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Running Title: A Single Dose Human Mass Balance study of Vismodegib by AMS

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<td>AME study</td>
<td>Absorption, Metabolism and Excretion study; AEs - Adverse Events; AMS - accelerator mass spectrometry; AUC - area under the concentration-time curve; BCC - basal cell carcinoma; BLQ - below the limit of quantitation; cGMP - current good manufacturing practice; CID spectra - collision-induced dissociation spectra; CTCAE - common terminology criteria for adverse events; CYPs - cytochromes P450; ESI – electrospray ionization; FA – formic acid; Gluc – glucuronide; Hh – Hedgehog; HPIs – Hedgehog pathway inhibitors; HPLC – high pressure liquid chromatography; HPLC-UV – high pressure liquid chromatography with UV detector; IDA – information-dependent acquisition; IV – intravenous; LC – liquid chromatography; LC-MS/MS – liquid chromatographic - tandem mass spectrometry; LP – liquid paraffin; LSC – liquid scintillation counting; MS – mass spectrometry; MS/MS – tandem mass spectrometry; PTCH1 – Patched; PEG – polyethylene glycol; PK – pharmacokinetics; SD – standard deviation; SMO – Smoothend</td>
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ABSTRACT

Vismodegib (GDC-0449), a small molecule Hedgehog Pathway Inhibitor, was well tolerated in patients with solid tumors and showed promising efficacy in advanced basal cell carcinoma in a Phase I trial. The purpose of the present study was to determine routes of elimination and extent of vismodegib metabolism, including assessment and identification of metabolites in plasma, urine and feces. Six healthy female subjects of non-childbearing potential were enrolled; each received a single 30 ml oral suspension containing 150 mg of vismodegib with 6.5 μg of ^14C-vismodegib to yield a radioactivity dose of approximately 37 kBq (1000 nCi). Blood, urine and feces samples were collected over 56 days, to permit sample collection for up to 5 elimination half-lives. Non-radioactive vismodegib was measured in plasma using liquid chromatographic-tandem mass spectrometry (LC-MS/MS) and total radioactivity in plasma, urine and feces was measured using accelerator mass spectrometry. Vismodegib was slowly eliminated by a combination of metabolism and excretion of parent drug, the majority of which was recovered in feces. The estimated excretion of the administered dose was 86.6%, on average with 82.2% and 4.43% recovered in feces and urine, respectively. Vismodegib was predominant in plasma, with concentrations representing greater than 98% of the total circulating drug-related components. Metabolic pathways of vismodegib in human included oxidation, glucuronidation, and uncommon pyridine ring cleavage. We conclude that vismodegib and any associated metabolic products are mainly eliminated through feces following oral administration in healthy volunteers.
INTRODUCTION

The Hedgehog (Hh) signaling pathway is a novel and potentially beneficial target for cancer therapy (Rubin and de Sauvage, 2006; Scales and de Sauvage, 2009). Extracellular Hh ligand binds to Patched (PTCH1), a 12-pass transmembrane cell surface receptor, relieving the inhibitory effect of PTCH1 on Smoothened (SMO), a 7-pass transmembrane domain protein in the G-protein–coupled receptor superfamily. Signal transduction by SMO causes activation and nuclear localization of GLI1 transcription factors and induction of Hh target genes, many of which are involved in proliferation, survival, and angiogenesis.

A role of aberrant Hh signaling in cancer was initially discovered in patients with Gorlin syndrome, a rare genetic disorder associated with predisposition to basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma (Hahn et al., 1996; Johnson et al., 1996; Raffel et al., 1997; Epstein, 2008). More recently, tumor-derived Hh ligand was found to activate the Hh pathway in adjacent tumor stroma (Yauch et al., 2008).

Vismodegib (GDC-0449) is an orally bioavailable small molecule inhibitor of the Hh signaling pathway (Rudin et al., 2009; Von Hoff et al., 2009). In a Phase I trial, vismodegib was well tolerated in patients with solid tumors and showed promising efficacy in advanced BCC (Von Hoff et al., 2009). Vismodegib is currently undergoing evaluation in Phase II clinical trials and represents the most advanced of the Hh pathway inhibitors (HPis) presently in clinical development (Scales and de Sauvage, 2009; Tremblay et al., 2009).

In preclinical studies with vismodegib, the PK was favorable with low clearance in mouse, rat, and dog and moderate clearance in monkeys (Wong et al., 2009). In vitro protein binding experiments indicated strong and reversible binding to both alpha-1-acid glycoprotein (AAG) and Human Serum Albumin (HSA), with approximately 10-fold greater affinity for AAG than HSA (Giannetti et al., 2011). Blood-to-plasma partitioning of vismodegib in pooled human whole blood ranged from approximately 0.6 to 0.8 and was not concentration dependent (Wong et al., 2009). Vismodegib was highly stable but formed two primary oxidative metabolites M1...
and M3, following *in vitro* incubations with human liver microsomes. These were principally formed by CYP3A4/5 and CYP2C9, respectively (Wong et al., 2009). In a $^{14}$C mass balance study in rats and dogs, vismodegib underwent extensive metabolism through oxidation of the 4-chloro-3-(pyridin-2-yl)-phenyl moiety followed by Phase II glucuronidation or sulfation. Further, three uncommon metabolites with pyridine ring opening (M9, M13 and M18) have been identified in rat or dog feces ((Yue et al., 2011)).

