Impact of P-glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) on the Brain Distribution of a novel B-RAF Inhibitor: Vemurafenib (PLX4032)

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Running Title: P-gp and Bcrp restrict brain distribution of vemurafenib

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List of abbreviations:
BBB, blood-brain-barrier; CNS, central nervous system; P-gp, p-glycoprotein; BCRP, breast cancer resistance protein; Mdr1, gene encoding the murine p-glycoprotein; Bcrp1, gene encoding the murine breast cancer resistance protein; MDR1, gene encoding the human p-glycoprotein; ABC, ATP-binding cassette; MDCKII, Madin-Darby canine kidney II; A to B, apical to basolateral; B to A, basolateral to apical; LY335979 (zosuquidar), \((R)-4-((1\alpha R,6R,10bS)-1,2-difluoro-1,1a,6,10b-tetrahydrodibenzo-(a,e)cyclopropa(c)cycloheptan-6-yl)-\alpha-((5-quinoloyloxy) methyl)-1-piperazine ethanol, trihydrochloride; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino(1',2':1,6) pyrido(3,4-b)indole-3-propanoic acid 1,1-dimethylethyl ester; PLX4720, Propane-1-sulfonic acid [3-(5-chloro-1H-pyrrolo[2,3-b]pyridine-3-carbonyl)-2,4-difluorophenyl]amide; \(P_{app}\), apparent permeability; FVB, Friend Leukemia Virus Strain B; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPMC, hydroxyl propyl methyl cellulose; DMSO, dimethyl sulphoxide; B/P, brain to plasma; AUC, area under the curve.
Abstract:

Vemurafenib (PLX4032) is a novel small molecule BRAF inhibitor, recently approved by US Food and Drug Administration (FDA) for the treatment of patients with metastatic melanoma with a BRAF\textsuperscript{V600E} mutation. The objective of this study was to investigate the role of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) on the distribution of vemurafenib to the central nervous system. \textit{In vitro} studies conducted in transfected Madin-Darby canine kidney-II cells show that the intracellular accumulation of vemurafenib is significantly restricted due to active efflux by P-gp and BCRP. Bidirectional flux studies indicated greater transport in basolateral-to-apical direction than apical-to-basolateral direction due to active efflux by P-gp and BCRP. Selective P-gp and BCRP inhibitors zosuquidar and Ko143 were able to restore the intracellular accumulation and bidirectional net flux of vemurafenib. The \textit{in vivo} studies revealed that the brain distribution coefficient (area under the concentration time profile of brain / area under the concentration time profile of plasma) of vemurafenib was 0.004 in wild-type mice. The steady-state brain-to-plasma ratio of vemurafenib was 0.035 \pm 0.009 in \textit{Mdr1a/b\textsuperscript{-/-}} mice, 0.009 \pm 0.006 in \textit{Bcrp1\textsuperscript{-/-}} mice, 1.00 \pm 0.19 in \textit{Mdr1a/b\textsuperscript{-/-} Bcrp1\textsuperscript{-/-}} mice, compared to 0.012 \pm 0.004 in wild type mice. These data indicate that the brain distribution of vemurafenib is severely restricted at the blood-brain barrier due to active efflux by both P-gp and BCRP. This finding has important clinical significance given the ongoing trials examining the efficacy of vemurafenib in brain metastases of melanoma.
INTRODUCTION:

Melanoma is a neoplasm that originates in the pigment producing cells of the skin. The incidence of melanoma is escalating. For example, in 2011, approximately 70,000 individuals were expected to be diagnosed with melanoma in the United States, and ~8800 were predicted to die from melanoma (Siegel et al., 2011). After lung and breast cancers, malignant melanoma is the third most common neoplasm that metastasizes to the brain (Johnson and Young, 1996). Approximately 50-75% of melanoma patients are found to have brain metastases at autopsy (Fife et al., 2004). Once the lesions have become established in the central nervous system (CNS), the median survival is less than 6 months (Fife et al., 2004; Raizer et al., 2008).

The current therapeutic options for melanoma patients include surgery, radiotherapy, and chemo or immunotherapy. The standard therapy using high dose interleukin-2 and dacarbazine proves to be unsuccessful in metastatic melanoma, with response rates of only 10 to 20% (Comis, 1976; Atkins et al., 1999; Garbe et al., 2011). The gene encoding the serine-threonine protein kinase B-RAF (BRAF) was found to be mutated in ~40-60% of melanomas (Wan et al., 2004). BRAF is an important component of RAF/MEK/ERK signaling pathway which regulates cell proliferation and growth (McCubrey et al., 2008). The mutated BRAF gene results in signaling pathways that promote tumor cell proliferation, invasion, and resistance. Among the BRAF mutations approximately 80% exhibit a valine to glutamic acid substitution (V600E; BRAF<sup>V600E</sup>) resulting in constitutive expression of kinase activity (Davies et al., 2002). A recent study indicated that BRAF<sup>V600E</sup> is associated with poor patient survival (Long et al., 2011), and further studies show that the incidence of BRAF<sup>V600E</sup> mutation in brain
metastases of melanoma is similar to that found in peripheral sites (Capper et al., 2011). Given the prevalence of \( \text{BRAF}^{\text{V600E}} \) mutations in a large number of melanomas, BRAF has been an attractive treatment target for melanoma patients with V600E mutation, and as such many small-molecule inhibitors of BRAF have been developed.

