# Investigations into the Mechanisms of Pyridine Ring Cleavage in Vismodegib<sup>S</sup>

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## ABSTRACT

Vismodegib (Erivedge, GDC-0449) is a first-in-class, orally administered small-molecule Hedgehog pathway inhibitor that is approved for the treatment of advanced basal cell carcinoma. Previously, we reported results from preclinical and clinical radiolabeled mass balance studies in which we determined that metabolism is the main route of vismodegib elimination. The metabolites of vismodegib are primarily the result of oxidation followed by glucuronidation. The focus of the current work is to probe the mechanisms of formation of three pyridine ring-cleaved metabolites of vismodegib, mainly M9, M13, and M18, using in vitro, ex vivo liver perfusion and in vivo rat studies. The use of stable-labeled ( $^{13}C_{2}$ ,  $^{15}N$ )vismodegib on the pyridine ring exhibited that the loss of carbon observed in both M9 and M13 was from the C-6 position of pyridine. Interestingly, the source of the nitrogen atom in the amide of M9 was from the pyridine. Evidence for the formation of aldehyde intermediates was observed using trapping agents as well as  $^{18}O$ -water. Finally, we conclude that cytochrome P450 is involved in the formation of M9, M13, and M18 and that M3 (the major mono-oxidative metabolite) is not the precursor for the formation of these cleaved products; rather, M18 is the primary cleaved metabolite.

## Introduction

Vismodegib is an orally active, small molecule that is among the new generation of targeted therapies for oncology. This drug targets the Hedgehog signaling pathway that is activated in certain types of cancer and is currently approved for treatment of metastatic or locally advanced basal cell carcinoma (Scales and de Sauvage, 2009). Further investigations are under way for other indications. Vismodegib was optimized for suitable potency; selectivity; and absorption, distribution, metabolism, and excretion properties (Robarge et al., 2009; Wong et al., 2009; Castanedo et al., 2010). Its unique absorption, distribution, metabolism, and excretion properties contribute to vismodegib's prolonged circulating half-life (Wong et al., 2010; Giannetti et al., 2011; Graham et al., 2011a, 2012). Here we report on studies to determine the mechanism of formation of three ring-opened metabolites formed as a result of metabolism of the pyridine moiety.

We previously reported the identification and characterization of vismodegib's metabolites (Wong et al., 2009, 2010; Graham et al., 2011b; Yue et al., 2011). After a single dose of <sup>14</sup>C-vismodegib, the radioactivity was predominately excreted in feces (80%–90% in rat, dog, and healthy human subjects), partly as unabsorbed material. Urine accounted for less than 5% of the dose in all species tested. The same studies showed that absorbed vismodegib underwent extensive oxidative

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and conjugative metabolic reactions on the basis of metabolite profile analyses. The amount of unchanged drug present in urine and bile accounted for less than 1% in rats and less than 5% in dogs. In humans, the percentage of absorbed drug could be estimated only on the basis of absolute bioavailability studies and human mass balance studies, and it was clear that hepatic elimination predominates for vismodegib.

The major primary metabolites of vismodegib were oxidative, with the oxidation taking place mainly on the pyridine and chlorophenyl rings, followed by glucuronide, sulfonate, or, to a lesser extent, glucose conjugation. The major metabolites were qualitatively similar in all species. The phase 2 conjugates were observed in the urine and bile samples from rats and dogs and were not detected in feces, which is consistent with gut bacteria hydrolysis of these conjugates to their respective oxidative metabolites. None of these metabolites was circulating in plasma at significant levels (<1% of total drug-related material).

In addition to simple oxidative products on the pyridine and chlorophenyl rings to form phenol-type metabolites, three pyridine ring cleavage metabolites were found in excreta (Yue et al., 2011) (Fig. 1). The literature precedence for such a reaction is limited. These metabolites were designated M9, M13, and M18 and were characterized using high-resolution mass spectrometry (MS) and, in the case of M13, also by <sup>1</sup>H NMR (Yue et al., 2011). Different amounts of these metabolites were found in the excreta of each species. In the rat, M9 was 4.4% of the administered dose, and M18 and M13 were barely detectable (<1%), whereas in dog and human, M13 was the major metabolite, making up 10% and 3% of the dose, respectively. M18 constituted 4.5% and 2% of the dose in dogs and humans, respectively, and M9 was not detected in either species.

**DRUG METABOLISM & DISPOSITI** 

ABBREVIATIONS: ACN, acetonitrile; ABT, 1-aminobenzyltriazole; FA, formic acid; MS, mass spectrometry; TBA, thiobarbituric acid.

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Fig. 1. Chemical structures of vismodegib, M3, M9, M13, and M18.