The pharmacokinetics (PK) of vismodegib was assessed in patients and healthy subjects. A half-life of approximately 10-14 days was reported following a single 150-mg oral dose administration to healthy volunteers (Ding et al., 2010). Unique and non-linear properties were observed after continuous daily dosing in patients, and were explained by high-affinity plasma protein binding, nonlinear absorption, and slow metabolic elimination properties (Von Hoff et al., 2009; Ding et al., 2010; Graham et al., 2011). Vismodegib plasma concentration correlated strongly with alpha-1-acid glycoprotein (AAG) levels, showing parallel fluctuations of AAG and total drug over time (Giannetti et al., 2011; Graham et al., 2011), suggesting protein binding plays a key role in the overall PK of vismodegib. During routine Phase I and Phase II drug development an exploratory analysis of human urine in patients receiving vismodegib, revealed only small amounts of parent drug and metabolites (data on file). Based on available preclinical data and the presence of minimal vismodegib in urine we hypothesized that the route of elimination of vismodegib is primarily hepatic, however, considering the high *in vitro* metabolic stability of vismodegib the relative contribution of metabolism and excretion of unchanged drug in humans remained to be determined.

To further understand the disposition of vismodegib, an open-label, Phase I, single-center AME study was conducted in healthy female volunteers of non-childbearing potential; this population was selected due to possible teratogenic effects of vismodegib. The Hh pathway plays a key role in embryogenesis (Ingham and McMahon, 2001) and cyclopamine, a naturally occurring inhibitor of SMO, has known teratogenic effects (Binns et al., 1972; Keeler, 1990).
The primary objective of the mass balance analysis was to determine the routes of excretion and extent of vismodegib metabolism following single oral dose administration. The secondary objective was to identify vismodegib metabolites in plasma, urine, and feces.

Based on the long half-life of vismodegib, we anticipated its low amount of excretion per day in urine/feces with less likelihood of sufficient recovery of the administered dose. It was observed that for long half-life drugs, the recovery of the dose in mass balance studies is historically low and at least in some cases, the cause is expected to be due to inadequate analytical sensitivity (Roffey et al., 2007). For enhanced analytical sensitivity, we applied a tracer radiocarbon labeling approach that included assessment using the ultrasensitive analytical technology of accelerator mass spectrometry (AMS), providing detection of radioactivity over a long duration after administration of only tracer levels (Garner, 2000; Garner et al., 2000; Lappin and Garner, 2003; Beumer et al., 2007). The use of AMS allowed for assessment of the excretion over a 56-day study period, the major part of this period was covered by intermittend sample collection intervals, which provided sufficient data to estimate the excretion of administered radioactivity. Here we report the results of a single dose human mass balance study with vismodegib, using AMS.
METHODS

General Chemicals

HPLC grade methanol, water, acetonitrile, and ammonium acetate as well as concentrated HCl were purchased from Fisher Scientific (Loughborough, UK). Liquid paraffin (LP), copper oxide wire (ACS), cobalt powder (100 mesh, 99.9% w/w), zinc powder (100 mesh, 99%, w/w), and titanium (II) hydride (325 mesh, 98% w/w) were purchased from Sigma-Aldrich Chemical Co. (Poole, Dorset, UK). All glassware for sample graphitization was purchased from York Glassware Ltd. (York, UK) and was pre-baked at 500°C. Australian National University sugar (certified value = 150.61 percent modern carbon) was used as an AMS standard.

Synthetic graphite (200 -325 mesh, 99.9999% w/w) was obtained from Alfa Aesar (Karlsruhe, Germany) and aluminum powder (99.99% w/w) from Acros Organics (Geel, Belgium). Solid aluminum cathodes were purchased from National Electrostatics Corporation (Middletown, WI, USA). Materials used for carbon, hydrogen, nitrogen analysis were purchased from Elemental Microanalysis Ltd. (Okehampton, Devon, UK).

Radiolabeled drug, reference compound and formulation

\[^{14}C\] vismodegib (Lot 802389) was synthesized by Selcia Limited (Essex, UK) as a free base with a chemical purity of > 99% and a radiochemical purity of > 99% as determined by HPLC-UV and radio-HPLC analysis. The radiolabel, \(^{14}C\), was evenly distributed in the middle phenyl ring of vismodegib (specific activity = 153.3 μCi/mg) (Figure 1). Unlabeled vismodegib (Lot 707845) was synthesized at Siegfried Ltd. (Zofingen, Switzerland).

The radiolabeled drug product was a 30 mL single-dose oral suspension containing 1000 nCi \[^{14}C\] vismodegib and 150 mg unlabeled vismodegib (specific activity = 14747 dpm/mg). The formulation used Ora-Blend (a commercial suspending vehicle for pharmacy compounding) as the suspending vehicle and contained approximately 10% v/v polyethylene
glycol (PEG) 400 and 0.3% v/v ethanol. The total volume was 30 mL. A stock solution of 
$^{14}$C-vismodegib was prepared in ethanol, and an appropriate volume of the solution was 
accurately transferred to an appropriate container so that an accurate dose of $^{14}$C-vismodegib 
was obtained. PEG 400 was used to dissolve vismodegib. This step ensured that, after the
addition of the Ora Blend, a homogeneous suspension of $^{14}$C-vismodegib and unlabelled 
vismodegib was obtained. The suspension was prepared according to cGMP standard and 
administered within 24 h of preparation.

