Brain Distribution and Active Efflux of Three panRAF Inhibitors: Considerations in the Treatment of Melanoma Brain Metastases

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ABSTRACT

Targeted inhibition of RAF and MEK by molecularly targeted agents has been employed as a strategy to block aberrant mitogen-activated protein kinase (MAPK) signaling in melanoma. While the use of BRAF and MEK inhibitors, either as a single agent or in combination, improved efficacy in BRAF-mutant melanoma, initial responses are often followed by relapse due to acquired resistance. Moreover, some BRAF inhibitors are associated with paradoxical activation of the MAPK pathway, causing the development of secondary malignancies. The use of panRAF inhibitors, i.e., those that target all isoforms of RAF, may overcome paradoxical activation and resistance. The purpose of this study was to perform a quantitative assessment and evaluation of the influence of efflux mechanisms at the blood-brain barrier (BBB), in particular, Abcb1/P-glycoprotein (P-gp) and Abcg2/breast cancer resistance protein (Bcrp), on the brain distribution of three panRAF inhibitors: CCT196969 [1-(3-(tert-butyl)-1-phenyl-1H-pyrazol-5-yl)-3-(2-fluoro-4-(3-oxo-3,4-dihydropyrido[2,3-b]pyrazin-8-yl)oxy)phenyl]urea], LY3009120 1-(3,3-Dimethylbutyl)-3-(2-fluoro-4-methyl-5-(7-methyl-2-(methylamino)pyrido[2,3-d]pyrimidin-6-yl)phenyl]urea], and MLN2480 [4-pyriminedicarboxamide, 6-amino-5-chloro-N-[(1R)-1-[5-[[5-chloro-4-(trifluoromethyl)-2-pyridinyl]amino]carbonyl]-2-thiazolyl]ethyl]-]. In vitro studies using transfected Madin-Darby canine kidney II cells indicate that only LY3009120 and MLN2480 are substrates of Bcrp, and none of the three inhibitors are substrates of P-gp. The three panRAF inhibitors show high nonspecific binding in brain and plasma. In vivo studies in mice show that the brain distribution of CCT196969, LY3009120, and MLN2480 is limited, and is enhanced in transgenic mice lacking P-gp and Bcrp. While MLN2480 has a higher brain distribution, LY3009120 exhibits superior in vitro efficacy in patient-derived melanoma cell lines. The delivery of a drug to the site of action residing behind a functionally intact BBB, along with drug potency against the target, collectively play a critical role in determining in vivo efficacy outcomes.

Introduction

Deregulated signaling of the mitogen-activated protein kinase (MAPK) pathway is commonly associated with melanomas, and often occurs due to activating mutations in BRAF (~50%) and NRAS (~20%) (Davies et al., 2002; Hocker and Tsao, 2007; Hodis et al., 2012). Inhibition of MAPK signaling by targeting RAF and MEK has been recognized as an important treatment strategy in BRAF-mutant melanomas (Samatar and Poulikakos, 2014). The improved clinical outcomes with BRAF and MEK inhibitors, either as single agents or in combination, has led to the Food and Drug Administration approval of BRAF inhibitors, vemurafenib, dabrafenib, and encorafenib, and MEK inhibitors, cobimetinib, trametinib, and binimetinib (Chapman et al., 2011; Flaherty et al., 2012a,b; Ascierto et al., 2013; Larkin et al., 2014; Long et al., 2014; Delord et al., 2017; Dummer et al., 2017, 2018).

A persistent clinical challenge in the management of BRAF-mutant melanomas is resistance to BRAF inhibitor therapy, both intrinsic and acquired (Samatar and Poulikakos, 2014; Shi et al., 2014). One approach to overcoming resistance is combination therapy with BRAF and MEK inhibitors, causing a vertical blockade of the MAPK pathway at two centralnodes. Although such combinations have improved clinical responses, patients still progress due to therapeutic resistance (Wagle et al., 2014; Welsh et al., 2016). Multiple mechanisms of acquired resistance have been identified,
and reactivation of the MAPK pathway by acquisition of secondary mutations, such as mutations in RAS, is a common resistance mechanism (Shi et al., 2014; Johnson et al., 2015; Welsh et al., 2016).

In addition, BRAF inhibitors have been attributed to cause a paradoxical activation of MAPK signaling in RAS-mutant melanomas, triggering the development of secondary malignancies (Hatzivassiliou et al., 2010; Heidorn et al., 2010; Poulikakos et al., 2010). By occupying one partner in homodimers or heterodimers of RAF, particularly in wild-type (WT) BRAF and RAS-mutant melanomas, many BRAF inhibitors promote transactivation of the drug-free partner, leading to paradoxical activation of the MAPK pathway (Poulikakos et al., 2010; Samatar and Poulikakos, 2014). Consequently, BRAF inhibitors are contraindicated in wild-type BRAF melanomas (Hatzivassiliou et al., 2010). Inhibitors targeting all isofoms of RAF, i.e., panRAF inhibitors, may show benefits by occupying and inhibiting both partners in RAF dimers and causing a more effective blockade of the MAPK signaling. Given this multiple isoform blockade, panRAF inhibitors may be useful in first-line treatment of BRAF- and NRAS-mutant melanomas, and also as second-line treatment options in drug-resistant melanomas (Girotti et al., 2015). Furthermore, the panRAF/MEK inhibitor combination may be a rational treatment strategy, especially in patients with acquired or intrinsic resistance to MAPK inhibitors (Atefi et al., 2015; Whittaker et al., 2015).

Another challenge is the effective management of melanoma patients with brain metastases. Approximately 70% of patients with metastatic melanoma will develop brain metastases, and after diagnosis of brain metastases the median overall survival is less than 8 months (Gupta et al., 1997; Barnholtz-Sloan et al., 2004; Spagnolo et al., 2016). Several anticancer therapies have a restricted ability to penetrate an intact blood-brain barrier (BBB), leading to the establishment of a pharmacological sanctuary for tumor cells, thereby limiting their efficacy in the treatment of tumors in the brain (Gampa et al., 2016, 2017; Kim et al., 2018). A key mechanism responsible for restricting drug delivery to the brain is active efflux at the BBB, often mediated by P-glycoprotein (P-gp), Abcb1/P-gp and breast cancer resistance protein (Bcrp), Abcg2/Bcrp (Gampa et al., 2016, 2017; Kim et al., 2018). Previous studies have indicated that vemurafenib, dabrafenib, encorafenib, trametinib, cobimetinib, and binimetinib will have limited brain distribution due to efflux by P-gp and/or Bcrp (Mittapalli et al., 2012, 2013; Choo et al., 2014; Vaidhyathanathan et al., 2014; de Gooijer et al., 2018; Wang et al., 2018). The drug delivery to brain tumors with heterogeneous disruption of the BBB can be highly variable for compounds that show a limited BBB penetration. While the BBB in the core of larger tumors may be relatively compromised, there can be regions within larger tumors, micro-metastases, and infiltrative tumor sites with a functionally intact BBB, and thus have restricted drug distribution (Lockman et al., 2010; Osswald et al., 2016). Given this, developing potent targeted therapies capable of permeating an intact BBB is critical to improving clinical outcomes in patients with melanoma brain metastases (MBM).