To probe the mechanisms for formation of these pyridine ring cleavage products, several studies were carried out, including in vitro studies using liver microsomes and long-lived hepatocyte cultures. Except in the monkey, the very low intrinsic clearance in rat, dog, and human liver microsomes posed a significant challenge for effectively studying the formation of these metabolites in vitro; therefore, we experimented with various systems. These studies included ex vivo rat liver perfusion and in vivo studies under various conditions, including pretreatment with 1-aminobenzotriazole (ABT; a broad cytochrome P450 inactivator). Stable-labeled vismodegib with two <sup>13</sup>C atoms and one <sup>15</sup>N atom on the pyridine [( $^{13}C_2$ ,  $^{15}N$ )vismodegib] (Fig. 2) was synthesized to examine the source of the atoms that constitute the cleaved side chain. In addition, we investigated whether the other oxidative products are the precursors for the formation of these cleaved products.

## Materials and Methods

## Chemicals

High-performance liquid chromatography grade acetonitrile (ACN), ammonium formate, ammonium hydroxide, diethyl ether, ethyl acetate, formic acid (FA), methanol, and water were purchased from either Mallinckrodt Baker, Inc. (Phillipsburg, NJ) or EM Science (Gibbstown, NJ). Ammonium acetate (analytical grade), 1-aminobenzyltriazole (ABT), methoxylamine, thiobarbituric acid (TBA), nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), bacitracin, neomycin sulfate, and streptomycin sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). <sup>18</sup>O-Water (97% isotopic purity) was



Fig. 2. Chemical structure of (<sup>13</sup>C<sub>2</sub>,<sup>15</sup>N)vismodegib.

obtained from Santa Cruz Biotechnology (Dallas, TX). Sprague-Dawley rat (Lot 62547), cynomolgus monkey (Lot 61370), and human (Lot 38289) liver microsomes were obtained from BD Biosciences (San Jose, CA). Vismodegib, ( $^{13}C_2$ ,  $^{15}N$ )vismodegib, *N*-oxide of vismodegib, and M3 (Supplemental Method) were synthesized at Genentech, Inc. (South San Francisco, CA).

## In Vitro Studies

Liver Microsomal Incubations. Vismodegib (5  $\mu$ M), *N*-oxide of vismodegib (5  $\mu$ M), or M3 (5  $\mu$ M), was incubated in liver microsomes (1.0 mg/ml), NADPH (1 mM), and MgCl<sub>2</sub> (2 mM) in potassium phosphate buffer (100 mM, pH 7.4) with or without ABT (1 mM). Vismodegib (5  $\mu$ M) was also incubated in cynomolgus monkey liver microsomes (1.0 mg/ml), NADPH (1 mM), and MgCl<sub>2</sub> (2 mM) in potassium phosphate buffer (100 mM, pH 7.4) with methoxylamine (5 mM) or thiobarbituric acid (5 mM) or incubation mixture containing approximately 50:50 <sup>18</sup>O·<sup>16</sup>O-water. The liver microsomes were warmed at 37°C for 5 minutes before reaction initiation by addition of the test article and cofactor mixture. The incubation lasted 60 minutes at 37°C and was quenched with three volumes of cold acetonitrile. Samples were centrifuged at 3000g for 5 minutes, and the supernatant was analyzed as described as follows.

**Hydrolysis of M18.** An aliquot of a rat bile sample was extracted with ethyl acetate, and the organic phase was removed and dried as previously reported (Yue et al., 2011). The sample was reconstituted in <sup>18</sup>O-water with or without sulfuric acid (0.5 N). The mixture was incubated for 1 hour at 50°C and extracted with ethyl acetate. The organic fraction was dried and reconstituted in the mobile phase before analysis.

HepatoPac Assays. Vismodegib (10  $\mu$ M) was incubated with cocultured HepatoPac as described previously by Wang et al. (2010) for 7 days. Micropatterned cocultures were generated from Sprague-Dawley rat, beagle dog, cynomolgus monkey, and human hepatocytes. Cultured hepatocytes for all species except dog were cryopreserved, whereas dog hepatocytes were freshly isolated. At the end of the incubation, cell viabilities were assessed using the Cell Titer-Glo Luminescent cell viability assay (Promega, Madison, WI) according an established protocol (Crouch et al., 1993). The culture medium was removed from the well and analyzed as described as follows.

