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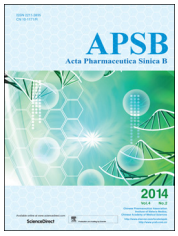

REVIEW

The pharmacological impact of ATP-binding cassette drug transporters on vemurafenib-based therapy

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KEY WORDS

ABC transporter;
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Abstract Melanoma is the most serious type of skin cancer and one of the most common cancers in the world. Advanced melanoma is often resistant to conventional therapies and has high potential for metastasis and low survival rates. Vemurafenib is a small molecule inhibitor of the BRAF serine-threonine kinase recently approved by the United States Food and Drug Administration to treat patients with metastatic and unresectable melanomas that carry an activating BRAF (V600E) mutation. Many clinical trials evaluating other therapeutic uses of vemurafenib are still ongoing. The ATP-binding cassette (ABC) transporters are membrane proteins with important physiological and pharmacological roles. Collectively, they transport and regulate levels of physiological substrates such as lipids, porphyrins and sterols. Some of them also remove xenobiotics and limit the oral bioavailability and distribution of many chemotherapeutics. The overexpression of three major ABC drug transporters is the most common mechanism for acquired resistance to anticancer drugs. In this review, we highlight some of the recent findings related to the effect of ABC drug transporters such as ABCB1 and ABCG2 on the oral bioavailability of vemurafenib, problems associated with treating

Abbreviations: ABC, ATP-binding cassette; AML, acute myeloid leukemia; BBB, blood–brain barrier; CNS, central nervous system; CSCs, cancer stem cells; GI, gastrointestinal; MAPK, mitogen-activated protein kinase; MDR, multidrug resistance; NBDs, nucleotide-binding domains; PFS, longer progression-free survival; PKIs, protein kinase inhibitors; TKIs, tyrosine kinase inhibitors; TMDs, transmembrane domains

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melanoma brain metastases and the development of acquired resistance to vemurafenib in cancers harboring the BRAF (V600E) mutation.

1. Introduction

Melanoma is the most serious type of skin cancer. It originates in pigment-producing melanocytes[1]. Melanoma has become one of the most common cancers in the world. Due to its high potential for metastasis, individuals with this disease have a poor prognosis and low survival rates[2]. Melanoma at advanced stages is often resistant to conventional radiation therapy and chemotherapy as a result of multiple mechanisms, including increased DNA repair and alterations of several key regulatory genes or proteins[3,4]. Therefore, therapeutic approaches directed at specific signaling pathways or mutations in melanoma have been employed[5,6]. One of the targets is the RAS-activated serine-threonine protein kinase B-raf (BRAF). It plays a central role in the regulation of the mitogen-activated protein kinase (MAPK) signaling pathway that regulates cell division, proliferation and differentiation in melanoma[7,8]. The consequence of mutations is the constitutive activation of the BRAF kinase and downstream MAPK signaling that promotes unregulated cell proliferation and cell invasion. In melanoma patients, the BRAF(V600E; valine to glutamate) substitution is the most common mutation[9], which is associated with poor clinical outcome[10] and brain metastases[11]. Since this mutation is found in approximately 40–60% of melanoma patients[8], improved clinical outcome is expected for melanoma patients with inhibition of BRAF(V600E) signaling[8–10].

2. Vemurafenib treatment for BRAF (V600E) mutation patients with advanced or metastatic melanoma

Vemurafenib (PLX4032, Zelboraf®) is a small molecule inhibitor of the cytoplasmic BRAF serine-threonine kinase (chemical structure given in Fig. 1), which in 2011 was approved by the US Food and Drug Administration (FDA) for treatment of metastatic and unresectable melanomas that carry an activating BRAF(V600E) mutation[12–14]. Moreover, in addition to treat unresectable BRAF(V600E) mutant melanomas[12], studies on evaluating the effectiveness of vemurafenib in brain metastases of melanoma (ClinicalTrials.gov identifier NCT01378975), colorectal cancer[15,16] (ClinicalTrials.gov identifier NCT00405587) and thyroid cancer[17] (ClinicalTrials.gov identifier NCT01709292) are ongoing. Unfortunately, acquired drug resistance to vemurafenib and relapse among patients were reported frequently within months of therapy[12,14]. Identifying and overcoming mechanisms that lead to acquired clinical resistance to vemurafenib presents a significant therapeutic challenge[18].

