Activation Status of the Pregnane X Receptor Influences Vemurafenib Availability in Humanized Mouse Models

A. Kenneth MacLeod, Lesley A. McLaughlin, Colin J. Henderson, and C. Roland Wolf

Abstract

Vemurafenib is a revolutionary treatment for melanoma, but the magnitude of therapeutic response is highly variable, and the rapid acquisition of resistance is frequent. Here, we examine how vemurafenib disposition, particularly through cytochrome P450-mediated oxidative pathways, could potentially influence these outcomes using a panel of knockout and transgenic humanized mouse models. We identified CYP3A4 as the major enzyme involved in the metabolism of vemurafenib in vitro assays with human liver microsomes. However, mice expressing human CYP3A4 did not process vemurafenib to a greater extent than CYP3A4-null animals, suggesting that other pregnane X receptor (PXR)-regulated pathways may contribute more significantly to vemurafenib metabolism in vivo. Activation of PXR, but not of the closely related constitutive androstane receptor, profoundly reduced circulating levels of vemurafenib in humanized mice. This effect was independent of CYP3A4 and was negated by cotreatment with the drug efflux transporter inhibitor elacridar. Finally, vemurafenib strongly induced PXR activity in vitro, but only weakly induced PXR in vivo. Taken together, our findings demonstrate that vemurafenib is unlikely to exhibit a clinically significant interaction with CYP3A4, but that modulation of bioavailability through PXR-mediated regulation of drug transporters (e.g., by other drugs) has the potential to markedly influence systemic exposure and thereby therapeutic outcomes.

Introduction

The BRAF gene is mutated in approximately 50% of all malignant melanomas (1). In three quarters of these cases, mutation results in a valine to glutamic acid substitution at codon 600 (V600E), conferring an enhancement of kinase activity that promotes cellular proliferation and tumorigenesis (1-4). Vemurafenib (RG7204, PLX4032) was the first therapeutic agent clinically approved for the treatment of BRAFV600E-positive malignant melanoma. A highly specific inhibitor of the mutant kinase, vemurafenib elicits an objective tumor response in 48% of all patients with BRAFV600E-positive metastatic melanoma when given as a single agent, with increases in both overall and progression-free survival when compared with conventional chemotherapy with dacarbazine (5-7).

Vemurafenib is both a revolutionary treatment for melanoma and, along with the companion diagnostic assay (8), a widely cited example of the potential for therapeutic stratification based on mutational analysis. However, as with many recently developed clinically approved kinase inhibitors, drug resistance is a major problem. The magnitude of initial response to vemurafenib is highly variable (5-7). Although approximately half of patients meet the RECIST threshold for an objective response after 14 weeks of therapy, the remainder occupy a spectrum from nonobjective response to disease progression, suggestive of varying degrees of innate resistance (7). Moreover, the median duration of response is only 6.7 months due to the acquisition of resistance (6). A large number of potential mechanisms of both innate and acquired resistance have been identified, most of which invoke the plasticity of signaling networks within the tumor to circumvent blockade to BRAFV600E function (9, 10). The majority of these studies have been conducted in cultured cell lines and tumor biopsy samples. There has been comparatively little effort to investigate whether the high interindividual variability in drug exposure at steady state, with coefficients of variation of both Cmax and AUC of 30% to 40%, is a determinant of therapeutic outcome and/or resistance, despite the trends toward an exposure–response relationship in both overall and progression-free survival identified by population pharmacokinetic (PK) analyses (11-13), is a determinant of therapeutic outcome and/or resistance, with the notable exception of a recent study in which low circulating vemurafenib concentrations were associated with disease progression (14).

During preclinical development, vemurafenib was found to be a substrate and possible inducer of CYP3A4, which was the major enzyme involved in its metabolism in vitro (11, 12, 15). Moreover, CYP3A4-generated monohydroxy-vemurafenib (OH-vemurafenib) was detected in a mass balance analysis in patients at steady state (11, 12, 15). In this latter study, OH-vemurafenib levels were low compared with the parental compound but, as the absolute bioavailability of vemurafenib is unknown, it is impossible to draw any conclusions as to the relative contribution of metabolism in general, and CYP3A4 in particular, to elimination of the...
drug. Therefore, if only a small percentage of vemurafenib reaches the systemic circulation, metabolism may yet constitute a major route of elimination and an important determinant of drug exposure. In addition to these metabolic considerations, post-approval studies using knockout mouse lines have identified phase III drug efflux transporters as critical determinants of vemurafenib bioavailability (16, 17).

CYP3A4 shows high inter- and intraindividual variation in expression, with differences in excess of 100-fold (18). The primary mode of CYP3A4 regulation is transcriptional, with activation of two nuclear hormone receptors in particular, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), by exogenous ligands, endogenous ligands, or upstream signaling pathways known to increase its levels (19). In the present study, we have assessed the role of these metabolic factors in mediating vemurafenib disposition in both in vitro assays and in humanized mouse models in vivo. We have compared the relative contributions of metabolic and efflux pathways with vemurafenib PK, and have investigated the potential of vemurafenib to influence its own disposition.