Vemurafenib (previously known as PLX4032) is a small molecule \( \text{BRAF}^{\text{V600E}} \) inhibitor that was developed using a structure-guided drug discovery approach (Tsai et al., 2008). It was approved by U.S. Food and Drug Administration (FDA) in August 2011 for late-stage melanoma patients with \( \text{BRAF}^{\text{V600E}} \) mutation. Clinical trials with vemurafenib have shown remarkable responses in a high percentage of BRAF mutant melanoma cases (Ribas et al., 2011), with improved overall and progression-free survival (Chapman et al., 2011). A clinical trial evaluating the efficacy of vemurafenib in brain metastases of melanoma is currently recruiting patients (ClinicalTrials.gov identifier: NCT01378975). Whether or not vemurafenib will show clinical activity in brain metastases of melanoma is an important question that remains to be answered. In this context, it is crucial to determine the mechanisms influencing the brain distribution of vemurafenib to further support the clinical investigations.

A major factor contributing to the rapid and near 100% mortality in melanoma patients with brain metastases has been the presumed limited permeability of chemotherapeutics across the blood-brain barrier (BBB). The BBB is a highly evolved vasculature structure which limits most molecules from distributing into the brain from the blood compartment. Anatomically, the vasculature of the BBB is unique in that it is comprised of endothelial cells, that are circumferentially sealed together by tight-junction protein complexes, that form the lumen of the vessel. Further, active efflux
transporters that are present on the luminal side of capillary endothelium efficiently pump out the drugs from the brain to the blood circulation. Adenosine triphosphate-binding cassette proteins (ABC) such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are major members of the efflux transporters present on the luminal membrane of the brain capillary endothelial cells (Schinkel and Jonker, 2003). Studies have shown that many therapeutic agents are substrates of these transporters and as a result have very limited brain distribution (Loscher and Potschka, 2005).

Vemurafenib can be considered a “sea-change” in the treatment of melanoma patients. However, important questions still remain regarding resistance and effective delivery to all sites of melanoma metastases, particularly in brain. In this regard, there is a paucity of data regarding the delivery of anti-melanoma agents to brain metastases. Given the remarkable activity of the novel targeted BRAFV600E inhibitors in peripheral disease, it becomes critical to examine the mechanisms that may limit their delivery to brain metastases. Whether vemurafenib can cross the BBB to achieve therapeutic levels in the CNS remains unknown. This has motivated us to examine the interaction of vemurafenib with the two main BBB efflux transporters, namely P-gp and BCRP. Herein, using in vitro models, we show that vemurafenib is an avid substrate for both P-gp and BCRP. In vivo studies using genetic knockout mice indicate both transporters play a significant role in limiting the CNS distribution of vemurafenib.
MATERIALS AND METHODS:

Chemicals: Vemurafenib (PLX4032) was purchased from Chemietek (Indianapolis, IN). [\(^3\)H]-Vinblastine was purchased from Moravek Biochemicals (La Brea, CA). [\(^3\)H]-Prazosin was purchased from Perkin Elmer Life and Analytical Sciences (Waltham, MA). KO143 was a generous gift from Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and zosuquidar [LY335979, \((R)-4-((1aR, 6R,10bS)-1,2-difluoro-1,a,6,10b-tetrahydrodibenzo-(a,e) cyclopropa(c)cycloheptan-6-yl)-(5-quinoloyloxy) methyl)-1-piperazine ethanol, trihydrochloride] was kindly provided Eli Lilly and Co.(Indianapolis, IN). All other chemicals used were of high performance liquid chromatography or reagent grade and were obtained from Sigma-Aldrich (St. Louis, MO).

In vitro studies:

All the in vitro studies were performed using polarized Madin-Darby canine kidney-II (MDCKII) cells. MDCKII-wild type (WT) and MDR1-transfected (MDCKII-MDR1) cell lines were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute). MDCKII-WT and Bcrp1-transfected (MDCKII-Bcrp1) cells were a kind gift from Dr. Alfred Schinkel (The Netherlands Cancer Institute). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100 U/mL; streptomycin, 100 µg/mL; and amphotericin B, 250 ng/mL). Cells were grown in 25 mL tissue culture treated flasks before seeding for the experiments and were maintained at 37\(^{\circ}\) C in a humidified incubator with 5% CO\(_2\).
In vitro accumulation studies: The intracellular accumulation of vemurafenib was performed in 12-well polystyrene plates (Corning Inc. Corning, NY) as described previously (Agarwal et al., 2010; Agarwal et al., 2011). Briefly, cells were seeded at a density of 2 x 10^5 cells and were grown until the cells were ~80% confluent. On the day of experiment the culture media was aspirated and the cells were washed two times with cell assay buffer (122 mM NaCl, 25 mM NaHCO₃, 10 mM glucose, 10 mM HEPES, 3 mM KCl, 2.5 mM MgSO₄, 1.8 mM CaCl₂, and 0.4 mM K₂HPO₄). Then the cells were preincubated with assay buffer for 30 min, after which the buffer was aspirated and the experiment was initiated by adding 1 mL of vemurafenib (2 µM) to each well and further incubated for 60 min. The assay plates were incubated at 37º C on an orbital shaker (60 rpm) for the entire duration of the experiment. After the incubation period, the drug solution was aspirated and the cells were washed twice with ice cold PBS. Then the cells were lysed by adding 500 µL of 1% Triton X to each well. A 400 µL of solubilized cell fraction was sampled from each well and the concentration of vemurafenib was determined using LC-MS/MS as described below.