3. The impact of ATP-binding cassette transporter-mediated drug transport on cancer chemotherapy

Generally, the success of cancer chemotherapy depends on several key factors. For an anticancer agent to be effective, a sufficient amount of the drug must be distributed to the target site(s), which is dependent on the chemical and biological properties of the therapeutic agent, as well as the location of the target site(s). Cancer cells can often acquire resistance through adaptation or spontaneous induction of certain key regulatory genes during the course of chemotherapy, which is dependent on the patient, cancer type, stage of the disease and treatment strategy[4,19]. Collectively, drug absorption, distribution and acquired resistance may result in poor response to chemotherapy and unfavorable patient outcome. Among various adverse factors in cancer chemotherapy, energy dependent drug efflux and drug compartmentalization are the most common ways that cancer cells evade drug absorption and drug penetration[20,21].

Normally, the first line of cellular defense against xenobiotics is to rapidly reduce the intracellular concentration of xenobiotics by means of a transporter-mediated efflux system. Unfortunately, cancer cells can utilize the same protective mechanism by up-regulating some of the drug transporters that reduce drug sensitivity in patients, many of whom eventually relapse with multidrug-resistant forms of cancer[19]. One of the most common causes of acquired drug resistance in cancer is energy-dependent drug efflux by members of the human ATP-Binding Cassette (ABC) protein superfamily. Human ABC proteins are subdivided into seven families (ABCA-ABCG), based on structural and sequence similarities[20]. Several ABC proteins are

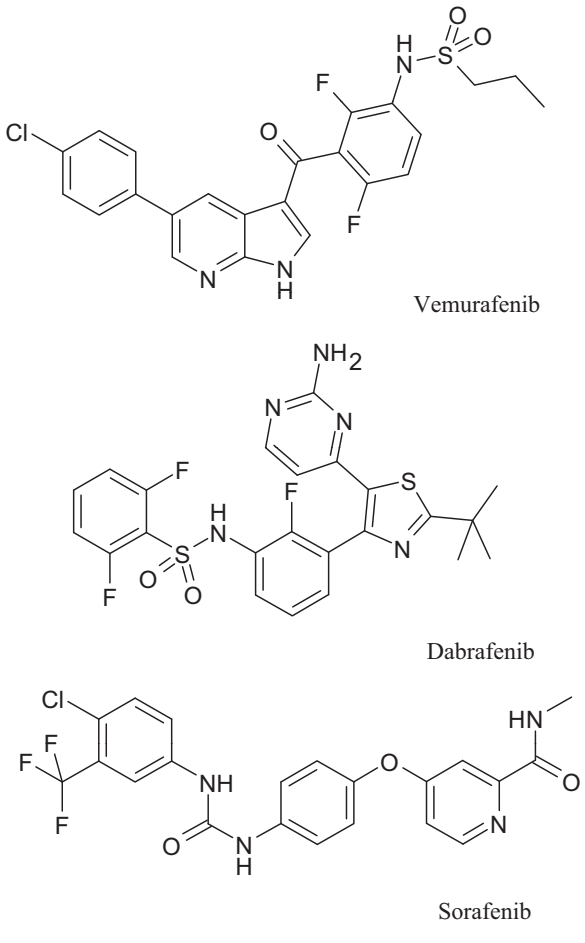

Figure 1 Chemical structures of vemurafenib, dabrafenib and sorafenib.