## **Ex Vivo Studies**

**Rat Liver Perfusion.** Rats (n = 2) were pretreated i.p. 2 hours before dosing of the test article with or without ABT at 100 mg/kg, using 50 mg/ml solution (dose volume 2 ml/kg). Vismodegib or M3 (each at 20  $\mu$ M) was perfused separately with isolated rat livers for 3 hours. The isolated liver perfusion was performed as described previously (Mehvar and Reynolds, 1995). Briefly, adult male

Sprague-Dawley rats were anesthetized by i.p. injection of a ketamine/xylazine cocktail and, after opening the abdominal cavity, the hepatic portal vein was catheterized with a 14-gauge i.v. catheter to serve as the inlet. After cutting the diaphragm, another i.v. catheter, inserted into the thoracic inferior vena cava, served as the outlet. The liver was then excised and transferred to a temperaturecontrolled perfusion tray. The isolated livers were perfused using a commercial perfusion apparatus MX Perfuser II from MX International (Aurora, CO) in a recirculating manner. The perfusate consisted of a Krebs-Henseleit bicarbonate buffer (pH 7.35-7.45) containing glucose (5.0 g/l), bovine serum albumin (4%, w/v), and bovine red blood cells (20%, v/v) delivered at a flow rate of 12 ml/min. The perfusate was oxygenated with an O2:CO2 (95:5) mixture for at least 30 minutes before entering the liver. Perfusate and bile samples were collected, and the liver was harvested. Liver samples were homogenized with a 4-fold volume of water, and aliquots of liver homogenates and the perfusate were subjected to liquidliquid extraction with ethyl acetate. The extracts were dried down and reconstituted in 30% ACN in water followed by centrifugation at 3000g for 2 minutes. Bile samples were centrifuged at 3000g at 4°C for 10 minutes to remove undissolved solids. The viability of the liver was confirmed through the overall macroscopic appearance of the liver, the color of the inlet and outlet perfusate, and the extent of perfusate and bile flow. Samples were analyzed as described as follows.

#### In Vivo Studies

**Stable-Labeled Vismodegib In Vivo Metabolites.** One rat was orally administered a single dose of stable-labeled vismodegib at a target dose of 50 mg/kg formulated in 0.5% methylcellulose and 0.2% Tween 80 in reverse osmosis water (pH 3.0). The animal was treated as described previously (Wang et al., 2010). Feces and urine were collected from metabolic cages on dry ice at 0-8, 8-24, and 24-48 hours after the dose and stored at  $-70^{\circ}$ C before analysis.

#### Liquid Chromatography-Mass Spectometry Analysis

The liquid chromatography-mass spectrometry system consisted of an Accela UPLC (Thermo Fisher Scientific, San Jose, CA) and an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Separation was achieved on a Hypersil Gold C18 column (100  $\times$  2.1 mm, 1.9  $\mu$ m; Thermo Fisher Scientific) at a flow rate of 0.4 ml/min. A binary mobile phase consisted of water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The highperformance liquid chromatography gradient was initiated at 5% B for 1 minute, increased to 20% B in 11 minutes, followed by a gradient to 40% B over 9 minutes, and then changed to 60% over 2.9 minutes. The gradient was rapidly ramped to 95% B in 0.1 minute and remained at 95% B over 1.8 minutes. The gradient was then returned to the initial condition of 5% B in 0.2 minute and equilibrated at 5% B for 4 minutes before the next injection. The total run time was 30 minutes. A longer gradient was used to achieve better separation of methoxylamine or thiobarbituric acid trapped adducts from the incubation of vismodegib with cynomolgus monkey liver microsomes. The gradient was initiated at 5% B for 2 minutes, increased to 25% B in 38 minutes, followed by a gradient to 45% B over 10.5 minutes. The gradient was rapidly ramped to 95% B in 0.5 minute and remained at 95% B over 4.8 minutes. The gradient was then returned to the initial condition of 5% B in 0.2 minute and equilibrated at 5% B for 4 minutes before the next injection. The total run time was 60 minutes.

The LTQ-Orbitrap mass spectrometer was operated in the positive ion mode with electrospray ionization source. The ion source conditions were as follows: spray voltage, 4.0 kV; sheath and auxiliary gas (N<sub>2</sub>) flow, 60 and 10 (arbitrary units), respectively; capillary temperature, 300°C; and source heater temperature, 150°C. The scan event cycle consisted of a full-scan mass spectrum at a resolving power of 30,000 and the corresponding data-dependent tandem MS (MS/MS) and multistage mass spectrometry (MS<sup>3</sup>) scans acquired at a resolving power of 7500 with collision energy of 30% and an isolation mass window of 2 Da. To separate the isotope peaks of <sup>37</sup>Cl- from <sup>18</sup>O-, the full-scan mass spectra were acquired at a mass resolving power of 100,000. Data were processed using Xcalibur software 2.2 SP1 (Thermo Fisher Scientific).