Directional transport across MDKCII monolayers: The bidirectional flux studies were performed using 6-well Transwell® plates (Cornings Inc. Lowell, MA). The cells were seeded at a density of 2 x 10^5 cells per well and the media was changed every other day until confluent monolayers were formed. On the day of experiment, the culture medium was aspirated and the cells were washed twice with assay buffer and after 30 min preincubation, the experiment was initiated by adding the vemurafenib solution (2 µM) in assay buffer to the donor compartment. Samples (200 µL) were collected from the receiver compartment at 0, 30, 60, 90, 120, and 180 minutes and replaced with drug-
free assay buffer. Similarly, at the beginning of the experiment a 200 µL sample was
drawn from the donor compartment and replaced with 200 µL of drug solution. The
Transwell® plates were incubated at 37 °C on an orbital shaker for the duration of
experiment except for the brief sampling times. When an inhibitor was used, the
inhibitor was present in the both compartments during the pre and post incubation
period.

The apparent permeability (P_{app}) for the directional transport was calculated as
previously described (Agarwal et al., 2011). The permeabilities were calculated using
the following equation

\[
P_{app} = \frac{dQ}{dt} / (A \times C_0)
\]

Where, \( (dQ/dt) \) is the slope obtained from the initial linear range from the amount
transported versus time plot, A is the area of the Transwell® membrane, and C_0 is the
initial donor concentration. The efflux ratio and corrected efflux ratio were calculated
using eqs. 2 and 3, respectively,

\[
\text{Efflux ratio} = \frac{P_{app}(B \rightarrow A)}{P_{app}(A \rightarrow B)}
\]

\[
\text{Corrected Flux ratio} = \frac{\left( \frac{B \rightarrow A}{A \rightarrow B} \right)_{Transfected \ line}}{\left( \frac{B \rightarrow A}{A \rightarrow B} \right)_{Wild-type \ line}}
\]

where A→B represents permeability in apical to basolateral and B→A represents
permeability in basolateral to apical direction.
Competition assays using P-gp and Bcrp probes: Competition assays were performed using the prototypical probe substrates prazosin for BCRP and vinblastine for P-gp. Intracellular accumulation of these substrates at 60 min was determined in MDCKII-Bcrp1 or MDCKII-MDR1 cells in the presence of different concentrations of vemurafenib ranging from 1 µM to 25 µM. The increase in probe accumulation relative to control (i.e., no vemurafenib treatment) was reported as a function of vemurafenib concentration.

In vivo Studies:

Animals: All of the in vivo studies were performed in FVB (wild type), Mdr1a/b⁻/⁻ (P-gp knockout), Bcrp1⁻/⁻ (BCRP knockout), and Mdr1a/b⁻/⁻Bcrp1⁻/⁻ (triple knockout) mice of a FVB genetic background (Taconic Farms, Germantown, NY). All animals were 8 to 10 weeks old at the time of experiment. Animals were maintained in a 12 hr light/dark cycle with an unlimited access to food and water. All studies were carried out in accordance with the guidelines set by the Principles of Laboratory Animal Care (National Institutes of Health, Bethesda, MD) and approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Brain Distribution of vemurafenib in FVB mice: The dosing formulation of vemurafenib was prepared either in a vehicle containing 1% Tween 20 and 1% HPMC (for oral dosing) or in a vehicle containing 40% DMSO, 40% propylene glycol, and 20% saline (for i.v. studies). All vemurafenib formulations were freshly prepared on the day of experiment. In the first study, wild type, Mdr1a/b⁻/⁻, Bcrp1⁻/⁻, and Mdr1a/b⁻/⁻Bcrp1⁻/⁻ mice
received an oral dose of 25 mg/kg and blood and brain samples were collected 1 and 4 hr post dose. At the end of the desired time point, the animals were euthanized using a CO$_2$ chamber. Blood was collected via cardiac puncture and collected in heparinized tubes. Plasma was obtained by centrifuging the blood at 7500 rpm for 10 min. Brains were rapidly removed from the skull, rinsed with ice cold PBS followed by a flash freeze in liquid nitrogen. Brain and plasma samples were stored at -80º C until further analysis.

In the i.v. dosing study, wild type and $Mdr1a/b^{-/-}$ $Bcrp1^{-/-}$ mice were administered vemurafenib via the tail vein at a dose of 2.5 mg/kg. Blood and brain samples were processed after 5, 30, 90, 180, 300, and 480 minutes ($n=4$ for each time point) as mentioned above.