transporters that can utilize energy derived from ATP to mediate direct drug efflux. These ABC transporters are membrane proteins, consisting of transmembrane domains (TMDs) and distinctive nucleotide-binding domains (NBDs). The TMDs form substrate-binding pockets, while the NBDs generate energy from ATP hydrolysis to actively transport a wide range of substrates, including anticancer agents, across biological membranes, reducing intracellular drug concentration and eventually resulting in multidrug resistance (MDR)[22]. ABCA9, ABCB1, ABCB5, ABCB8, ABCC2, ABCD1 and ABCG2 are some of the ABC proteins that have been identified in melanoma cells[23–28]. In this review, we focus mainly on the potential roles of ABCB1, ABCG2 and ABCB5 in limiting the absorption, distribution and penetration of vemurafenib, as well as in the development of resistance to this drug in cancer cells expressing a BRAF(V600E) mutation.

3.1. *ABCB1*

The 170 kDa cell membrane ABCB1 (also known as P-glycoprotein, P-gp) was the first member of the mammalian ABC protein family to be identified[29]. ABCB1 consists of two transmembrane domains, each containing six α-helices, both linked to ATP-binding domains that provide energy by hydrolyzing ATP to transport drug substrate across cell membranes. A large number of classical anticancer agents including taxanes, *Vinca* alkaloids, etoposide, teniposide, camptothecins, methotrexate, colchicines, actinomycin D, anthracyclines and mitoxantrone are well-known drug substrates of ABCB1. More importantly, many of the newly developed targeted therapy drugs such as tyrosine kinase inhibitors (TKIs), have been identified as substrates of ABCB1 as well[30]. ABCB1 is expressed in endothelial cells at the blood–brain barrier (BBB) sites in normal brain tissue and also in primary brain tumors, and it functions to limit penetration of the brain by many chemotherapeutics[31,32]. In addition, ABCB1 is highly expressed in many normal tissues, including those of the liver and intestinal walls, signifying the physiological and pharmacological importance of ABCB1[20]. Moreover, ABCB1 is known to be overexpressed in many types of cancer and is linked to the MDR phenotype[33]. Considering the wide tissue distribution and substrate specificity of ABCB1, it is not surprising that ABCB1 plays a key role in limiting the oral bioavailability of anticancer drugs, preventing drug distribution and penetration through the blood–brain barrier and affecting therapeutic outcome in patients[19]. In terms of melanomas, endogenous ABCB1 mRNA has been detected in the melanoma cell lines SK-MEL-28, SK-MEL-5 and M16[23,34], as well as non-cutaneous melanomas[35,36]. ABCB1 was also detected in a subpopulation of human melanoma cells that co-express ABCB5, hTERT, and Nanog, and has high self-renewal capacity, representing characteristics of melanoma stem cells[37]. Interestingly, though the MDR phenotype has been shown in human BRO melanoma cells transfected with human ABCB1[38], the relevance of endogenous ABCB1 in conferring drug resistance in melanomas has not been demonstrated yet.

