DISPOSITION AND METABOLISM OF DARAPLADIB, A LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A2 INHIBITOR, IN HUMANS

Authors:
Mehul Dave, Mike Nash, Graeme C Young, Harma Ellens, Mindy Magee, Andrew D Roberts, Maxine A Taylor, Robert W Greenhill and Gary W Boyle

Department of Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Research & Development, Ware, United Kingdom (M.D., M.N., G.C.Y., A.D.R., M.A.T., G.W.B.); Department of Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Research & Development, Upper Merion, Philadelphia, USA (H.E.); Clinical Pharmacology, Modeling and Simulation, GlaxoSmithKline Research & Development, Upper Merion, Philadelphia, USA (M.M.); Department of Safety Assessment, GlaxoSmithKline Research & Development, Ware, United Kingdom (R.W.G)

Primary Laboratory of Origin:
Drug Metabolism and Pharmacokinetics, GlaxoSmithKline Research & Development Ltd., Park Road, Ware
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Corresponding author:
Gary W Boyle, DMPK, GlaxoSmithKline, Ware, UK.
Telephone: +44 1920 882465
Fax: +44 1920 884374
Email: Gary. W. Boyle@gsk.com

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Abbreviations: darapladib (SB-480848), N-[2-(diethylamino)ethyl]-2-(2-[(4-fluorophenyl)methyl]thio)-4-oxo,5,6,7-tetrahydro-1H-cyclopenta[d]pyrimidin-1-yl)-N-[(4'-trifluoromethyl)-4-biphenyl]methyl)acetamide; lipoprotein-associated phospholipase A2, Lp-PLA2; HPLC, high performance liquid chromatography; LSC, liquid scintillation counting; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; AMS, accelerator mass spectrometry; NOESY, nuclear overhauser effect spectroscopy; M3 (SB-823094), N-[2-(diethylamino)ethyl]-2-(2-[(4-fluorophenyl)methyl]thio)-5-hydroxy-4-oxo,5,6,7-tetrahydro-1H-cyclopenta[d]pyrimidin-1-yl)-N-[(4'-trifluoromethyl)-4-biphenyl]methyl)acetamide; M4 (SB-553253), N-[2-(ethylamino)ethyl]-2-(2-[(4-
fluorophenyl)methyl]thio)-4-oxo-4,5,6,7-tetrahydro-1H-cyclopenta[d]pyrimidin-1-yl)-N-[[4'-(trifluoromethyl)-4-biphenyl]methyl]acetamide; M7 (SB-735258), N-[2-(diethylamino)ethyl]-2-[2-(methyloxy)-4-oxo-4,5,6,7-tetrahydro-1H-cyclopenta[d]pyrimidin-1-yl]-N-[[4'-(trifluoromethyl)-4-biphenyl]methyl]acetamide; M10 (SB-554008), N-[2-(diethylamino)ethyl]-2-[2,4-dioxo-2,3,4,5,6,7-hexahydro-1H-cyclopenta[d]pyrimidin-1-yl]-N-[[4'-(trifluoromethyl)-4-biphenyl]methyl]acetamide; M11 (GSK219147), N-[2-(diethylamino)ethyl]-2-(4-oxo-2-thioxo-2,3,4,5,6,7-hexahydro-1H-cyclopenta[d]pyrimidin-1-yl)-N-[[4'-(trifluoromethyl)-4-biphenyl]methyl]acetamide.
ABSTRACT:

The absorption, metabolism and excretion of darapladib, a novel inhibitor of lipoprotein-associated phospholipase A2, was investigated in healthy male subjects using \(^{14}\text{C}\)-radiolabeled material in a bespoke study design. Disposition of darapladib was compared following single intravenous (i.v.) and both single and repeated oral administrations. The anticipated presence of low circulating concentrations of drug-related material required the use of accelerator mass spectrometry as a sensitive radiodetector. Blood, urine and feces were collected up to 21 days post radioactive dose, and analysed for drug-related material. The principal circulating drug-related component was unchanged darapladib. No notable metabolites were observed in plasma post-i.v. dosing, however metabolites resulting from hydroxylation (M3) and N-deethylation (M4) were observed (at 4-6% of plasma radioactivity) following oral dosing, indicative of some first pass metabolism. In addition, an acid catalysed degradant (M10) resulting from pre-systemic hydrolysis was also detected in plasma at similar levels of ~5% of radioactivity post oral dosing. Systemic exposure to radioactive material was reduced within the repeat dose regimen, consistent with the notion of time-dependent pharmacokinetics resulting from enhanced clearance or reduced absorption. Elimination of drug-related material was predominantly via the feces, with unchanged darapladib representing 43-53% of the radioactive dose, and metabolites M3 and M4 also notable accounting for ~9 and 19% of the dose, respectively. The enhanced study design has provided an increased understanding of the ADME properties of darapladib in humans, and substantially influenced future work on the compound.
Introduction

Despite contemporary multidrug therapies (treatment with statins, anti-platelet therapy, renin-angiotensin aldosterone system and/or beta adrenergic blockade and diabetes control) there is still a large unmet medical need to develop new strategies for the treatment of atherosclerotic vascular disease. Lipoprotein-associated phospholipase A2 (Lp-PLA₂) has been identified as a risk marker in atherosclerosis (The Lp-PLA₂ Studies Collaboration, 2010) and is therefore a target for therapeutic intervention. Darapladib, SB-480848 or N-[2-(diethylamino)ethyl]-2-[(4-fluorophenyl)methyl][thio]-4-oxo-4,5,6,7-tetrahydro-1H-cyclopenta[d]pyrimidin-1-yl)-N-[(4'-trifluoromethyl)-4-biphenylyl][methyl]acetamide is a novel, selective, orally active inhibitor of Lp-PLA₂, and is in late phase clinical development as a potential anti-atherosclerotic agent when administered in combination with current standard of care treatment for patients with acute coronary syndrome or coronary heart disease (Mohler et al., 2008; Serruys et al., 2008). It is generally safe and well tolerated in clinical studies completed to date. A dose of 160 mg produced significant and sustained inhibition of plasma Lp-PLA₂ activity and halted necrotic core progression, a key determinant of plaque vulnerability (Serruys et al., 2008). This dose was therefore selected for the ongoing pivotal outcomes studies.

This current study was designed to characterize the disposition and metabolism of darapladib in healthy adult male volunteers following single doses of intravenous (i.v.) as well as oral [¹⁴C]darapladib (Fig. 1). Early in preclinical development an N-desethyl metabolite (M₄, SB-553253) was identified and found to be pharmacologically active, and this metabolite has been assayed in all subsequent work, including this current study.
Preclinical excretion and metabolism studies in rats and dogs estimated that darapladib absorption, based on the sum of biliary secretion and urinary excretion of radioactive drug-related material, was relatively low at approximately 4% of the dose with the majority of the dose eliminated apparently as unabsorbed drug via the feces (M Dave, M Nash, B Squillaci, data on file). The major route of elimination of absorbed drug was via biliary secretion of metabolites (resulting predominantly from N-deethylation and oxidation), and unchanged darapladib, while direct intestinal secretion of darapladib (post i.v. dosing) was also observed. Darapladib recovered in feces following an oral dose in human may therefore represent both biliary and/or intestinal secretion, or simply unabsorbed drug. Administration of the i.v. dose in this current study facilitates elucidation of the processes involved in excretion, i.e. the extent to which darapladib is eliminated in feces and urine as parent or metabolites.

In early clinical studies it was noted that darapladib accumulates (at 30-60%) in the systemic circulation after multiple oral doses. This was however, less than was predicted from the single dose PK data which suggested accumulation of 130-200%. The ideal way to investigate the reasons for this would be to administer radiolabeled darapladib until steady-state conditions are achieved (10 days) and compare the results to that following single dose. Nonetheless, radioactive exposure following this dosing regimen and utilizing conventional radioactive doses would have been unacceptable. Therefore, non-radiolabeled darapladib was administered for 10 days and on day 11 a single oral dose of [14C]darapladib was administered, followed by three more doses of non-radiolabeled darapladib. This allowed examination of the relative metabolic profile of [14C]darapladib at steady-state compared to that following a single dose.
The dose levels of 8 mg (i.v. infusion), 80 mg (radiolabeled oral solution) and 160 mg (non-radiolabeled enteric tablet) were selected based on each providing similar systemic exposure. Accelerator Mass Spectrometry (AMS) was used to determine levels of total radioactivity in plasma samples (post oral administration) and plasma metabolite profiles from all phases due to the very low levels of radioactivity in these samples.