Materials and Methods

Chemicals and reagents

Crystalline vemurafenib and PLX4720 were purchased from Selleck chemicals. Microcrystallized bulk powder (MPB) vemurafenib was obtained through the Genentech Research Projects and Reagents Program (Genentech). Klucel LF Pharm was obtained from Ashland Inc. Elacridar hydrochloride was purchased from Sequoia Research Products. NADPH was purchased from Melford Chemicals and reagents. Enzyme activities were determined using a FluoTime 300 spectrofluorimeter (Thermo Fisher Scientific) and a Fluoroskan Ascent FL fluorimeter (Thermo Fisher Scientific).

Animal lines and husbandry

The generation and characterization of Cyp3aKO/Cyp3a13−/−, huCYP3A4/3A7, huPXR/huCAR/huCYP3A4/3A7, and huPXR/huCAR mice have been described previously (20, 21). Briefly, the Cyp3aKO/Cyp3a13−/− line bears a deletion of seven of the eight murine Cyp3a genes and exhibits low and undetectable oxidative metabolism of the CYP3A4 probe substrates triazolam, dextromethorphan, midazolam, and diazepam (20). The huCYP3A4/3A7 line carries a large genomic insertion of human CYP3A4 and CYP3A7 in place of the Cyp3a locus (20). In this line, CYP3A4 is maintained at a low basal level, but can be upregulated following ligand-activated nuclear translocation of the transcription factor, Pxr, most effectively with a synthetic glucocorticoid, 5-pregnenolone-3β-ol-20-one-16β-carbonitrile (PCN; ref. 20). The huPXR/huCAR/huCYP3A4/3A7 line has been similarly humanized for CYP3A4/3A7, with additional humanizations for Pxr/PXR and Car/CAR (20). In this line, CYP3A4 can be induced by rifampicin (RIF), a ligand activator of human PXR (20). The huPXR/huCAR line carries the human transcription factors, but retains the murine Cyp3a cluster (21). All animals were maintained under standard animal house conditions, with free access to food (RM1 diet, Special Diet Services) and water, and a 12-hour light/12-hour dark cycle. All animal work was carried in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

Subcellular fractionation

Livers were excised and snap-frozen in liquid nitrogen for storage at −80°C until processing. Briefly, samples were thawed by the addition of 3 volumes of KCl buffer (1.15% w/v potassium chloride, 10 mmol/L potassium phosphate, pH 7.4) and homogenized by rotor-stator. Debris was pelleted by centrifugation (11,000 × g at 4°C for 15 minutes) and the supernatant withdrawn for ultracentrifugation (100,000 × g at 4°C for 60 minutes). After ultracentrifugation, the supernatant (cytosolic fraction) was retained and the pellet (microsomal fraction) was resuspended in KCl buffer containing 0.25 mol/L sucrose. Protein content of microsomal and cytosolic fractions was quantified by Bradford assay (Bio-Rad).

In vitro studies

All in vitro analyses were carried out with crystalline vemurafenib in 50 mmol/L HEPES pH 7.4 containing 30 mmol/L MgCl2. Incubations were initiated by the addition of NADPH to a final concentration of 1 mmol/L and terminated by the addition of 1x volume of ice cold acetonitrile. Microsomal stability assays were performed in triplicate with 1 mmol/L compound and 0.2 mg/mL pooled human liver microsomes (HLM, 150 donors; Life Technologies) with or without NADPH for a period of up to 50 minutes. HLM and mouse liver microsome (MLM, pooled from 3 wild-type mice) incubations for formation of the OH-vemurafenib metabolite contained 0.4 mg/mL microsomal protein and 60 μmol/L vemurafenib. Incubations with HLM from individual donors for Spearman’s rank correlation were performed with 50 μmol/L vemurafenib and 0.1 mg/mL protein for 6 minutes. Initial incubations with recombinant P450s were carried out with 60 μmol/L compound and 400 pmol/mL enzyme for 15 minutes. Assays to determine the apparent kinetic parameters of OH-vemurafenib formation in HLM and MLM were performed in triplicate under conditions of linearity for time and protein: 10 minutes at 0.2 mg/mL for HLM, 15 minutes at 0.4 mg/mL for MLM, 6 minutes at 160 pmol/mL for recombinant CYP3A4, and 9 minutes at 160 pmol/mL for CYP2C9. For inhibition studies, pooled HLM were incubated at 0.2 mg/mL with 60 μmol/L vemurafenib for 10 minutes in the presence of 1 μmol/L ketoconazole, 2 μmol/L sulfaphenazole, or both together. Inhibition of 7-benzoxyl quinoline (BQ) turnover by vemurafenib was measured using a BQ concentration of 30 μmol/L and 0.2 mg/mL HLM with increasing concentrations of vemurafenib, and the fluorescent product measured using a Fluoroskan Ascent FL fluorimeter (Thermo Fisher Scientific).

PXR-transactivation assay

Cells stably transected with the PXR gene and a reporter construct with luciferase under the control of PXR-responsive elements of the CYP3A4 promoter (DPX2 cells; Puracyp Inc.) were treated with a range of concentrations of vemurafenib (0.06, 0.17, 0.51, 1.54, 4.62, 13.89, 41.67, and 125 μmol/L) in triplicate for 48 hours and assayed for luminescence and fluorescence as per the manufacturer’s instructions. The top two concentrations of vemurafenib were highly toxic so data from these points were excluded from the analysis. PXR activation (fold change) was calculated by dividing luminescence by fluorescence and normalizing to vehicle control.