3.2. *ABCG2*

ABCG2 (also known as breast cancer resistance protein, BCRP; or placenta-specific ABC transporter, ABCP; or mitoxantrone resistance protein, MXR) was identified in 1998[39,40]. In contrast to ABCB1, ABCG2 consists of a single ATP-binding domain followed by a transmembrane domain with six α-helices in a reverse orientation[41]. A functional unit of ABCG2 is a dimer or a multimer. Similar to ABCB1, ABCG2 is overexpressed in many cancers, and is linked to reduced drug accumulation and to the development of MDR in patients with advanced non-small cell lung cancer or acute myeloid leukemia (AML)[42,43]. ABCG2 is capable of transporting a large variety of anticancer agents such as etoposide, docetaxel, topotecan, CPT-11, SN-38, methotrexate, flavopiridol, anthracyclines, mitoxantrone, and similar to ABCB1, many tyrosine kinase inhibitors including imatinib, nilotinib, saracatinib and ponatinib[30,44,45]. ABCG2 also has a physiological and pharmacological impact on drug bioavailability, drug distribution, protection of cells or tissues from xenobiotics and the transport of porphyrins and sterols[33]. Similar to ABCB1, ABCG2 has been detected at the luminal membrane of brain capillaries and the BBB, protecting the brain from xenobiotics and chemotherapeutics[46,47]. Studies have shown that both the protein expression and function of ABCG2 are up-regulated in neuro-epithelial tumors, restricting penetration of chemotherapeutics and leading to the development of MDR[48,49]. ABCG2 is believed to play a protective role in cancer stem cells (CSCs) or "side population" cells, with self-renewal properties and critical roles in tumorigenesis, metastasis and relapse[50]. Since ABCG2 is expressed in a wide range of human stem cells, it is considered as a biomarker for stem cells. ABCG2, along with CD133 and nestin, have been detected in melanomas[25,51–53], but the potential contribution of ABCG2 to chemoresistance in melanomas remains to be determined. Recently, ABCG2 has been linked to the disease gout, as mutations (for example Q141K) in this transporter result in decreased efflux of urate from kidney epithelial cells[54,55].

3.3. *ABCB5*

ABCB5 is predominantly expressed in pigment-producing (melanogenic) melanoma cells[23,24]. The melanogenesis-related vesicles, called "melanosomes" are derived from lysosomes and represent a unique feature of melanomas[56,57]. Structurally, ABCB5 has 73% sequence homology with ABCB1 protein[24,26]. In contrast to ABCB1, which mediates drug efflux from cells, ABCB5 is thought to confer chemoresistance to cisplatin, doxorubicin and daunorubicin by intracellular drug sequestration[16,24,57,58]. Furthermore, studies have reported that ABCB5 protein expression is up-regulated upon exposure to the chemotherapeutic drugs dacarbazine (DTIC) and doxorubicin[28,59]. Both ABCB1 and ABCG2 are known to be present in cancer stem cells, and hence are used as stem cell markers[60]. Similar cancer stem cell properties were discovered in metastatic melanoma cells, in which ABCB5 was present[61]. These ABCB5-positive melanoma stem cells are not only drug-resistant, but also possess self-renewal, differentiation and tumorgenic capabilities[58,62]. Interestingly, a recent study showed that ABCB5-expressing cells are resistant to temozolomide, dacarbazine and vemurafenib, suggesting that ABCB5 may contribute to the drug resistance mechanism, and thus is a potential therapeutic target for melanoma chemotherapy[28]. However, ABCB5-mediated transport of these drugs in melanoma patient samples has not yet been demonstrated.

4. The pharmacological impact of ABC drug transporters on the bioavailability and distribution of vemurafenib

Reports have shown a high incidence of melanoma metastases in the brain[63,64]. Prior to the discovery of vemurafenib, a patient's response to the standard therapy of interleukin-2 and dacarbazine was extremely poor[14,65]. However, in order for vemurafenib to be effective against brain metastases of melanoma, sufficient amounts of vemurafenib must first be absorbed in the gastrointestinal (GI)

tract (Fig. 2A), be distributed, and also penetrate the BBB and accumulate in the brain (Fig. 2B). The vasculature structure of the BBB consists of tightly sealed tight-junction protein complexes combined with overexpression of several ABC transporters that actively transport chemotherapeutics back into the bloodstream (Fig. 2B), making drug penetration of the brain a major obstacle in chemotherapy[66].