Materials and Methods

Chemicals. $^{[14]}$C]Darapladib, darapladib, $^{[13]}$C$_6$darapladib (stable labeled in the phenyl ring) and a number of synthetic standards were supplied by Chemical Development, GlaxoSmithKline Research and Development Ltd. (Stevenage, UK). Metabolite standards were: $N$-[2-(diethylamino)ethyl]-2-(2-[(4-fluorophenyl)methyl]thio)-5-hydroxy-4-oxo-4,5,6,7-tetrahydro-$^{1}$H-cyclopenta[d]pyrimidin-1-yl)-$N$-[4′-(trifluoromethyl)-4-biphenyl]methyl]acetamide (coded M3, SB-823094); $N$-[2-(ethylamino)ethyl]-2-(2-[(4-fluorophenyl)methyl]thio)-4-oxo-4,5,6,7-tetrahydro-$^{1}$H-cyclopenta[d]pyrimidin-1-yl)-$N$-[4′-(trifluoromethyl)-4-biphenyl]methyl]acetamide (coded M4, SB-553253) and $^{[13]}$C$_6$SB-553253 (stable labeled in the phenyl ring); $N$-[2-(diethylamino)ethyl]-2-[2-(methylthio)-4-oxo-4,5,6,7-tetrahydro-$^{1}$H-cyclopenta[d]pyrimidin-1-yl)-$N$-[4′-(trifluoromethyl)-4-biphenyl]methyl]acetamide (coded as M7, SB-735258); $N$-[2-(diethylamino)ethyl]-2-(2,4-dioxo-2,3,4,5,6,7-hexahydro-$^{1}$H-cyclopenta[d]pyrimidin-1-yl)-$N$-[4′-(trifluoromethyl)-4-biphenyl]methyl]acetamide (coded as M10, SB-554008); $N$-[2-(diethylamino)ethyl]-2-(4-oxo-2-thioxo-2,3,4,5,6,7-hexahydro-$^{1}$H-cyclopenta[d]pyrimidin-1-yl)-$N$-[4′-(trifluoromethyl)-4-
biphenyl[methyl]acetamide (coded as M11, GSK219147). All these materials were used as chromatographic, mass spectrometric and/or nuclear magnetic resonance (NMR) spectroscopy standards during the study (Fig 1). Commercially obtained chemicals and solvents were of high-performance liquid chromatography (HPLC) or analytical grade. Liquid scintillation cocktails were obtained from PerkinElmer LAS (UK) Ltd. (Beaconsfield, Bucks, UK).

Sucrose (IAEA-C6) standard (certificated value = 1.506 times modern), from the International Atomic Energy Agency, Vienna, Austria, was used as the source of graphite (6.5 mg sugar for each graphite produced) for use as the AMS instrument normalisation and process control standard.

Synthetic graphite 200 Mesh, (99.9999%), from Alfa Aesar, Heysham, UK, was used as the AMS instrument background determination standard. All of the reagents used in preparation of samples to graphite prior to analysis by AMS were supplied by Sigma-Aldrich Company Ltd., UK.

**Darapladib formulations.** The i.v. dose was prepared by taking 8 ml of $[^{14}\text{C}]$darapladib (at 1 mg/ml; 6.25 μCi/ml) and diluting to 40 ml with normal saline to give a final i.v. dose of 8 mg darapladib. The 40 ml i.v. solution was subsequently infused over a period of 3 h. The radiolabeled oral dose was prepared by mixing 3.2 ml of $[^{14}\text{C}]$darapladib (1 mg/ml; 6.25 μCi/ml) injection solution with 76.8 ml of non-radiolabeled darapladib (1 mg/ml solution) to give a final oral solution dose of 80 mg darapladib. Radiochemical purity determination of the dose formulations was conducted and was >98% for the i.v. dose and >99% for each oral radiolabelled dose. Non-radiolabeled darapladib (micronized) was supplied as enteric-coated 160 mg white tablets.
Study Design and Subjects. The clinical study (GlaxoSmithKline SB-480848/015) was performed at Covance Laboratories Inc. in conjunction with the Covance Clinical Research Unit (CRU, Madison, Wisconsin, USA) in accordance with Good Clinical Practice and the guiding principles of the 1996 version of the Declaration of Helsinki. The protocol including the proposed radioactive dose was reviewed by the GlaxoSmithKline Global Safety Board and by the Covance CRU Institutional Review Board. The total radioactive dose of 90 μCi was estimated using rat whole body autoradiography and excretion balance data and corresponded to an effective dose of ≤1mSv, which is approximately one-third of the annual average background effective dose and within risk Category IIa, in accordance with the International Commission on Radiological Protection (ICRP). The amount of radioactivity used for each phase also incorporated the anticipated analytical requirements and study objectives. Written consent was obtained from all subjects before any protocol-specific procedures were conducted.

Eight healthy male subjects (four white, three african american, one mixed race), between 30 and 44 years of age, bodyweight >50 kg, and with a body mass index between 19 and 32 kg/m², were enrolled in this study with seven completing all sessions (one subject was excluded from the final session due to low recovery of radioactivity in the previous session). The study design was an open-label, non-randomized, three-session study where darapladib was administered to each subject over three sessions in the following order: a single radiolabeled i.v. dose (session 1); a single radiolabeled oral dose (session 2); and as a series of non-radiolabeled repeat oral doses with a radiolabel oral dose administered on Day 11 of 14 days of dosing (session 3). There was a minimum of a 21 day washout period between radioactive doses in each session. Subjects were in good health, with no history of drug or alcohol
abuse, and were on no other medication at the time of the study, with no prescribed medication within 7 days (or 5 half-lives, whichever was longer) of the study commencing.

**Study Procedures.** On the morning of Day 1 for Session 1, all subjects received a single i.v. infusion at a target dose of 8 mg (approximately 50 μCi) of $[^{14}C]$darapladib in 40 ml of normal saline over 3 h. After a washout period, on the morning of Day 1 for Session 2, all subjects received a single oral administration at a target dose of 80 mg (approximately 20 μCi) of $[^{14}C]$darapladib as an 80 ml solution, and were instructed to rinse their mouth with a further 100 ml of water and swallow the water. After a further washout period, on the morning of Day 1 for Session 3, seven subjects received a 160 mg dose of enteric-coated darapladib tablet, and subsequently received daily doses of 160 mg enteric-coated darapladib tablet on Days 2 to 10. On the morning of Day 11, subjects received a single oral administration at a target dose of 80 mg (approximately 20 μCi) of $[^{14}C]$darapladib as an 80 ml solution, with a further 100 ml of water. Daily dosing of 160 mg enteric-coated darapladib tablet continued on Days 12 to 14.

After i.v. administration, blood samples (7 ml) for plasma total radioactivity, unchanged darapladib and SB-553253 analysis were collected into EDTA tubes at predose and 0.5, 1, 2, 3 (immediately prior to termination of infusion), 3.5, 4, 5, 6, 7, 9, 12, 18, 24, 32, 48, 72 and 96 h after the start of infusion. Following oral administration in Session 2 and on Day 11 of Session 3, blood samples (7 ml) were collected at predose and 0.5, 1, 2, 3, 4, 6, 9, 12, 18, 24, 32, 48, 72 and 96 h postdose. Additional samples (30 ml) were also collected for metabolite analysis from subjects at 3, 12, 24 and 48 h post start of i.v. infusion or after oral administration (Session 2 or following dosing on Day 11 of Session 3). Blood samples were mixed,
immediately chilled on crushed ice, and centrifuged for 15 min at 1500g at approximately 4°C to obtain plasma. Total radioactivity was measured using aliquots of plasma (0.06 to 0.5 ml); the remaining plasma was stored at -70°C or less before the assay for darapladib and SB-553253, or metabolite profiling by HPLC.