CCT196969 [1-(3-(tert-butyl)-1-phenyl-1H-pyrazol-5-yl)-3-(2-fluoro-4-((3-oxo-3,4-dihydroprydino[2,3-b|pyrazin-8-yl]oxy)phenyl)urea], LY3009120 1-(3,3-Dimethylbutyl)-3-(2-fluro-4-methyl-5-(7-methyl-2-(methylamino)pyrido[2,3-d]pyrimidin-6-yl)phenyl)urea, and MLN2480 [4-pyrimidinecarboxamide, 6-amino-5-chloro-N-[(1R)-1-[5-[[[5-chloro-4-(trifluoromethyl)-2-pyridinyl]amino][carbonyl]-2-thiazolyl]ethyl]-] (Fig. 1; Table 1) are panRAF inhibitors that inhibit MAPK signaling with minimal paradoxical activation (Rasco et al., 2013; Girotti et al., 2015; Henry et al., 2015). CCT196969 has low nanomolar IC50 against BRAF and CRAF, and also is a potent inhibitor of SRC (Fig. 2). CCT196969 shows efficacy in melanoma cells and patient-derived xenografts that are resistant to BRAF inhibitors and BRAF/MEK inhibitor combinations (Girotti et al., 2015). LY3009120 exhibits low nanomolar potency against ARAF, BRAF, and CRAF, and shows activity in preclinical melanoma models (Fig. 2) (Henry et al., 2015; Peng et al., 2015; Chen et al., 2016). MLN2480 has a low nanomolar IC50 against BRAF and CRAF, and shows activity in BRAF-mutant tumors, including melanoma, in preclinical models (Elenbaas et al., 2010; Sun et al., 2017). LY3009120 and MLN2480 are currently in phase I and II clinical testing (https://clinicaltrials.gov).

The objective of this study is to evaluate the brain distribution of CCT196969, LY3009120, and MLN2480, and examine the role of P-gp and/or Bcrp in limiting their brain delivery. Anti-RAF therapy is routinely used in combination with MEK inhibitors for the management of melanoma patients. The newer panRAF inhibitors have advantages over the traditional BRAF inhibitors, and as such panRAF/MEK inhibitor combinations may result in potential benefits in melanoma patients. However, to be effective in brain metastases, drug delivery to target sites in the brain is a key consideration. Given this, evaluation of the distribution of panRAF inhibitors to the brain is important to understand their utility in the treatment of MBM.

**Materials and Methods**

**Chemicals**

CCT196969, vemurafenib, and dabrafenib were purchased from ChemieTek (Indianapolis, IN). LY3009120 was purchased from

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**Fig. 1.** Chemical structures of (A) CCT196969, (B) LY3009120, and (C) MLN2480.
TABLE 1
Physico-chemical properties of panRAF inhibitors
The reported properties were calculated using ChemAxon (http://www.chemicalize.com).

<table>
<thead>
<tr>
<th>Molecular formula</th>
<th>CCT196969</th>
<th>LY3009120</th>
<th>MLN2480</th>
</tr>
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<tbody>
<tr>
<td>Molecular weight (g/mol)</td>
<td>513.53</td>
<td>424.52</td>
<td>506.29</td>
</tr>
<tr>
<td>Solubility (mg/ml)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Log P</td>
<td>5.5</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>Log D (pH 7.4)</td>
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<td>4.2</td>
</tr>
<tr>
<td>pKa</td>
<td>10.36</td>
<td>11.61</td>
<td>10.29</td>
</tr>
<tr>
<td>Topological polar surface area (Å²)</td>
<td>123</td>
<td>92</td>
<td>136</td>
</tr>
<tr>
<td>Hydrogen bond donor count</td>
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<tr>
<td>Rotatable bond count</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
</tbody>
</table>

*The values represent the strongest acidic pKa value reported by ChemAxon.

In Vitro Accumulation Studies
Polarized Madin-Darby canine kidney II (MDCKII) cells were used for in vitro cell accumulation studies. MDCKII-WT (vector control) and human P-glycoprotein (MDR1)-transfected (MDCKII-MDR1) cell lines were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute). MDCKII-WT (vector control) and human P-glycoprotein (MDR1)-transfected (MDCKII-MDR1) cell lines were kindly provided by Dr. Alfred Schinkel (The Netherlands Cancer Institute). MDCKII-WT and Bcrp1-transfected (MDCKII-Bcrp1) cell lines 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% fetal bovine serum, and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml). Cells were grown in 25 ml tissue culture flasks before seeding for the intracellular accumulation experiments, and were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

The intracellular accumulation assays were performed in 12-well polystyrene plates (Corning Glassworks, Corning, NY). In brief, cells were seeded at a density of 2 x 10⁵ cells and grown until ~80% confluent. On the day of experiment, culture media were aspirated and cells were washed two times with warm 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). The cells were then preincubated with cell assay buffer for 30 minutes, after which the buffer was aspirated and the experiment was initiated by adding 1 ml assay buffer containing 2 μM drug into each well, with further incubation for 60 minutes in an orbital shaker (ShelLab, Cornelius, OR) maintained at 37°C and 60 rpm. The drug solution was aspirated after 60-minute incubation, followed by washing twice with ice-cold PBS, and addition of 500 μl of 1% Triton-X100 to each well for cell lysis. When an inhibitor was used, it was included in both preincubation and accumulation steps. The concentration of the drug of interest in solubilized cell fractions was analyzed using liquid chromatography–tandem mass spectrometry (LC-MS/MS), as described subsequently, and normalized to protein content that was analyzed using a BCA protein assay (Thermo Scientific Pierce).

In VitroBinding Assays for Determination of Unbound (Free) Fractions
The unbound fractions of the panRAF inhibitors in plasma, brain, and serum-containing cell culture media (10% fetal bovine serum, w/v) were determined by performing rapid equilibrium dialysis (RED) experiments as per the protocol described by the manufacturer, with some modifications suggested in the literature (Kalvass and Maurer, 2002; Fridén et al., 2007). Preliminary experiments conducted at two concentrations (5 and 10 μM) and two time points (4 and 6 hours) suggested that the unbound fractions were linear across these concentrations and equilibrium was achieved for 4 hours for each of the three inhibitors. Consequently, unbound fractions determined at the 5 μM concentration and 4-hour time point were used for estimation of unbound (free) brain-to-plasma ratio/unbound partition coefficient (Kpu,unbound) and free concentrations. A RED base plate (Thermo Fisher Scientific, Waltham, MA), and single-use RED inserts (Thermo Fisher Scientific) with 8 kDa molecular weight cutoff were used for these experiments. Briefly, fresh plasma and brain homogenates (prepared in three volumes of PBS, w/v) isolated from WT Friend leukemia virus strain B (FVB) mice, and cell culture media were used. The drug stock in DMSO (1 mg/ml) was spiked in each matrix to obtain final concentrations of 5 μM. Then, 300 μl of 5 μM drug spiked matrix was placed in the sample chamber (donor), and 500 μl of 1x PBS (at pH 7.4; 100 mM sodium phosphate and 150 mM sodium chloride) was placed in the receiver chamber (acceptor). The unbound fractions of the panRAF inhibitors in plasma, brain, and serum-containing cell culture media were determined by performing rapid equilibrium dialysis (RED) experiments as per the protocol described by the manufacturer, with some modifications suggested in the literature (Kalvass and Maurer, 2002; Fridén et al., 2007). Preliminary experiments conducted at two concentrations (5 and 10 μM) and two time points (4 and 6 hours).
placed in the buffer chamber (receiver) of the RED inserts (in triplicate). The inserts were placed in a base plate, and the assembly was covered with sealing tape and incubated on an orbital shaker (ShellLab) at 37°C and 300 rpm for 4 hours. The samples were collected after dialysis and stored at −80°C until subsequent LC-MS/MS analysis.