A recent study by Mohammed et al.[67] reported that the delivery of vemurafenib to the brain is restricted due to its direct transport by human ABCB1 and mouse Abcg2 at the blood–brain barrier. In their *in vitro* experiments, the intracellular accumulation of vemurafenib was reduced in MDCKII cells transfected with ABCB1 or ABCG2, as a direct result of ABCB1 and ABCG2-mediated transport of vemurafenib. Moreover, the ABCB1 and ABCG2-mediated transport of vemurafenib can be inhibited by zosuquidar and Ko143, respectively. Furthermore, in their knockout mouse model, the brain-to-plasma ratios of vemurafenib were increased significantly when Abc1a/1b and Abcg2 were both absent. The authors concluded that vemurafenib is a substrate of both ABCB1 and ABCG2, and both transporters play a significant role in limiting the central nervous system (CNS) distribution of vemurafenib. The findings by Mohammed et al. were later supported by an independent group. Durmus et al.[68] reported that inhibition of both ABCB1 and ABCG2 could significantly improve the bioavailability (Fig. 2A) and brain penetration (Fig. 2B) of vemurafenib. In their *in vitro* experiments, vemurafenib transport mediated by either ABCB1 or ABCG2 was demonstrated by using MDCK II cells transduced with either human ABCB1 or ABCG2. The ABCB1- and ABCG2-mediated transport of vemurafenib was inhibited completely by the ABCB1 inhibitor zosuquidar and the ABCG2 inhibitor Ko143[68]. *In vivo*, the dual Abcb1a/1b and Abcg2 inhibitor elacridar significantly elevated the plasma levels of vemurafenib and brain accumulation in WT mice to the same levels as in *Abc1a/1b*$^{-/-}$; *Abcg2*$^{-/-}$ mice. Interestingly, Durmus et al.[68] found that Abcg2 is responsible for reducing the intestinal uptake of vemurafenib, but limited to a lower oral dose. In contrast, Abcb1a/1b is accountable for reducing plasma levels of vemurafenib at later stages. This particular observation is in accordance with findings by Chapman et al.[14], that in BRAF(V600E) mutant A375 melanoma cells, ABCG2 behaves as a high-affinity but low capacity transporter of vemurafenib.