## Results

The results from vismodegib incubations with liver microsomes were the formation of three pyridine ring–opened metabolites (M9, M13, and M18; Table 1). In liver microsomes from rat, dog, and human, no

Generation of the cleaved metabolites M9, M13, and M18, from incubations of vismodegib with liver microsomes, hepatocytes, and HepatoPac from various species

TABLE 1

Matrix	Species	Cofactor	M9	M13	M18
Liver microsomes	Rat	+N	_	_	_
	Dog	+N	-	-	-
	Monkey	-N	-	-	-
		+N	+	-	+
		+N+ABT	-	-	-
	Human	+N	-	-	-
HepatoPac	Rat	NA	-	-	_
	Dog	NA	-	-	_
	Monkey	NA	-	-	+
	Human	NA	_	-	+

-, No formation of metabolite; +, formation of metabolite; ABT, 1-aminobenzotriazole; +N, with NADPH; -N, without NADPH; NA, not applicable.

cleaved products were detected. On the other hand, in monkey liver microsomes, M9 and M18 were detected, but M13 was not. Formation of these metabolites was NADPH-dependent and decreased by more than 90% after ABT preincubation, suggesting that cytochrome P450 enzymes are involved in their formation. We also examined whether other metabolites could be the precursor for the formation of the openring metabolite. For this reason, *N*-oxide vismodegib and M3 were incubated in cross-species liver microsomes but did not result in the formation any of the pyridine-cleaved metabolites.

In monkey liver microsomes, to test the formation of aldehyde intermediate, aldehyde trapping agents (methoxylamine and TBA) were coincubated with vismodegib. With methoxylamine, one trapped metabolite was detected with a protonated molecular ion at m/z 511.06021, indicating that its empirical formula is  $C_{21}H_{21}Cl_2N_4O_5S$  (mass accuracy, -0.41 ppm). The product ion mass spectrum of m/z 511 contains fragment ions at m/z480, 479, 449, and 448 (Fig. 3A; Supplemental Fig. 1). The fragment ions at m/z 480 and 479 resulted from loss of methoxyl radical and methanol from the protonated molecular ion, respectively. The fragment ions at m/z449 and 448 were further loss of methoxyl radical and methanol from the fragment ion at m/z 480, which indicated two methoxylamine molecules reacted with two aldehydes. The  $MS^3$  spectrum of m/z 449 resulted in fragment ions at m/z 431, 422, 414, 395, 370, 232, and 217 (Fig. 3A; Supplemental Fig. 1A). The characteristic fragment ion at m/z 217 was the same as unchanged vismodegib, suggesting that 2-chloro-4-methylsulfonyl benzene moiety remained unchanged. The fragment ion at m/z 370 resulted from loss of methylsulfonyl benzene radical (78.93538 Da). Mass accuracies for all fragment ions were within  $\pm$  5 ppm of the proposed structures, and the mechanism of its formation is shown in Supplemental Fig. 2. TBA reacted with an  $\alpha,\beta$ -unsaturated aldehyde intermediate to form a cyclic conjugate indicative of a ring-opened intermediate (Fig. 3B; Supplemental Fig. 1B). It had a protonated molecular ion at m/z 578.99528, which is consistent with a molecular formula of C23H16Cl2N4O6S2 (mass accuracy -1.43 ppm). The product ion mass spectrum of m/z 579 produced a fragment ion at m/z 551 by loss of a carbon monoxide. The MS<sup>3</sup> spectrum of m/z 551 resulted in fragment ions at m/z 534, 475, and 449 by further loss of ammonia, thiourea, and 4-thioxo-1,3-diazetidin-2-one, respectively. Mass accuracies for all fragment ions were within  $\pm 4$  ppm of the proposed structures and the mechanism of its formation is shown in Supplemental Fig. 2.

Vismodegib was also incubated with monkey liver microsomes in 50:50 of <sup>16</sup>O-water:<sup>18</sup>O-water, which resulted in incorporation of one <sup>18</sup>O atom into the carboxylic acid of M18. This was evident by the doublet peaks at m/z 471.01725 and 473.02141 with approximately equal intensity (Supplemental Fig. 3). Similarly, the presence of doublet peaks at m/z 443.02237 and 445.02659 suggested that one



Fig. 3. Interpretation of fragment ions of (A) methoxylamine-trapped conjugates at molecular ion at m/z 511 and (B) thiobarbituric acid (TBA) trapped conjugate at m/z 579. Vismodegib (5  $\mu$ M) was incubated with monkey liver microsomes (1 mg/ml) fortified with NADPH and methoxylamine (5 mM) or TBA (5 mM).

<sup>18</sup>O- atom was incorporated into M9. Using high-resolution MS at a mass resolving power of 100,000, the contribution of <sup>37</sup>Cl- from <sup>18</sup>O- in M9 and M18 were separated (Supplemental Fig. 3).