Urine samples from all three sessions were collected predose and between 0 to 6, 6 to 12, and 12 to 24 h after drug administration and then at 24-h intervals until 504 h post i.v. infusion (Session 1), 216 h post single oral dose (Session 2) and 264 h post Day 11 dosing (Session 3). For each collection period, after thorough mixing, the weight and pH were measured and recorded. A single subsample (50 ml) was removed from the bulk sample and set aside for metabolite profiling and identification and stored at -20°C. Total radioactivity was measured using triplicate aliquots (approximately 1 ml) of urine.

Feces samples for all three sessions were collected quantitatively predose and at 24-h intervals for the same time periods described for urine. Feces from each collection interval were weighed, mixed with deionised water (approximately 1:1), and the total weight of sample recorded prior to homogenization. Total radioactivity was measured using triplicate aliquots (0.3 g) of each feces homogenate. A single subsample (30 g) was removed from the bulk sample and set aside for metabolite profiling and identification and stored at -20°C.

**Assay of Total Radioactivity by LSC.** Aliquots (0.5 ml) of plasma samples from Session 1 were mixed with Ultima Gold scintillation fluid (PerkinElmer LAS [UK] Ltd.) and analysed by liquid scintillation counting (LSC) using a low level scintillation counter (PerkinElmer LAS [UK] Ltd.). The LLQ was 1.0 darapladib ng equivalents per ml. Triplicate aliquots (1 ml) of urine were also subjected to LSC following mixing with Ultima Gold scintillation fluid. Triplicate aliquots (0.3 g) of
feces homogenates were weighed into Combustocones containing Combustopads for oxidation using a Packard Tri-Carb 307 automatic sample oxidizer (PerkinElmer LAS [UK] Ltd.). The \(^{14}\)CO\(_2\) generated was collected by absorption in CarboSorb (8 ml) to which Permafluor was added. Radioactivity from urine and feces was quantified using a liquid scintillation counter (Packard Instrument Company, Downers Grove, IL.) with automatic quench correction using an external standard method (Botta et al., 1985). Prior to calculation of individual results a background count rate was determined and subtracted from each sample count rate. The LLQ for liquid scintillation counting of urine samples or those derived from feces was 4 - 110 ng of darapladib.

**Assay of Total Radioactivity by AMS.** Aliquots (0.06 ml) of plasma from Sessions 2 and 3 samples were analysed by AMS and were first prepared so that the carbon within the samples was harvested and converted to graphite. This involved a 2 stage process of sample combustion (oxidation) followed by graphitisation (reduction) as detailed in a previously published method (Young et al., 2008).

The carbon analysis was carried out using a Costech (26074 Avenue Hall, Suite 14, Valencia, CA 91355, USA) Elemental Combustion System (Model 4010) CHNS-O Analyser, supplied by Pelican Scientific Ltd. Chester, UK. The AMS instrument was manufactured by National Electrostatics Corp., Middleton, Wisconsin, USA. The AMS instrument was a 250 kV Single Stage Accelerator Mass Spectrometer, and was operated via NEC proprietary “AccelNET” software on a Linux operating system. Post acquisition data processing was performed using the NEC software “abc”. Further details of the operating conditions have been previously published (Young et al., 2008).
The predose samples from Session 1 were also analysed by AMS and used as background values for samples from Sessions 2 and 3 (LLQ was 0.1 darapladib ng equivalents per ml).

**Darapladib and SB-553253 (M4) Quantification.** Plasma concentrations of darapladib and SB-553253 were quantified using a validated analytical method based on protein precipitation with acetonitrile-ammonium formate pH 3.0, followed by HPLC-tandem mass spectrometry (MS/MS) analysis. The lower limit of quantification (LLQ) for darapladib and SB-553253 was 0.1 ng/ml and 0.25 ng/ml, respectively, using a 50 μl aliquot of human plasma with a higher limit of quantification (HLQ) of 50 ng/ml for both compounds. Acetonitrile-ammonium acetate pH 3.0 (75:25, v/v) containing internal standards ([13C₆]darapladib and [13C₆]SB-553253, at concentrations of 2 and 5 ng/ml, respectively) was added to plasma samples. After vortex mixing, the deproteinized samples were centrifuged for 20 min at approximately 6800g. The supernatant was analyzed using a TurboIonSpray Interface and multiple reaction monitoring. The chromatography was performed using a 50 x 2.0 mm i.d. Mac Mod ACE C18 (3μ) column and eluted at a flow rate of 0.75 ml/min. The isocratic mobile phase consisted of 30% 2 mM ammonium formate (to pH 3.0 with formic acid), 55% acetonitrile and 15% water. The mass spectrometer used was a Sciex API-4000 triple quadrupole mass spectrometer (Applied Biosciences, Ontario, Canada) operated in positive ion mode. The temperature of the probe was maintained at 650°C with a curtain gas setting of 20 and collision gas setting of 12. Darapladib and SB-553253 were monitored by multiple reaction monitoring of 667 to 391 and 639 to 235, respectively. [13C₆]Darapladib and [13C₆]SB-553253 were monitored by multiple reaction monitoring of 673 to 397 and 645 to 241, respectively.
The concentrations of darapladib and SB-553253 in plasma samples were calculated from calibration plots, constructed from analysis of calibration standards prepared at known concentrations of darapladib and SB-553253 in human plasma. A weighted 1/x² linear regression was applied in each case over the range 0.1 to 50 ng/ml for darapladib and 0.25 to 50 ng/ml for SB-553253.

Quality control (QC) samples, prepared at three different analyte concentrations and stored with study samples, were analyzed with each batch of samples against separately prepared calibration standards. QC samples and calibration standards were prepared using independently prepared stock solutions of darapladib and SB-553253 reference materials. For the analysis to be acceptable, no more than one third of the QC results were to deviate from the nominal concentration by more than 15%, and at least 50% of the results from each QC concentration were to be within 15% of nominal.

Quantification and Profiling of Metabolites in Plasma, Urine and Feces. Plasma samples taken at 3, 12 and 24 h postdose were pooled using volumes in proportion to the time interval between individual samples to provide a single pool of plasma, representative of the pharmacokinetic area under the curve (AUC) described by these time points, for each volunteer and for each dose session, (according to the method described by Hop et al., 1998). Plasma samples at 48 h postdose were not examined due to the very low levels of radioactivity present. Representative samples of feces were pooled by total weight ratio for each volunteer, in order to obtain a pool containing 90% or greater of the radioactivity excreted via that route. For Session 1 (i.v. dosing) this ranged between 0- to 72-h and 0- to 240-h collections; for session 2 (single oral dose) this ranged between 0- to 72-h and 0- to 240-h collections; for session 3 (following dosing on Day 11) this ranged between 0- to 96-h and 0- to 120-h
collections. Urine was not analysed from any session due to the very low levels of drug-related material excreted in this matrix.

All plasma sample pools were analysed following dilution, rather than solvent extraction, prior to HPLC-AMS analysis. Each pooled plasma sample was diluted 20-fold (plasma-50 mM ammonium acetate [native pH]-10% methanol [aq], 1:18:1, v/v) incorporating authentic non-radiolabeled standards each at concentrations of 10 μg/ml. Each diluted plasma sample was individually fractionated at a frequency of 0.2 minutes by HPLC, a carbon carrier (2.6 μl of liquid paraffin; a source of carbon essentially containing no $^{14}$C) added to each fraction, followed by conversion to graphite and analysis of radioactivity by AMS.

Fecal homogenates (approximately 2 g) were extracted by vortex-mixing for 1 min then rotary-mixing for 30 min with 6 ml of methanol. After centrifugation, the supernatant was removed, and the process was repeated. Weighed aliquots were taken from the individual supernatants and assayed by LSC. The two extracts for each sample were subsequently combined, and the residual pellets were oxidized to determine the extent of any unextracted radioactivity. Prior to radio-HPLC analysis, aliquots (0.5 ml) of each pooled fecal extract were diluted 1:1 with 50 mM ammonium acetate (native pH). All spiked control and blank control samples were subjected to the same methods of pretreatment as detailed for test samples.