**Steady state brain distribution of vemurafenib:** To determine the steady state brain and plasma concentrations of vemurafenib, Alzet osmotic mini pumps (Durect Corporation, Cupertino, CA) were loaded with vemurafenib (25 mg/mL dissolved in DMSO) to be released for 48 hrs at a rate of 1µL/hr. After vemurafenib loading, mini pumps were primed overnight in sterile saline at 37 º C. Pumps were implanted in the peritoneal cavity of wild type, $Mdr1a/b^{-/-}$, $Bcrp1^{-/-}$, and $Mdr1a/b^{-/-}Bcrp1^{-/-}$ mice as previously described (Agarwal et al., 2010; Agarwal et al., 2011). Briefly, mice were anesthetized using isofluorane and the abdominal cavity was shaved. A small midline incision was made in the lower abdomen under the rib cage. Then a small incision was made directly in the peritoneal membrane and the primed pump was inserted in the cavity. The incision was sutured and the skin was closed using surgical clips. The animals were allowed to recover on a heating pad and, once recovered, moved to their
original cages. The animals were sacrificed 48 hrs after the implantation of the pumps, and brain and plasma samples were processed as described above.

**Analysis of vemurafenib using LC-MS/MS:** The concentrations of vemurafenib in cell lysates, assay buffer, plasma and brain homogenate were determined using a sensitive and specific liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) assay. For brains, 3 volumes of 5% bovine serum albumin was added and homogenized to get a uniform homogenate. For analysis of unknowns, an aliquot of cell lysate, cell assay buffer, brain homogenate or plasma was spiked with 50 ng of PLX4720 as an internal standard and alkalinized by addition of 2 volumes of pH 11 buffer (1 mM sodium hydroxide, 0.5mM sodium bicarbonate). The samples were then extracted by addition of 10 volumes of ethyl acetate followed by vigorous shaking for 5 min and centrifuged at 7500 rpm for 5 min at 4ºC to separate the organic layer. The organic layer was transferred to microcentrifuge tubes and dried under nitrogen. Samples were reconstituted in 100 µL of mobile phase and transferred into HPLC glass vials. Chromatographic analysis was performed using an AQUITY UPLC® system (Milford, MA, USA). The chromatographic separation was achieved using an Agilent Technologies Eclipse XDB-C18 column (4.6 x 50 mm) with 1.8 µm Zobrax Rx-SiL as the stationary phase. The mobile phase consisted of 20 mM ammonium formate with 0.1% formic acid and acetonitrile (30:70 v/v), and was delivered at a flow rate of 0.25 mL/min.

The column effluent was monitored using a Waters/Micromass Quattro™ Ultima mass spectrometer (Waters, Milford, MA). The instrument was equipped with an
electrospray interface, and controlled by the Masslynx (Version 4.1) data system. The samples were analyzed using an electrospray probe in the negative ionization mode operating at a spray voltage of 2.96 kV for both vemurafenib and PLX4720 (internal standard). Samples were introduced into the interface through a heated nebulized probe where the source and desolvation temperatures were set at 100 °C and 275 °C, respectively. The spectrometer was programmed to allow the [MH]- ion of vemurafenib at \( m/z \) 488.23 and that of internal standard at \( m/z \) 412.26 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 27V for vemurafenib and 25V for PLX4720. The product ions for vemurafenib (\( m/z \) 380.89) and the internal standard PLX4720 (\( m/z \) 304.82) were monitored through the third quadrupole (Q3). The retention time for vemurafenib and the internal standard PLX4720 was 4.2 and 2.9 min, respectively. The assay was sensitive and linear over a range of 1.2 ng/mL to 1.2 µg/mL, with the coefficient of variation being less than 15% over the entire range.

**Pharmacokinetic Calculations:** Pharmacokinetic parameters and metrics were calculated by noncompartmental methods using Phoenix WinNonlin (Version 6.1) (Pharsight, Mountain View, CA). The area under the concentration time curve in both plasma and brain was calculated using the trapezoidal rule, and the area under the curve was determined up to the last measured time point (AUC \( 0-t_{last} \)). AUC \( 0-t_{last} \) was used in determining brain to plasma distribution ratio. The area under the curve from time zero to infinity was also determined and the area extrapolated was less than 20% of the total area under the concentration curve. The terminal rate constants were determined using all the data points in the brain and the last three data points in the
plasma. In plasma, the concentration at zero time was extrapolated using the terminal rate constant to measure the area under the curve at time zero to the first measured time point.

**Statistical Analysis:** Data in all experiments represent mean ± SD unless otherwise indicated. Comparisons between two groups were made using an unpaired t-test. One way ANOVA, followed by Bonferonni’s multiple comparisons test, was utilized to compare multiple groups. A significance level of p <0.05 was used for all experiments. (GraphPad Prism 5.01 software, San Diego, CA, USA).