In Vitro Cytotoxicity Assays in Patient-Derived Melanoma Cell Lines

Short-term cultured human primary melanoma cells (BRAF-mutant M12, BRAF-mutant M27, or NRAS-mutant M15) were maintained through serial passages in mice via subcutaneous flank implantation in immune-deficient mice (Carlson et al., 2011). Dulbecco’s modified Eagle’s medium, supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml), was used to grow the explant cultures that were maintained at 37°C in a humidified incubator with 5% carbon dioxide.

For the determination of in vitro drug potency, melanoma cells (M12, M27, or M15) were seeded into 96-well, black clear-bottom plates (Corning Incorporated, Corning, NY) at a density of 3500 cells per well in 100 μl of culture media. At 24 hours following plating (~80% confluency), the cells were treated with nine concentrations of drug in media (n = 6 per concentration). The plates were incubated for 5 days after treatment. The cell viability in each well was determined by a CyQuant cell proliferation assay (Invitrogen) and fluorescence measurement using a BioTek Synergy HT plate reader. The relative survival of cells in the presence of drugs was normalized to the untreated controls.

In Vivo Studies

Animals. FVB WT and triple knockout (Mdr1a/b−/− Bcrp1−/−, P-gp, and Bcrp deficient) mice, balanced for sex, were used in the in vivo studies (Taconic Farms, Germantown, NY). All mice used were 8–16-week-old adults, approximately 15–35 g at the time of experiments. Mice were maintained in a 12-hour light/dark cycle with unlimited access to food and water. All studies carried out were in agreement with the guidelines set by Principles of Laboratory Animal Care (National Institutes of Health, Bethesda, MD), and approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Plasma and Brain Pharmacokinetics Following Intravenous and Oral Administration of panRAF Inhibitors. All dosing solutions were prepared on the day of the experiment. A single intravenous dose of 5 mg/kg CCT196969 (vehicle: dimethyl sulfoxide, Tween 80, and distilled water in a volume ratio of 10:2.5:87.5) was administered to FVB WT mice. Blood and brain samples were harvested at 0.17, 0.5, 1, 2, 4, 8, and 14 hours postdose (n = 4 at each time point). In another oral dosing study, FVB WT mice received a single dose of 10 mg/kg CCT196969 via oral gavage (vehicle: 5% dimethyl sulfoxide; percentage of grams per volume). Brain and blood samples were collected at 0.17, 0.5, 1, 2, 4, 6, 8, 11, and 14 hours postdose (n = 4 per time point).

Brain distribution studies following single intravenous and oral doses of LY3009120 were also conducted. A single intravenous dose of 5 mg/kg LY3009120 (vehicle: dimethyl sulfoxide, propylene glycol, Cremophor EL, and distilled water in a volume ratio of 30:20:10:40) was administered to FVB WT mice. Blood and brain samples were harvested at 0.17, 0.5, 1, 2, 4, 7, and 11 hours postdose (n = 4 at each time point). In the oral dosing study, FVB WT mice received a single dose of 25 mg/kg LY3009120 via oral gavage (vehicle: 20% hydroxyl propyl β cyclodextrin; percentage of grams per volume). Brain and blood samples were collected at 0.17, 0.5, 1, 2, 4, 8, and 15 hours postdose (n = 4 per time point).

The brain distribution of MLN2480 was determined following administration of a single intravenous dose of 5 mg/kg MLN2480 (vehicle: dimethyl sulfoxide, Tween 80, and distilled water in a volume ratio of 10:2.5:87.5) to FVB WT mice. Blood and brain samples were harvested at 0.17, 0.5, 1, 2, 4, 8, and 14 hours postdose (n = 4 at each time point).

In another vivo brain distribution study, FVB WT and Mdr1a/b−/− Bcrp1−/− mice were administered a single oral dose of CCT196969 (5 mg/kg), LY3009120 (10 mg/kg), or MLN2480 (10 mg/kg). Blood and brain samples were harvested 1 hour postdose (n = 4).

A serial sacrifice (destructive sampling) design was employed for sampling in the pharmacokinetic studies. At the desired sampling time point, mice were euthanized using a carbon dioxide chamber. Blood was collected by cardiac puncture and transferred to heparinized tubes. The whole brain was removed from the skull and rinsed with ice-cold distilled water, and superficial meninges were removed by blotting with tissue paper. Plasma was separated by centrifugation of whole blood samples at 3500 rpm and 4°C for 15 minutes. Both plasma and brain samples were stored at −80°C, until analysis for drug concentrations by LC-MS/MS. The concentrations in brain were corrected for residual drug in brain vasculature, assuming a vascular volume of 1.4% in mouse brain (Dai et al., 2003).

Steady-State Brain Distribution of panRAF Inhibitors

The determination of steady-state plasma and brain concentrations of panRAF inhibitors was accomplished by implanting Alzet osmotic minipumps (model 1003D; Durect Corporation, Cupertino, CA) loaded with CCT196969 (5 mg/ml in dimethyl sulfoxide), LY3009120 (3 mg/ml in dimethyl sulfoxide), or MLN2480 (5 mg/ml in dimethyl sulfoxide) in the peritoneal cavity of WT and Mdr1a/b−/− Bcrp1−/− mice to deliver 1 μl/h drug solution as a constant infusion. The minipumps were loaded with drug solution on the day before the experiment and primed overnight in sterile PBS at 37°C. The pumps were implanted into the peritoneal cavity as described previously (Agarwal et al., 2013). Briefly, mice were anesthetized using isoflurane, and the hair on the abdominal cavity was removed. A small incision was made in the skin on the lower right abdomen, followed by an incision in the exposed peritoneal membrane under the cutaneous opening, and the primed pump was inserted into the peritoneal cavity. The peritoneal membrane was sutured with absorbable sutures, and the opening in the skin was sealed with surgical clips. The whole procedure was performed on a heating pad until the animals fully recovered from the anesthesia. Forty-eight hours following pump implantation, the mice were sacrificed, and blood and brain samples were collected. Plasma was obtained by centrifugation of whole blood samples at 3500 rpm and 4°C for 15 minutes. Samples were stored at −80°C until analysis by LC-MS/MS.