**Subject Selection**

Females of non-child bearing potential between 18 and 65 years of age were enrolled at 
a study center in the Netherlands (PRA International, Zuidlaren, The Netherlands). The Clinical 
Study Protocol and its amendment, the Informed Consent Forms, and any information given to 
the subject and relevant supporting information were submitted for review and approval by the 
Independent Ethics Committee of the ‘Stichting Beoordeling Ethiek Bio-Medisch Onderzoek’ 
(PO Box 1004, 9400 BA Assen, The Netherlands) prior to the eligibility screening. All 
participants were informed verbally and in writing regarding the objectives, procedures, and 
risks of study participation. All subjects signed the ethics committee-approved study consent 
form during the pre-study screening visit before any study related procedures were started. 
Participants were excluded if any of the following criteria were applicable: history of stomach or 
intestinal surgery, stomach disease, or resection that would potentially alter absorption and/or 
excretion of orally administered drugs; irregular defecation pattern or acute constipation 
problems within 3 weeks prior to Day 1; use of any prescription medications/products, including 
known enzyme-inducing/inhibiting agents, over-the-counter medication, or other non-
prescription preparations within 2 weeks prior to Day 1; smoking, use of tobacco, or nicotine-
containing/nicotine-replacement products for $\geq$6 months before start of study. The study was 
conducted in accordance with the principles of the Declaration of Helsinki including
amendments in force up to and including the time the study was conducted. The study was conducted in compliance with the International Conference on Harmonization (ICH) E6 Guideline for Good Clinical Practice (CPMP/ICH/135/95) and compliant with the European Union Clinical Trial Directive: Directive 2001/20/EC.

Excluded and Concomitant Therapies

While on-study, subjects could not take prescription medications/products, over-the-counter medication, any known enzyme-inducing/inhibiting agents (e.g., rifampin, St. John’s wort, or other hypericum perforatum-containing substance, ketoconazole, etc.), investigational drugs, or other non-prescription preparations, with the exception of hormone-replacement therapy. Subjects could not receive vaccinations or blood products. Subjects could not take nicotine-containing or nicotine-replacement products, alcohol or methylxanthine-containing beverages or food (coffee, tea, cola, chocolate, “powerdrinks”) until Day 14; after day 14, these substances were not permitted within 24 h before a study visit, unless deemed acceptable by the Medical Investigator. In addition, subjects were advised not to engage in strenuous activity until completion of the study.

Study Design and Data Collection

Six healthy female subjects meeting study entry criteria, were dosed with vismodegib. All subjects arrived for admission to the clinical research facility in the afternoon preceding the day of drug administration. Upon admission to the clinical research facility, the inclusion and exclusion criteria were re-checked and the drug screen (including alcohol) was repeated. The occurrence of baseline complaints or adverse events (AEs) and the use of concomitant medication were checked and recorded.

On Day 1, subjects received a 30-mL oral suspension containing 150 mg of vismodegib with 6.5 μg of ^14^C- vismodegib to give a radioactivity of approximately 37 kBq (1000 nCi). After the suspension was administered the container was rinsed 3 times with approximately 50 mL of
bottled water (150-mL total volume of water), which was also orally administered to the subject. The solution, including the rinse, was consumed within 5 minutes. Serial pharmacokinetic blood, urine, and feces samples for vismodegib (plasma) and total radioactivity (whole blood, plasma, urine, and feces) analysis, were collected at pre-determined times after the single oral dose administration, up to 312 h (Day 14) during which time the subjects were confined to the clinical research facility (Figure 2). Subjects also checked into the clinic for 24-h overnight visits on Days 21, 28, 35, 42, 49, and 56 (± 1 day). During these visits, blood samples for PK as well as total radioactivity analysis and all urine and feces for total radioactivity analysis, were collected. Subjects were instructed to start feces collection 24 h before checking in for the overnight visits (i.e., on Days 20, 27, 34, 41, 48, and 55 (± 1 day)) and to continue collection at home until the next visit, if no feces was produced in the targeted 48-h period. The study completion visit took place on Day 56 (± 1 day).

Safety data for all subjects who had received any amount of vismodegib were analyzed in the safety analyses. Safety was assessed by summaries of adverse events (AEs), changes in laboratory test results, vital signs, ECG and physical examination findings.

Bioanalytical Methods

Radioanalysis: \(^{14}\text{C}-\text{radioanalysis in whole blood, plasma, urine, and feces was performed using AMS by Xceleron (Xceleron, York, UK). Whole blood (20 \mu L), undiluted plasma (60 \mu L), pooled plasma samples (60 \mu L), diluted urine (100 \mu L) and freeze-dried feces (~4 mg) were placed in sample tubes containing pre-baked copper oxide wire (50±10 mg). Briefly, sample pretreatment consisted of the conversion of the carbon within the samples to graphite via a two-step process of oxidation and reduction (Vogel, 1992; Lappin and Garner, 2004). Each sample was counted in the AMS instrument for a minimum of 100 s, and this was repeated for each sample at least three times. AMS data were converted to either disintegrations per minute (dpm) either per milliliter or per gram taking into account the carbon
content of the sample and any added carbon carrier (LP). The natural background $[^{14}C]/[^{12}C]$ level, measured in pre-dose samples of each matrix, was subtracted to obtain the amount of $^{14}$C- vismodegib -related $^{14}$C. Total carbon content and the specific activity of $^{14}$C- vismodegib in the dose were used to convert the $[^{14}C/^{12}C]$ ratio to vismodegib mass equivalents (pg eq/mL or pg eq/g). Mass concentration data were converted to molar concentrations or % administered dose values, as appropriate.

Freeze dried feces samples at levels of approximately 250 dpm/g and above were analysed by liquid scintillation counting (LSC) only. Aliquots (150-200 mg in duplicate) were combusted in a sample oxidizer (Canberra Packard 307 Sample Oxidizer, Perkin Elmer, Cambridge, Cambridgeshire, UK) and the resulting CO$_2$ was trapped and analyzed in a carbosorb and permafluor mixture. A scintillant blank (in duplicate) was used to provide a background sample. The vials were capped, shaken and left to dark adapt in the LSC (Packard Tri-Carb TR/SL 2770, Perkin Elmer, Cambridge, Cambridgeshire, UK). The samples were counted for one cycle of 60 minutes or 2% $\sigma$ whichever came first.