In vivo studies

All animal work was carried out on 8- to 12-week-old male mice (except for those shown in Fig. 7, where female mice were used). PCN was suspended in corn oil at 1 mg/mL for administration by i.p. injection at 10 mg/kg (10 μL/g body weight).
RIF was administered in the same way at 60 mg/kg. Phenobarbital (PB) was dissolved in PBS for i.p. administration at 80 mg/kg. For PK analyses, crystalline vemurafenib was first dissolved in DMSO and then further diluted in a mixture of Polysorbate 80, ethanol, and water (20:13:67) for immediate administration by oral gavage at a final dose of 50 mg/kg, as described by Durmus and colleagues (16). The same method was used for administration of elacridar at 100 mg/kg (16). The same method was used for administration of elacridar at 100 mg/kg (16). For sample collection, 10 μL of whole blood was withdrawn from the tail vein at the indicated time points. Samples were immediately added to a tube containing 100 μL heparin solution (100 IU/mL) and stored at −20°C until processing. For induction studies, MBP vemurafenib was suspended in water containing 2% Klucel LF Pharm, which had been adjusted to pH 4.0 by the addition of hydrochloric acid, and administered twice daily (8 hours apart) by oral gavage, as described by Yang and colleagues (22).

**Sample processing for LC-MS/MS**

Water (100 μL) containing 100 μg/mL internal standard (PLX4720) was added to in vitro and in vivo samples, followed by 500 μL of diethyl ether (Thermo Fisher Scientific). This mixture was incubated at room temperature for 30 minutes with gentle mixing. Samples were centrifuged for 10 minutes at 16,000 × g and the organic solvent phase withdrawn to a fresh tube for drying under a vacuum (20 minutes at 30°C). The dried extract was redissolved in 100 μL of acetonitrile (Sigma) at 37°C with agitation for 5 minutes and samples were added to 96-well plates for LC-MS/MS.

**LC-MS/MS**

Analysis of in vitro incubation and in vivo blood PK samples was carried out on a Waters Acquity UPLC and Micromass Quattro Premier mass spectrometer (both Micromass). LC separation was performed on a Kinetex 1.7 μm C18 100A, 50 × 2.1 mm column (Phenomenex) at a temperature of 45°C with an injection volume of 5 μL and flow rate of 0.6 mL/min. Mobile phases were water containing 0.1% (v/v) formic acid (A) and acetonitrile containing 0.1% (v/v) formic acid (B). Isocratic elution was carried out at 35%/65% A/B for 1 minute. Multiple reaction monitoring data in ESI-positive mode were acquired for vemurafenib (m/z 490.10 > 255.04, cone voltage (CV) = 65 V, collision energy (CE) = 40 kV), OH-vemurafenib (m/z 505.99 > 255.24, CV = 65 V, CE = 40 kV) and PLX4720 (m/z 414.20 > 306.97, CV = 60 V, CE = 37 kV). Acquired data were analyzed in Qunalyx relative to analyze standard curves spanning the range of concentrations under study.

**Data analysis**

In vitro data exhibited non-Michaelis–Menten kinetics, and therefore was fitted with the Hill equation using GraFit version 5 (Eiríkur Software). Standard deviations given are from the fit of the calculated curve. PK parameters of in vivo data were calculated with a simple non-compartmental model using WinNonLin software, v4.1 (Pharsight) and are shown with SDs. P values were calculated using an unpaired t test.

**Western blotting**

Microsomal and cytosolic samples were adjusted to 1 mg/mL in LDS sample buffer (Life Technologies) for electrophoresis through 10% acrylamide gels for 1 hour at 150 V, followed by transfer onto nitrocellulose membranes. Primary antibodies used for immunoblotting included anti-CYP3A4 (458234; BD Biosciences), Cyp1a, Cyp2b, Cyp2c, Cyp3a/CYP3A, and Pgr (23, 24). Non-GRP78 (ab21685; Abcam) and anti-GAPDH (G9545, Sigma) were used as loading controls for microsomal and cytosolic fractions, respectively. In blots for CYP3A4/CYP3A and CYP3A4, pooled HLM material was the same as used in microsomal incubations, whereas individuals with low, medium, and high expression were taken from the panel of 15 characterized donors based on testosterone 6β-hydroxylation activity.

**mRNA analysis**

Total RNA was isolated from snap-frozen small intestinal samples using TRIzol (Life Technologies). The purity of this RNA was increased by processing with the RNasy Mini Kit (Qiagen) followed by treatment with RQ1 DNase (Promega). Conversion to cDNA was carried out using the ImProm-II Reverse Transcription System with random primers (Promega). The relative levels of mRNA species were determined by TaqMan RT-PCR (Life Technologies) using the following assays: Abcb1a (Mm00440761_m1), Abcb1b (Mm00440736_m1), Abcg2 (Mm00496364_m1), and CYP3A4 (Hs00604506_m1). Fold changes were calculated by the comparative Ct method with 18S rRNA as an endogenous control (4319413E; Life Technologies).