LC-MS/MS Analysis

The concentrations of drugs in all samples from in vitro and in vivo studies were determined using specific and sensitive LC-MS/MS assays. Brain samples were homogenized using a mechanical homogenizer (PowerGen 125; Thermo Fisher Scientific) following the addition of three volumes of 5% bovine serum albumin to obtain uniform homogenates. For analysis of unknowns, an aliquot of sample was spiked with 250 ng of MLN2480, 50 ng of dabrafenib, or 10 ng of CCT196969 as internal standards for analysis of CCT196969, LY3009120, and MLN2480, respectively. Liquid-liquid extraction was performed by addition of 5–10 volumes of ethyl acetate, followed by vigorous shaking for 5 minutes and centrifugation at 7500 rpm and 4°C for 5 minutes. The organic layer was separated, transferred to microcentrifuge tubes, and dried under nitrogen gas. The dried residue was reconstituted in 100 μl of mobile phase and transferred into high-performance liquid-chromatography glass vials with disposable microliter. The chromatographic analysis was performed on an AQUITY UPLC system (Waters, Milford, MA) using a Phenomenex Gemini 3 μm NX-C18 110A column (50 mm length × 4.6 mm i.d.; Torrance, CA). The mobile phase (0.1% formic acid in water (A) and 0.1% formic acid in methanol (B)) was delivered at a constant flow rate.
of 0.35 mL/min. An isocratic method (43% A and 57% B) was employed for C5719696 and MLN2480 analysis and a gradient method was employed for LY3009120 analysis. The gradient for LY3009120 was as follows: started with 50% B at 0 minutes, held at 50% B for 2 minutes, increased to 90% B over 1 minute, maintained at 90% B for 1 minute, decreased to 50% B over 0.25 minutes, and maintained at 50% B for the remainder of 7 minutes. The column effluent was monitored using a Micromass Quattro Ultima mass spectrometer (Waters). The instrument was equipped with an electrospray interface, and controlled by the MassLynx (version 4.1; Waters) data system. The samples were analyzed using an electrospray probe in negative-ionization mode operating at a spray voltage of 4.5 kV for C5719696 and MLN2480 and positive-ionization mode operating at a spray voltage of 5 kV for LY3009120 and dabrafenib. Samples were introduced into the interface through a heated probe, in which the source temperature and desolvation temperature were set at 100 and 350°C, respectively. The mass-to-charge (m/z) transitions were 511.98–270.85, 425.08–324.00, 505.90–284.23, and 519.88–306.91 for C5719696, LY3009120, MLN2480, and dabrafenib, respectively. The retention times for C5719696, LY3009120, MLN2480, and dabrafenib were 3.23, 3.59, 3.10, and 4.65 minutes, respectively. The runtimes were 5.5 minutes for C5719696 and MLN2480 and 7 minutes for LY3009120. Three quality controls, representing low, medium, and high concentration ranges in the calibration curve, were used to determine the between-run (interday) precision and accuracy for each of the three compounds. Precision was expressed as the CV percentage, while accuracy is expressed as the percentage of bias. For the LC-MS/MS assays of C5719696, LY3009120, and MLN2480, the percentages of bias were less than 7%, 11%, and 6%, and the CV percentages were less than 12%, 15%, and 7%, respectively.

Calculations

The unbound (free) fractions ($f_u$) in plasma, brain homogenate, and serum-containing cell culture media were calculated as the ratio of concentrations of the compound under investigation in buffer to matrix (Kalvass and Maurer, 2002)

$$f_u,\text{diluted} = \frac{\text{Drug concentration in buffer (receiver)}}{\text{Drug concentration in matrix (donor)}}$$

(1)

The unbound fraction in brain was determined from the measured unbound fraction in diluted brain homogenate ($f_u,\text{diluted}$), using the following equation (Kalvass and Maurer, 2002)

$$f_u,\text{brain} = \frac{1/D}{(1/f_u,\text{diluted} - 1) + 1/D}$$

(2)

where D represents the dilution factor accounting for the diluted brain homogenate, which is equal to 4.

The brain-to-plasma ratio ($K_p$) was calculated as the area under the curve (AUC) ratio of AUCbrain to AUCplasma

$$K_p = \frac{\text{AUC}_{\text{brain}}}{\text{AUC}_{\text{plasma}}}$$

(3)

A comparison of relative drug exposure in the brains of WT and knockout (Mdr1a/b−/−/Bcrp1−/−) mice was made using the distribution advantage (DA), which was calculated as the $K_p$ value in the strain under consideration normalized by the $K_p$ value in WT mice

$$\text{DA} = \frac{K_p,\text{knockout}}{K_p,\text{wild type}}$$

(4)

The unbound partition coefficient, referred to as $K_{p,uu}$, was determined using the following equation:

$$K_{p,uu} = \frac{\text{AUC}_{\text{brain}} \times f_u,\text{brain}}{\text{AUC}_{\text{plasma}} \times f_u,\text{plasma}}$$

(5)

The data from in vitro cytotoxicity experiments were fitted to the log(inhibitor) versus normalized response–variable slope equation using GraphPad Prism version 6.04 software program (GraphPad, La Jolla, CA) to determine the IC50 values of the compounds of interest in the cell lines tested.

Pharmacokinetic Data Analysis

Pharmacokinetic parameters from the concentration-time profiles in plasma and brain were obtained by noncompartmental analysis performed using Phoenix WinNonlin version 6.4 (Certara USA, Inc., Princeton, NJ). The areas under the concentration-time curve (AUC) for plasma (AUCplasma) and brain (AUCbrain) were calculated using the linear trapezoidal method. The S.E. around the mean values of the AUC and $c_{\text{max}}$ were estimated using the sparse sampling module in WinNonlin (Nedelman and Jia, 1998).

Statistical Analysis

The GraphPad Prism version 6.04 software program (GraphPad) was used for statistical analysis. The sample sizes used were based on previous work and were determined based on approximately 80% power to detect 50% difference between groups. Data from all experiments are represented as mean ± S.D. or mean ± S.E.M., unless otherwise indicated. Comparisons between two groups were made using an unpaired t test. Comparisons between multiple groups were made using one-way ANOVA, followed by Bonferroni’s multiple comparisons test. A significance level of $P < 0.05$ was used for all statistical analyses.

Results

In Vitro Accumulation of panRAF Inhibitors in MDCKII-Bcrp1 and MDCKII-MDR1 Cells. The intracellular accumulation of C5719696, LY3009120, and MLN2480 in MDCKII-WT (vector control), Bcrp1-transfected (MDCKII-Bcrp1), and P-gp-transfected (MDCKII-MDR1) cell lines is summarized in Fig. 3. [3H]-Prazosin and [3H]-vinblastine were used as positive controls for Bcrp1 and MDR1 function, respectively. The cellular accumulation of [3H]-prazosin is significantly lower in Bcrp1-transfected cells compared with WT controls (WT: 100% ± 8%; Bcrp1: 24% ± 2%; $P < 0.0001$), and such an effect is abolished in the presence of a specific Bcrp1 inhibitor. The cellular accumulation of [3H]-vinblastine is also significantly lower in MDR1-transfected cells than in WT controls (WT: 100% ± 1%; MDR1: 9% ± 4%; $P < 0.0001$), and such an effect is abolished in the presence of 1 μM Ko143, a specific Bcrp1 inhibitor. The cellular accumulation of [3H]-vinblastine is also significantly lower in MDR1-transfected cells than in WT controls (WT: 100% ± 1%; MDR1: 9% ± 4%; $P < 0.0001$), and such an effect is abolished in the presence of 1 μM Ko143, a specific Bcrp1 inhibitor. The results confirm a significant elevation in functional activity of efflux transporters in the pertinent transfected cell lines.