**Bioanalysis of unlabelled vismodegib:** Non-radiolabelled vismodegib plasma concentrations were determined by Tandem Labs (Salt Lake City, UT) using a validated solid phase extraction LC-MS/MS method, using reverse-phase chromatography coupled with a TurbolonSpray$^TM$ interface validated over a calibration curve range of 5 to 5000 ng/mL (0.012—11.9 µM) (Ding et al., 2010).

**Metabolite Profiling Analysis**

**Sample preparation:** Plasma was pooled (by equal volumes) across all subjects at selected time points (i.e., 4, 24, 168, and 312 h) and spiked with a solution containing vismodegib (4.1 mg/mL) as a chromatographic marker. Pooled plasma (500 µL) and acetonitrile (ACN) (1500 µL) were combined, vortex-mixed and sonicated (15 minutes) followed by centrifugation at 2095 x $g$ for 10 minutes at +4ºC. The supernatant layer was removed and the
pellet re-extracted two additional times with ACN (1500 µL). The supernatants were pooled and evaporated to complete dryness under a stream of nitrogen gas. The extract was reconstituted with methanol followed by 0.1% formic acid (FA).

Homogenized feces samples were pooled (by equivalent percent weight) across all subjects for selected time periods (0-72 h and 72-312 h) and spiked with a solution containing non-radiolabeled vismodegib and prepared as described above, however the matrix:solvent ratio differed slightly (~1g feces homogenate was extracted with 2 mL ACN). Urine samples were pooled (by equivalent percent volume) across all subjects for the selected time period (0-312 h) and the pooled sample was spiked with a solution containing non-radiolabeled vismodegib prior to direct injection onto the HPLC system. Aliquots of extracted samples were analyzed by AMS to determine extraction recovery of total radioactivity (ie all vismodegib-related material).

**HPLC conditions:** Pooled urine and extracts from pooled plasma and homogenized feces were profiled using an Agilent 1200 Diode Array Detector HPLC system equipped with a 96-well plate fraction collector (Agilent Technologies, Stockport, Cheshire, UK). Chromatography was performed on a Synergi 4 μ Hydro 250×4.6 mm RP 80A column. The mobile phase consisted of mobile phase A: 10 mM ammonium acetate in water with 0.1% FA, and mobile phase B: 10 mM ammonium acetate in 90% ACN in water containing 0.1% FA. The flow rate was 1 mL/min. The HPLC gradient was held at 10% B for 3 minutes, increased to 20% B at 10 minutes (this and all other gradient changes were linear), to 35% B at 60 minutes, to 55% B at 70 minutes, then to 98% B at 80 minutes, held for 10 minutes, decreased to 10% B at 91 minutes, and equilibrated for 9 minutes. HPLC eluent fractions were collected every 15 seconds. Fractions were pooled across 2 minute bands and analyzed by AMS. Subsequently, individual fractions from 2 minute bands in areas of particular interest were also analyzed by AMS. Fractions and fraction pools (~250-400 µL) were placed in sample tubes containing LP
(2.5 µL; equivalent to ca 2 mg carbon) and pre-baked copper oxide wire (50±10 mg), dried under vacuum, then converted to graphite as described above.

HPLC column recoveries were determined for selected samples by collecting the total HPLC column eluate for the appropriate run as a single sample and assaying the radioactivity content using AMS.

Structure Identification of Metabolites

The same pooled plasma (4, 24, 168 and 312 h), urine (0-312 h), and feces (0-72 and 72-312 h) samples as used for metabolite profiling analysis were selected for metabolite identification. This was performed on an Accela HPLC system with a built-in degasser and autosampler coupled with a high resolution mass spectrometer LTQ-Orbitrap from Thermo Fisher Scientific, Inc. (San Jose, CA). The HPLC conditions were the same as described above for metabolite profiling. The LTQ-Orbitrap mass spectrometer was equipped with a Max-ESI source and operated in the positive electrospray ionization (ESI) mode. The electrospray ion source voltage was 4.5 kV. The heated capillary temperature was 350°C. Auxiliary and sheath gases were set to 10 and 25 units, respectively. The scan-event cycle consisted of a full-scan mass spectrum at a resolving power of 30,000 (at m/z 400) and the corresponding data-dependent tandem mass spectrometry (MS^n) scans acquired at a resolving power of 7500. Accurate mass measurements were performed using external calibration.

A 5500 QTRAP® linear ion trap mass spectrometer (AB SCIEX, Foster City, CA) equipped with a TurbolonSpray source and a Shimadzu® LC-20AD HPLC System (Shimadzu Scientific Instruments, Columbia, MD) was also used to obtain enhanced product ion mass spectra for the metabolites of interest. The LC column, mobile phases, and gradient were the same as described as above. Precursor information-dependent acquisition (IDA), multiple
reaction monitoring-IDA, and neutral loss IDA scans were used to search for potential metabolites.

Metabolites were identified on the basis of accurate masses of protonated molecular ions and their collision-induced dissociation (CID) fragmentation. Authentic standards, when available, were used to compare chromatographic retention times and fragmentation patterns. The same metabolites identified in rat and dog radiolabeled bile and feces samples and in samples of vismodegib incubated with CYP3A4 and CYP2C9 recombinant enzymes were compared in the assignment of metabolite structures (Yue et al., 2011)).

**Pharmacokinetic Analysis**

Individual and mean plasma concentration-time data were plotted. PK parameters were calculated using a non-compartmental model with WinNonlin® (Pharsight Corporation, version 5.2.1, Mountain View, CA). Plasma concentration values in ng or ng equivalents per mL were converted to µM using the molecular weight of vismodegib (421.3 g/mol). All area under the concentration-time curve (AUC) values were calculated using the linear trapezoidal method when the concentrations were rising and by the logarithmic trapezoidal method when the concentrations were declining (Linear up/log down rule in WinNonlin®). Below the limit of quantitation (BLQ) values at pre-dose were considered as zero for PK analysis. Actual blood collection time was used to calculate PK parameters and the parameters were summarized by mean and standard deviation (SD). Total radioactivity measured by AMS was converted to dpm/mL. Specific activity of the administered dose was determined from the specific activity of 14C- vismodegib reference standard and the amount of non-labeled vismodegib added to make up the dose suspension. The specific activity of the administered dose was used to calculate the drug concentration in plasma in ng equivalents per mL.