**Results**

Vemurafenib is metabolized by CYP3A4 in vitro

Metabolic stability of vemurafenib was assessed in pooled HLM (Fig. 1A). At 1 μmol/L, the compound was highly stable in both the presence and absence of NADPH; however, formation of OH-vemurafenib was detectable in the presence of NADPH (Fig. 1B). Apparent kinetic parameters of OH-vemurafenib formation by HLM and MLM were found to be sigmoidal, and therefore were fitted using the Hill equation. The V_{max} for HLM was approximately 10-fold higher than that observed in MLM (4879 ± 108 vs. 467 ± 54 peak area/min/mg, respectively) whereas the S_{50} was similar (23.2 ± 0.8 vs. 23.8 ± 4.9 μmol/L, respectively, Fig. 1C). Hill coefficients are detailed in Supplementary Table S1, alongside parameters calculated using the Michaelis–Menten model. In a panel of HLM from 15 donors that had been characterized by the vendor with regard to their activity with probe substrates for individual CYP isoforms, formation of OH-vemurafenib correlated most strongly with that of 6β-hydroxy-testosterone, the marker for CYP3A4 (Supplementary Table S2). However, this was deemed artefactual as CYP3A4 and CYP2A6 probe activities within the HLM panel correlate (data not shown), an observation that has previously been made in a separate batch of samples (23). Indeed, in incubations with recombinant CYP containing high concentrations of both protein and substrate, OH-vemurafenib was formed by CYP3A4, CYP2C9 and, to a lesser extent, by CYP2C8 and CYP1A1, but not by CYP2A6 (Fig. 1E). The in vitro apparent kinetics of OH-vemurafenib formation by CYP3A4 and CYP2C9 were, similarly to HLM and MLM, sigmoidal in nature. CYP3A4 had a 2-fold higher affinity for vemurafenib (S_{50} = 10.0 ± 0.9 vs. 20.4 ± 1.7 μmol/L) and a 3.4-fold higher V_{max} (11693 ± 471 vs. 3481 ± 216 peak area/min/nmol) than CYP2C9. As with the hepatic microsomal preparations, Hill coefficients and Michaelis–Menten analyses for recombinant P450s are detailed in Supplementary Table S1. In pooled HLM, coinubation with the CYP3A4 inhibitor, ketoconazole, significantly inhibited...
and Methods. Twenty-four hours after the third dose of PCN, CYP3A4 is expressed in the liver of huCYP3A4/3A7 at a level similar to that observed in pooled HLM (data not shown). As stated above, Cyp3aKO/Cyp3a13/−/+ mice are essentially null for Cyp3a/CYP3A activity, both basal and in response to activation of PXR. Therefore, any difference in the PK of vemurafenib between huCYP3A4/3A7 PCN and Cyp3aKO/Cyp3a13/−/+ PCN is attributable to the activity of CYP3A4. Although not statistically significant, there was a decrease in both Cmax and AUC0α in huCYP3A4/3A7 mice, and an increase in CL, in the huCYP3A4/3A7 PCN group, relative to the Cyp3aKO/Cyp3a13/−/+ PCN group (Fig. 2A; Supplementary Table S3). More pronounced, however, were these same changes in the PCN groups of both genotypes, relative to their respective vehicle control groups. The fact that PCN treatment enhanced clearance equally well in Cyp3a null and huCYP3A4/3A7 animals suggests that PXR-regulated pathways other than CYP3A4 predominate in the clearance of vemurafenib in vivo. Immediately after the final blood sample collection, livers were harvested and Western blotting carried out to confirm that CYP3A4 had been effectively induced by PCN in the huCYP3A4/3A7 mice (Fig. 2B). Because of this harvest occurring 48 hours after the last dose of PCN, CYP3A4 expression was lower than in pooled HLM due to turnover of the enzyme.

Exposure to vemurafenib is decreased following activation of PXR/PXR, but unaffected by activation of CAR

As PCN pretreatment decreased the systemic concentration of vemurafenib independently of CYP3A4 (Fig. 2A; Supplementary Table S3), we further investigated the role of nuclear hormone receptor activity in mediating disposition to vemurafenib. HuPXR/huCAR/huCYP3A4/3A7 mice were pretreated with Rif, a potent activator of human PXR, and thus inducer of CYP3A4, leading to a marked reduction in the AUC0α of vemurafenib from 432 ± 169 to 121 ± 18 h·μg/mL (P = 0.0106, Fig. 3A; Supplementary Table S4).

Figure 1.

Vemurafenib is metabolized by CYP3A4 and CYP2C9 in vitro. A, microsomal stability of vemurafenib with HLM in the absence (open circles) and presence (filled circles) of NADPH. B, formation of OH-vemurafenib from vemurafenib when incubated with HLM in the absence (open circles) and presence (filled circles) of NADPH. C, vemurafenib kinetics with HLM (open circles) and MLM (filled circles) fitted with the Hill equation. D, correlation of conversion of 6β-OH-testosterone formation in characterized liver microsomes from 15 individuals with formation of OH-vemurafenib from vemurafenib. E, generation of OH-vemurafenib by recombinant CYP. F, vemurafenib kinetics with recombinant CYP2C9 (filled circles) and CYP3A4 (open circles) fitted with the Hill equation. G, inhibition of OH-vemurafenib formation in pooled HLM by ketoconazole and sulfaphenazole. H, inhibition of BQ activity in pooled HLM by vemurafenib (IC50 = 14.9 μmol/L). In all cases, data comprise mean ± SD of triplicate incubations.