In the same experiment, incubation with 2 μM solution of each of the three panRAF inhibitors showed that the accumulation is significantly different in Bcrp1 cells when compared with corresponding WT controls for LY3009120 (WT: 100% ± 12%; Bcrp1: 30% ± 7%; $P < 0.001$), and MLN2480 (WT: 100% ± 10%; Bcrp1: 49% ± 10%; $P < 0.01$). The addition of 0.2 μM Ko143 to the Bcrp1 cells caused a reversal of Bcrp1-mediated efflux of LY3009120 and MLN2480. For C5719696, the cellular accumulation is not significantly different in Bcrp1 cells when compared with corresponding WT controls (WT:
Fig. 3. In vitro intracellular accumulation of panRAF inhibitors. (A) The accumulation of prazosin (Bcrp probe substrate; positive control), CCT196969, LY3009120, and MLN2480 in MDCKII-WT and Bcrp1-transfected cells with and without Bcrp inhibitor Ko143 (0.2 μM). (B) Accumulation of vinblastine (probe substrate for P-gp/MDR1; positive control), CCT196969, LY3009120, and MLN2480 in WT and MDR1-transfected cells with and without P-gp inhibitor \((R)-4-\{1\alpha R, 6R, 10b\}-1,2\text{-difluoro}-1,6,10b\text{-tetrahydrodibenzo[\alpha,\epsilon]/cyclopropa[c]/cycloheptan-6-yl}-\alpha\{5\text{-quinololxyloxy}\} \text{methyl}\}-1\text{-piperazine ethanol, trihydrochloride (1 μM). Data represent the mean ± S.D.; } n = 3 \text{ for all data points. } ^{\#}P < 0.05 \text{ compared with the untreated transfected cell line; } ^{\ast}P < 0.001 \text{ compared with the untreated transfected cell line; } ^{**}P < 0.0001 \text{ compared with respective WT controls; } ***P < 0.0001 \text{ compared with respective WT controls.}
100% ± 35%; Bcrp1: 60% ± 20%; ns, P = 0.16), and the addition of 0.2 μM Ko143 to Bcrp1 cells did not lead to significant differences in intracellular accumulation. Incubation with 2 μM drug solution shows no significant difference in cellular accumulation in MDR1 cells when compared with corresponding WT controls for CCT196969 (WT: 100% ± 32%; MDR1: 83% ± 15%; ns, P = 0.56), LY3009120 (WT: 100% ± 27%; MDR1: 72% ± 40%; ns, P = 0.46), and MLN2480 (WT: 100% ± 3%; MDR1: 48% ± 25%; ns, P = 0.07). Also, the addition of 1 μM LY335979 to MDR1 cells did not lead to significant differences in intracellular accumulation when compared with transfected cells without inhibitor. These in vitro results indicate that only LY3009120 and MLN2480 are substrates of Bcrp1, and none of the three panRAF inhibitors are substrates of P-gp.

### Unbound (Free) Fractions of panRAF Inhibitors in Matrices of Interest.

The in vitro RED technique was employed for the determination of the unbound fraction (eqs. 1 and 2) of panRAF inhibitors in plasma, brain, and serum-containing cell culture media. The percentages of unbound fractions for CCT196969, LY3009120, and MLN2480 in plasma, brain, and cell culture media are as reported in Table 2. The three panRAF inhibitors exhibit high binding in plasma and brain. The estimated unbound fractions in plasma and brain were used for the determination of the unbound partition coefficient, \(K_{puu}\), and the unbound fraction in cell culture media was used in the estimation of the free IC\(_{50}\) value of the three panRAF inhibitors in each of the cell lines tested.

### In Vitro Efficacy in Patient-Derived Melanoma Cell Lines.

The in vitro efficacy studies were performed in BRAF-mutant M12, BRAF-mutant M27, and NRAS-mutant M15 patient-derived melanoma cell lines. The total IC\(_{50}\) values for vemurafenib (control), CCT196969, LY3009120, and MLN2480 were estimated using the total drug concentration-response curves determined in the three patient-derived cell lines.
melanoma cell lines. The free fractions in cell culture media for CCT196969, LY3009120, and MLN2480 were used together with the total IC\textsubscript{50} estimates to determine the free IC\textsubscript{50} values. The dose-response curves for vemurafenib, CCT196969, LY3009120, and MLN2480 in the three cell lines tested are shown in Fig. 4 and the IC\textsubscript{50} values are listed in Table 3. The results suggest that M12 is the most sensitive line to treatment with the four inhibitors, and LY3009120 exhibits higher potency in each of the three cell lines tested.

**Plasma and Brain Pharmacokinetics in FVB Mice.** The pharmacokinetic profiles of panRAF inhibitors were determined in FVB WT and transporter-deficient (knockout) mice following intravenous and oral drug administration. The brain and plasma concentration time profiles and brain-to-plasma ratios in FVB WT mice following a single intravenous bolus dose of 5 mg/kg CCT196969, LY3009120, and MLN2480 are as shown in Figs. 5–7. The total brain concentrations at the indicated time points are significantly lower than the total plasma concentrations for all three compounds. Table 4 summarizes the estimated pharmacokinetic parameters following intravenous drug administration. The brain-to-plasma AUC ratios (K\textsubscript{pu}; eq. 3) for CCT196969, LY3009120, and MLN2480 are 0.006, 0.05 and 0.20, respectively, and the corresponding K\textsubscript{pu,uu} (eq. 5) are 0.03, 0.02 and 0.05, respectively.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>IC\textsubscript{50} (95% CI)</th>
<th>Free IC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M12</td>
<td>M27</td>
</tr>
<tr>
<td>Vemurafenib</td>
<td>0.14 (0.10–0.19)</td>
<td>0.38 (0.28–0.52)</td>
</tr>
<tr>
<td>CCT196969</td>
<td>0.19 (0.13–0.27)</td>
<td>0.53 (0.41–0.70)</td>
</tr>
<tr>
<td>LY3009120</td>
<td>0.002 (0.001–0.003)</td>
<td>0.001 (0.07–0.002)</td>
</tr>
<tr>
<td>MLN2480</td>
<td>3.59 (3.00–4.29)</td>
<td>3.83 (3.11–4.71)</td>
</tr>
</tbody>
</table>

CI, confidence interval.
—, not determined for vemurafenib.

**Fig. 5.** Pharmacokinetic profiles of CCT196969 in FVB WT mice following intravenous and oral drug administration. Plasma and brain concentrations (A) and brain-to-plasma concentration ratios (B) of CCT196969 following administration of a single intravenous bolus dose of 5 mg/kg. Plasma and brain concentrations (C) and brain-to-plasma concentration ratios (D) of CCT196969 after a single oral dose of 10 mg/kg. Data represent mean ± S.D., n = 4.
The concentration time profiles and brain-to-plasma ratios in FVB WT mice following a single oral dose of 10 mg/kg CCT196969 and 25 mg/kg LY3009120 are shown in Figs. 5 and 6. The estimated pharmacokinetic parameters are summarized in Table 5. The brain-to-plasma AUC ratios ($K_p; \text{eq. 3}$) for CCT196969 and LY3009120 are 0.01 and 0.04, respectively, and are similar to that observed in the intravenous dosing studies. The corresponding $K_{p,\text{uu}}$ for CCT196969 and LY3009120 (eq. 5) are 0.05 and 0.02, respectively. The bioavailability (F) following oral administration is 0.77 for CCT196969 and 0.04 for LY3009120.