Cumulative excretion was expressed as a percent of the administered dose and was determined in urine and feces over the study duration. Cumulative recovery was calculated as
\[ A_{0-56}^e = A_{0-14}^e + A_{15-56}^e \] where \( A_{0-14}^e \) is the cumulative excretion through Day 14 calculated by summation of the percent recovery at each collection interval and \( A_{15-56}^e \) was calculated as the area under the excretion rate versus time curve through Day 56 (trapezoidal method, excretion rate in terms of percent recovery per collection interval). Total recovery was described as percent recovery derived from the amount recovered expressed as a percent of the administered dose.
RESULTS

Subject Disposition

All 6 subjects completed the study with no reported adverse events that were greater than Common Terminology Criteria for Adverse Events (CTCAE) Grade 1 or that were considered to be related to vismodegib.

Plasma Pharmacokinetics

Plasma concentration–time curves for unlabeled vismodegib (LC-MS/MS) and total radioactivity (AMS) are shown in Figure 3. Following a single dose of an oral suspension, non-radiolabelled vismodegib and total radioactivity appeared to be absorbed over two days followed by a very slow terminal elimination phase with mean half-life values of 9.33 and 9.46 days, respectively. Plasma levels of unlabelled vismodegib and total radioactivity were detected over the entire 56-day study period. On average, $C_{\text{max}}$ and $AUC_{(0-\text{inf})}$ for total radioactivity were higher than for unlabeled vismodegib (1.51-fold and 1.35-fold, respectively), however the elimination half-lives ($t_{1/2}$) were similar for both analytes (Table 1). The AUC ratio of unlabeled vismodegib to total radioactivity was variable, ranging from 0.469 to 0.949. Over the 56-day plasma sampling period, AUC and terminal elimination rate constant were adequately determined despite the long $t_{1/2}$ with the extrapolated AUC represented by only 3.2% and 3.6% of the $AUC_{0-\text{inf}}$ (total radioactivity and unlabeled vismodegib, respectively).

Blood to Plasma Partitioning

Blood to plasma partitioning was evaluated at 4, 24, 144, and 312 h after vismodegib administration. In general, the blood to plasma partition ratio was similar at each sampling time and the mean values (by sampling time) ranged from 0.687 to 0.709 (data on file); a result that is consistent with preclinical studies using human pooled whole blood (Wong et al., 2009).

Mass Balance
As shown in Figure 4, approximately 50% of the administered dose was recovered in feces by 7-days post dose with the remainder recovered through the end of the study. All subjects provided at least one feces sample during all collection intervals; either from at home feces collections or during the overnight clinic visits, or both. Urinary recovery was consistently low throughout the study. During the 56 day collection period the estimated average recovery of vismodegib-related radioactivity was 86.6% (range, 72.7% to 95.0%) of the administered dose with 82.2% (67.2% to 91.9%) recovered in feces. Recovery of radioactivity in urine was a relatively low 4.43% (range, 2.89 to 6.03%) (Table 2). The estimated total excretion of the administered dose was greater than or equal to 84.7% in five of the six subjects. Drug-related total radioactivity was still detectable in plasma on Day 56 for all subjects.

Metabolite Profile and Identification

**Plasma:** The mean extraction recovery of total radioactivity from human plasma was 84.3% (83.5% at 4h, 94.6% at 24h, 83.4% at 168h and 75.8% at 312 h). A representative plasma radiochromatogram obtained from AMS analysis is presented in Figure 5A. The HPLC column recovery was greater than 95.9% for all selected samples. Vismodegib was predominant (>98% of the total radioactivity) in human plasma at all time intervals (4, 24, 168, and 312 h). This result was confirmed by profiling plasma from subject S0012, the subject with the lowest AUC ratio of unlabeled vismodegib to total radioactivity (Supplementary Figure 1). Trace levels of oxidative metabolite M3 and glucuronides M4 and M5 were detected in 4 h post dose plasma by high-resolution MS only; notably similar results were obtained in the 24, 168 and 312 h post-dose plasma samples. Proposed structures and supporting spectra data are presented in Figure 6 and Table 3, respectively.

**Urine:** While no significant radioactive peaks were observed by AMS analysis, based on high-resolution MS and MS/MS, minor oxidative metabolites M1, M3, and M14; glucuronides
M4 and M5; pyridine ring cleavage metabolites M13 and M18; and vismodegib were observed in human urine (0–312 h).

**Feces:** The distribution of metabolites detected in feces is listed in Table 4 and HPLC radiochromatographic profiles of pooled feces are presented in Figures 5B and 5C. The mean extraction recovery of fecal radioactivity was 84.7% (83.7% to 87.6% at 0-72 h and 72-312 h post dose, respectively). In feces over 0-72 h post-dose, vismodegib was dominant, representing 21.7% of the dose while only one metabolite (M3) was detected by AMS representing 3.2% of the dose (Figure 5B). Over 72-312 h post-dose, vismodegib was 9.0% of the dose in feces and additional metabolites were detected during this period (Figure 5C). Metabolites M1, M3, M13, and M18 represented 1.1% 12.0%, 3.0%, and 2.0% of the dose in human feces, respectively.