**HPLC method.** The chromatographic instrument used consisted of an Agilent 1100 series binary pump and column oven (40°C) (Agilent Technologies, Santa Clara, CA), with an autosampler (CTC Analysis LC PAL; CTC Analytics AG, Zwingen, Switzerland) using a Waters Symmetry Shield RP8 column (25 cm x 4.6 mm, 5μ; Waters Corporation, Milford, MA). The mobile phase consisted of 50 mM ammonium acetate (native pH) (solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml/min.
A gradient was used, starting at 5% B and held for 5 min, then additional linear changes to 35% B by 10 min, to 50% by 35 min to 95% by 45 mins with these conditions being maintained for an additional 5 min.

HPLC column recoveries were determined on selected plasma samples only using AMS through comparison of pre-column injectates with total radioactivity of all fractions and recoveries were 95% or greater in all cases.

Radio-HPLC data were captured off-line (Bruin et al., 2006) with chromatographic fractions collected using a Gilson 222XL fraction collector into 96 deep-well microtitre LUMAPLATES™ containing yttrium silicate solid scintillant (Perkin Elmer LAS [UK] Ltd). Radioactivity determination was performed by scintillation counting using a Packard Topcount NXT counter (Perkin Elmer LAS [UK] Ltd. HPLC-AMS data analysis was captured as previously described.

Metabolites of interest were isolated by preparative HPLC (Agilent 1100 auto sampler, UV detector, and binary pump; Agilent Technologies, Santa Clara, CA) with a Zorbax SB C18 column (5 cm x 21.2 mm, 5 µ) (Agilent Technologies, Santa Clara, CA). The mobile phase consisted of 50 mM ammonium acetate (native pH) (solvent A) and acetonitrile (solvent B) at a flow rate of 20 ml/min. A gradient was used, starting at 5% B and held for 5 min, then additional linear changes to 35% B by 10 min, to 50% by 35 min and held for 10 mins, to 95% by 49.5 mins with these conditions being maintained for an additional 0.5 min. Metabolite-containing fractions were taken to dryness and reconstituted in 1:1 acetonitrile: D2O before being submitted for NMR analysis.

**Structural Identification of Metabolites.** Structural characterization was performed on selected samples by HPLC-MS using triple quadrupole Quattro Ultima and hybrid quadrupole/time-of-flight Q-TOF Premier mass spectrometers. (Waters MS
Electrospray ionization, in the positive ion mode, was used. The HPLC flow was split (1:3) between mass spectrometer and waste or fraction collector.

Metabolites were identified based on charged molecular ions, mass accuracy, and their collision-induced dissociation fragmentation (Oliveira and Watson, 2000). Authentic standards, when available, were used to compare chromatographic retention times (in particular for drug-related material in plasma) and fragmentation patterns. Supporting data from preclinical studies were also used in the assignment of metabolite structures. For many metabolites, confirmation of the structure has been obtained by $^1$H-NMR using a Bruker DRX-600 spectrometer (Bruker, Rheinstetten, Germany) equipped with an inverse 5-mm TCI Cryo-Probe (Bruker) ($^1$H/$^{13}$C/$^{15}$N) operating at 600.40 MHz under the control of Topspin. v1.3. $^1$H-NMR spectra were acquired using a standard NOESYPRESAT pulse sequence with spoil gradients for solvent suppression with time-shared double presaturation of the water and acetonitrile frequencies. In these experiments, typically 256 transients were acquired into 65536 data points over a spectral width of 12019 Hz (20 ppm) with an interscan delay of 2.4 s giving a pulse repetition time of 5 s. Acquisition time was extended up to 4096 transients for some metabolites to improve signal to noise. Fully characterized metabolites were designated by the letter M followed by a number; where a synthetic standard was available, a GSK (or SB) code number was assigned.

**Pharmacokinetic Analysis.** Actual blood collection times and the actual dose administered were used for all pharmacokinetic calculations. Analysis of plasma total radioactivity, darapladib and SB-553253 concentration-time data was conducted using the non-compartmental Models 200 and 202 of WinNonlin Professional Edition.
versions 4.1 and 5.2 (Pharsight Corporation, Mountain View, CA). Maximum plasma concentration \(C_{\text{max}}\) and time of \(C_{\text{max}}\) \(t_{\text{max}}\) were taken directly from the pharmacokinetic concentration-time data. Where data permit, the terminal plasma elimination rate constant \(\lambda_z\) was estimated from log-linear regression analysis of the terminal phase of the plasma concentration-time profile. The numbers of points included in the terminal phase was determined by visual inspection of the semi-log plots of the plasma concentration-time profiles. The associated \(t_{1/2}\) was calculated as \(\ln 2 / \lambda_z\). Values of \(\text{AUC}_{0-t}\) and \(\text{AUC}_{0-\infty}\) were calculated using linear trapezoidal method for each incremental trapezoids and the logarithmic trapezoidal method for each decremental trapezoid. After i.v. administration only, systemic clearance (CL) was calculated as Dose divided by \(\text{AUC}_{0-\infty}\); and the volume of distribution (Vd) was the product of CL and the mean residence time as determined by moment analysis. Concentration units for radioactivity are expressed as nanogram-equivalents of darapladib/milliliter (ng Eq/ml) and as dpm/ml.

Due to the relatively long plasma half-life of total radioactivity and the sensitivity of the AMS analysis, many predose plasma samples contained total radioactivity concentrations that were above LLQ (0.1 darapladib ng equivalents per ml). In many cases, the predose plasma radioactive concentrations (in dpm/ml) were > 5% of the observed maximum plasma concentration in the same profile, thus correction was made for each individual plasma total radioactivity concentration-time profile to subtract, at each sampling time, the residual plasma total radioactivity concentrations resulting from the previous dose. The correction process involved initial determination of the terminal elimination rate constant \(\lambda_z\) of total radioactivity in the plasma profile from the previous dose period (i.e., from the plasma profile before the profile to be corrected). Thus, PK calculations were first performed
using the original, uncorrected plasma concentration-time profiles to determine the required $\lambda z$ values. This initial $\lambda z$ value was then used to calculate a plasma concentration decay profile from the pre-dose total radioactivity concentration (above LLQ) for each plasma profile using the equation:

$$C_{ti} = C_{t0} \cdot e^{(-\lambda z \cdot t_i)}$$

where $C_{ti}$ is the concentration at the $i^{th}$ sampling time, with $C_{t0}$ as the pre-dose (time zero) concentration. The corrected plasma concentration time profile was then calculated by subtracting the residual concentration at each time point (per decay curve) from the original concentration.

**In-vitro reaction phenotyping.** $[^{14}\text{C}]$darapladib was incubated at 5 and 50 $\mu$M with human liver microsomes (HLMs), at 2 mg/ml of microsomal protein and 37° C, in phosphate buffer (50 mM, pH 7.4). Reactions were started by the addition of pre-warmed cofactor solution (an NADPH regenerating system containing: 1.7 mg of NADP, 7.8 mg of glucose-6-phosphate and 6 units of glucose-6-phosphate dehydrogenase per ml of 2% [w/v] sodium hydrogen carbonate) and performed in the presence and absence of the selective cytochrome P450 inhibitors; furafylline (CYP1A2), quercetin (CYP2C8), sulphaphenazole (CYP2C9), quinidine (CYP2D6) and ketoconazole (CYP3A4). $[^{14}\text{C}]$darapladib was also incubated with Supersomes™, overexpressing individual cytochrome P450 (CYP) enzymes 1A2, 2C8, 2C9, 2C19, 2D6 or 3A4. All incubations were terminated by addition of acetonitrile and were centrifuged (at approximately 13000 g), and supernatants removed for analysis by HPLC-MS.