The brain and plasma concentrations and brain-to-plasma ratios in FVB WT and $\text{Mdr1a/b}^{-/-} \text{Bcrp1}^{-/-}$ mice 1-hour post oral administration of panRAF inhibitors (5 mg/kg CCT196969, 10 mg/kg LY3009120, and 10 mg/kg MLN2480) are shown in Fig. 8. The brain concentrations are significantly lower than the plasma concentrations in WT mice for all three panRAF inhibitors, and the brain-to-plasma concentration ratios for CCT196969, LY3009120, and MLN2480 are 0.01, 0.05, and 0.24, respectively. The brain-to-plasma concentration ratios in $\text{Mdr1a/b}^{-/-} \text{Bcrp1}^{-/-}$ mice are significantly higher than in WT mice for CCT196969, LY3009120, and MLN2480, and are 0.02, 0.32, and 2.66, respectively.

Steady-State Brain Distribution of panRAF Inhibitors. The steady-state brain-to-plasma ratios for CCT196969, LY3009120, and MLN2480 are 0.01 $\pm$ 0.002, 0.01 $\pm$ 0.01, and
The pharmacokinetic/metric parameters of panRAF inhibitors in FVB WT mice following administration of a single intravenous dose of 5 mg/kg.

Data are presented as mean or mean ± S.E.M. (n = 4).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CCT196969</th>
<th>LY3009120</th>
<th>MLN2480</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Brain</td>
<td>Plasma</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>2.79</td>
<td>3.37</td>
<td>2.35</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty} (\mu g \cdot h/\text{ml}))</td>
<td>150.23 ± 6.08</td>
<td>0.92 ± 0.02</td>
<td>10.86 ± 0.59</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty} (\mu g \cdot h/\text{ml}))</td>
<td>155.07</td>
<td>0.97</td>
<td>10.88</td>
</tr>
<tr>
<td>(\text{CL} (\text{ml/min per kilogram}))</td>
<td>0.54</td>
<td>—</td>
<td>7.66</td>
</tr>
<tr>
<td>(V_d (l/kg))</td>
<td>0.13</td>
<td>—</td>
<td>1.56</td>
</tr>
<tr>
<td>(K_p (\text{AUC}_{0-\infty} \text{ (ratio)}))</td>
<td>—</td>
<td>0.006</td>
<td>—</td>
</tr>
<tr>
<td>(K_{p,\text{uni}} (\text{AUC}_{0-\infty} \text{ (ratio)}))</td>
<td>—</td>
<td>0.03</td>
<td>—</td>
</tr>
</tbody>
</table>

\(\text{AUC}_{0-\infty}\), area under the curve from zero to the time of the last measured concentration; \(\text{AUC}_{0-\infty}\), area under the curve from zero to time infinity; \(\text{CL}\), clearance; \(K_p\), ratio of \(\text{AUC}_{0-\infty}\) (brain) to \(\text{AUC}_{0-\infty}\) (plasma) using total drug concentrations; \(K_{p,\text{uni}}\), ratio of \(\text{AUC}_{0-\infty}\) (brain) to \(\text{AUC}_{0-\infty}\) (plasma) using free drug concentrations; \(V_d\), volume of distribution. —, not relevant in these tissues.

0.34 ± 0.10 in WT mice and 0.05 ± 0.02, 0.27 ± 0.12, and 2.88 ± 0.31 in \(\text{Mdr} \text{1a/b}^{-}\) \(\text{Bcrp} \text{1}^{-}\) mice, respectively (Fig. 9; Table 6). The brain-to-plasma ratios are about 5-, 27-, and 9-fold higher for CCT196969, LY3009120, and MLN2480, respectively, in \(\text{Mdr} \text{1a/b}^{-}\) \(\text{Bcrp} \text{1}^{-}\) mice when compared with WT mice. These results are similar to the observations from the brain distribution studies in FVB WT and \(\text{Mdr} \text{1a/b}^{-}\) \(\text{Bcrp} \text{1}^{-}\) mice that evaluated the brain-to-plasma ratios 1-hour post oral drug administration. Collectively, the in vivo studies show that the delivery of the three panRAF inhibitors to the brain is restricted, and active efflux by P-gp and/or Bcrp plays a role in limiting their brain distribution.

**Discussion**

The small molecule molecularly targeted therapies and immunotherapies approved for the treatment of melanoma have shown improvements in survival by a few months in patients with MBM (Long et al., 2012; Margolin et al., 2012; Dummer et al., 2014; Ahmed et al., 2016; Goldberg et al., 2016, Spagnolo et al., 2016; Tawbi et al., 2018). While such improvements in survival bring new optimism, the management of metastatic disease that has spread to the brain is still challenging (Gampa et al., 2017). The modest efficacy of these compounds in patients with MBM may be related to factors that include inadequate drug delivery to tumor cells in the brain and specific brain microenvironment-driven alterations in gene expression. As a consequence, both drug delivery and resistance concerns should be addressed to improve treatment outcomes in patients with MBM. A novel class of compounds, the panRAF inhibitors, may address both of these issues.

The panRAF inhibitors may show benefits over mutant-BRAF inhibitors in the treatment of melanoma, either as single agents or in combination with a MEK inhibitor, due to their ability to overcome paradoxical activation of the MAPK pathway (Girotti et al., 2015; Peng et al., 2015). Several anticancer drugs have restricted brain delivery due to active efflux at the BBB, mainly by P-gp and Bcrp (Gampa et al., 2017; Kim et al., 2018). The transport of substrate compounds back into systemic circulation by efflux transporters can prevent drugs with a potential to be efficacious from reaching the target tumor cells residing behind an intact BBB. Given this, an evaluation of the brain distribution and efflux liability of therapies intended for use in central nervous system disorders is important. Herein, we report the findings of the brain distribution, binding, and in vitro efficacy studies for three panRAF inhibitors, i.e., CCT196969, LY3009120, and MLN2480. The results provide important information on the ability of these compounds to distribute across the BBB, and also allow a correlation of the in vivo concentrations with in vitro efficacy in patient-derived melanoma cell lines that will help inform in vivo efficacy studies.