**RESULTS:**

**Intracellular accumulation of vemurafenib:** The intracellular accumulation of vemurafenib was studied in MDCKII WT and P-gp or Bcrp overexpressing cell lines. [3H]-prazosin and [3H]-vinblastine were used as positive controls for Bcrp and P-gp, respectively. As expected, the accumulation of [3H]-prazosin ([Fig. 1A]) was significantly lower in Bcrp overexpressing cell lines (WT: 100.0 ± 8.7; Bcrp: 8.0 ± 2.4, p<0.0001). Similarly, the accumulation of [3H]-vinblastine ([Fig. 1B]) in P-gp overexpressing cells was ~80% lower compared to its WT line (WT: 110.0 ± 16.4; MDR1: 13.8 ± 4.1, p = 0.0004). Vemurafenib accumulation was ~ 77% lower in Bcrp overexpressing cell line compared to its WT line (WT: 100.0 ± 5.0; Bcrp: 12.9 ± 0.3, P<0.0001). The difference in accumulation was abolished when a specific Bcrp inhibitor Ko143 was added (Bcrp: 8.0 ± 2.4; Bcrp with Ko143: 69.8 ± 3.0, p<0.05). Similarly, the accumulation of vemurafenib was ~20% lower in P-gp overexpressing line compared to its WT control.
(WT: 100.0 ± 2.6; MDR1: 73.6 ± 5.5, p<0.05), and the difference in accumulation was abolished (Fig. 1B) when a specific P-gp inhibitor LY335979 was added (MDR1: 73.6 ± 5.5; MDR1 with LY: 110.8 ± 2.9). The aggregate of these data indicates that vemurafenib is a substrate for both P-gp and Bcrp in vitro.

**Competition assays:** The in vitro competition assays for vemurafenib were performed in MDCKII cells using vinblastine and prazosin as P-gp and Bcrp prototype probe substrates, respectively. The addition of increasing concentrations of vemurafenib resulted in a gradual increase in accumulation of prazosin and vinblastine in MDCKII-Bcrp1 cells and MDCKII-MDR1 cells, respectively. The fold increase in prazosin accumulation in MDCKII-Bcrp1 cells at 10 µM of vemurafenib was no different than the effect seen with 0.2 µM Ko143, a potent Bcrp inhibitor (Fig. 2A). Similarly, at 25 µM of vemurafenib, the fold increase in vinblastine accumulation was no different than the effect seen with the potent P-gp inhibitor LY335959 (Fig. 2B) in MDCKII-MDR1 cells. These data suggest that vemurafenib may share the same binding sites on the transporter proteins as these prototypical probe substrates.

**Bidirectional flux across MDCKII monolayers:** The in vitro transport (apical to basolateral, A to B; and basolateral to apical, B to A) of vemurafenib was studied in MDCKII wild type, and P-gp or Bcrp overexpressing cell lines. Fig. 3A and 3B demonstrate the transport of vemurafenib in the A to B and B to A direction in Bcrp1 and corresponding wild-type cell monolayers. In the wild type cells, there was minimal transport (less than 2.3% in 1.5 hrs) of vemurafenib in either direction (Fig. 3A). In case of the MDCKII-Bcrp1 cells, the permeability of vemurafenib in the B to A direction was significantly higher than the permeability in the A to B direction [A to B: (0.02 ± 0.003 ×
The addition of 0.2 μM Ko143, a specific Bcrp inhibitor, resulted in partial inhibition of Bcrp mediated vemurafenib transport in these cells (Fig. 3C).

The transport of vemurafenib in the A to B and B to A direction in MDR1 and corresponding wild-type cell monolayers is depicted in Fig. 3D and 3E. In the case of the MDCKII-MDR1 transfected line, the permeability of vemurafenib in B to A direction was significantly greater compared with permeability in A to B direction [A to B: (0.21 ± 0.16 × 10⁻⁶ cm/s); B to A: (16.7 ± 2.1 × 10⁻⁶ cm/s); p <0.05; Fig. 3F] resulting in an efflux ratio of ~80. The addition of 1 μM LY335979, a potent inhibitor of P-gp, resulted in partial inhibition of P-gp mediated vemurafenib transport in these cell lines (Fig. 3F). The B to A permeability of vemurafenib was not significantly different from the A to B permeability in the corresponding wild-type cells. The corrected flux ratio was found to be approximately 34 in control and 2 in cells treated with the P-gp inhibitor LY335979.

**Brain distribution of vemurafenib in FVB wild type and Mdr1a/b⁻/⁻Bcrp1⁻/⁻ mice after intravenous administration:** The brain distribution of vemurafenib was studied in FVB wild type mice after an i.v. dose of 2.5 mg/kg via the tail vein. Fig. 4 shows the concentration time profile of vemurafenib in both plasma and brain at 5, 30, 90, 180, 300, and 480 minutes of post i.v dose. The plasma concentrations of vemurafenib were significantly higher (~3 log units) at all-time points compared to its brain concentrations, indicating the severely restricted brain distribution of vemurafenib. The brain to plasma partitioning (AUCbrain / AUCplasma) was found to be ~0.004 in FVB wild type mice. The pharmacokinetic parameters are depicted in Table 1.
In a separate study, the brain distribution of vemurafenib in *Mdr1a/b<sup>-/-</sup>*Bcrp<sup>1/-</sup> mice was examined after an i.v. dose of 2.5 mg/kg. As shown in Fig. 5B, the brain concentrations of vemurafenib in *Mdr1a/b<sup>-/-</sup>*Bcrp<sup>1/-</sup> mice were significantly higher than in wild type mice. The plasma concentrations were not different between the two types of mice (Fig. 5A). The brain to plasma (B/P) ratio of vemurafenib in wild-type mice was ~0.4%, significantly lower than the B/P ratio in *Mdr1a/b<sup>-/-</sup>*Bcrp<sup>1/-</sup> mice (Fig. 5C).