OH-vemurafenib formation (Fig. 1G). The CYP2C9 inhibitor, sulfaphenazole, effected a partial decrease in OH-vemurafenib formation. As expected, coinubation with both inhibitors completely ablated OH-vemurafenib formation. Also in pooled HLM, vemurafenib inhibited the activity of the CYP3A4 probe drug substrate BQ with an IC50 value of 14.9 μmol/L (Fig. 1H).

Single-dose vemurafenib is not extensively metabolized by CYP3A4 in vivo

To study the contribution of CYP3A4 to metabolism of vemurafenib in vivo, Cyp3aKO/Cyp3a13/−/+ and huCYP3A4/3A7 mice were administered either PCN or vehicle (CO) alone for 3 days before administration of vemurafenib, as described in Materials

Figure 2.

CYP3A4 has a modest effect on the PK profile of a single dose of vemurafenib in vivo. A, PK profiles of vemurafenib administered to huCYP3A4/3A7 PCN (filled circles; n = 5), Cyp3aKO/Cyp3a13/−/+ PCN (filled squares; n = 4), huCYP3A4/3A7 vehicle (open circles; n = 5), and Cyp3aKO/Cyp3a13/−/+ vehicle (open squares; n = 4) mice. Mean data ± SEM are shown. B, Western blot analysis of hepatic microsomal fractions from Cyp3aKO/Cyp3a13/−/+ and huCYP3A4/3A7 mice dosed with either PCN or CO vehicle alone, alongside pooled HLM.

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Vemurafenib Pharmacokinetics in Humanized Mice

Figure 3.
Activation of PXR reduces exposure to vemurafenib independently of CYP3A4 activity. A, PK profiles of vemurafenib in huPXR/huCAR/huCYP3A4/3A7 vehicle (open circles; n = 4), huPXR/huCAR/huCYP3A4/3A7 RIF (closed circles; n = 4), huPXR/huCAR vehicle (open squares; n = 3), and huPXR/huCAR RIF (closed squares; n = 3) mice. Mean data ± SEM are shown. B, Western blot analysis of hepatic microsomal fractions from huPXR/huCAR/huCYP3A4/3A7 mice dosed with either RIF or CO vehicle alone, alongside HLM preparations; pooled and low, medium, and high CYP3A4 activity. C, TagMan RT-PIC analysis of Abcb1a, Abcb1b, and Abcg2 mRNA levels in whole small intestine of huPXR/huCAR/huCYP3A4/3A7 mice dosed with vehicle or RIF.

Western blotting confirmed induction of CYP3A4 in the liver and, due to the high initial magnitude of this induction, CYP3A4 levels remained several fold higher than pooled HLM (and equivalent to HLM expressing high CYP3A4) despite 48 hours having elapsed after administration of the final dose of RIF (Fig. 3B). In addition, we observed a 4.7-fold increase in mRNA for Abcb1a, but no change for Abcb1b or Abcg2, in the small intestine of huPXR/huCAR/huCYP3A4/3A7 mice treated with RIF (Fig. 3C). Activation of PXR in the huPXR/huCAR mouse line led to a similar decrease in AUC_{all} of vemurafenib from 349 ± 24 to 75 ± 33 h·µg/mL (P = 0.0003, Fig. 3A; Supplementary Table S4).

To determine whether CAR might play a role in modulating vemurafenib disposition, huPXR/huCAR/huCYP3A4/3A7 mice were dosed for 3 days with PB, a CAR activator (25), before vemurafenib administration. This had no effect on any of the kinetic parameters assessed (Fig. 4A, Supplementary Table S5).

Figure 4.
Activation of CAR does not alter vemurafenib PK. A, PK profiles of vemurafenib in huPXR/huCAR/huCYP3A4/3A7 vehicle (open circles; n = 3), PB (closed circles; n = 3), and RIF (closed squares; n = 3). Mean data ± SEM are shown. B, Western blots of hepatic microsomal fractions from huPXR/huCAR/huCYP3A4/3A7 mice dosed with either vehicle (PBS), PB, or RIF, alongside HLM preparations; pooled and low, medium, and high CYP3A4 activity.

Activation of PXR reduces vemurafenib exposure through induction of efflux transporters

Activation of PXR by RIF in huPXR/huCAR/huCYP3A4/3A7 mice decreased the AUC_{all} of vemurafenib (Figs. 3A, 4A, and 5A; Supplementary Tables S3–S5). This decrease was almost completely ablated by pretreatment of mice, 90 minutes before administration of vemurafenib, with elacridar, an effective inhibitor of Abcb1 and Abcg2, but not CYP3A4, at the dose used (100 mg/kg orally, Fig. 5A; refs. 16, 26, 27). Relative to mice dosed with RIF alone, AUC_{all} of vemurafenib increased from 90 ± 13 to 330 ± 85 h·µg/mL (P = 0.0085) in animals dosed with RIF followed by elacridar, whereas C_{max} increased from 11.1 ± 1.8 to 21.3 ± 2.2 µg/mL (P = 0.0032; Supplementary Table S6). Moreover, huPXR/huCAR/huCYP3A4/3A7 mice, which had been predosed with vehicle (CO) alone, were subject to a similar magnitude of increase in exposure to vemurafenib following elacridar pretreatment, with AUC_{all} increasing from 391 ± 112 to 684 ± 85 h·µg/mL (P = 0.0224) and C_{max} increasing from 29.1 ± 4.3 to 37.8 ± 3.5 µg/mL (P = 0.0507, Fig. 5A). Again, effective activation of PXR by RIF was confirmed by Western blot analysis of CYP3A4 in liver samples (Fig. 5B).

