agents, and it may depend in part on level of expression as to whether transport is sufficient to confer drug resistance[42]. Because laboratory experiments are typically conducted in cell lines with high levels of transporter expression, it has been difficult to determine which transporters deserve the most scrutiny as mechanisms of drug resistance, and the reader is referred to several reviews compiling substrate lists of a range of transporters[43–45]. One detailed compilation notes that 19 of the 48 ABC transporters have been shown to efflux anticancer agents in some context[43]. This review will focus on the subset of ABC transporters that were first reported as multidrug efflux pumps—ABCB1, ABCC1 and ABCG2—what we have learned about their basic science, how clinical application has faltered, and what might constitute a path forward. More recent work in this massive research field will be the focus of our review due to space constraints. In Figure 1A, we present the most recent 3D structures for ABCB1, ABCC1 and ABCG2, and list common substrates of the transporters. Additionally, we provide a description of the pumping action of ABCB1 in Figure 1B.

The physiologic roles of ABCB1, ABCC1, and ABCG2 appear to be in excretory and/or protective capacities by transporting substrates across biological membranes. In the blood-brain barrier (BBB), the blood-testis, and blood-placental barriers, expression of the pumps in the capillary endothelial cells serves to prevent entry of exogenous molecules[46,47]. A consequence of these protective roles is that these transporters can affect pharmacokinetic parameters of drug absorption, distribution, metabolism, excretion, and toxicity. Inhibition of ABC transporters often leads to toxicities or pharmacokinetic changes due to drug-drug interactions[48] and the FDA now requires that investigational drugs be characterized with regard to their ability to interact with ABC transporters.

The ABCB1 hypothesis

Nearly 40 years of findings from cell culture and animal models implicate multidrug efflux pumps as a cause of multidrug resistance. Their potential importance in MDR is illustrated by the numerous anti-cancer agents that have been identified as substrates, including anthracyclines, taxanes, vinca alkaloids, camptothecins, epipodophyllotoxins, and tyrosine kinase inhibitors[1,49]. While there is considerable overlap among the substrate profiles for the various multidrug efflux pumps, there are some distinct differences. ABCC1 has been shown to transport various neutral and anionic hydrophobic compounds and products of Phase II drug metabolism, including many glutathione and glucuronide conjugates[50]. ABCG2 transports the anti-cancer drugs methotrexate, mitoxantrone, topotecan, irinotecan, and flavopiridol[51].

In addition to studies of ABC transporter structure and function, efforts have been made to define their clinical roles in drug resistance, primarily for ABCB1, the first transporter to be discovered. These studies generally used RNA or immunohistochemical methods of detection and examined association with outcomes[52]. Numerous studies reported the presence of *ABCB1* mRNA and protein in clinical samples—in leukemias and in kidney, colon, breast, and lung cancers—and typically *ABCB1* expression portended a poor response to chemotherapy[52–54]. These results led to the development of clinical trials to test the ABCB1 hypothesis: that inhibitors of ABCB1 could improve response to chemotherapy and outcome via increased drug accumulation mediated by inhibition of drug transport.[55,56]

Failure of ABCB1 inhibitors in clinical trials

After the discovery of ABCB1, a number of "first generation" inhibitors including verapamil, quinidine, amiodarone, and cycloposorin A were identified and added to chemotherapy regimens. However, these agents were not very potent or were toxic in their own right and their ability to inhibit ABCB1 was not verified in patients[5]. The second-generation agents valspodar and dexverapamil were more potent ABCB1 inhibitors[55], and surrogate assays were 7used to confirm that serum concentrations of the inhibitors were able to inhibit rhodamine 123 transport from ABCB1 positive circulating CD56+ cells after inhibitor administration[57,58]. However, neither the expression nor function of ABCB1 in tumors was verified, nor whether the inhibitors actually blocked efflux in tumor cells. Additionally, pharmacokinetic interactions with the inhibitors, such as the inhibition of cytochrome P450 by valspodar, required chemotherapy dose reductions, leading to potential underdosing of patients[59]. A third generation of inhibitors including dofequidar, zosuquidar, tariquidar, elacridar and biricodar were developed specifically as ABCB1 inhibitors. These were more potent and displayed less pharmacokinetic interactions, but caused some chemotherapy toxicity in combination, potentially due to inhibition of normal tissue ABCB1[56]. After the discovery of ABCG2, elacridar and tariquidar were found to inhibit both ABCB1 and ABCG2[60,61] while cyclosporine A and biricodar were found to inhibit ABCB1, ABCC1 and ABCG2[62,63]. Interestingly, inclusion of cyclosporine A to chemotherapy led to increased response in AML in one study[64]; however, these results could not be duplicated with other inhibitors[65]. Unfortunately, despite a few early successes, the

majority of clinical trials with ABCB1 inhibitors, even third-generation inhibitors, did not confirm clinical benefit[56].

From a clinical oncology perspective, the result was a nearly complete shutdown of study in the field after these trials were completed, and the clinical validation and development of specific inhibitors for other ABC transporters potentially involved in MDR was not pursued. Further clinical trials using ABCB1 inhibitors were vehemently discouraged[66]. However, some work continued on more mechanistic and functional aspects of the transporters, including work defining substrates and inhibitors; defining the role of transporters in the blood-brain barrier; and defining their roles in pharmacology. As noted above, the FDA now requires determination of the interaction of drugs with ABC transporters during development, given that transporters can be critical determinants of drug pharmacokinetics, including oral availability, drug-drug interactions, and drug toxicity. The finding in clinical trials that inhibition of ABC transporters increased chemotherapy toxicity has newer parallels in combinations with tyrosine kinase inhibitors. Recent reports of unexpected toxicity of combinations of targeted kinase inhibitors could be explained by interactions with ABC transporters[67–69]. Indeed, several targeted small molecules have been shown to interact with one or more ABC transporters, usually as an inhibitor[70–72], but also as a substrate for transport[73–75]. In some cases, these interactions might be beneficial. When patients were treated with oral topotecan in combination with the tyrosine kinase inhibitor pazopanib, total topotecan exposure was 1.7-fold higher than patients treated with topotecan alone, primarily due to the fact that pazopanib inhibits ABCG2, thus increasing the absorption of orally administered topotecan[76].