While the results from pooled feces samples collected at 0-72 h and 72-312 h intervals described above were informative, it was difficult to discern between unabsorbed vismodegib and excreted radioactivity of absorbed vismodegib (i.e., unchanged vismodegib or metabolites). To this end, additional feces samples were pooled across all subjects over 24 h intervals post-dose through 312 h (Table 5). Over the first 72 h post-dose, most of the total radioactivity was associated with vismodegib with up to 5% represented by M3. In the 72-96 h collection interval, the vismodegib contribution to total radioactivity in feces had decreased considerably and approximately 10% of the total radioactivity was from M3. From 96-120 h through the 288-312 h collection interval, the majority of the total radioactivity was represented by components other than vismodegib and M3.
DISCUSSION

The primary aim of the present study was to determine the disposition and excretion of vismodegib, however given the long single dose half-life, the study design posed challenges different from those of traditional human mass balance studies. We considered a traditional mass balance approach for vismodegib, however this would have required high levels of radioactivity (relative to AMS) to be administered to healthy subjects with the radioactivity persisting in plasma for a long duration (due to the long half life) with inherent concerns for cumulative radioactive exposure. Furthermore, we expected a small fraction of radioactivity to be excreted per day in amounts that would be insufficient for detection by conventional means. Thus, there would be a low likelihood of sufficient recovery of the administered dose, particularly at later timepoints. Therefore, we proposed to utilize AMS to maximize the probability of technical success for the present study (i.e., obtaining an acceptable recovery of the administered dose) with the added benefit of minimizing the exposure of subjects to radioactivity over the long study duration.

Following administration of $^{14}$C-vismodegib oral suspension the average total estimated excretion was >85% of the administered dose over a collection period of 56 days, with the majority of vismodegib-related radioactivity recovered in feces and low recovery in urine. Notably, detectable drug-related material remained in plasma, urine and feces in all subjects on Day 56, which suggests that recovery could have been even more complete if urine and feces collections would have continued beyond Day 56. The lack of more complete recovery is likely due to inadequate study duration, losses via other routes than urine and feces (notably perspiration), weekly collection intervals, and/or the excretion estimation method necessitated by the long half-life of vismodegib.

The PK parameters of vismodegib in the present study were generally similar to those reported previously in healthy volunteers following a 150 mg oral dose despite differences in the formulations (oral suspension versus solid capsule) (Ding et al., 2010). Single dose and
multiple dose IV and oral pharmacokinetics in healthy volunteers administered the to-be-marketed formulation, will be reported in a separate publication. Herein we reported only those PK measures that are relevant to the mass balance study, including the AUC ratio for plasma vismodegib to total radioactivity.

Profiling results showed that unchanged vismodegib was predominant in plasma, corresponding to greater than 98% of the total circulating radioactivity at 4, 24, 168, and 312 h. The average AUC ratio for unlabeled vismodegib (corrected for extraction efficiency) and total radioactivity in plasma was 0.757, which is not entirely consistent with the plasma profiling results. In other words, the AUC ratio was expected to be closer to 1, on average. This was in fact the case for 3 of 6 subjects (S0007, S0008 and S0010), with AUC ratios of 0.878, 0.988, and 0.898, respectively; however AUC ratios were much lower for S0009, S0011 and S0012 (0.637, 0.671, and 0.468, respectively). Inspection of the individual concentration versus time profiles suggests that the lower AUC ratio for these three subjects was propagated from lower unlabeled vismodegib plasma concentrations over the entire time course and not in a particular phase of the profile. An issue with comparing AUC ratios in this study is that two different bioanalytical techniques (AMS and LC-MS/MS), each with their own inherent measurement error, could be expected to give rise to variability in this parameter. Nonetheless, the inconsistency between AUC ratio and plasma profiling results has no impact on the overall interpretation of the study results because the primary route of elimination and extent of metabolism were clear from the totality of the data.

Seven minor metabolites were identified from human pooled plasma, urine and feces, including oxidative metabolites (M1, M3, and M14), glucuronides (M4 and M5) and pyridine ring cleavage metabolites (M13 and M18). The structures of the metabolites were characterized by high resolution MS and CID spectra with mass accuracy better than 1 ppm. The HPLC retention time and mass spectra of human metabolites confirmed that these metabolites matched those present in preclinical species. All metabolites identified were consistent with
those observed in rat and dog mass balance studies (Yue et al., 2011). Of note, two uncommon pyridine ring opening metabolites M13 and M18, which represented 5% of the dose in total in human feces (also observed in rats and dogs) were absent from in vitro hepatic incubations (Yue et al., 2011). These observations may be explained by either very slow hepatic turnover of vismodegib observed in microsomes and hepatocytes or the involvement of extra-hepatic enzymes in their formation. Further investigations are underway to study the mechanism of M13 and M18 formation.

Vismodegib was slowly eliminated by a combination of extensive metabolism and excretion of parent drug, the majority of which was recovered in feces. Over the first three days after dose administration, the majority of the recovered radioactivity in feces likely represented unabsorbed vismodegib. However, a substantial portion of the estimated excreted radioactivity in feces was recovered in the period following the first 3 days. From Day 6 through Day 14, less than 15% (on average) of the total radioactivity per day recovered in feces was attributed to parent vismodegib suggesting that after GI transit (2-3 days) (Davies and Morris, 1993), the recovered radioactivity in feces likely represents drug that had been absorbed and excreted unchanged or as metabolites.