Vemurafenib is a strong inducer of PXR in vitro

We tested the capacity of crystalline vemurafenib to activate a PXR-driven luciferase reporter construct in the DPX2 cell line (Puracyp Inc.). The reported aqueous solubility of this form is in the range of 0.01 to 10 µg/mL (approximately 20 to 20 µmol/L) at physiologic pH (12). An EC_{50} value of 8.13 µmol/L and an E_{max} of 8.54-fold (Fig. 6) was determined. The positive control, rifampicin, gave an EC_{50} value of 1.01 µmol/L and an E_{max} of 14.10-fold (data not shown).
Vemurafenib is a weak inducer of PXR in vivo. We administered the high bioavailability MBP form of vemurafenib at a dose of 100 mg/kg twice daily for 4 days to huPXR/huCAR/huCYP3A4/3A7 mice, as described in Materials and Methods. The PK parameters of a single 100-mg/kg dose (Supplementary Table S7) were in good agreement with those calculated in a previous study (22). Western blotting of liver proteins demonstrated weak induction of CYP3A4 and Cyp2b, and moderate induction of Cyp1a (Fig. 7A). Analysis of small-intestinal mRNA revealed no change in expression in CYP3A4, Abcb1a, or Abcb1b (Fig. 7B).

Discussion

Vemurafenib is a revolutionary treatment for advanced melanoma and, along with the companion diagnostic test for BRAF mutation (8), is a successful example of cancer therapy stratification based on molecular-level information. As with other oncogenic kinases, however, clinical response to inhibition of BRAF\textsuperscript{V600} is highly variable, and the acquisition of resistance in patients initially responsive to therapy is usually rapid (5–7). A wide variety of mechanisms of innate and acquired resistance have been identified, most of which are a function of the redundancy of the pro-proliferative signaling cascade being targeted (9, 10, 28). Data generated during the preclinical development of vemurafenib suggested several points of interaction with proteins involved in xenobiotic metabolism. These have the capacity to alter PK parameters, and hence the therapeutic effectiveness of this agent (11, 12).

In agreement with the preclinical data (11, 12), we found that CYP3A4 was the major CYP isoform involved in vemurafenib metabolism in vitro, but that the rate of metabolism was low.

Figure 5.

PXR regulates vemurafenib bioavailability through the modulation of efflux transporters. A, PK profiles of vemurafenib in huPXR/huCAR/huCYP3A4/3A7 mice treated with vehicle/vehicle (open circles; n = 3), vehicle/elacridar (filled circles, dashed line; n = 3), RIF/vehicle (open squares; n = 3), and RIF/elacridar (filled squares, dashed line; n = 3). Mean data ± SEM are shown. B, Western blots of hepatic microsomal fractions from huPXR/huCAR/huCYP3A4/3A7 mice dosed with either CO (vehicle) or RIF, then with either DMSO/Tween 80/ethanol/water (vehicle) or elacridar 90 minutes before vemurafenib, alongside HLM preparations; pooled and low, medium, and high CYP3A4 activity.

Vemurafenib is a weak inducer of PXR in vivo.

We administered the high bioavailability MBP form of vemurafenib at a dose of 100 mg/kg twice daily for 4 days to huPXR/huCAR/huCYP3A4/3A7 mice, as described in Materials and Methods. The PK parameters of a single 100-mg/kg dose (Supplementary Table S7) were in good agreement with those calculated in a previous study (22). Western blotting of liver proteins demonstrated weak induction of CYP3A4 and Cyp2b, and moderate induction of Cyp1a (Fig. 7A). Analysis of small-intestinal mRNA revealed no change in expression in CYP3A4, Abcb1a, or Abcb1b (Fig. 7B).

Figure 6.

Vemurafenib activates PXR in vitro. DPX2 cells were used to calculate the EC\textsubscript{50} value (8.13 μmol/L) and E\textsubscript{max} (8.54-fold) for vemurafenib-mediated transactivation of PXR.

Figure 7.

Vemurafenib activates PXR and possibly other transcription factors in vivo. A, Western blots of hepatic microsomal fractions from huPXR/huCAR/huCYP3A4/3A7 mice dosed with either vehicle (4% Klucel LF, pH 4.0, n = 4) or MBP vemurafenib (n = 4) twice daily at 100 mg/kg (8 hours apart) for 4 days. B, TaqMan RT-PCR of CYP3A4, Abcb1a and Abcb1b in duodenum (Duo), jejunum (Jej), and ileum (Ile) of huPXR/huCAR/huCYP3A4/3A7 mice dosed with either vehicle or vemurafenib. Data shown are mean of triplicate analyses ± SD.
also found that vemurafenib has the capacity to inhibit CYP3A4, with an \( IC_{50} \) value for BQ activity of 14.9 \( \mu \text{mol/L} \). In addition, and in conflict with the manufacturer’s findings (11, 12), we identified a minor role of CYP2C9 in generating OH-vemurafenib, evidenced by a small but statistically significant decrease in OH-vemurafenib in pooled HLM coincubated with the 2C9-specific inhibitor, sulfaphenazole.