**In-vitro bacterial incubations.** $[^{14}\text{C}]$darapladib was incubated at a concentration of 5 $\mu$M in an anaerobic environment at 37°C for 72 hours in the presence of individually cultured bacteria; *Enterococcus faecalis*, *Escherichia coli*, *Bifidus adolescentis*, *Lactobacillus acidophilus*.
bifidobacterium longum, Lactobacillus casei, Bacteroides vulgatus and Bacteroides thetaiotaomicron. These bacteria were selected based on their known presence in the human gastrointestinal tract and their availability. After the incubations, an equal volume of acetonitrile was added to each and the resultant samples were centrifuged (at approximately 3300 g) and the supernatants removed for analysis by HPLC-MSn.

**In-vitro bacterial mutation assay.** Both darapladib and SB-554008 (M10) were assessed in the bacterial mutation assay (or Ames test). Genetically modified strains of Salmonella typhimurium (TA98, TA100, TA1535 and TA1537) and Escherichia coli WP2 uvrA (pKM101) were used, in the presence and absence of an exogenous mammalian oxidative metabolism system (Arochlor or phenobarbital-induced rat liver post mitochondrial S9 fraction) using established methodologies (Ames et al, 1973; Maron & Ames, 1983; Green, 1984). The final volume of the S9 mix used was 500 µl/plate, with darapladib tested up to 5000 µg/plate and SB-554008 tested up to 500 µg/plate (concentration limited by precipitation). An additional assessment of drug-related components present under the conditions of the darapladib Ames test was conducted, using a comparable mammalian oxidative metabolism system to that described above, to assess the metabolism of darapladib (at concentrations of 25 and 100 µM).

**Results**

**Demographic, Safety and Tolerability data.** Eight healthy male subjects (four white, three african american, one mixed race) were entered into the study, and seven subjects completed all three sessions with one individual excluded due to lack of compliance with sample collection during Session 2. Following all three sessions the
compound was regarded as generally safe and well tolerated, with any adverse events (AEs) reported, typically mild in nature (e.g. diarrhoea, flatulence, dizziness, etc).

Mass Balance of Total Radioactivity. The actual doses of i.v. and oral \(^{14}\)C-darapladib administered ranged from 6.62 to 7.91 mg (41.4- 49.5 \(\mu\)Ci, session 1), 75.3 to 78.8 mg (18.6-19.9 \(\mu\)Ci, session 2) and 79.2 to 79.4 mg (19.2-20.0 \(\mu\)Ci, session 3), respectively. Mean cumulative excretion balance data obtained from up to eight healthy male subjects following doses of \(^{14}\)C-darapladib (i.v. and oral routes) are detailed in Table 1.

Following both i.v. and oral administration, total radioactivity was eliminated almost exclusively in the feces, accounting for 85% of the dose post i.v. infusion and 93 to 95% following oral administration. Total radioactivity recovered in urine was negligible (<1%) following all dosing regimens over the respective collection periods (up to 216 or 504 h postdose). Mean recoveries of approximately 90 to 94% of the radioactive dose were reached by 120 h post oral administration, with the remaining radioactivity recovered up to 216 or 264 h postdose. Slower elimination of total radioactivity was observed post i.v. administration with only approximately 62% recovered by 120 h postdose and collections continuing to 504 h, recovering 86% of the dose.

Pharmacokinetics. A summary of the plasma pharmacokinetic parameters for darapladib, SB-553253 (M4) and total radioactivity after a single i.v. dose, a single oral dose and repeat dose oral administration is presented in Table 2. Due to the relatively long half-life of total radioactivity, many predose plasma samples in sessions 2 and 3 still contained levels of radioactivity indicating that a small amount of circulating radioactivity remained after the i.v. dose. Subtraction of the residual plasma radioactivity was performed (as described in the Materials and Methods.
section), and resulting parameters are summarized in Table 2. Mean (± standard deviation) plasma concentrations of darapladib, SB-553253, and total radioactivity are shown in Fig. 2. After i.v. administration maximum darapladib concentrations were achieved, as expected, at the end of the 3 h infusion, with volume of distribution estimated at >800 L and low plasma clearance of 17 L/h. After i.v. and single oral dose administration, the terminal half-life of darapladib was similar, and relatively long at ~40 h. After single and repeat dose oral administrations, darapladib solutions were absorbed slowly with a median $T_{\text{max}}$ of 5 h (single dose) and 6 h (repeat dose). The absolute bioavailability of darapladib solution was 12%, on average, comparing dose normalized exposures following single dose i.v. and single dose oral administration. Due to the study design, no estimate of oral bioavailability of the tablet was conducted within this study. The mean AUC(0-$\tau$) following repeat oral dosing was approximately 50% greater than AUC(0-$\tau$) following single oral dose indicating accumulation; however, this degree of accumulation was less than expected from single dose exposure data (approximately 270% accumulation was predicted from AUC(0-$\infty$)/AUC(0-$\tau$) following single dose oral data). This less than expected accumulation was consistent with results observed from previous pharmacokinetic studies in healthy volunteers (data on file).

The metabolite, SB-553253, was rapidly formed following both i.v. and oral administration of darapladib with $T_{\text{max}}$ similar to that of parent compound, although plasma concentrations of SB-553253 were much lower, with exposure of SB-553253 (as estimated by AUC) being 0.2%, 1% and 5% of parent darapladib following single i.v., single oral and repeat oral administration, respectively.

AUC(0-$\infty$) and half-life parameters for total radioactivity were not reported as the sampling duration of 96 h was too short relative to the approximated half-life of
radioactivity to adequately describe the terminal elimination phase of plasma total radioactivity resulting in large extrapolation area. Pharmacokinetic parameters for total radioactivity (nanogram-equivalents) following repeat oral dosing could not be generated due to the unknown dilution effect of non-radiolabeled darapladib from dosing on days 1 through 10.

**Metabolite Profiles. Plasma.** Representative reconstructed radiochromatogram profiles of individual time-adjusted pooled plasma samples from each dose regimen are shown in Fig. 3. Quantification of drug-related material in plasma from each dose regimen is summarised in Table 3.

Following i.v. infusion of $[^{14}\text{C}]$darapladib, the predominant component in plasma across all volunteers was unchanged darapladib which represented a mean of 89% of plasma radioactivity. The only other radiolabeled component detected was SB-553253 (M4), which accounted for approximately 1% of plasma radioactivity.

Following single oral administration of $[^{14}\text{C}]$darapladib, unchanged darapladib was again the principal radiolabeled component in plasma representing a mean of 75% of plasma radioactivity. Circulating metabolites were more notable and included M3 (SB-823094) resulting from hydroxylation of the cyclo penta pyrimidinone moiety, SB-553253, M10 (SB-554008) a uracil derivative formed following removal of the fluorobenzylthiol group, and a hydroxylated N-desethyl metabolite (M16), each of which accounted for approximately 4 to 5% of plasma radioactivity. Any remaining metabolites each accounted for <1% of plasma radioactivity.

The principal radiolabeled component in plasma samples obtained following repeat oral administration was unchanged darapladib, which represented a mean of 64% of plasma radioactivity. Circulating metabolites observed included SB-823094 (M3), SB-553253 and SB-554008 (M10) which individually accounted for means of 4
to 6% of plasma radioactivity. Other metabolites were observed, each accounting for 1% or less of the plasma radioactivity. Proposed structures and supporting spectral data are shown in Table 4.

**Urine.** Negligible amounts (1% or less of the dose) were recovered in urine across all three regimens and therefore this matrix was not examined further.

**Feces.** Radio-HPLC analysis of fecal extracts from i.v. and oral administration of [14C]darapladib were similar. Representative radiochromatograms of pooled fecal extracts are shown in Fig 4. Quantification of drug-related material in feces from each dose regimen is summarised in Table 3.