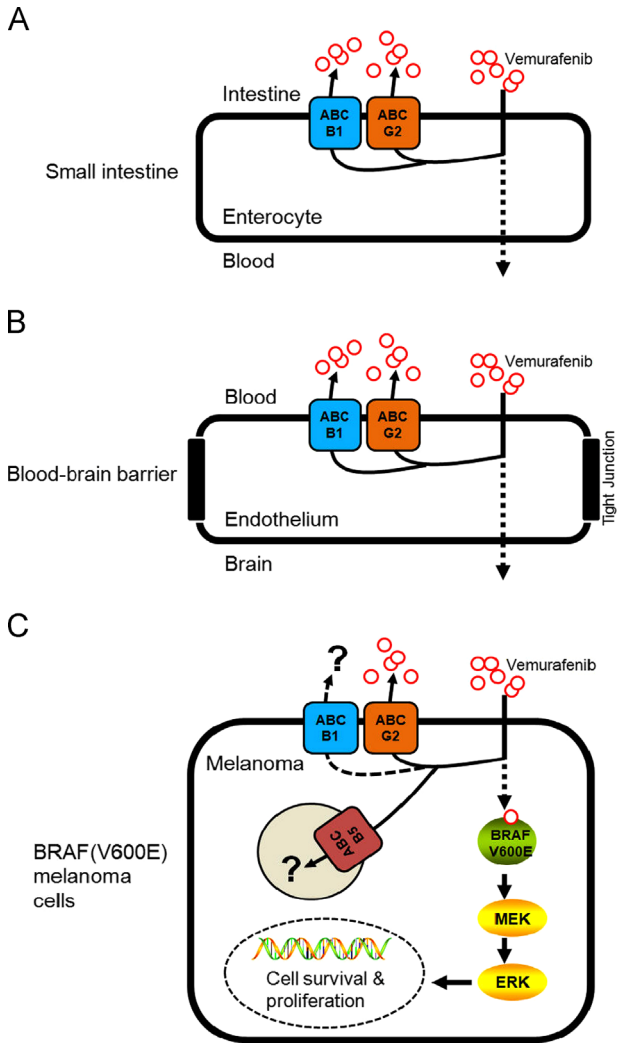

Figure 2 The potential role of multidrug resistance-associated ABC drug transporters in the oral bioavailability, brain penetration and therapeutic efficacy of vemurafenib in melanoma and other cancer cells harboring V600E mutation in BRAF kinase. (A) Highly active ABCB1 and ABCG2 transporters in intestinal epithelial cells can significantly limit the absorption of vemurafenib into the blood stream, reducing its bioavailability. (B) The presence of both ABCB1 and ABCG2 at the blood–brain barrier restricts vemurafenib penetration of the brain, reducing its effectiveness in patients with brain metastatic melanoma. (C) The presence of ABCG2 confers resistance to vemurafenib in BRAF(V600E) mutant A375 melanoma cells. The role of the ABCB5 transporter in melanoma remains to be evaluated.

5. The potential impact of ABC drug transporters on vemurafenib-based treatment of advanced or metastatic melanoma

Initial success at using vemurafenib to treat patients with metastatic and unresectable melanomas or other cancers that carry an activating BRAF(V600E) mutation was short lived. The rapid development of acquired resistance to vemurafenib is now becoming a major obstacle in the treatment of patients diagnosed with BRAF(V600E)-positive cancer[12,14]. Multiple mechanisms involving the reactivation of the mitogen-activated protein kinase (MAPK) pathway have been reported in vemurafenib-resistant BRAF(V600E) mutant cancer cells. Up-regulation of CRAF[69,70] and overexpression of Tpl2/COT[69], RAS activation[38,71], enhanced activation of the FGFR3/RAS pathway[72], pathways that lead to reactivation of ERK signaling[73] and activation of RTK signaling pathways such as IGF-1R or PDGFRβ[25,71,74] have all been shown to contribute to acquired resistance to vemurafenib, depending on the cancer type[17,26].

Recently, we have discovered that in addition to a RAF isoform switch and activation of various compensatory survival pathways[25,38,69–74], the overexpression of ABCG2 could also contribute to the development of acquired resistance to vemurafenib in BRAF (V600E) mutant cancer cells (Fig. 2C)[6]. This is not surprising since the overexpression of ABC transporters is one of the most common mechanisms of acquired resistance to anticancer agents[33]. In our study, the interactions of vemurafenib with three major MDR-associated ABC drug transporters, ABCB1, ABCC1 and ABCG2 were investigated. Results showed that vemurafenib binds directly to the substrate binding pockets of ABCG2, inhibits its function and stimulates ATP hydrolysis. Similar interactions between vemurafenib and ABCB1 were observed, but the binding affinity and the

stimulation of ATP hydrolysis were significantly lower. We found that since vemurafenib binds to the drug binding site of human ABCG2 with relatively high affinity, it effectively inhibited ABCG2-mediated transport of other drug substrates. Moreover, at non-toxic concentrations, vemurafenib was able to restore chemosensitivity of ABCG2-overexpressing HEK293 cells to anticancer agents such as mitoxantrone and topotecan. Similarly, vemurafenib also restored the sensitivity of drug-resistant ABCG2-overexpressing and also expressing (V600E) mutant BRAF A375 melanoma cells to mitoxantrone[6]. In contrast, no interaction was detected between vemurafenib and ABCC1 protein. Moreover, 72 h of vemurafenib treatment had no significant effect on the expression of ABCB1, ABCC1 or ABCG2 protein in cancer cells expressing wild-type BRAF. Surprisingly, while overexpression of human ABCG2 had no effect on the chemosensitivity of wild-type BRAF cancer cells to vemurafenib, the ectopic expression of human ABCG2 led to vemurafenib resistance in A375 melanoma cells harboring the BRAF(V600E) mutation. We found that in A375 melanoma cells, BRAF kinase inhibition by vemurafenib was significantly reduced in the presence of functional ABCG2, implicating ABCG2-mediated efflux as a mechanism of resistance for vemurafenib[6]. Unfortunately, it is still unknown whether prolonged treatment with vemurafenib leads to overexpression of ABC drug transporters in BRAF(V600E) melanoma, thyroid or colorectal cancers. Furthermore, the potential impact of ABCB1 or ABCB5 or other MDR-associated ABC drug transporters on the therapeutic outcome using vemurafenib in melanomas or other cancers harboring the BRAF(V600E) mutation needs to be determined.