Unfortunately, these studies left a fundamental question unanswered: what is the role, if any, of transporter-mediated reduced drug accumulation in clinical drug resistance? Newer technologies that were not available when ABCB1 was discovered some 30 years ago—such as gene expression, gene mutation and SNP studies—have provided evidence supporting a re-examination of ABC transporters in clinical drug resistance. Additionally, genetically engineered mouse models (GEMMs) have provided new impetus for examining the role of transporters in acquired resistance to PARP inhibitors. Imaging studies and mouse models examining the role of transporters in the GI tract and at the BBB have consistently demonstrated that ABCB1 and ABCG2 are a significant impediment to anticancer agent oral absorption and brain exposure. A summary of the new evidence supporting the role of ABC transporters in drug resistance is given below.

The complex role of transporters in MDR

ABCB1 overexpression leads to resistance in some tumor subsets.

Many of the clinical trials designed to test the ABCB1 hypothesis were conducted in acute myeloid leukemia (AML). Although largely failures, we now recognize their design was optimistic and success unlikely given the focus on only ABCB1 and the lack of selection for a subset of patients with ABCB1 as a mechanism of resistance. One study reported gene expression profiling in 170 AML samples, finding that a refractory subset with ABCB1 or ABCG2 expression comprised only 13% of samples[77]. A recent report of whole genome sequencing of 92 patients with high-grade serous ovarian carcinoma (HGSOC) with primary

and matched resistant disease concluded that CCNE1 amplification and BRCA1/2 reversions were potential mechanisms of resistance. However, the authors also found recurrent promoter fusions associated with overexpression of *ABCB1*[78]. This discovery, occurring in about 8% of recurrent disease samples, suggests *ABCB1* overexpression via a mechanism first reported more than a decade earlier in cell culture models and in clinical samples from patients with refractory acute lymphoblastic leukemia[79,80]. As shown in Figure 2, gene rearrangements can occur that place a more active promoter 5' to the *ABCB1* transcript. These events are mono-allelic, with the remaining alleles being transcribed normally. While these striking results strongly suggested the fusions were selected as part of the drug-resistant phenotype (paclitaxel and docetaxel, the mainstays of treatment of ovarian cancer, are classic ABCB1 substrates), the low occurrence rate argues that the rearrangements may occur in a particular molecular background. We previously reported that, using a Taqman low-density array, high levels of *ABCB1* after drug treatment are found in only a small percentage of ovarian cancers[81], suggesting any clinical trial targeting ABCB1 would be futile without identifying patients *a priori* with ABCB1 expressing tumors.

In a recent anecdotal observation, investigators identified unexpectedly high levels of *ABCB1* in samples obtained from a patient whose anaplastic lymphoma kinase (ALK)-rearranged lung cancer had developed resistance to ceritinib, a drug used to treat lung cancers with mutations in the ALK gene, without a new ALK mutation. The investigators established cell lines from different metastatic sites and found that both the post-ceritinib treated tumor and cell lines derived from it exhibited high levels of P-gp[73]. The authors also found P-gp was highly expressed in 3 of 11 ALK-rearranged refractory lung cancers in which secondary ALK mutations were absent. Lung cancer studies conducted with P-gp inhibitors in the 1990s[2] could not have envisioned the subset stratification that would be needed to find the patients in whom P-gp could confer drug resistance.

Expression of multiple transporters.

It is now clear that multiple ABC transporters may be expressed in a single tumor type. A study that examined expression of all 48 human ABC transporters in 281 acute myeloid leukemia samples found in a multivariate analysis, *ABCB1*, *ABCG1*, and *ABCG2* linked to overall survival and the overall survival decreased with increasing number of transporter genes co-expressed[82]. Similar results were observed in childhood AML, where ABCA3, ABCB1, ABCC3, and ABCG2 were measured by RT-PCR, and the increasing number of co-expressed transporters conferred shorter relapse free-survival[83]. Finally, in a set of 11 paired samples taken from patients with AML at diagnosis and relapse, a 2-fold overexpression of at least one ABC transporter capable of transporting anthracyclines or vinca-alkaloids was found in 10 of the paired samples[84]. Together, these results suggest that ABCB1 alone may not be the prevalent mechanism of resistance in AML, and that coexpression of transporters may require inhibition of multiple transporters to achieve clinical benefit. This may be especially true for primitive leukemic CD34+/38- cells, the putative "stem cell" population. These cells have been shown to express high levels of ABCG2 as well as ABCB1 and ABCC1 in some patient samples[85]. Expression of ABCG2 and ABCB1 in this population has been found to correlate with response to chemotherapy both clinically and when measured *ex vivo* in leukemic blast samples[86].

Support for the idea that multiple transporters might underlie the resistance phenotype in some solid tumors has also emerged, suggesting that coordinate expression of transporters in normal tissues will lead to expression of multiple transporters in cancer. Whether all, or a subset of these pumps may be involved in anticancer drug efflux remains to be determined. A study of all ABC transporters in pancreatic cancer found significant up-regulation of *ABCB4*, *ABCB11*, *ABCC1*, *ABCC3*, *ABCC5*, *ABCC10*, and *ABCG2* at the RNA level in tumors relative to normal pancreas[87]. ABCB1, physiologically at high levels in a normal pancreas, was not upregulated beyond normal pancreatic tissue expression. Upregulation of transporter expression beyond the high levels found in normal tissue counterparts may not matter, as in drug-refractory hepatocellular carcinoma, where expression of multiple transporters has been reported, albeit in different studies[88,89].