**Brain distribution of vemurafenib after oral administration:** The brain distribution of vemurafenib was examined 1 and 4 hr post oral dose (25 mg/kg) of vemurafenib in FVB wild type, *Mdr1a/b<sup>1/-</sup>*, *Bcrp<sup>1/-</sup>* , and *Mdr1a/b<sup>1/-</sup>*Bcrp<sup>1/-</sup> mice. As shown in Fig. 6A, the brain concentrations of vemurafenib were significantly lower than plasma after 1 hr post dose in wild type, *Mdr1a/b<sup>1/-</sup>* , and *Bcrp<sup>1/-</sup>* mice with a B/P ratio of < 0.02. It should be noted that the brain concentrations were not corrected for the vascular content as the total brain distribution of vemurafenib was approximately equal to the vascular volume indicating the very limited brain distribution of vemurafenib. However, the B/P ratio of vemurafenib was significantly (p <0.0001) higher in *Mdr1a/b<sup>1/-</sup>*Bcrp<sup>1/-</sup> mice [Fig. 6A and 6B, B/P ratio at 1hr: 0.090 ± 0.036; at 4hr: 0.36 ± 0.07] than the wild type and single knockout mice at both 1 and 4 hr post dose. Importantly, the B/P ratio of *Mdr1a/b<sup>1/-</sup>*Bcrp<sup>1/-</sup> mice was 8-fold higher than wild type at 1 hr and 30-fold higher than wild type at 4hrs. These data show the important roles of both P-gp and Bcrp in restricting the delivery of vemurafenib across the BBB.

**Steady state brain distribution of vemurafenib:** The steady state brain distribution of vemurafenib was studied after a constant intraperitoneal infusion using the Alzet...
osmotic pumps for 48 hrs. As shown in Fig. 7A, the steady state brain concentrations of vemurafenib were significantly lower in wild type, Mdr1a/b\(^{-/-}\), and Bcrp1\(^{-/-}\) mice compared to their respective steady state plasma concentrations. The steady state B/P plasma ratio of vemurafenib in Mdr1a/b\(^{-/-}\)Bcrp1\(^{-/-}\) mice equaled approximately 1 (Fig. 7B), which is 80 fold (p< 0.0001) greater than the wild type and single knockout mice. [Wild type: 0.012 ± 0.004; Mdr1a/b\(^{-/-}\): 0.035 ± 0.009; Bcrp1\(^{-/-}\): 0.009 ± 0.006; Mdr1a/b\(^{-/-}\)Bcrp1\(^{-/-}\): 1.00 ± 0.19].
Discussion:

The development of vemurafenib, a potent BRAF\(^{V600E}\) inhibitor, yields new hope for the melanoma patients who harbor this mutational status. However, the durable efficacy of vemurafenib depends on overcoming resistance (Wagle et al., 2011) and ensuring adequate delivery to all sites of metastases in melanoma patients, particularly the brain. Given the remarkable success in the early clinical trials (Chapman et al., 2011; Ribas et al., 2011), it is of particular interest to examine mechanisms that may limit the CNS distribution of vemurafenib to support further clinical trials. In the current study, we have evaluated how BBB efflux transporters influence the CNS distribution of vemurafenib using both \textit{in vitro} cell culture models and \textit{in vivo} using genetic knockout mice. We demonstrate that vemurafenib is a substrate for the ABC transporters P-gp and BCRP, and that active efflux by these two transporters at the BBB severely restricts the CNS distribution of vemurafenib.

Studies performed \textit{in vitro} using MDCKII cells that overexpress human P-gp or murine BCRP revealed that vemurafenib is an avid substrate for the two efflux transporters. Using prototypical probe substrates (prazosin for BCRP and vinblastine for P-gp), we have seen a concentration-dependent increase in cellular accumulation of the probe with increasing concentrations of vemurafenib. We have examined the vectorial transport of vemurafenib across monolayers formed from MDCKII wild-type, MDCKII-MDR1 transfected cells and MDCKII-Bcrp1 transfected cells. The corrected flux ratio for vemurafenib in the MDR1 transfected line was ~34, and that ratio in the BCRP transfected line was 150, indicating that vemurafenib subject to significant efflux by each transporter across the monolayers. The selective P-gp and BCRP inhibitors
zosuquidar and Ko143 were able to restore the intracellular accumulation and bidirectional net flux of vemurafenib. These in vitro results conclusively show that vemurafenib is a substrate for these two efflux transporters.