In **in vitro** intracellular accumulation studies in transfected MDCKII cells overexpressing either murine Bcrp or human P-gp, both important BBB efflux transporters, suggest that only LY3009120 and MLN2480 are substrates of Bcrp.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CCT196969 (10 mg/kg)</th>
<th>LY3009120 (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Brain</td>
</tr>
<tr>
<td>Half-life (h)</td>
<td>3.64</td>
<td>3.97</td>
</tr>
<tr>
<td>(T_{max} (h))</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>(C_{max} (\mu g/ml))</td>
<td>31.09 ± 7.15</td>
<td>0.40 ± 0.04</td>
</tr>
<tr>
<td>(\text{AUC}_{0-\infty} (\mu g \cdot h/ml))</td>
<td>222.01 ± 18.94</td>
<td>2.18 ± 0.17</td>
</tr>
<tr>
<td>(\text{CL} (\text{ml/min per kilogram}))</td>
<td>0.53</td>
<td>—</td>
</tr>
<tr>
<td>(V_d (l/kg))</td>
<td>0.16</td>
<td>—</td>
</tr>
<tr>
<td>(F (%))</td>
<td>77</td>
<td>—</td>
</tr>
<tr>
<td>(K_p (\text{AUC}_{0-\infty} \text{ (ratio)}))</td>
<td>—</td>
<td>0.01</td>
</tr>
<tr>
<td>(K_{p,\text{uni}} (\text{AUC}_{0-\infty} \text{ (ratio)}))</td>
<td>—</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(\text{AUC}_{0-\infty}\), area under the curve from zero to the time of last measured concentration; \(\text{AUC}_{0-\infty}\), area under the curve from zero to time infinity; \(\text{CL}\), clearance; \(F\), absolute bioavailability ratio of the oral dose-corrected \(\text{AUC}_{0-\infty}\) to the intravenous dose-corrected \(\text{AUC}_{0-\infty}\); \(K_p\), ratio of \(\text{AUC}_{0-\infty}\) (brain) to \(\text{AUC}_{0-\infty}\) (plasma) using total drug concentrations; \(K_{p,\text{uni}}\), ratio of \(\text{AUC}_{0-\infty}\) (brain) to \(\text{AUC}_{0-\infty}\) (plasma) using free drug concentrations; \(T_{max}\), time to reach the maximum concentration; \(V_d\), volume of distribution. —, not relevant in these tissues.
however, none of the three inhibitors were substrates of P-gp (Fig. 3). In vitro RED experiments indicate that the three panRAF inhibitors exhibit high, nonspecific binding in brain and plasma (Table 2). The results of the in vitro efficacy testing reveal that M12, a BRAF-mutant melanoma brain metastasis patient-derived model, is the most sensitive line to treatment with CCT196969, LY3009120, MLN2480, and vemurafenib (Fig. 4; Table 3). LY3009120 has a low
Fig. 9. Steady-state brain distribution of pan-RAF inhibitors in FVB WT and $\text{Mdr1a/b}^{-/-}$ $\text{Bcrp1}^{-/-}$ mice. (A) Steady-state plasma and brain concentrations of CCT196969 and (B) brain-to-plasma ratios. (C) Steady-state plasma and brain concentrations of LY3009120 and (D) brain-to-plasma ratios. (E) Steady-state plasma and brain concentrations of MLN2480 and (F) brain-to-plasma ratios. **$P < 0.01$ compared with WT brain concentration, ***$P < 0.001$ compared with WT brain concentration; **$P < 0.01$ compared with WT brain-to-plasma ratio; ###$P < 0.001$ compared with WT brain-to-plasma ratio, for statistical comparison by unpaired t test. Data represent mean ± S.D., $n = 4$. 

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nanomolar IC\(_{50}\) value, and is the most potent inhibitor in each of the three cell lines tested.

Subsequently, experiments were conducted in mice to test the influence of P-gp and/or Bcrp on brain distribution of CCT196969, LY3009120, and MLN2480 in vivo. Pharmacokinetic studies in mice following a single intravenous or oral dose indicate that CCT196969, LY3009120, and MLN2480 all have restricted brain delivery (Figs. 5–7). The bioavailability following oral administration of CCT196969 was promising (~77%) using the current formulation. On the other hand, the oral bioavailability of LY3009120 was poor (<5%) (Table 5). Reports indicate that formulating LY3009120 as an amorphous solid dispersion improves the bioavailability by overcoming solubility limitations (Henry et al., 2015; Peng et al., 2015). The results of steady-state studies show that the brain distribution of CCT196969, LY3009120, and MLN2480 is enhanced in mice lacking P-gp and Bcrp (Fig. 9; Table 6). Also, studies evaluating the brain distribution 1-hour post oral administration of the three panRAF inhibitors in WT and triple-knockout mice (\(Mdr1a/b^{-/-}\) Bcrp1\(^{-/-}\)) show similar results (Fig. 8). Together, the in vivo brain distribution studies in WT and transgenic knockout mice suggest that the delivery of CCT196969, LY3009120, and MLN2480 to brain is limited due to active efflux by P-gp and/or Bcrp at the BBB, with MLN2480 showing greater brain distribution. Furthermore, MLN2480 exhibits higher brain distribution than vemurafenib and dabrafenib (Table 7). However, MLN2480 shows relatively modest in vitro efficacy, and LY3009120 exhibits superior in vitro efficacy when compared with CCT196969 and MLN2480 in patient-derived melanoma cell lines. These studies highlight the fact that the combination of drug delivery to target site in the brain and drug potency against the target will eventually influence the in vivo efficacy outcomes.

Given the importance of both drug delivery as well as drug potency in dictating treatment outcomes, a correlation of the observed in vivo concentrations of panRAF inhibitors with in vitro potencies is valuable. The average total drug concentrations post intravenous dosing at the measured time points were used together with the unbound fractions in brain and plasma to obtain unbound concentration-time profiles for CCT196969, LY3009120, and MLN2480 in WT mice. The unbound concentration-time profiles were then compared with in vitro potency estimates (free IC\(_{50}\)) in M12, to evaluate the potential of CCT196969, LY3009120, and MLN2480 in the treatment of MBM (Fig. 10). The selection of cell line was guided by the results of in vitro efficacy experiments, which indicate M12 to be the most sensitive line to treatment with CCT196969, LY3009120, and MLN2480 (Fig. 4; Table 3). The unbound concentration-time profiles in brain and plasma for MLN2480 show that the unbound concentrations are substantially below the free IC\(_{50}\) estimate in M12, when administered at a dose of 5 mg/kg, i.v. Assuming that the administration of MLN2480 at the maximum reported oral dose of 30 mg/kg in mice (Sun et al., 2017) will result in a 6-fold increase in concentrations (this assumes linear kinetics and oral bioavailability of 100%), the resulting unbound plasma concentrations will just reach the free IC\(_{50}\) value, and the brain concentrations will still be substantially below the free IC\(_{50}\) estimate in M12. For CCT196969, the unbound concentrations in brain are lower and those in plasma are higher (for about 1 hour) than the free IC\(_{50}\) values following a dose of 5 mg/kg, i.v. Considering that the maximum reported oral dose in mice for CCT196969 is 25 mg/kg (Girotti et al., 2015) and the oral bioavailability is 77%, the concentrations will be 4-fold higher (again assuming linear kinetics) following an intravenous dose (20 mg/kg) that will result in similar exposure to an oral dose of 25 mg/kg. The 4-fold increase in concentrations will lead to unbound plasma concentrations higher than the free IC\(_{50}\) value for about 7 hours; however, the unbound brain concentrations will still be substantially below the free IC\(_{50}\) value. In the case of LY3009120, the unbound concentrations in brain reach levels higher than the free IC\(_{50}\) value for approximately 7 hours, and the unbound concentrations in brain are higher than the free IC\(_{50}\) estimate in M12 for about 2 hours postdose after a dose of 5 mg/kg, i.v. An intravenous dose of 10 mg/kg LY3009120 was not tolerable in FVB mice. These findings suggest that CCT196969 and LY3009120 will possibly show superior in vivo efficacy in the treatment of systemic melanoma, since the unbound plasma concentrations reach levels higher than the free IC\(_{50}\).