For the three dose regimens mean recovery of radioactive material from feces following solvent extraction ranged from 96 to 99%. Radio-HPLC analysis of fecal extracts from all dose regimens revealed the predominant radiolabeled component was unchanged darapladib, although several other drug-related peaks were also evident. Following i.v. administration unchanged darapladib represented a mean of approximately 55% of the fecal radioactivity (43% of the dose). The predominant metabolites observed across all subjects were SB-553253 (M4) and SB-823094 (M3) accounting for means of 23% and 11% of the fecal radioactivity (18% and 9% of the administered dose, respectively). Following both single and repeat dose oral administration unchanged darapladib accounted for a mean of 53% of the radioactive dose administered. As for i.v. administration the predominant metabolites post oral dosing were SB-553253 and SB-823094 accounting for approximately 18 and 11% of the administered radioactive dose. A number of minor metabolites were also observed across the dose regimens representing <4% of the dose and included SB-735268 (M7), SB-554008 (M10), M16 (hydroxylated SB-553253) and M19 (N-oxide). Proposed structures and supporting spectral data are shown in Table 4.
**In-vitro incubations.** Reaction phenotyping. Incubations of [14C]darapladib with HLMs showed NADPH-dependent metabolism to several metabolites including SB-553253 (M4) and SB-823094 (M3). Ketoconazole, a CYP3A inhibitor, caused marked inhibition of darapladib metabolism whereas other CYP inhibitors had no or minimal effects. Metabolism of darapladib with expressed enzymes showed extensive metabolism (up to 85% of parent metabolised) with CYP3A4, with minor turnover in other expression enzymes, most notably CYP2C8.

**Bacterial incubations.** Incubations of darapladib with various human gut bacteria under anaerobic conditions produced no evidence of metabolism with any of the strains used.

**Ames bacterial mutation assay.** Darapladib did not induce point (gene) mutation in Salmonella typhimurium (TA98, TA100, TA1535, TA1537), either in the presence or absence of metabolic activation, when tested up to 200 µg/plate (as limited by toxicity). Darapladib did not induce point (gene) mutation in Escherichia coli (WP2 pKM101 and WP2 uvr pKM10), either in the presence or absence of metabolic activation, when tested up to 5000 µg/plate. Similarly, SB-554008 (M10) showed no increases in the numbers of revertant colonies observed at any of the concentrations tested (up to 500 µg/plate), in either the presence or absence of metabolic activation. The metabolite, SB-823094, was identified under the incubation and metabolic activation conditions of the Ames assay of darapladib and was not genotoxic.

**Discussion.** Following i.v. and oral administration of [14C]darapladib to humans, total radioactivity was eliminated almost exclusively in the feces (86 to 95% of the dose). Recovery of the dose was considered complete (Roffey et al., 2007) across all regimens (86 to 95% of the administered dose) but elimination was somewhat
protracted, especially following i.v. administration. Following oral dosing, between 90 and 94% of the dose had been recovered by 120 h postdose, whereas recovery of the i.v. dose was only around 62% at this time, with notable amounts of dose continuing to be eliminated slowly thereafter. The volume of distribution in humans was very high (>800 L) suggesting notable tissue distribution. With a pKa of 8.4 (tertiary amine) and a Log P > 3.4, darapladib is a cationic amphiphilic drug (CAD), a common feature of which is sequestration in lysosomes by a mechanism called pH partitioning (Funk and Krise, 2012). As a result CADs often have a large volume of distribution, protracted elimination and inhibit lysosomal lipid metabolism resulting in phospholipidosis (Reasor and Kacew, 2001), which was observed in rats and mice following dosing of darapladib at toxicological doses. It is therefore considered that the slower elimination of a proportion of the radioactive dose post i.v. dosing may represent more widespread tissue exposure to darapladib via direct administration into the blood, when compared to oral dosing where a large proportion of the dose is either unabsorbed or removed via first pass metabolism or elimination of parent compound prior to entering the systemic bloodstream (consistent with the low absolute bioavailability of 12%). The plasma elimination half-lives of darapladib post i.v. and oral dosing were consistent with prolonged elimination, each around ~40 h.

The principal radiolabelled component observed in human plasma following both i.v. and oral solution dosing of radiolabeled compound was unchanged darapladib. The only metabolite observed in plasma following i.v. dosing was SB-553253 (M4), albeit at very low levels (1% of plasma radioactivity). Following oral administration circulating metabolites were more evident, including SB-823094 (M3), SB-553253 and SB-554008 (M10), each of which were present at mean concentrations of around 5% of plasma radioactivity, and at low absolute
concentrations of less than 2 ng equivalents/ml. The fact that metabolites are more prevalent in the circulation after oral than after i.v. administration is indicative of first pass metabolism, and consistent with the notable difference (50-60%) in plasma AUC and C_{max} for total radioactivity compared to unchanged darapladib. Incubations with HLMs showed that CYP3A is the main enzyme responsible for darapladib metabolism, which is consistent with intestinal and/or hepatic first pass metabolism. Notably, SB-554008 was not detected in any \textit{in vitro} incubations using HLMs, but is the major acid degradant of darapladib (e.g. dosed in solution) \textit{in vivo}. Moreover, the clinical formulation of darapladib has been developed as an enteric coated tablet to minimise acid hydrolysis in the stomach to SB-554008. Nonetheless, because of the nature and objectives of this study, and the technical issues of developing a protected radiolabeled formulation, \textsuperscript{[14C]}darapladib was formulated as a solution, and administered with food to minimise any acid degradation. Since SB-554008 was seen in plasma following a single oral dose but not post-i.v. administration, it is inferred a proportion of SB-554008 is being generated pre-systemically. Bacterial incubations with darapladib showed negligible metabolism, and therefore SB-554008 observed post oral dosing of \textsuperscript{[14C]}darapladib solution is likely the result of acid hydrolysis in the stomach and is greater than would be expected following administration of an enterically coated darapladib tablet.

While SB-553253 (M4) was quantified in plasma in preclinical toxicology and clinical studies, SB-554008 had not been previously observed in preclinical species in either plasma or excreta, and SB-823094 (M3) had not been previously quantified in plasma, preclinically or clinically. Therefore SB-554008 (M10) was potentially human specific and SB-823094 potentially disproportionate (i.e. present at higher plasma concentrations in humans than in preclinical species), albeit present at very low concentrations.
low levels in plasma. Both metabolites were therefore evaluated for their genotoxicity potential prior to entering the large phase III program, (as recommended in the MIST guidance). The presence of SB-823094 was demonstrated under the conditions of the darapladib Ames test, but SB-554008 was absent and required direct testing, which proved negative. Safety margins for general toxicity were also established for both metabolites prior to the start of phase III. Specific HPLC-MS/MS assays were developed and the exposures of SB-554008 and SB-823094 were determined in humans post repeat dosing of the enteric clinical formulation, and in rats and dogs (at no adverse effect dose levels) with preclinical exposure margins for SB-823094 being established in both rats (~1.5-fold) and dogs (~14-fold). Given the chiral nature of SB-823094 the formation of each enantiomer was also confirmed, with no marked differences between rats, dogs and humans. Safety margins for SB-554008 were attained in the dog only (~1.5-fold), while in the rat this metabolite was present at lower concentrations than in humans, which is consistent with the physiological pH of the stomach in rat being higher than dog in both fed and fasted states (McConnell et al, 2008; Sagawa et al, 2009). Interestingly, SB-554008 was not observed at all systemically after a single oral dose of the enteric tablet in humans (LLQ 0.1 ng/mL), while after a single oral solution dose of [14C]darapladib the mean individual pooled plasma concentration of SB-554008 was up to 4.5 ng equivalents/ml, clearly demonstrating that SB-554008 concentrations are substantially greater after oral dosing of solution than an enteric coated tablet, which protects darapladib from the low pH environment in the stomach. SB-554008 is detectable at low concentrations after dosing to steady state which is ascribed to the fact that even at neutral pH there is a very slow rate of hydrolysis of darapladib.
The pharmacological activity of SB-823094 was similar to that of darapladib and SB-553253, while that of SB-554008 was about 100-fold less. Since exposure to the metabolites in humans at steady state was much lower than darapladib (means of ≤5% of parent based on AUC), these metabolites are not expected to contribute significantly to the pharmacological activity. Based on the genotoxicity evaluation, general safety cover and pharmacological activity, no further metabolite work was conducted.