Vismodegib was incubated for 7 days in HepatoPac cocultures. During this period, no changes in cell viability of the cocultures were observed. Supernatants were profiled, and the metabolites formed were matched to in vivo samples on the basis of accurate masses and retention times. M18 was detected in cocultures containing human and monkey hepatocytes but not in rat or dog cocultures. Formation of this metabolite was greatest in monkeys (normalized to 100%) and was 36% in humans on the basis of the MS signal.

To examine whether M18 was the primary metabolite leading to formation of M13 and M9, a bile sample from a rat dosed with vismodegib was incubated under acidic conditions. The ethyl acetate extract from rat bile, which contained M18 and M13 among other metabolites of vismodegib, was dried and incubated in <sup>18</sup>O-water in the presence of 0.5 N sulfuric acid for 1 hour at 50°C. Under these conditions, one <sup>18</sup>O atom was incorporated into the carboxylic acid of M13 and no changes to M9 (Fig. 4). For M13 formed from the incorporation of one <sup>18</sup>O atom, we observed the generation of m/z463.03660 ( $[M + NH_4]^+$ ) for addition of <sup>18</sup>O versus *m/z* 461.03250 for the addition of <sup>16</sup>O. Using high-resolution MS, we were also able to separate the contribution of <sup>35</sup>Cl/<sup>37</sup>Cl from <sup>16</sup>O/<sup>18</sup>O. For M13 generated from M18, two atoms of <sup>18</sup>O atoms were incorporated, resulting in the molecular ion at m/z 465.04084. Of the two <sup>18</sup>O atoms incorporated into M13, one was at the carboxylic acid and one other incorporated into the ketone (Fig. 4). With high-resolution MS, the mass difference showing the incorporation of two <sup>18</sup>O atoms confirmed the formation of M13 from M18. In summary, under these experimental conditions, M18 did lead to the formation of M13 but not M9.

To understand the role of the liver in the formation of M9, M13, and M18, rat liver perfusion studies were conducted. Vismodegib was continuously perfused to the isolated rat liver for 3 hours. Detectable levels of M18, but not M13 or M9, plus other oxidative metabolites of vismodegib that were reported previously were found in the perfusate and bile samples. Interestingly, another cleaved metabolite, MA, which had not been previous detected, was discovered. Formation of these metabolites (M18 and MA) decreased after the pretreatment of rats with ABT (Fig. 5). MA has the same elemental composition as M9, but it has a different retention time and fragment ion spectrum under collision-induced

dissociation. MA eluted at 17.6 minutes (whereas M9 eluted at 16.6 minutes) and had a protonated molecular ion at m/z 443.0225, indicating that its empirical formula is  $C_{18}H_{17}O_5N_2Cl_2S$  (-1.06 ppm; Fig. 5). The product ion scan at m/z 443 from MA produced fragment ions at m/z 425.99814 (loss of NH<sub>3</sub>), 425.01404 (loss of H<sub>2</sub>O), 397.01899 (a loss of formic acid), and 385.01910 (loss of oxiran-2-one) (Fig. 6). The MS<sup>3</sup> spectrum of m/z 425 gave characteristic fragment ions at m/z 216.97319 and 154.99118, which suggests that the 2-chloro-4-methylsulfonyl benzene moiety remained unchanged and that the modification occurred on the pyridine-chloroaniline moiety. The loss of formic acid from m/z 443 to form m/z 397 indicates that MA has an aliphatic carboxylic acid moiety.

Stable-labeled vismodegib was synthesized by incorporating two <sup>13</sup>C atoms at the C-2 and C-6 positions plus one <sup>15</sup>N atom on the pyridine (Fig. 2). Metabolites excreted in feces from intact rats dosed orally with this labeled vismodegib were compared with those excreted from rats dosed with vismodegib in previous studies. Table 2 summarizes the findings from this study. As expected, the molecular ion of labeled vismodegib is 3 Da higher than that of nonlabeled vismodegib. The fragment ions from labeled vismodegib also include the stable labels. M9 is the amide metabolite, and the interpretation of its collision-induced dissociation is shown in Fig. 7. The difference of 2 Da between M9 and stable-labeled M9 is indicative of a loss of one of the labeled atoms from the original parent. The neutral loss of ammonia (NH<sub>3</sub>) from M9 and its stable-labeled analog lead to the formation of m/z 426 and 427, respectively. This suggests that the nitrogen atom on the amide was the same nitrogen atom originally located on the pyridine ring. The other fragment ions confirmed that the labeled carbon retained on the molecule was at the C-2 position, suggesting that the labeled carbon at the C-6 position was lost. Metabolite M13 is a carboxylic acid metabolite, and there is a 1 Da difference between M13 and its stablelabeled analog. Based on the fragment ion interpretation, only one label was retained at the C-2 position. M18 has a molecular ion consistent with all three stable-labeled atoms being retained in the compound. The summary of these findings is described in Fig. 1.