In a previous study, mass balance analysis in 7 patients at steady state found that, in serum samples withdrawn up to 48 hours after administration of a radiolabeled dose of vemurafenib, 95% of recovered radioactivity was in the parental form (11, 12, 15). There was an increase in (the CYP3A4-generated form of) OH-vemurafenib from 0.5% to approximately 4% over this time period. It took 18 days for 95% of the radioactive dose to be recovered; 94% in feces, 1% in urine (15). Metabolic profiling was carried out on the 68.5% of the total dose that was recovered within the first 96 hours. Over the 0- to 48-hour period, >94% of the radioactivity recovered was in the parental form. Over the 48- to 96-hour period, however, this value decreased to 55.5% ± 20.1%. OH-vemurafenib accounted for 13.7% of the radioactivity recovered from the 48- to 96-hour samples (3.4% of the total dose).

Conjugated (glucosylated and glucuronidated) forms of vemurafenib were the only other metabolites definitively identified in these samples (11, 12, 15). Although the percentage of vemurafenib excreted in the form of metabolites was low relative to the parent compound, it was acknowledged that a large proportion of the parent compound eliminated during the first 48 hours might constitute drug that had not been absorbed, and a significant proportion of vemurafenib excreted during the 0- to 96-hour post-dose period might have been generated by hepatobiliary recirculation. Indeed, it was also reported that metabolites predominated over the parent compound in a bile sample from one of the patients receiving vemurafenib (15). As the absolute bioavailability of vemurafenib is unknown, and hence the contribution of metabolism to its elimination cannot be calculated, phase IV clinical trials assessing the effect of an inhibitor and an inducer of CYP3A4 in the PK of vemurafenib have been requested by both the FDA and EMA. At present, an inhibitor (ketoconazole) study (NCT01765556) has been withdrawn and an inhibitor (rifampicin) study (NCT01765543) is recruiting (clinicaltrials.gov). Despite the occurrence of CYP-dependent hydroxylation of vemurafenib in vitro, we found that the in vivo effect of CYP3A4 in modulating the systemic levels of a single dose of the compound in humanized mice was small. Far more apparent was a decrease in \( AUC_{\text{CYP3A4}} \) following activation of PXR, which was independent of CYP3A4 as it occurred in both the Cyp3aK0/Cyp3a13/+/− and huCYP3A4/3A7−/− lines. This decrease was even more pronounced following activation of PXR in the huPXR/huCAR and huPXR/huCAR/huCYP3A4/3A7 mouse lines with Rif. The likely explanation for the differing magnitudes of this decrease is that, at the doses used, PCN-mediated activation of mPXR (and thereby induction of the downstream effector proteins) is less effective than Rif-mediated activation of hPXR (20).

Preincubation of vemurafenib-resistant clones of the BRAF\(^{V600E}\) positive A375 melanoma cell line with verapamil, an ABCB1 inhibitor, partially increased their sensitivity to the drug (29). Studies with the MDCK-ABC1 cell line demonstrated that vemurafenib was both an ABCB1 substrate and inhibitor (12), and a post-authorization report mentions evidence of an interaction with ABCG2 (30). These observations have been confirmed independently, and studies in mice null for these transporters either separately or in combination have found that they modulate both intestinal bioavailability and permeability at the blood–brain barrier (16, 17, 31). This synergistic interaction of Abcb1 and Abcg2 is a phenomenon that has been shown to modulate the bioavailability of other therapeutics (32). ABCB1 is a transcriptional target of PXR in human cells (33, 34), and this interaction is thought to be conserved for Abcb1a in the mouse (35, 36). There is some evidence that PXR also regulates Abcg2 (37). Our measurements of Abcb1a, Abcb1b, and Abcg2 mRNA levels in the small intestine of huPXR/huCAR/huCYP3A4/3A7 mice demonstrate that, of these, only the first was induced following Rif treatment. Activation of CAR by PB induced Cyp2b10 to the same extent as activation of PXR by Rif, while also weakly inducing CYP3A4, but this pretreatment had no effect on the PK parameters of vemurafenib. Taken together, these observations suggest that the Pxr/PXR-dependent modulation of vemurafenib PK is likely to be due to the upregulation of Abcb1a, and hence a decrease in the bioavailability of vemurafenib, rather than to induction of drug metabolism enzymes. Although PXR regulates the transcription of other drug-metabolizing enzymes in humanized mice, such as UDP-glucuronosyltransferases (UGT) of the 1a subfamily (38), our contention that the observed effects are due to the modulation of transport are supported by the finding that administration of the Abcb1/Abcg2 inhibitor, elacridar, 90 minutes before administration of vemurafenib completely ablated the effect of Rif pretreatment in huPXR/huCAR/huCYP3A4/ 3A7 mice. Furthermore, elacridar pretreatment of uninduced (i.e., not pretreated with Rif) huPXR/huCAR/huCYP3A4/3A7 mice increased \( AUC_{\text{CYP3A4}} \) of vemurafenib, further supporting a role for PXR in the regulation of vemurafenib PK. These observations suggest that inhibition of efflux transporters in the basal state can profoundly increase vemurafenib bioavailability. It should be noted that, although elacridar is often cited as a specific inhibitor of ABCB1/Abcb1 and ABCG2/ Abcg2, this is not the case. At the dose used in this study, the predicted plasma concentration of elacridar is an order of magnitude below the \( IC_{50} \) value for CYP3A4 and other P450s (26, 27). However, this compound is known to inhibit SLCO1B1 (39) and is highly likely to inhibit other transporters (40). Therefore, although we cannot conclusively state that PXR-directed modulation of Abcb1a level in the small intestine is the pivotal determinant of \( AUC_{\text{CYP3A4}} \) of vemurafenib, this remains the most likely explanation. In support of this, studies in the Abcb1a/b and Bcrp knockout mouse lines suggested a predominant role for the former transporter (16). Increased drug efflux mediated by ABC transporter proteins in the cancer cell is a mechanism of multidrug resistance that has been understood for nearly 40 years (41). As a class of therapeutics, tyrosine kinase inhibitors, such as vemurafenib, are no less vulnerable to this process than conventional chemotherapeutics (42). The demonstration that drug transporters can markedly affect \( in vivo \) vemurafenib PK implies that changes in their expression in the tumor itself will also be a factor in drug response. Surprisingly, to date, this possibility has not been investigated.