With the advent of genomic analysis, we have an unbiased approach to measuring ABC transporter mRNA expression. Figures. 3A and 3B show the range of *ABCB1* and *ABCG2* expression, respectively, in RNA-Seq data from The Cancer Genome Atlas (TCGA) Bioportal. Figure 3C shows the broad range of expression of both *ABCB1* and *ABCG2* in a variety of tumor types. Their expression ranges over 1000-fold, a far greater range of expression than was reported in studies using older technologies. Although a frequent criticism of early ABCB1 studies was that *in vitro* levels of *ABCB1* did not reflect levels in the patient tumors[90], this broad range suggests very high levels in some tumors. It is interesting that tumor types most refractory to chemotherapy are among those with the highest levels of expression. In Figure 3C, hepatocellular and kidney cancers have the highest expression of both *ABCB1* and *ABCG2*. While tyrosine kinase inhibitors are approved for both, these cancers remain enigmatically resistant to the mainstay chemotherapeutics that are substrates for ABC transporters and form the backbone of treatment for almost every other tumor type. The ability to detect RNA levels more accurately highlights the single most important deficiency in our understanding of MDR— how do we show that ABC transporters measurably reduce anti-cancer drug accumulation in cancer cells, and in a clinically significant way? The centrality of this question is highlighted when one considers also that the quantitative RNA methods generally do not isolate cancer cells from the normal tissue stroma surrounding them. Indeed, it has been reported that in the case of breast cancer, ABCB1 expression is found primarily in tumor-associated macrophages and not in the tumor samples themselves[91]. Discordance between RNA and immunohistochemical data is thought to be responsible for this problem in some cases[92], but antibody specificity questions have also been raised[93].

What is clear in the cases outlined above is that only a fraction of cancers express levels of an ABC transporter that might lead to drug resistance—for ABCB1, 13% of AML, 8% of ovarian cancer and 30% of the relatively rare ALK+ NSCLC—suggesting that ABCB1 should have been targeted as cancer mutations are today. And, expression of more than one transporter is common. Clinical studies without molecular characterization such as those conducted in the past could never achieve statistical significance and could be potentially confounded by inhibiting normal ABCB1 in patients who did not have ABCB1-expressing tumors.

SNP studies implicate transporters as critical factors to chemotherapy response.

One way to answer the question of whether ABCB1 matters in cancer drug resistance is to evaluate the impact of polymorphic variants on chemotherapy response or cancer outcome. Polymorphic variants of ABC transporter genes that impair substrate efflux could be associated with a higher cancer incidence, due to decreased xenobiotic efflux and impaired normal tissue protection because of decreased transporter efficacy. But they could also be associated with better outcome once a cancer is diagnosed, because of reduced chemotherapy drug efflux. However, multiple variables confound such associations—not all cancers are linked to xenobiotic exposure; not all cancer chemotherapeutics are substrates for transporters; current methods provide incomplete information on the transport activity of polymorphic variants; and clinical outcome may be impacted by coexisting polymorphic variants in multiple genes. As a result, the literature for outcomes associated with polymorphic variants of *ABCB1* is contradictory[94–97].

The largest SNP association study to date was based on RNA sequence data found in the TCGA database. This study examined sequence and expression data from 4616 ovarian cancer patients who had received any form of adjuvant chemotherapy. Johnatty et al. examined the correlation of three common coding SNPs tagging either C1236T (rs1128503), G2677T/A (rs2032582), or C3435T (rs1045642) of ABCB1 in patients with prior chemotherapy[98] and found only a marginal association of C1236T with improved overall survival; although in 143 serous ovarian tumors *ABCB1* over-expression was associated with a worse prognosis in sub-optimally debulked patients[98].

Transporter expression in mouse models of acquired resistance.

Expression of ABC transporters emerged as the principal mechanism of resistance in an elegant series of studies published by the groups of Borst and Rottenberg involving a genetically engineered mouse model (GEMM) of hereditary breast cancer that arises in the mammary epithelium of mice deficient for *Brca1* and *tp53*. Treatment of animals from this model with doxorubicin led to a robust response, but when tumors recurred, upregulation of the *Abcb1a* and *Abcb1b* (the murine orthologs of *ABCB1*) was found[99]. These resistant tumors were additionally resistant to docetaxel, another substrate of ABCB1. Transport of 99mTc-MIBI was also demonstrated in resistant tumors that overexpressed Abcb1a/b and was used to predict the efficacy of the ABCB1 inhibitor tariquidar[100]. Similar results were obtained when the mice were treated with the PARP inhibitor olaparib: tumors responded initially, and recurring tumors overexpressed *Abcb1a/b* as a mechanism of resistance[101]. In tumors where *Abcb1a/b* was upregulated, the addition of tariquidar to olaparib treatment resensitized tumors to olaparib[101]. In the mouse tumors, even moderate increases in ABCB1 expression—as little as five-fold compared to treatment-naïve tumors—conferred resistance that could be reversed by the ABCB1 inhibitor tariquidar[102].

In a separate study using the same model, when tumors arising from this model were treated with the topoisomerase I inhibitor topotecan, some, but not all, resistant tumors expressed higher levels of Abcg2 as a mechanism of resistance, suggesting that this transporter is not as readily upregulated[103]. Of note, expression of Abcc1 and Abcc4, which have also been implicated in topotecan resistance, were not found to be increased[103]. While the ABCG2

inhibitor Ko143 was not able to completely overcome Abcg2-mediated resistance in topotecan-resistant tumors, treatment of mice with pegylated SN-38 was found to be effective[104].