Darapladib was eliminated both by metabolism and as unchanged drug, via the feces. The radiolabeled metabolite profiles in fecal extracts from all three dosing regimens were very similar, qualitatively and quantitatively, and were independent of the route and duration of administration. Darapladib was the primary component in feces representing 43-53% of the dose, while SB-823094 and SB-553253 accounted for 9-19% of the dose. Several minor metabolites, each accounting for less than 4% of the dose, were also observed. Based on the structure of the metabolites, a simplified metabolic scheme is shown in Fig. 5.

Minimal absorption can be estimated in humans from urinary excretion post oral dosing or, where data exists, by comparing urinary excretion following i.v. and oral administrations of a radiolabeled drug (Gibaldi and Perrier, 1982). However, due to the negligible amounts of radioactive drug-related material in urine following [14C]darapladib administration neither approach was undertaken. Alternatively, absorption can be estimated from oral fecal metabolite profiles, assuming unchanged darapladib observed in these profiles (53% of the dose) is unabsorbed, and given the negligible degradation of darapladib by gastro-intestinal microbiota. Possible acid degradation of darapladib to SB-554008 (up to 4% of the dose) was also considered and together suggest absorption of darapladib is at least 43% of the dose. Moreover,
i.v fecal profiles showed a large proportion of the dose (43%) is secreted unchanged (via bile and/or direct gut) into feces and therefore oral absorption may well be substantially higher than the lower estimate of 43%. Absorption is defined as crossing the apical membrane of the enterocytes, and as darapladib is both a CYP3A and PgP substrate (as are metabolites SB-823094 and SB-553253) it is postulated that the oral fecal profiles observed in humans reflect metabolism and subsequent elimination at the level of the enterocyte (Hochman et al, 2000; Paine et al, 2006). Previous lower estimates of absorption from rats and dogs (based on urinary and biliary excretion of drug-related material) do not allow for this gut wall elimination, although a re-examination of oral fecal data in dogs suggested a higher absorption of at least 41%, however this information is not available for rats. The similar levels of SB-823094 and SB-553253 in feces following oral and i.v. dosing are thought to reflect the varying contributions of hepatic and intestinal CYP3A to the overall metabolic clearance of darapladib depending on the route of administration.

The systemic exposure to radioactivity following a single oral radioactive dose during steady state dosing was about 30% lower than that following the first single oral radioactive dose. This observation is consistent with, and an independent confirmation of, the lower than expected accumulation of darapladib on repeat dosing. Given its CYP3A and PgP substrate status, the lower exposure to radioactivity may indicate lesser absorption of darapladib across the enterocyte after repeat oral dosing combined with greater extent of first pass metabolism and/or efflux transport as a result of induction of enzyme and/or efflux transporter activity after repeat dosing.

Due to the complexity of AMS analysis and the decision to only profile time-adjusted pooled plasma samples to 24 h, it was not possible to confirm whether enzyme induction is the mechanism underlying the time dependent pharmacokinetics. The
question of CYP3A induction will be addressed in a planned DDI study with midazolam.

In summary, this unusual study design utilising different routes of administration, low level AMS detection and a repeat dose oral leg, has allowed an enhanced understanding of the relative absorption, metabolism and elimination of darapladib in humans, despite the absence of absolute metabolite quantification post repeat dose.

The increased availability and acceptance of AMS within the pharmaceutical industry (Bowers et al, 2013; Harrell et al, 2013; Iyer et al, 2012; Lappin et al, 2012; Smith 2011; Vuong et al, 2012) does facilitate replacing the traditional single dose human radiolabeled study with a design using repeated therapeutic doses incorporating trace levels of radioactivity amenable to AMS detection, which in conjunction with MS and NMR techniques, would allow complete determination of metabolism and disposition under the more relevant steady state conditions.

**Acknowledgements.** The authors are grateful to Jackie Bloomer for her input into the study design, to Will Ellis, Clive Felgate and Steven Corless for providing technical assistance with AMS, Bianca Squillaci for technical assistance with metabolite identification and Dave Lundberg, John Kratz and Sherry Wang for bioanalysis of darapladib and SB-553253 (M4). The authors would also like to acknowledge Simon Harwood for the synthesis of $[^{14}\text{C}]$darapladib.

**Authorship Contributions**

*Participated in research design:* Young, Ellens, Magee, Boyle

*Conducted experiments:* Dave, Nash, Young, Roberts, Taylor
Performed data analysis: Dave, Nash, Young, Magee, Roberts, Taylor

Wrote or contributed to the writing of the manuscript: Dave, Nash, Young, Ellens, Magee, Greenhill, Boyle
References


Legends for Figures

Figure 1:
Structure of [14C]darapladib and Synthetic Metabolites

Figure 2:
Mean (± SD) concentrations of total radioactivity, darapladib and SB-553253 following (a) single i.v. (b) single oral and (c) repeat dose oral administration of [14C]darapladib to human volunteers, and (d) comparative total radioactivity profiles following single and repeat oral dose of [14C]darapladib to human volunteers.

Figure 3:
Representative radiochromatograms of human plasma following (a) single i.v. (b) single oral and (c) repeat dose oral administration of [14C]darapladib to human volunteers.

Figure 4:
Representative radiochromatograms of human fecal extracts following (a) single i.v. (b) single oral and (c) repeat dose oral administration of [14C]darapladib to human volunteers.

Figure 5:
Simplified metabolic scheme for darapladib in humans
**TABLE 1**

*Mean cumulative total radioactivity excreted in urine and feces (% of dose) after i.v. and oral administration of [*14C*]darapladib to healthy volunteers*

<table>
<thead>
<tr>
<th>Elapsed Time from Dose (h)</th>
<th>Intravenous</th>
<th>Single Oral</th>
<th>Repeat dose Oral</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Urine</td>
<td>Faeces</td>
</tr>
<tr>
<td>Predose</td>
<td>8</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0-24</td>
<td>8</td>
<td>0.4</td>
<td>8.0</td>
</tr>
<tr>
<td>0-48</td>
<td>8</td>
<td>0.5</td>
<td>21.9</td>
</tr>
<tr>
<td>0-72</td>
<td>8</td>
<td>0.6</td>
<td>37.5</td>
</tr>
<tr>
<td>0-96</td>
<td>8</td>
<td>0.7</td>
<td>57.8</td>
</tr>
<tr>
<td>0-120</td>
<td>8</td>
<td>0.7</td>
<td>61.5</td>
</tr>
<tr>
<td>0-144</td>
<td>8</td>
<td>0.8</td>
<td>69.1</td>
</tr>
<tr>
<td>0-168</td>
<td>8</td>
<td>0.8</td>
<td>74.2</td>
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<tr>
<td>0-192</td>
<td>8</td>
<td>0.8</td>
<td>74.8</td>
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<tr>
<td>0-216</td>
<td>8</td>
<td>0.8</td>
<td>77.9</td>
</tr>
<tr>
<td>0-240</td>
<td>8</td>
<td>0.9</td>
<td>79.7</td>
</tr>
<tr>
<td>0-504</td>
<td>8</td>
<td>0.9</td>
<td>85.5</td>
</tr>
</tbody>
</table>

*a- Fecal data from subject 101 have been excluded from the calculation of fecal mean due to low balance recovery during single oral dose regimen.

b-Subject 101 excluded from this regimen due to low balance recovery during single oral administration regimen.

c- Intravenous group samples collected for 504 hours, both single and repeat oral dose final sampling point was 264 hours post (radioactive) dose.