In summary, vismodegib was absorbed and slowly eliminated as a combination of the unchanged parent form and its metabolites after oral dosing in healthy volunteers. The majority of the administered radioactivity was recovered in feces indicating hepatic elimination predominates for vismodegib.
ACKNOWLEDGEMENTS

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Vismodegib was discovered by Genentech and was jointly validated through a series of preclinical studies performed under a collaborative agreement between Genentech, Inc. (South San Francisco, CA) and Curis, Inc. (Cambridge, MA).
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Graham, Lum, Morrison, Chang, Jorga, Dean, Shin, Malhi, Xie, Low, and Hop

Conducted experiments: Yue and Mulder

Contributed new reagents or analytic tools:

Performed data analysis: Graham, Malhi, Yue, Mulder, and Shin

Wrote or contributed to the writing of the manuscript: Graham, Lum, Morrison, Chang, Jorga, Dean, Shin, Yue, Mulder, Malhi, Xie, Low, and Hop
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1: The Chemical Structure of [14C]Vismodegib. The star (*) denotes the location of the uniformly distributed 14C-radiolabel in the phenyl ring of the 4-chlorine-aniline moiety.

Figure 2: Study Design. Frequent blood sampling occurred over the first 84 h post-dose to characterize the absorption phase, including 10 samples within the first 24 h, 4 samples between 24-40 h, 2 samples between 48-60 h, and two samples between 72-84 h. Subsequently, a single blood sample was collected each day through day 14, followed by weekly collection thereafter, as depicted in the figure. *At Days 21, 28, 35, 42, 49, and 56 subjects collected feces at home over a 24-h period the day before the overnight weekly visits.

Figure 3: Comparison of Unlabeled Vismodegib and Total Radioactivity in Plasma After Administration of 150 mg Vismodegib and 6.5 µg 14C-Vismodegib Oral Suspension. Open symbols represent total radioactively and closed symbols represent unlabelled Vismodegib. (A) The three subjects for which the AUC ratio was <0.7; S0009, S0011, and S0012 represented by circles, triangles, and squares, respectively. (B) The three subjects for which the AUC ratio was ~1; S0007, S0008, and S0010 represented by circles, triangles, and squares, respectively. The following time points are not represented on the graph to allow for visualization of the absorption phase following drug administration (0.5, 10, 14, 16, 30, 35, 40, 60 and 84 h post-dose).

Figure 4: Mean Cumulative Recovery of Total Radioactivity in Feces and Urine After Administration of 150 mg Vismodegib and 6.5 µg 14C-Vismodegib Oral Suspension.


**Figure 5:** (A) Metabolite Profiling from Pooled Plasma Samples at 4 h Post-Dose (B) Metabolite Profiling from Pooled Feces at 0-72 h Post-Dose and (C) Metabolite Profiling from Pooled Feces at 72-312 h Post-Dose.

**Figure 6:** Proposed Major Metabolic Pathways of Vismodegib in Human Subjects following an Oral Dose of 150 mg Vismodegib.
Table 1: Values of Pharmacokinetic Parameters Identified in Study

<table>
<thead>
<tr>
<th>Treatment Subject</th>
<th>Plasma Vismodegib</th>
<th>Plasma Total Radioactivity</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cmax (µM)</td>
<td>AUC₀⁻inf (µM*µM)</td>
<td>t₁/₂ (h)</td>
</tr>
<tr>
<td>S0007</td>
<td>8.12</td>
<td>1940</td>
<td>157</td>
</tr>
<tr>
<td>S0008</td>
<td>9.49</td>
<td>4180</td>
<td>269</td>
</tr>
<tr>
<td>S0009</td>
<td>7.31</td>
<td>4510</td>
<td>329</td>
</tr>
<tr>
<td>S0010</td>
<td>6.08</td>
<td>2540</td>
<td>277</td>
</tr>
<tr>
<td>S0011</td>
<td>7.56</td>
<td>2040</td>
<td>182</td>
</tr>
<tr>
<td>S0012</td>
<td>7.50</td>
<td>1550</td>
<td>128</td>
</tr>
<tr>
<td>Mean</td>
<td>7.67</td>
<td>2790</td>
<td>224</td>
</tr>
<tr>
<td>SD</td>
<td>1.12</td>
<td>1250</td>
<td>79.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> AUC₀⁻inf is Ratio equal to plasma vismodegib AUC₀⁻inf /plasma total radioactivity AUC₀⁻inf
Table 2: Estimated Excretion of Total Radioactivity over the 56-Day Study Period

<table>
<thead>
<tr>
<th>Percent of Dose Recovered (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subject</td>
</tr>
<tr>
<td>---------</td>
</tr>
<tr>
<td>S0007</td>
</tr>
<tr>
<td>S0008</td>
</tr>
<tr>
<td>S0009</td>
</tr>
<tr>
<td>S0010</td>
</tr>
<tr>
<td>S0011</td>
</tr>
<tr>
<td>S0012</td>
</tr>
<tr>
<td>Mean ± SD</td>
</tr>
</tbody>
</table>
Table 3: Accurate masses and Major Fragment Ions in Product Ion Spectra of Vismodegib and its Metabolites