In vivo studies using FVB wild-type mice demonstrated that the CNS distribution of vemurafenib is significantly restricted across the blood-brain barrier. The brain concentrations of vemurafenib in FVB wild-type mice are significantly (~3 log units; Fig. 5) lower than the plasma concentrations. However, the brain concentrations were approximately ~8-30 fold higher in Mdr1a/b–/–Bcrp1–/– mice than in the wild-type mice (Fig. 7). Steady state brain to plasma ratios increased from approximately 0.01 in the wild-type mice to approximately 1 in the triple knockout mice. This remarkable 80-fold increase in targeted brain distribution of vemurafenib in Mdr1a/b–/–Bcrp1–/– mice indicates the significant impact of P-gp and BCRP on CNS penetration of vemurafenib. It should be noted that the brain distribution of vemurafenib did not increase in Bcrp1–/– mice, it is increased by ~3-fold in Mdr1a/b–/– mice, indicating the “cooperative” role of these transporters at BBB. This type of “synergistic” effect of P-gp and BCRP was seen with other drugs; such as topotecan (de Vries et al., 2007), lapatinib (Polli et al., 2009), dasatinib (Chen et al., 2009), gefitinib (Agarwal et al., 2010) and sorafenib (Agarwal et al., 2011). One of the assumptions for this disproportional increase in brain distribution of the compounds is that the compensatory up- or down-regulation of active efflux and influx transporters in the single knockout (mice lacking either P-gp or BCRP) mice. However, in our previous study, using a quantitative proteomics approach, we have shown no compensatory changes in the BBB expression of relevant transporters in the
single and combined knockout mice (Agarwal et al., 2012). The exact functional compensation between P-gp and BCRP at the BBB needs further investigation.

Although vemurafenib shows high initial response rates in melanoma patients with BRAF<sup>V600E</sup> mutation, and has been shown to yield a durable response in a melanoma brain metastasis in one recent case study (Rochet, 2011), the development of resistance can occur quickly (Wagle et al., 2011). At the present time, several resistance mechanisms against BRAF inhibitors have been documented, some of which include the upregulation of NRAS, PDGFR, and IGFR-1/PI3K signaling (Johannessen et al., 2010; Nazarian et al., 2010; Villanueva et al., 2010). Therefore, the development of more effective combination therapies has been suggested (Vultur et al., 2011; Ji et al., 2012). In this context, the interaction of drugs such as vemurafenib with ABC transporters could be of great relevance for the rational design of therapeutic strategies in clinical setting. In the current study, we have shown that the brain distribution of vemurafenib is severely restricted at the blood-brain barrier due to active efflux by both P-gp and BCRP. This finding is clinically significant considering the ongoing trials on the efficacy of vemurafenib in brain metastases of melanoma.

Given the remarkable success thus far with vemurafenib, it will be crucial to find both the drug resistance and drug delivery liabilities to further improve progression free survival rates through rational drug development and design. In this particular case, the lack of treatment options and the aggressive course of this malignancy suggest that
adjuvant treatment to improve delivery to the CNS through efflux inhibition may be a viable option to improve survival.


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Authorship Contributions

Participated in research design: Mittapalli, Vaidhyanathan, Sane, Elmquist

Conducted experiments: Mittapalli, Vaidhyanathan, Sane

Performed data analysis: Mittapalli, Elmquist

Wrote or contributed to writing of the manuscript: Mittapalli, Vaidhyanathan, Elmquist
REFERENCES:


Footnotes

Rajendar K Mittapalli and Shruthi Vaidhyanathan contributed equally to this work.

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Reprint Requests

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Figure legends:

**Figure 1:** Intracellular accumulation of vemurafenib in MDCKII cells. Panel A shows that accumulation of vemurafenib is significantly lower in Bcrp transfected lines compared to its WT control. The difference in accumulation was abolished when a specific Bcrp inhibitor Ko143 was used. Panel B shows that the accumulation of vemurafenib is ~20% less in MDR1 transfected cells than the WT controls and difference was abolished when MDR1 specific inhibitor LY335979 was used. Data represent mean ± SD, n=3-6 for all data sets.

**Figure 2:** Competition assays for vemurafenib in MDCKII-MDR1 and MDCKII-Bcrp1 cells using [³H]-vinblastine and [³H]-prazosin as P-gp and Bcrp prototypical probe substrates respectively. The addition of increasing concentrations of vemurafenib resulted in an increased accumulation of [³H]-prazosin and [³H]-vinblastine in Bcrp1 (Panel A) and MDR1 (Panel B) cells respectively. The data represent mean ± SD, n = 3 for all data sets. A *, **, and *** indicates a p value of <0.05, <0.01, and <0.0001, respectively.

**Figure 3:** Directional flux of vemurafenib in MDCKII cell monolayers. Panel A and B show the transport of vemurafenib in wild-type and Bcrp1-transfected cells. Panel C shows the apparent permeability of vemurafenib in wild-type and Bcrp1 cells in both A-to-B and B-to-A direction. In the Bcrp1 transfected cells, the B-to-A permeability of vemurafenib was significantly greater than the A-to-B permeability (***, p < 0.05). The addition of 0.2 µM Ko143, a potent bcrp inhibitor, decreased this directionality in flux due to bcrp (#, p < 0.05). Panel D and E show the transport of vemurafenib in wild-type...
and MDR1-transfected cells. Panel F shows the apparent permeability of vemurafenib in wild-type and MDR1 cells in both A-to-B and B-to-A direction. In the MDR1 transfected cells, the B-to-A permeability of vemurafenib was significantly greater than the A-to-B permeability (***, p < 0.05). The addition of 1 µM LY335979, a potent P-gp inhibitor, abolished this directionality in flux due to P-gp (#, p < 0.05), such that there was no significant difference between in the permeability of vemurafenib in both directions. Data represent mean ± SE, n = 3-9 for all data sets.