Similar observations have been reported in other GEMM models. In a model of BRCA2-deficient cancer generated in *K14cre;Brca2F/F;p53F/F* mice that yields BRCA2-deficient carcinosarcomas, ABCB1 expression was high in chemo-naive tumors, resulting in intrinsic doxorubicin and olaparib resistance reversible by tariqudar[105]. Interestingly, in this same model, expression of ABCB1 was associated with an epithelial-to-mesenchymal transition (EMT) phenotype[105]. An examination of *ABCB1* expression in human triple negative breast cancer samples demonstrated that tumors with an EMT phenotype (claudin low) had high basal levels of *ABCB1* compared to basal-like tumors[106].

Despite these recent mouse studies implicating ABCB1 as a significant resistance mechanism in breast cancer, it is not understood why it has not been as clearly implicated in humans. This could be due to the fact that basal levels of ABCB1 are higher in rodents, leading to increased ABCB1 expression in response to drug treatment[107]. The use of human breast epithelial organoids may be one way to overcome this limitation associated with murine-derived tumors[108]. Alternatively, as relatively small increases in ABCB1 expression were linked to resistance, this increase might not have been detected in human patient samples with some techniques such as microarrays or by examining protein expression using immunohistochemistry[107].

Decreased oral bioavailability due to transporters.

Although not directly involved in drug resistance, transporter expression in the GI tract is known to affect the oral bioavailability of some chemotherapy drugs that are transporter substrates. This is especially true for taxanes and topotecan, which are not given orally due to interactions with ABCB1 and ABCG2, respectively. The dual ABCB1/ABCG2 inhibitor elacridar has been combined with oral taxol[109] or topotecan[110] to increase oral bioavailability in patients; however, the clinical efficacy of these combinations has not been reported. The potential for transporters in the gut to cause drug resistance has been suggested for imatinib, where both ABCB1 and ABCG2 were induced in Caco-2 intestinal cells chronically exposed to the drug[111]. If this were to occur in the gut, it would limit oral availability of the imatinib, resulting in lower serum concentrations and resistance to the drug. Although an intriguing possibility, this has yet to be demonstrated clinically.

Transporters at the BBB limit brain penetration.

One of the stunning discoveries made during the evolution of this field was the critical importance of ABCB1 at the blood brain barrier (BBB), first shown in mouse models developed by Schinkel et al. in which deletion of *Abcb1a* and *Abcb1b* resulted in CNS toxicity from ivermectin[112]. After the discovery of ABCG2, mice lacking *Abcb1a*, *Abcb1b* and *Abcg2* were generated, and a systematic series of investigations demonstrated the often overlapping and synergistic role of these two transporters in restricting the entrance of anticancer therapeutics to the brain in murine models in which brain concentration of chemotherapeutics is measured in wild-type versus transporter-deficient mice.

Fig. 4 presents a summary of published data for multiple agents, drawing on data from the many murine studies carried out by several groups. While the lack of either *Abcb1a/b* or *Abcg2* typically has only minimal effects on blood levels (left 4 columns), steady-state brain levels of substrates are markedly higher when both transporters are deleted rather than either alone. As one example, mice lacking *Abcb1a/b* had 2.3-fold higher steady-state brain levels of vemurafenib, while *Abcg2*-deficient mice had no change. However, brain concentrations in mice lacking both *Abcb1a/b* and *Abcg2* were approximately 43-fold higher than in wild-type mice[113]. This suggests a remarkable compensatory function for the two transporters. Similarly, a recent report on the ALK kinase inhibitor ceritinib demonstrated that brain concentrations of ceritinib are increased approximately 37-fold in the absence of *Abcb1a/b*, and 87-fold in the absence of both *Abcb1a/b* and *Abcg2*[114]. Interestingly, knockout of *Abcg2* alone did not increase brain accumulation. Presumably for these drugs, *Abcb1a/b* is able to compensate for the absence of *Abcg2*. Ceritinib, approved by the FDA in 2014 for the treatment of lung cancers harboring ALK-rearrangements and vemurafenib, approved in 2011 for the treatment of melanoma, are likely restricted from the brain by ABCB1 and ABCG2 and therefore cannot control early metastatic disease in the brain. This is particularly troubling for vemurafenib, as high serum concentrations are necessary for treatment[115].

Imaging demonstrates utility of transport inhibition at the BBB

Other *in vivo* models have used inhibitors to demonstrate the role of ABC transporters at the BBB. In mouse models, [^{11}C]erlotinib was restricted from the brain by both Abcb1 and Abcg2 and deletion of both transporters led to the greatest increase in brain penetration[116]. In non-human primates, co-administration of [^{11}C]erlotinib with elacridar resulted in a 3.5-fold increase in brain penetration[117]. Similarly, in healthy human subjects, significant increases in the brain of the ABCB1-specific substrate, (R)-[^{11}C]verapamil, were demonstrated during ABCB1 inhibition with high doses of tariquidar. By contrast, high doses of tariquidar did not lead to increased brain levels of [^{11}C]tariquidar, a substrate of both ABCB1 and ABCG2, owing to the ability of ABCG2 to compensate for inhibition of ABCB1 function[118]. More marked increases were observed when individuals carrying the ABCG2 polymorphic variant known to reduce protein level and function were tested. These studies align with the animal data and validate work in clinical medicine.

Innis and colleagues studied [N-methyl-^{11}C] N-desmethyl-loperamide (d-loperamide, dLop) as a PET imaging agent for the central nervous system (CNS). Like loperamide, its metabolite, dLop, is also transported exclusively by ABCB1[119]. Mice lacking Abcb1a/b had a 3.5-fold higher brain uptake of [^{11}C]dLop compared to wild-type mice by PET measurements; in non-human primates, inhibition of ABCB1 with DCPQ yielded a 7-fold increase in brain penetration of [^{11}C]dLop[119]. Clinical studies of [^{11}C]dLop showed that it had low brain retention[120] and co-administration of tariquidar with [^{11}C]dLop resulted in a 2- to 4-fold increase in the human brain[121].