<table>
<thead>
<tr>
<th>Analyte</th>
<th>RT (min)</th>
<th>Experimental [M + H]^+ (m/z)</th>
<th>Theoretical [M + H]^+ (m/z)</th>
<th>Mass Accuracy (ppm)</th>
<th>Formula</th>
<th>Major Fragment Ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vismodegib</td>
<td>75.0</td>
<td>421.0176</td>
<td>421.0175</td>
<td>0.34</td>
<td>C_{19}H_{15}O_{3}N_{2}Cl_{2}S</td>
<td>403, 342, 313, 306, 217, 204, 188, 155, 139</td>
</tr>
<tr>
<td>M1</td>
<td>70.4</td>
<td>437.0126</td>
<td>437.0124</td>
<td>0.37</td>
<td>C_{19}H_{15}O_{4}N_{2}Cl_{2}S</td>
<td>419, 340</td>
</tr>
<tr>
<td>M3</td>
<td>71.0</td>
<td>437.0125</td>
<td>437.0124</td>
<td>0.23</td>
<td>C_{19}H_{15}O_{4}N_{2}Cl_{2}S</td>
<td>419, 358, 322, 217, 155, 139</td>
</tr>
<tr>
<td>M4</td>
<td>38.3</td>
<td>613.0447</td>
<td>613.0445</td>
<td>0.40</td>
<td>C_{25}H_{23}O_{10}N_{2}Cl_{2}S</td>
<td>437, 419</td>
</tr>
<tr>
<td>M5</td>
<td>42.9</td>
<td>613.0447</td>
<td>613.0445</td>
<td>0.40</td>
<td>C_{25}H_{23}O_{10}N_{2}Cl_{2}S</td>
<td>437</td>
</tr>
<tr>
<td>M13</td>
<td>69.1</td>
<td>461.0336^a</td>
<td>461.0335^a</td>
<td>0.07</td>
<td>C_{18}H_{19}O_{6}N_{2}Cl_{2}S</td>
<td>426, 398, 217</td>
</tr>
<tr>
<td>M14</td>
<td>51.7</td>
<td>437.0126</td>
<td>437.0124</td>
<td>0.37</td>
<td>C_{19}H_{15}O_{4}N_{2}Cl_{2}S</td>
<td>383, 304, 155</td>
</tr>
<tr>
<td>M18</td>
<td>52.2</td>
<td>471.0179</td>
<td>471.0179</td>
<td>-0.08</td>
<td>C_{19}H_{17}O_{6}N_{2}Cl_{2}S</td>
<td>453, 443, 426, 425, 397</td>
</tr>
</tbody>
</table>

[M+H]^+=protonated molecule ion; m/z=mass to charge ratio; RT=retention time.

^a [M+NH_4]^+.
Table 4: Detection of Vismodegib and Metabolites in Pooled Urine and Feces following Oral Administration of [14C]Vismodegib to Human Subjects

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Percent of Dose (%)</th>
<th>Urine</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–312 h</td>
<td>0–72 h</td>
</tr>
<tr>
<td>Vismodegib</td>
<td>D (^a)</td>
<td>21.7</td>
<td>9.0</td>
</tr>
<tr>
<td>M1</td>
<td>D</td>
<td>D</td>
<td>1.1</td>
</tr>
<tr>
<td>M3</td>
<td>D</td>
<td>3.2</td>
<td>12.0</td>
</tr>
<tr>
<td>M4</td>
<td>D</td>
<td>ND(^b)</td>
<td>ND</td>
</tr>
<tr>
<td>M5</td>
<td>D</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>M13</td>
<td>D</td>
<td>D</td>
<td>3.0</td>
</tr>
<tr>
<td>M14</td>
<td>D</td>
<td>ND</td>
<td>D</td>
</tr>
<tr>
<td>M18</td>
<td>D</td>
<td>D</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^a\) D = detected by MS only;  
\(^b\) ND = not detected by MS.
Table 5: Percent of Total Radioactivity represented by Vismodegib, M3, and other metabolites in pooled feces samples collected over 24 h intervals.

<table>
<thead>
<tr>
<th>Collection Period (h)</th>
<th>% Total $^{14}$C</th>
<th>Vismodegib</th>
<th>M3</th>
<th>Other$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-24</td>
<td></td>
<td>117</td>
<td>0.638</td>
<td>-</td>
</tr>
<tr>
<td>24-48</td>
<td></td>
<td>92</td>
<td>3.52</td>
<td>4.2</td>
</tr>
<tr>
<td>48-72</td>
<td></td>
<td>82.1</td>
<td>5.13</td>
<td>12.8</td>
</tr>
<tr>
<td>72-96</td>
<td></td>
<td>67.7</td>
<td>8.40</td>
<td>23.9</td>
</tr>
<tr>
<td>96-120</td>
<td></td>
<td>37.7</td>
<td>10.7</td>
<td>51.6</td>
</tr>
<tr>
<td>120-144</td>
<td></td>
<td>13.2</td>
<td>21.3</td>
<td>65.5</td>
</tr>
<tr>
<td>144-168</td>
<td></td>
<td>12.8</td>
<td>7.28</td>
<td>79.9</td>
</tr>
<tr>
<td>168-192</td>
<td></td>
<td>14.0</td>
<td>6.82</td>
<td>79.1</td>
</tr>
<tr>
<td>192-216</td>
<td></td>
<td>10.6</td>
<td>8.83</td>
<td>80.6</td>
</tr>
<tr>
<td>216-240</td>
<td></td>
<td>22.0</td>
<td>8.39</td>
<td>69.6</td>
</tr>
<tr>
<td>240-264</td>
<td></td>
<td>10.9</td>
<td>5.82</td>
<td>83.3</td>
</tr>
<tr>
<td>264-288</td>
<td></td>
<td>18.0</td>
<td>6.41</td>
<td>75.6</td>
</tr>
<tr>
<td>288-312</td>
<td></td>
<td>13.9</td>
<td>13.4</td>
<td>72.7</td>
</tr>
</tbody>
</table>

$^a$ Vismodegib and M3 were measured over each 24 h interval, while “Other” was calculated by subtracting the sum of vismodegib and M3 from total $^{14}$C.
Figure 2

*At Days 21, 28, 35, 42, 49 and 56, subjects collected feces at home over a 24-hour period the day before the overnight weekly visits.
Figure 3

A

B
Figure 4

Graph showing cumulative percent of administered dose over time intervals (days). The graph compares feces, urine, and total cumulative doses.
Figure 6

- Vismodegib

M1 \( R_1 = H, R_2 = OH, R_3 = H \)
M3 \( R_1 = OH, R_2 = H, R_3 = H \)
M14 \( R_1 = H, R_2 = H, R_3 = OH \)
M4 \( R_1 = H, R_2 = O\text{Glc} \)
M5 \( R_1 = O\text{Glc}, R_2 = H \)
M18
M13