6. Impact of ABC drug transporters on treatment with other BRAF inhibitors (dabrafenib and sorafenib)

Dabrafenib (GSK2118436) is a new BRAF inhibitor (Fig. 1) designed to target melanomas expressing V600E and V600K mutant BRAF. Good clinical response rates have been observed in metastatic melanoma (including brain metastases) patients receiving dabrafenib[75,76], but cases of acquired resistance to dabrafenib have also been reported[77–79]. Although the link between ABC drug transporters and acquired resistance to dabrafenib is still lacking, a recent study using MDCKII cells indicated that dabrafenib is a substrate of both ABCB1 and ABCG2[80]. Moreover, Mittapalli et al.[80] showed that in both *in vivo* and intact BBB models, the dabrafenib brain distribution is limited by the function of both ABCB1 and ABCG2. In contrast to vemurafenib and dabrafenib, sorafenib is a nonselective BRAF inhibitor (Fig. 1) that targets both BRAF and CRAF, and inhibits other multiple kinases[81]. A phase I/II clinical trial reported that in metastatic melanoma patients, combination therapy of sorafenib, carboplatin and paclitaxel demonstrated a better response rate and longer progression-free survival than with standard chemotherapy[82]. Like vemurafenib and dabrafenib, the interactions between sorafenib, ABCB1 and ABCG2 have been demonstrated by several independent groups. Other studies have reported that sorafenib is transported by both ABCB1[83,84] and ABCG2, but more efficiently by ABCG2[84], and consistent with these findings the penetration of the brain by sorafenib was significantly higher in *Abcg2*$^{-/-}$ mice than in WT[84,85].

7. Conclusions

Collectively, the actions of ABCB1 and ABCG2 in the GI tract and at the BBB contribute significantly to reduced oral bioavailability and limit the penetration of the brain by vemurafenib (Fig. 2A and B), which is a major obstacle when treating patients with melanoma brain metastases. The clinical application of a dual ABCB1 and ABCG2 inhibitor such as elacridar could possibly provide a solution to increase the oral bioavailability and enhance brain penetration of vemurafenib in patients with brain metastatic melanoma[86]. At the cellular level, the presence of MDR-associated ABC drug transporters may present new therapeutic challenges when treating cancers expressing the V600E mutant version of BRAF kinase. The ability of ABC drug transporters to effectively reduce the intracellular concentration of vemurafenib in cancer cells can potentially lead to acquired resistance to this drug (Fig. 2C). Moreover, the reported high affinity of vemurafenib for binding to ABCG2 suggests the potential use of vemurafenib as a chemosensitizer that would work alongside classical anticancer agents to treat ABCG2-positive MDR cancers. Consistent with these findings, vemurafenib was found to dock in the drug-binding pocket of the homology model of human ABCB1 and ABCG2 and also modulate the function of the ABCC10 (MRP 7) transporter[87]. Thus, we propose that simultaneous administration of vemurafenib and protein kinase inhibitors targeting key signaling pathways that are involved in the development of acquired resistance to vemurafenib[25,38,69–74], as well as inhibiting the actions of ABC drug transporters in BRAF(V600E) mutant cancers[33], may offer great promise for effective treatment of melanoma patients.

Acknowledgments

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