Finally, D-luciferin, the substrate for firefly luciferase, was shown to be a substrate of ABCG2[122], and was subsequently shown to be selectively transported by ABCG2 rather than ABCB1 or ABCC1[123]. When firefly luciferase was expressed in a mouse model in glia

behind the BBB under the control of the GFAP promoter, co-administration of D-luciferin and an ABCG2 inhibitor resulted in increased bioluminescent signal in murine brains as compared to mice administered D-luciferin alone[123]. This is shown in Figure 5. These studies pave the way for use of radiotracers in patients to confirm the ability of inhibitors to increase brain penetration of substrate drugs, and as possible biomarkers for assessing the MDR status of patient tumors.

Studies of the blood-brain barrier have addressed the ability to target ABC transporters in patients to increase drug distribution into sanctuaries such as the brain[121]. Some investigators have attempted to demonstrate this in human cancers using radiolabeled substrates, particularly [^{94}Tc]-Sestamibi by either planar or PET imaging in patients with ABCB1-expressing cancers before and after administration of an ABCB1 inhibitor. Except for a small number of patients[124], these studies have never shown the type of differential observed in laboratory models[125,126]. But the caveats mentioned above concerning patient selection and inhibitor choice apply to these studies as well.

While data from imaging is limited, it represents the one method that could be further developed to both demonstrate the impact of drug efflux and the ability to alter it, and act as a biomarker for clinical trials. Such studies have been needed for a very long time, even in the absence of a strategy to inhibit ABCB1.

Conclusions

We have asked whether sufficient evidence exists to re-open investigation of a question encumbered by the weight of a succession of negative clinical trials. We would answer the question with a qualified yes. Yes, because there are clear examples of ABCB1 limiting drug accumulation in selected cancers in patients, of ABCB1 or ABCG2 associated with poor clinical outcomes, and of gene rearrangements resulting in high levels of ABCB1 in patients whose tumors exhibit drug resistance.

Certainly, adequate and consistent drug delivery has never been documented in clinical oncology, and results of the few studies to determine this suggest highly variable drug penetration[127]. Whether ABC transporters play a role in variable drug delivery is at present unknown. Clinical trials examining the effectiveness of inhibitors of ABC transporters were conducted without selection of patients whose tumors had high levels of transporter expression. If personalized medicine does nothing else, it should be possible to identify patients whose cancer cells overexpress a transporter that reduces drug efficacy—and who would not be expected to benefit from therapies with agents that are substrates for transporters. So that while we may not enhance efficacy with inhibitors of drug transporters, it is entirely plausible, and indeed expected, that we should predict a lack of efficacy and we would argue this would be a valuable accomplishment.

But while we should be able to successfully predict failure, our yes remains qualified, because we do not yet have the means to reliably detect the presence or importance of subsets of cells expressing ABC transporters in the clinic, and second, we do not have definitive proof that inhibitors could increase drug accumulation in cancers in a clinical

setting without unacceptable toxicity. We need a clinically validated method to detect the proteins (or a highly correlated biomarker), and we need a validated imaging assay to detect function in tumors. However, we can't accomplish this without reigniting interest in the transporter field. If we can first answer the question of when and where drug efflux matters, we can then focus on whether clinical outcomes can be improved by adding inhibitors to chemotherapy again.

Acknowledgements

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A

B
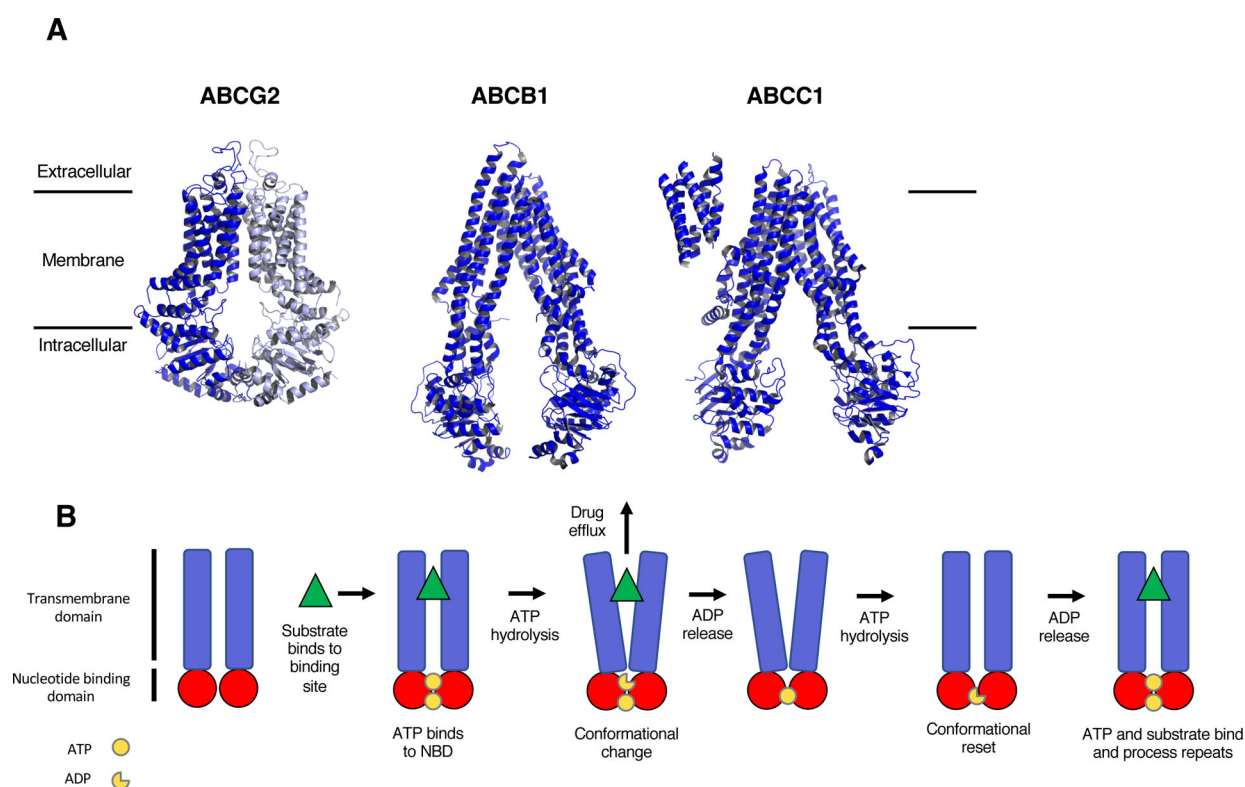
Figure 1:

Structure and mechanism of 3 ABC transporters. **A**, High-resolution 3D structures of ABCG2[25] (PDB accession 5NJ3), ABCB1[23] (PDB accession 5KPI) and ABCC1[24] (PDB accession 5UJ9). As ABCG2 forms a homodimer, the second dimer of the full protein is shown in light blue. Common substrates and inhibitors are listed. (Although the structure for ABCC1 is that of *Bos Taurus*, the protein identity is 91% and the structure is likely similar to human ABCC1). **B**, Schematic representation of the proposed pumping action of ABCB1. The substrate binds to the binding pocket and ATP binds to the two binding sites in the NBDs. This is followed by the hydrolysis of ATP that generates a conformational change, allowing the substrate to be released from the protein. The second molecule of ATP is hydrolyzed, allowing for a conformational reset, where substrate and ATP can bind again so the process can repeat.
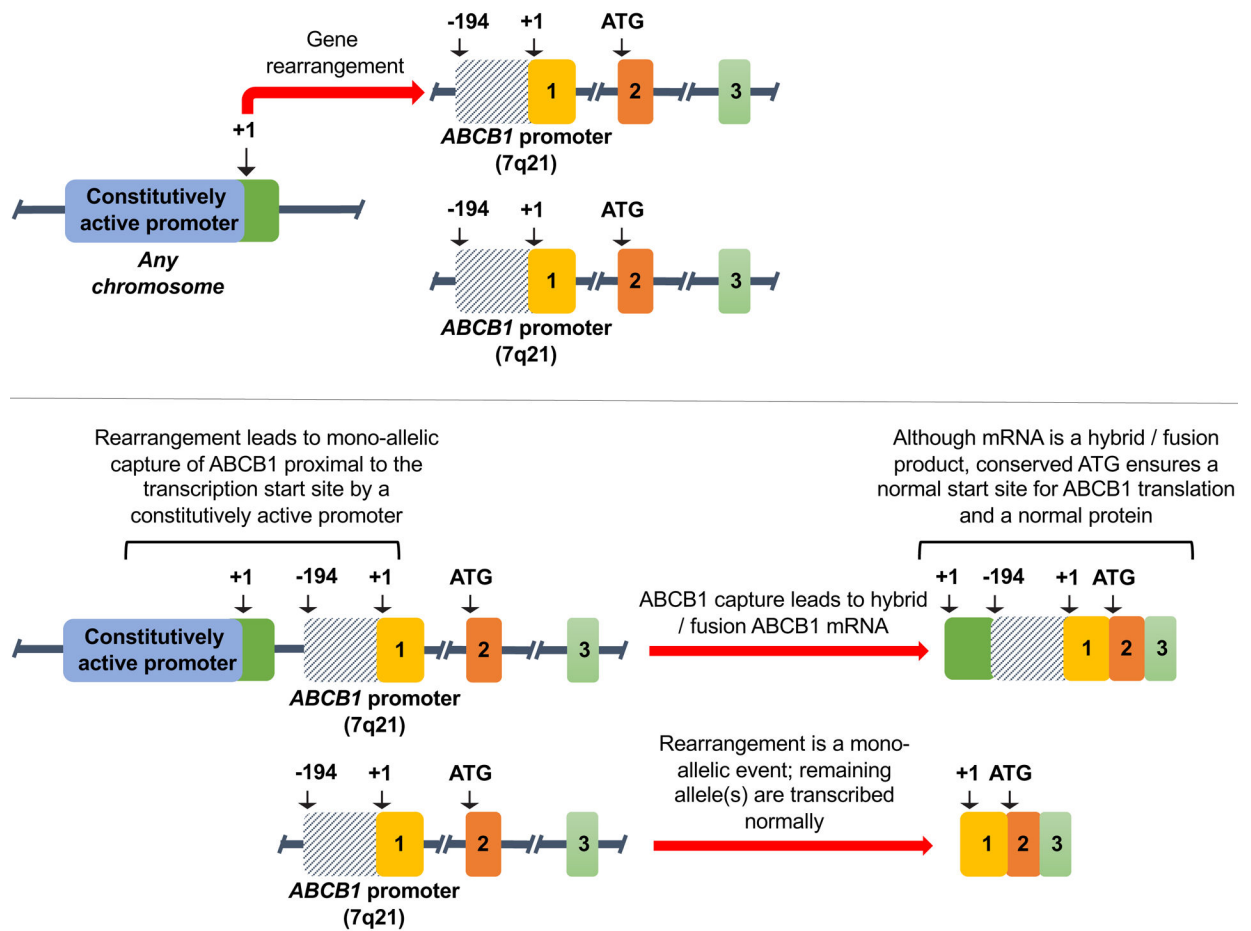
Figure 2:
Upregulation of ABCB1 via promoter capture. Adapted from Knutsen et al.[128]. Used by permission.
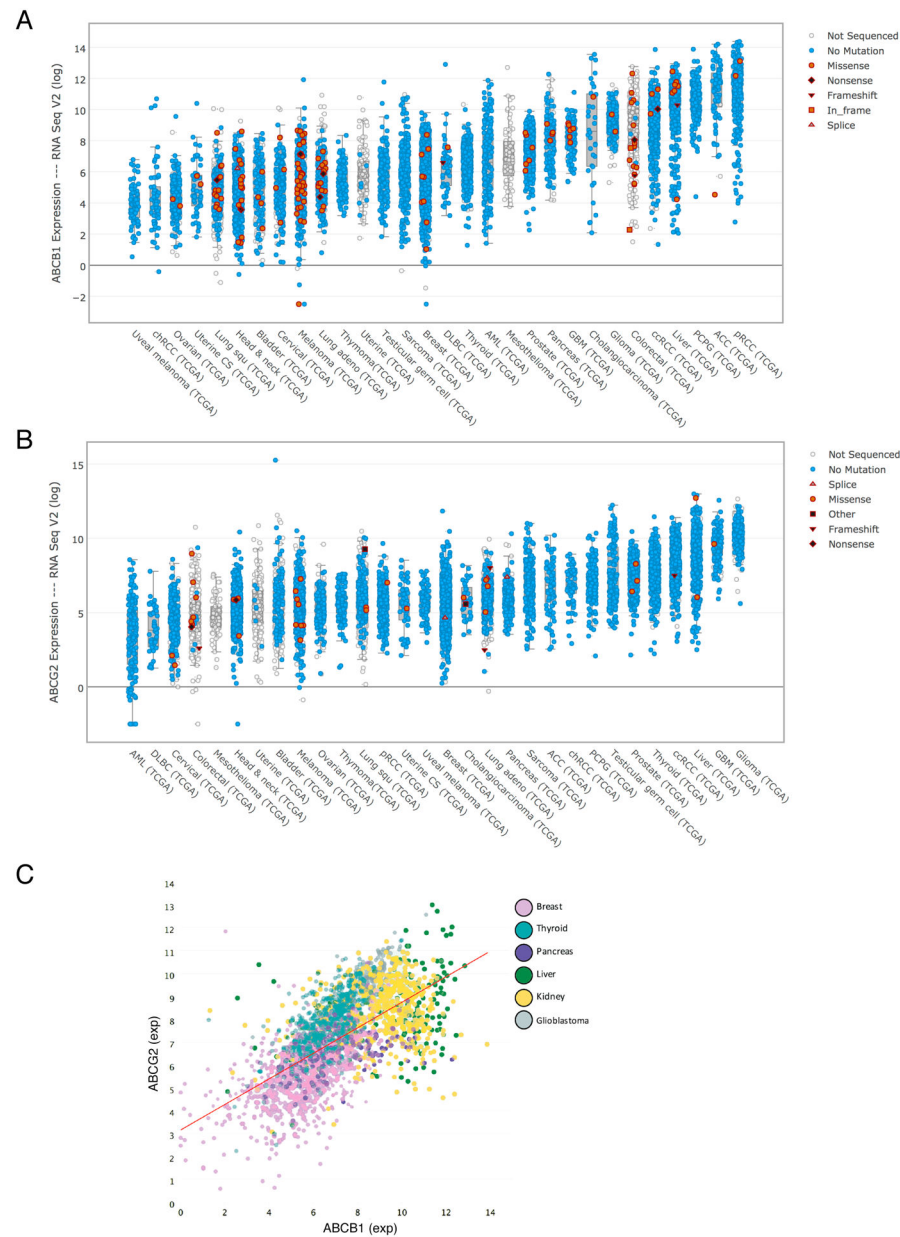
Figure 3:
Expression of *ABCB1* and *ABCG2* in patient tumor samples. Data from the cBioPortal website (www.cbioportal.org) showing expression of *ABCB1* (**A**) or *ABCG2* (**B**) in various tumor types. (**C**) Expression of both ABCB1 and ABCG2 in breast, thyroid, pancreas, liver, kidney and glioblastoma tumors taken from the TCGA database. The data are viewed with tcgaminer, an R Shiny-based program. The data shown in this figure are based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov.
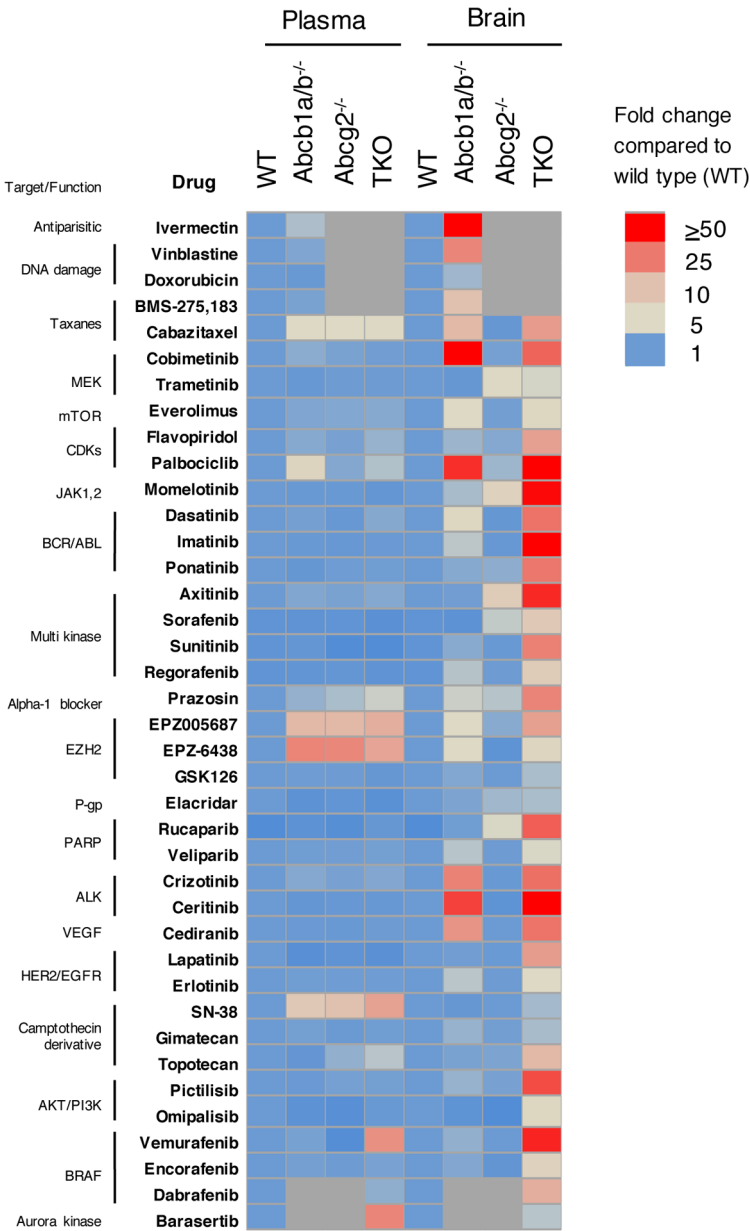
Figure 4:
Effect of transporter deletion on plasma or brain levels of drugs. Fold increase in plasma and brain drug levels in mice deficient for Abcb1a/b, Abcg2 or all three (TKO) transporters is compared to wild-type mice, which are assigned a value of 1. Grey blocks denote mice not studied. Adapted from Basseville et al[129] and compiled from references[112–114,130–159]. Used by permission.
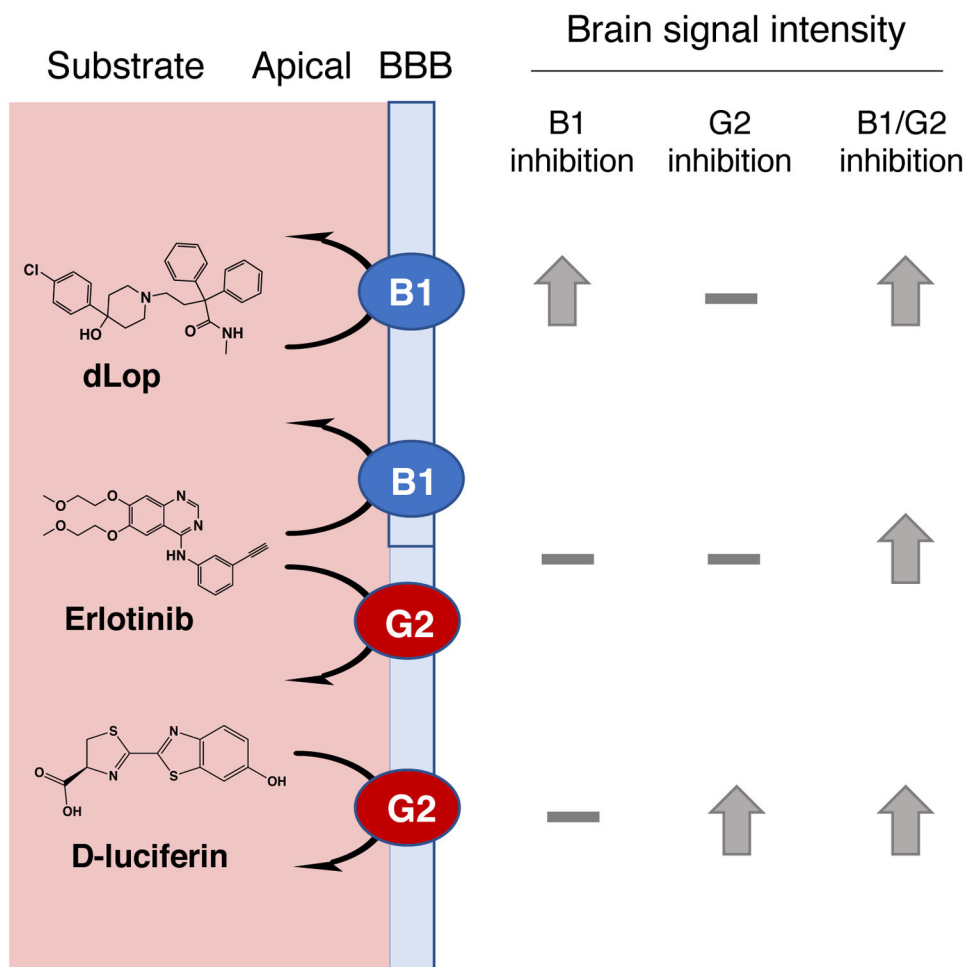
Figure 5:
The utility and function of PET radiotracers and other probes for imaging ABC transporter function, using the CNS as a model. Combination with inhibitors of known function, or administration to knockout mice, provides insight into function. ABCB1: des-methyl loperamide (dLop) is a specific substrate of ABCB1, producing almost no signal intensity under baseline conditions. Upon inhibition of ABCB1, high brain intensity is observed, though ABCG2 inhibition has no effect. In the instance of a dual substrate of both ABCB1 and ABCG2, such as erlotinib, specifically blocking either ABCB1 or ABCG2 results in a minimal increase in brain signal, and only dual inhibition or knockout produces an effect. An alternative imaging strategy using the specific ABCG2 substrate D-luciferin, with transgenic mice expressing firefly luciferase in astrocytes. Brain bioluminescence signal was low, and specific inhibition of ABCG2 but not ABCB1 produced elevated signal. Third-generation inhibitors such as tariquidar and elacridar are considered to primarily inhibit ABCB1, while Ko143 (which has not been used in humans) acts primarily on ABCG2. No gold-standard probe for dual inhibition of ABCG2 and ABCB1 exists. These imaging tools can act as the basis for studies of multidrug resistance in tumors, and efficacy and dose-optimization of new inhibitors.