**Figure 4:** Brain and plasma concentrations of vemurafenib after an i.v. dose of 2.5mg/kg in FVB wild type mice. Whole brain and plasma were collected at 5, 30, 90, 180, 300, and 480 minutes after dose and were analyzed using LC-MS/MS. The brain concentrations of vemurafenib were significantly lower than the plasma concentrations at all the time points. A *, **, and *** indicates a p value of <0.05, <0.01, and <0.0001 respectively. Data are mean ± SD; n=3-4 for all data points.

**Figure 5:** Comparison of vemurafenib brain distribution in wild-type and Mdr1a/b<sup>-/-</sup> Bcrp1<sup>-/-</sup> mice. Vemurafenib was given i.v. at 2.5mg/kg and the concentrations in brain and plasma were determined using LC-MS/MS. Panel A and Panel B shows the plasma and brain concentrations of vemurafenib in wild-type and Mdr1a/b<sup>-/-</sup> Bcrp1<sup>-/-</sup> mice respectively. The B/P ratio for vemurafenib in wild-type and Mdr1a/b<sup>-/-</sup> Bcrp1<sup>-/-</sup> mice was shown in Panel C. Data represent mean ± SD; n=3-4 for all data points.

**Figure 6:** Brain to plasma ratios of vemurafenib after an oral dose of 25mg/kg in FVB wild type, Mdr1a/b<sup>-/-</sup>, Bcrp1<sup>-/-</sup>, and Mdr1a/b<sup>-/-</sup> Bcrp1<sup>-/-</sup> mice. The mice were sacrificed after 1 (A) and 4hr (B) post dose of vemurafenib and the whole brain and plasma were...
analyzed using LC-MS/MS. The B/P ratios were significantly higher in \textit{Mdr1a/b}^{-/-} \textit{Bcrp1}^{-/-} mice than the wild type mice at both 1 and 4hr post dose. A *** indicates a p value of <0.0001. Data are mean ± SD; n=3-4 for all data points.

\textbf{Figure 7}: Steady state brain distribution of vemurafenib in FVB wild type, \textit{Mdr1a/b}^{-/-} (P-gp knockout), \textit{Bcrp1}^{-/-} (BCRP knockout), and \textit{Mdr1a/b}^{-/-} \textit{Bcrp1}^{-/-} (triple knockout) mice. Vemurafenib was delivered at a constant infusion for 48 hrs at a rate of 25µg/hr, and the brain and plasma concentrations were determined thereafter. Panel A shows the steady state brain and plasma concentrations of vemurafenib, and panel B shows the B/P ratio in all four type mice. The B/P ratios were not significantly different in single knockout mice when compared to wild type mice. However the B/P ratio in triple knockout mice was significantly higher than the wild type mice, indicating the “cooperative” role of P-gp and Bcrp at the blood-brain barrier. A *, and *** indicates a p value of <0.01 and <0.0001 respectively. Data are mean ± SD; n=3-4 for all data points.
Table 1:

Vemurafenib Pharmacokinetic Parameters after an I.V. dose of 2.5 mg/kg in FVB wild-type mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Terminal rate constant (min⁻¹)</td>
<td>0.0051</td>
<td>0.0047</td>
</tr>
<tr>
<td>Half-life (min)</td>
<td>136</td>
<td>148</td>
</tr>
<tr>
<td>Clearance (mL/min)</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>Volume of Distribution (mL)</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>$\text{AUC}<em>{0 \rightarrow t</em>{\text{last}}} \cdot \mu g/mL$</td>
<td>1663 ± 140*</td>
<td>6.5 ± 0.9*</td>
</tr>
<tr>
<td>$\frac{\text{AUC}<em>{\text{Brain}}}{\text{AUC}</em>{\text{Plasma}}}$</td>
<td>0.004</td>
<td></td>
</tr>
</tbody>
</table>

Plasma and brain pharmacokinetic parameters were analyzed using non compartmental analysis after an i.v. dose of 2.5 mg/kg in FVB wild-type mice. The $\frac{\text{AUC}_{\text{Brain}}}{\text{AUC}_{\text{Plasma}}}$ ratio of 0.004 indicates the severely restricted brain distribution of vemurafenib.

* Mean ± SE (standard error of the estimate)
Figure # 4

Vemurafenib Concentration (ng/gm; ng/mL) vs Time (min)

- **Brain**
- **Plasma**

Time (min):
0 100 200 300 400 500

Concentration (log scale):
1 10 100 1000 10000 100000

Legend:
- **Brain**: Circular symbols
- **Plasma**: Dot symbols

Significance Levels:
- *: p < 0.05
- **: p < 0.01
- ***: p < 0.001
**Figure # 7**

**A**

Steady state Concentrations of Vemurafenib (ng/mL or ng/gm)

- Wild Type
- Mdr1a/1b⁻/⁻
- Bcrp1⁻/⁻
- Mdr1a/1b⁻/⁻ Bcrp1⁻/⁻

**B**

Steady state Brain to Plasma Ratio of Vemurafenib (mL/g)

- Wild-type
- Mdr1a/1b⁻/⁻
- Bcrp1⁻/⁻
- Mdr1a/1b⁻/⁻ Bcrp1⁻/⁻