#### Discussion

In previous in vivo studies, vismodegib was shown to form three pyridine ring-opened metabolites M9, M13, and M18 (Graham et al.,



Source	Elemental Analysis of	Intensity	Observed	Theoretical	Accuracy <sup>b</sup>
	M13	(%)	$[M + NH_4]^+$	$[M + NH_4]^+$	(ppm)
			( <i>m/z</i> ) <sup>a</sup>	( <i>m/z</i> )	
No <sup>18</sup> O incorporation	C <sub>18</sub> H <sub>15</sub> NO <sub>6</sub> S <sup>35</sup> Cl <sub>2</sub>	96	461.03250	461.03354	-2.26
	C <sub>18</sub> H <sub>15</sub> NO <sub>6</sub> S <sup>35</sup> Cl <sup>37</sup> Cl	48	463.02965	463.03059	-2.03
	C <sub>18</sub> H <sub>15</sub> NO <sub>6</sub> S <sup>37</sup> Cl <sub>2</sub>	7	465.02729	465.02764	-0.75
M13→M13°	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>5</sub> <sup>18</sup> OS <sup>35</sup> Cl <sub>2</sub>	100	463.03660	463.03778	-2.55
	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>5</sub> <sup>18</sup> OS <sup>35</sup> Cl <sup>37</sup> Cl	52	465.03377	465.03483	-2.28
	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>5</sub> <sup>18</sup> OS <sup>37</sup> Cl <sub>2</sub>	6	467.03079	467.03188	-2.33
M18→M13 <sup>d</sup>	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>4</sub> <sup>18</sup> O <sub>2</sub> S <sup>35</sup> Cl <sub>2</sub>	20	465.04084	465.04203	-2.56
	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>4</sub> <sup>18</sup> O <sub>2</sub> S <sup>35</sup> Cl <sup>37</sup> Cl	14	467.03793	467.03908	-2.46
	C <sub>18</sub> H <sub>15</sub> N <sup>16</sup> O <sub>4</sub> <sup>18</sup> O <sub>2</sub> S <sup>37</sup> Cl <sub>2</sub>	2	469.03449	469.03613	-3.50

Fig. 4. Molecular ion of M13  $([M+NH_4]^{\dagger})$  from M18 and M13 present in a rat bile sample after incubation with  $H_2^{18}O$  under acidic conditions.

<sup>a</sup> The observed m/z values were based on the ammonium ion (NH<sub>4</sub><sup>+</sup>) adduct

 $^{\rm b}$  Accuracy was measured by subtracting the observed  $\it m/z$  from the theoretical

m/z and dividing by the theoretical mass multiplied by a million.

<sup>c</sup> One <sup>18</sup>O atom was incorporated into the acid of M13.

 $^{\rm d}$  Two  $^{\rm 18}{\rm O}$  atoms were incorporated into M13, one in the acid and the other in the ketone.

2011b; Yue et al., 2011). These metabolites were excreted mainly in feces, with the greatest amount in the dog (15%), followed by similar amounts in the excreta of human and rat ( $\sim$ 5%). M18 and M13 were detected in all species, but M9 was detected only in rats. Here we investigated the formation of these ring-opened metabolites to

understand the mechanisms of their formation as well as P450 involved in this biotransformation.

The biotransformation of a pyridine moiety resulting in a ring-opened metabolite is rarely reported. Pyridine is an aromatic heterocycle that is slightly basic in nature and is commonly found in key biologic systems





**Fig. 6.** Product ion spectra for metabolite MA at m/z 443 with (A) MS<sup>2</sup> and (B) MS<sup>3</sup>.

such as electron carriers (NADH and NADPH) and nucleotides, as well as in many pharmaceutical drugs. Pyridine moiety, compared with phenyl rings, is attractive to pharmaceutical scientists because it imparts increased solubility and metabolic stability against cytochrome P450 enzymes (St. Jean and Fotsch, 2012). This stability is due mainly to the electronegativity of nitrogen that makes the ring electron deficient and, therefore, less prone to electron abstraction. Other reports on metabolism of pyridine include N-oxidation, N-methylation (Damani et al., 1986), and N-glucronidation (Lin, 1999; Wiener et al., 2004). On the other hand, few cases of ring opening of pyridine have been reported, including microbial ring opening of pyridine derivatives (Kaiser et al., 1996), the formation of muconaldehyde from benzene mediated by cytochrome P450 (Latriano et al., 1986), and the formation of E-2-(acetaminomethylene) succinate from a vitamin B6 degradant (McCulloch et al., 2009). It is noteworthy that there are reports, albeit rare, on other six-membered aromatic heterocyclic ring-opening biotransformation, such as pyrimidine ring-cleaved metabolites of PF-00734200, a dipeptidyl peptidase inhibitor (Sharma et al., 2012).