### TABLE 6

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Strain</th>
<th>(K_{p,brain})</th>
<th>(K_{p,uu,brain})</th>
<th>Distribution Advantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCT196969</td>
<td>WT</td>
<td>0.01 ± 0.002</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>Mdr1a/b(^{-/-}) Bcrp1(^{-/-})</td>
<td>WT</td>
<td>0.05 ± 0.02</td>
<td>0.25</td>
<td>5</td>
</tr>
<tr>
<td>LY3009120</td>
<td>WT</td>
<td>0.01 ± 0.01</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>Mdr1a/b(^{-/-}) Bcrp1(^{-/-})</td>
<td>WT</td>
<td>0.27 ± 0.12</td>
<td>0.14</td>
<td>27</td>
</tr>
<tr>
<td>MLN2480</td>
<td>WT</td>
<td>0.34 ± 0.10</td>
<td>0.08</td>
<td>1</td>
</tr>
<tr>
<td>Mdr1a/b(^{-/-}) Bcrp1(^{-/-})</td>
<td>WT</td>
<td>2.88 ± 0.31</td>
<td>0.66</td>
<td>8.5</td>
</tr>
</tbody>
</table>

\(C_{ss,brain}\), Steady-state brain concentration; \(C_{ss,plasma}\), Steady-state plasma concentration; \(K_{p,brain}\), Ratio of \(C_{ss,brain}\) to \(C_{ss,plasma}\) using total drug concentrations; \(K_{p,uu,brain}\), ratio of \(C_{ss,brain}\) to \(C_{ss,plasma}\) using free drug concentrations; distribution advantage, ratio of \(K_{p}\) to \(K_{p,uu,\text{wild-type}}\).

### TABLE 7

Comparison of brain distribution of RAF inhibitors in WT mice

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Intravenous Dose</th>
<th>Brain Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_{p})</td>
<td>(K_{p,uu})</td>
</tr>
<tr>
<td>Vemurafenib(^a)</td>
<td>2.5</td>
<td>0.004</td>
</tr>
<tr>
<td>Dabrafenib(^b)</td>
<td>2.5</td>
<td>0.02</td>
</tr>
<tr>
<td>CCT196969</td>
<td>5</td>
<td>0.006</td>
</tr>
<tr>
<td>LY3009120</td>
<td>5</td>
<td>0.05</td>
</tr>
<tr>
<td>MLN2480</td>
<td>5</td>
<td>0.20</td>
</tr>
</tbody>
</table>

\(K_{p}\), ratio of AUC\(_{0\rightarrow\infty}\)\(_{\text{brain}}\) to AUC\(_{0\rightarrow\infty}\)\(_{\text{plasma}}\) using total drug concentrations; \(K_{p,uu}\), ratio of AUC\(_{0\rightarrow\infty}\)\(_{\text{brain}}\) to AUC\(_{0\rightarrow\infty}\)\(_{\text{plasma}}\) using free drug concentrations; NR, not reported.

\(^a\)As reported by Mittapalli et al. (2012).

\(^b\)As reported by Mittapalli et al. (2015).
Fig. 10. Plasma and brain unbound concentration-time profiles of CCT196969 (A), LY3009120 (B), and MLN2480 (C) in FVB WT mice following 5 mg/kg intravenous drug administration. The unbound concentrations were determined using the in vivo concentrations and the unbound fraction estimates from in vitro RED studies. The dashed lines represent the experimentally determined in vitro IC$_{50}$ (free) of the three panRAF inhibitors against the BRAF-mutant M12 melanoma cell line. Data represent mean ± S.D., n = 4.
estimates in M12 for both compounds. However, the unbound concentrations in brain achieve levels higher than the free IC_{50} estimate in M12 for LY3009120 alone, suggesting possible efficacy benefits with LY3009120 in the treatment of MBM. Given such insights, testing the efficacy of LY3009120 in preclinical models of MBM will be important to evaluate in vivo efficacy.

The responses to treatment with molecularly targeted therapies have been suboptimal and variable in patients with MBM. Such outcomes may be related to inadequate drug delivery to tumor cells in the brain and specific brain microenvironment-driven changes in gene expression, both critical challenges that need to be addressed to improve the quality of life of MBM patients. The panRAF inhibitors have advantages over mutant-BRAF inhibitors in overcoming resistance and preventing the paradoxical activation of the MAPK pathway. However, the delivery of panRAF inhibitors to the site of action in the brain and their potency against the target tumor cells are key determinants of in vivo efficacy in the treatment of tumors in the brain. A correlation of unbound plasma and brain drug concentrations with in vitro potency estimates in patient-derived melanoma cell lines for CČT196969, LY3009120, and MLN2484 suggests that LY3009120 is a novel panRAF inhibitor with low nanomolar in vitro potency against mutant-BRAF (M12 and M27) and NRAS (M15) patient-derived melanoma cell lines, and has adequate brain delivery to achieve therapeutically active concentrations in the brain. The novel mechanism involving inhibition of all isoforms of RAF, as well as an appropriate balance of potency and brain delivery, make LY3009120 a promising candidate for efficacy testing in preclinical models of MBM. Also, evaluating the use of a rational combination of panRAF/MEK inhibitors to achieve better vertical blockade of MAPK signaling and in vivo efficacy in the treatment of MBM will be of interest. When using combinations, all inhibitors in a combination regimen must have adequate brain delivery to achieve therapeutic concentrations at the sites of action in the brain in order to elicit the desired therapeutic response and minimize the emergence of resistance. Remarkable progress has been made in the treatment of melanoma; however, there still remains a need to develop better therapies for MBM, and drug delivery across a functionally intact BBB is an important challenge that needs to be addressed to fulfill this goal.

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Gampa G, Vaidhyanathan S, Resman BW, Parrish KE, Markovic SN, Sarkaria JN, Elquist WF (2017) Drug delivery to melanoma cell lines, and has adequate brain delivery to achieve therapeutic concentrations with in vitro potency estimates in patient-derived melanoma cell lines for CČT196969, LY3009120, and MLN2484 suggests that LY3009120 is a novel panRAF inhibitor with low nanomolar in vitro potency against mutant-BRAF (M12 and M27) and NRAS (M15) patient-derived melanoma cell lines, and has adequate brain delivery to achieve therapeutically active concentrations in the brain. The novel mechanism involving inhibition of all isoforms of RAF, as well as an appropriate balance of potency and brain delivery, make LY3009120 a promising candidate for efficacy testing in preclinical models of MBM. Also, evaluating the use of a rational combination of panRAF/MEK inhibitors to achieve better vertical blockade of MAPK signaling and in vivo efficacy in the treatment of MBM will be of interest. When using combinations, all inhibitors in a combination regimen must have adequate brain delivery to achieve therapeutic concentrations at the sites of action in the brain in order to elicit the desired therapeutic response and minimize the emergence of resistance. Remarkable progress has been made in the treatment of melanoma; however, there still remains a need to develop better therapies for MBM, and drug delivery across a functionally intact BBB is an important challenge that needs to be addressed to fulfill this goal.

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