During preclinical development, it was posited that decreases in the steady-state blood plasma concentrations of vemurafenib in dogs on a twice-daily dosing regimen between 29 and 92 days might be due to the induction of metabolism (11). Furthermore, in patients at steady state, vemurafenib decreased \( AUC_{\text{CYP3A4}} \) of the CYP3A4-specific probe, midazolam, by 39%, increased \( AUC_{\text{CYP3A4}} \) of the CYP1A2 probe, caffeine, by 160%, and increased \( AUC_{\text{CYP3A4}} \) of the CYP2D6 probe, dextromethorphan, by 47% (11, 12). This
defined vemurafenib as an inducer of CYP3A4, a moderate inhibitor of CYP1A2 and a weak inhibitor of CYP2D6. In agreement with these findings, we observed strong induction of CYP3A4-promoter–driven luciferase activity in a cell line–based reporter assay for PXR with crystalline vemurafenib. During the phase 1 clinical trial, vemurafenib was reformulated from a crystalline to an MBP form to improve bioavailability (5). With this formulation, AUC_{C6} is proportional to doses of between 240 and 960 mg twice daily (2, 5, 43). In the present study, we administered MBP vemurafenib to huPXR/huCAR/huCYP3A4/3A7 mice to assess the drug–drug interaction potential of this compound in this mouse model in vivo. Moreover, the AUC_{all} of 1,142 ± 242 h·μmol/L observed at this dose is approximately four times the AUC_{C6} of 300 h·μmol/L cited as being necessary to effect regression of COLO205 xenografts (44) and, with twice-daily dosing, was considered to be sufficiently representative of the steady state level of exposure in humans [AUC_{C6} = 1,741 ± 639 h·μmol/L; ref. 5]. In addition, the C_{max} of this single dose, at 121.7 ± 19.7 μmol/L is above the mean value of 86 ± 32 μmol/L reported in patients at steady state (5). With these parameters, in silico analysis predicted that steady-state concentrations in mice would be achieved within approximately 32 hours (i.e., with the fourth dose) on a schedule of two doses per day, 8 hours apart (data not shown). Under these conditions we observed induction of CYP3A4 in the liver of huPXR/huCAR/huCYP3A4/3A7 mice administered a high dose of the MBP formulation twice daily for 4 days, although the level of this induction was slight and highly variable. Nevertheless, our in vitro and in vivo data demonstrate that vemurafenib has the capacity to induce CYP3A4. In addition, we observed induction of Cyp1a and Cyp2b proteins in huPXR/huCAR/huCYP3A4/3A7 mice. These effects may, in part, be due to activation of PXR, but are likely also due to the activation of one or more additional transcription factors, such as the aryl hydrocarbon receptor (45). In addition to possible overall effects on drug transporters and the consequent modulation of bioavailability, these factors are likely to be a more important factor in defining disposition in vivo. In addition, we have demonstrated that vemurafenib has the capacity to activate PXR, and potentially other transcription factors, at clinically relevant concentrations. Taken together, our observations support the case for therapeutic monitoring of vemurafenib levels in patients over the course of therapy to evaluate whether low systemic drug concentrations coincide with innate or acquired resistance, and to determine whether modulators of PXR/CYP3A4/ABCB1 activity influence the outcome of vemurafenib therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: A.K. MacLeod, L.A. McLaughlin, C.J. Henderson, C.R. Wolf
Development of methodology: A.K. MacLeod, L.A. McLaughlin, C.R. Wolf
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. MacLeod, L.A. McLaughlin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. MacLeod, L.A. McLaughlin, C.R. Wolf
Writing, review, and/or revision of the manuscript: A.K. MacLeod, L.A. McLaughlin, C.J. Henderson, C.R. Wolf
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.A. McLaughlin, C.R. Wolf
Study supervision: C.J. Henderson, C.R. Wolf

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Vemurafenib Pharmacokinetics in Humanized Mice


Activation Status of the Pregnane X Receptor Influences Vemurafenib Availability in Humanized Mouse Models

A. Kenneth MacLeod, Lesley A. McLaughlin, Colin J. Henderson, et al.

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