ΤA	BL	Æ	2

Major collision-induced product ions of vismodegib and the stable-labeled vismodegib and their pyridine cleaved metabolites in feces of rats dosed orally with 50 mg/kg

Analyte	$[M + H]^+$	Major Fragment Ions <sup>a</sup>
	m/z	m/z
Vismodegib	421	342 (100), 313, 306, 217, 188, 155, 139
Labeled vismodegib	424	345 (100), 316, 309, 217, 191, 155, 139
M9	443	426 (100), 398, 380, 217, 155
Labeled M9	445	427 (100), 399, 381, 217, 155
M13	444	426 (100), 398, 380, 362, 301, 217, 155
Labeled M13	445	427 (100), 399, 381, 217, 155
M18	471	453 (100), 443, 426, 425, 397
Labeled M18	474	456 (100), 445, 427, 399

<sup>a</sup>Each base peak is labeled as 100 in parentheses.

Vismodegib provided an opportunity to further understand the mechanism of pyridine-ring cleavage; however, the compound posed a particular challenge for mechanistic studies. In vitro studies had limited utility because of the compound's low in vitro hepatic clearance in relevant species (rats, dogs, and humans); accordingly, these liver microsome studies generated small amount of metabolites. The monkey was the only species that generated an appreciable level of the ring-opened metabolites; however, the monkey was not one of the species studied for metabolism and preclinical toxicity because of the very high clearance. The mechanistic studies in monkey liver microsomes still provided the opportunity to further examine the mechanism of formation of the ring open metabolites. Aldehyde trapping agents methoxylamine and TBA confirmed the presence of two aldehyde moieties in an intermediate formed in the process of generating the open-ring metabolites (Fig. 3). <sup>18</sup>O-Water demonstrated that after the formation of ring-opened intermediate, there is an opportunity for exchange with water, most likely by aldehyde. The ring-opened metabolites were also generated by coculture hepatocytes, which can be maintained for a long incubation period. Even in this case, only small amounts of the M18 metabolite was generated in the human and monkey. We, therefore, complemented the in vitro data with studies using stable-labeled compound as well as ex vivo liver perfusion and in vivo studies.

The use of stable-labeled  $({}^{13}C_2, {}^{15}N)$ vismodegib (Fig. 2) allowed us to understand the source of the atoms in the ring-opened metabolites. Metabolites from the stable-labeled compound in the rat excreta confirmed that, as expected, M18 retained all three stable-labeled atoms, and M13 retained only one at the C-2 position. Surprisingly, M9 retained  ${}^{13}C$  atom at the C-2 position as well as the  ${}^{15}N$  at the primary amine. This means that the C-6 carbon (Fig. 1) was lost, followed by rearrangement of the pyridine nitrogen to form a terminal amide.

To understand the relationship between the three ring-opened metabolites, extracts from rat bile samples were treated with a low concentration of acid in the presence of  ${}^{16}$ O- or  ${}^{18}$ O-water. Under



Fig. 7. Product ion spectra and interpretations of (A) M9 and (B) stable-labeled M9 formed from the metabolism of vismodegib and its stable-labeled analog, respectively. The fragment ions displayed in the structures are based on theoretical masses.

these conditions, M18 converted to M13, but no or little conversion to M9 was observed. These results suggest that M18 conversion could be acid-catalyzed to M13, whereas the same was not true for the formation of M9 from M18. This finding was also consistent with the previous in vivo observations. If M9 were formed only by chemical degradation, then we would expect to detect M9 whenever M18 and M13 are detected; however, M9 is detected only in rats and not in humans or dogs despite M18 and M13 being present in measurable quantities.

Because of vismodegib's low rat hepatic intrinsic clearance, rat liver perfusion studies were used to study the formation of the ring-opened metabolites. In addition to finding detectable levels of M18 in the perfusate and bile samples, a new metabolite (MA) was identified that had not been previously observed in in vivo or in vitro studies. The role of P450 enzymes was established from pretreatment of ABT in a liver perfusion study. This pretreatment resulted in significantly decrease levels of M18 and MA, which is consistent with cytochrome P450 enzymes being involved in the formation of these metabolites.

To determine whether M18 might be formed via other metabolites, M3 and N-oxide vismodegib were synthesized and dosed in rats or incubated in vitro. M3 was a candidate to be the precursor to ringopened metabolites since this was the major oxidative metabolite detected in all species. *N*-oxide vismodegib, although not detected as a metabolite, could potentially be converted to the ring-opened metabolites. Neither of these compounds generated any of the ringopened metabolites that were not detected to a significant level in rat mass balance studies.

We also considered the involvement of gut microflora in the formation of these metabolites since pyridine ring-opening biotransformation has been reported (Kaiser et al., 1996). Preliminary studies with pretreatment of antibiotics did not result in diminished levels of the ring-opened metabolites. In addition, when vismodegib was incubated with rat feces under anaerobic conditions, the drug concentration remained stable and no formation of any metabolite was observed (data not shown).

On the basis of the results of these studies, we propose a mechanism in which M18 is the primary metabolite and the other cleaved products, M13 and M9, are formed from M18 via MA. The formation of M18 might occur through cytochrome P450–mediated epoxidation (Fig. 8, pathway A). The epoxide intermediate (Ia) could be hydrolyzed to a dihydrodiol intermediate and further oxidized that

could lead to ring-opened intermediate Ib. It is out of the scope of this manuscript to probe in this first step of oxidation for the formation of Ib, but one could also consider that cytochrome P450 enzymes act as a peroxidase (Vaz et al., 1998) and insert two oxygen atoms into the double bond between C-5 and C-6 to form a dioxetane Ia' intermediate (Fig. 8, pathway A). This is similar to the previously proposed mechanism, but not proven, for the formation of muconaldehyde from benzene oxidation by P450 (Latriano et al., 1986). The dioxetane intermediate (Ia') could rearrange to form the intermediate Ib (Fig. 8, pathway B). This intermediate could then rearrange by hydrogen cation abstraction to form a ketene intermediate Ie (pathway C). Alternatively, the Ib aldehyde could hydrate to form the Ic intermediate (pathway D), which could rearrange by hydrogen abstraction and eventually lead to formation of M18 (pathway F). Methoxylamine and TBA confirmed the presence of Ib and <sup>18</sup>O-water exchange confirmed intermediates Ic and Ie. M18 could undergo hydrolysis by loss of formic acid, leading to an amine and the generation of MA. M13 could be formed by further hydrolysis of M18 or MA (pathway H or I). The formation of M9 could possibly be enzyme mediated because this metabolite is detected only in the rat, despite M18 and M13 being present in measurable quantities in human and dog. We speculate that MA is the precursor for the formation of M9 since the amine has the ability to be more nucleophilic. This reaction could proceed in two possible ways. One is a nucleophilic attack of amine on C-5 to form 2-pyrroline. There are two considerations that could make this mechanism less likely. One is that the carboxylic acid is not usually susceptive to nucleophilic attack by amine under normal conditions; second, there is a chance that 2-pyrroline loses water and forms stable pyrrole, which was not detected. We therefore propose that the carboxylic acid could be activated similarly to the amino acid conjugation reactions (Knights et al., 2007). Once the carboxylic acid is activated (-ROX; Fig. 8, pathway J), amine could react with C-5 and eventually be converted to M9.

In summary, because of the limitations of in vitro tools, alternative methods, such as the use of stable-labeled vismodegib, in vivo and ex vivo studies, were used to investigate the vismodegib mechanism of ring cleavage in human and other preclinical species. M18 is the primary ring-opened metabolite formed in the liver through cyto-chrome P450 biotransformation. The subsequent hydrolysis of M18 leads to formation of MA, which could be the precursor for both M13 and M9. Under slightly acidic conditions, M13 is formed from M18;



Fig. 8. Proposed metabolic cleavage of vismodegib by cytochrome P450. Reactions with methoxylamine (CH3ONH2) and TBA are most likely with Ib intermediate.  $\times$  is to denote either hydroxyl or the modification of carboxylic acid to an activated form, perhaps similar to amino acid conjugation process via acetyl-CoA to form thioester.

however, M9 was not formed from M18 at significant levels, suggesting that the transformation to M9 may be enzymatic in nature.

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#### **Authorship Contributions**

Participated in research design: Khojasteh, Yue, Ma, Chen, Liu, Takahashi, Hop, Wong.

*Conducted experiments*: Yue, Ma, Castanedo, Chen, Mulder, Takahashi, Ly, Messick, Jia.

Contributed new reagents or analytic tools: Castanedo, Chen, Takahashi.

Performed data analysis: Khojasteh, Yue, Ma, Lyssikatos, Takahashi. Wrote or contributed to the writing of the manuscript: Khojasteh, Yue, Ma.

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