All urine and fecal radioactivity was determined using LSC.*
TABLE 2

Geometric mean (CV%) pharmacokinetic parameters of total radioactivity, darapladib and SB-553253 in plasma after i.v. and oral administration of $[^{14}C]$darapladib to healthy volunteers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Single Dose Intravenous (n = 8)</th>
<th>Single Dose Oral (n = 8)</th>
<th>Repeat Dose Oral (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total radioactivity</td>
<td>Darapladib</td>
<td>SB-553253</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;∞&lt;/sub&gt; (ng.h/mL)</td>
<td>491 (17.7)</td>
<td>603 (18.8)</td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→t&lt;/sub&gt; (ng.h/mL)</td>
<td>567 (16.1)</td>
<td>605 (17.2)</td>
<td>0.614&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0→τ&lt;/sub&gt; (ng.h/mL)</td>
<td>200 (13.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</td>
<td>34.9 (20.6)</td>
<td>25.3 (12.4)</td>
<td>0.296&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>2.98 (2 - 5)</td>
<td>2.97 (2 - 3.5)</td>
<td>2.98 (2 - 3.5)</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>40.9 (8.74)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL (L/h)</td>
<td>11.2 (17.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L)</td>
<td>632.3 (19.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup> parameter units for total radioactivity are ng equiv.h/mL for all regimens

<sup>2</sup> parameter units for total radioactivity are ng equiv/mL for all regimens

<sup>3</sup> median (range)

<sup>4</sup> AUC and C<sub>max</sub> corrected for pre-dose concentration (see detail in Materials and Methods section)

<sup>5</sup> n = 2

* Relative mean radioactive exposure (i.e. dpm only) was 320 dpm.h/mL on Day 11 (compared to 420 dpm.h/mL on Day 1)

Total radioactivity post iv administration was determined using LSC; total radioactivity post oral dosing was determined using AMS.
### TABLE 3

Mean percentage of radioactivity of darapladib and its metabolites in human plasma and feces after i.v. and oral administration of $[^{14}\text{C}]$darapladib to healthy volunteers

<table>
<thead>
<tr>
<th>Peak id$^1$</th>
<th>Mean Percentage Plasma Radioactivity</th>
<th>Mean % matrix radioactivity (mean % dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.v</td>
</tr>
<tr>
<td>Darapladib</td>
<td></td>
<td>89.2</td>
</tr>
<tr>
<td>M3 (SB 823094)</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>SB-553253 (M4)</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>SB-735258 (M7)</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M8</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M9</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>GSK219147 (M11) or M6$^5$</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>SB-554008 (M10)</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M14</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M16</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>M19</td>
<td></td>
<td>ND</td>
</tr>
</tbody>
</table>

1. Metabolites M9 and M14 coeluete
2. Metabolites M10, M16 and M19 coeluete (on a few occasions some of these peaks were resolved but have been quantified as a coeluting peak for consistency)
3. Metabolites M6 and M11 coeluete
4. Metabolites M10 and M16 coeluete
5. The repeat oral dose results - %dose results refers to only the radioactive dose administered on day 11.

BLQ – below limit of Quantification
ND- Not detected
Several metabolites including M2, M6, M12, M21 and M22 observed in fecal extracts were detected by mass spectrometry only. Plasma profiles were determined using HPLC-AMS, whereas fecal profiles utilised off-line radio-HPLC.
**TABLE 4**

*Structural information on darapladib and its notable metabolites following administration of $[^{14}C]$darapladib to human volunteers*

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Proposed Structure</th>
<th>[M+H]$^+$ ion and MS-MS Product ions</th>
<th>$^1$H-NMR (600Mhz, 1:1 ACN:D$_2$O) (where available)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL,FE</td>
<td><img src="image" alt="Darapladib" /></td>
<td>Accurate mass [M+H]$^+$&lt;br&gt;Observed: 667.2728&lt;br&gt;Calculated: 667.2730&lt;br&gt;594, 486, 391, 351, 317, 235, 109</td>
<td>7.75 (d, 2H), 7.67 (d, 2H), 7.53 (d, 2H), 7.38 (d, 2H), 7.30 (dd, 2H), 6.90 (t, 2H), 4.79 (s, 2H), 4.65 (s, 2H), 4.30 (s, 2H), 3.67 (bdt, 2H), 2.91 (t, 2H), 2.84 (q, 4H), 2.77 (t, 2H), 2.68 (t, 2H), 2.05 (m, 2H), 1.07 (t, 6H) Mixture of rotamers, major rotamer assigned</td>
</tr>
<tr>
<td>FE$^1$</td>
<td><img src="image" alt="M2" /></td>
<td>Accurate mass [M+H]$^+$&lt;br&gt;Observed: 611.2112&lt;br&gt;Calculated: 611.2104</td>
<td>No NMR</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Compound</th>
<th>Accurate mass [M+H]^+</th>
<th>Observed</th>
<th>Calculated</th>
<th>Peaks (m/z)</th>
<th>NMR Data</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PL',FE</strong></td>
<td></td>
<td>683.2681</td>
<td>683.2679</td>
<td>610, 557, 502, 484, 391, 351, 333, 315, 235, 207, 109</td>
<td>7.83 (d, 2H), 7.81 (d, 2H), 7.62 (d, 2H), 7.49 (d, 2H), 7.40 (dd, 2H), 7.08 (t, 2H), 5.18 (brm, 1H), 5.08 (s, 2H), 4.69 (s, 2H), 4.48 (s, 2H), 3.35 (t, 2H), 3.29 (ddd, 1H), 2.68 (ddd, 1H), 2.60 (t, 2H), 2.53 (q, 4H), 2.51 (m, 1H), 2.40 (m, 1H), 0.99 (t, 6H)</td>
<td>Mixture of rotamers, major rotamer assigned</td>
</tr>
<tr>
<td><strong>SB-823094 (M3)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PL',FE</strong></td>
<td></td>
<td>639.2408</td>
<td>639.2417</td>
<td>486, 431, 363, 323, 317, 235, 109</td>
<td>7.78 (d, 2H), 7.67 (d, 2H), 7.50 (d, 2H), 7.37 (d, 2H), 7.27 (dd, 2H), 6.88 (t, 2H), 4.67 (s, 2H), 4.59 (s, 2H), 4.27 (s, 2H), 3.77 (brt, 2H), 3.12 (t, 2H), 2.96 (q, 2H), 2.81 (t, 2H), 2.70 (t, 2H), 2.08 (m, 2H), 1.16 (t, 3H)</td>
<td>Mixture of rotamers, major rotamer assigned</td>
</tr>
<tr>
<td>FE&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Accurate mass [M+H]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>No NMR</td>
<td></td>
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<td></td>
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<tr>
<td>---</td>
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<td></td>
</tr>
</tbody>
</table>
| M6 | Observed: 573.2689  
Calculated: 573.2692  
391, 235, 205 | |
| **PL<sup>1</sup>,FE** | Accurate mass [M+H]<sup>+</sup> | 7.85 (d, 2H), 7.79 (d, 2H), 7.79, (d, 2H), 7.41 (d, 2H), 4.78 (s, 2H), 4.70, (s, 2H), 3.91 (s, 3H), 2.76 (t, 2H), 2.68 (t, 2H), 1.05 (t, 6H)  
Some resonances obscured | |
| SB-735238 (M7) | Observed: 557.2751  
Calculated: 557.2740  
484, 391, 361, 351, 347, 235, 207, 127 | |
PL', FE'

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mass Details</th>
<th>NMR Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>M8</td>
<td>635 [M+H]^+ 562, 235</td>
<td>No NMR</td>
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</tbody>
</table>

FE'

<table>
<thead>
<tr>
<th>Structure</th>
<th>Mass Details</th>
<th>NMR Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>M9</td>
<td>Accurate mass [M+H]^+ Observed: 515.2263 Calculated: 515.2270</td>
<td>No NMR</td>
</tr>
<tr>
<td>Structure</td>
<td>Compound</td>
<td>Accurate mass [M+H]+</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>-----------------------</td>
</tr>
<tr>
<td>SB-554008 (M10)</td>
<td>PL,FE</td>
<td>7.84 (d, 2H), 7.78 (d, 2H), 7.75, (d, 2H), 7.45 (d, 2H), 4.68 (s, 2H), 4.65, (s, 2H), 3.52 (t, 2H), 2.62 (t, 4H), 0.99 (t, 6H)</td>
</tr>
<tr>
<td>GSK219147 (M11)</td>
<td>PL,FE</td>
<td>7.84 (d, 2H), 7.79 (d, 2H), 7.76, (d, 2H), 7.56 (d, 2H) remaining resonances obscured</td>
</tr>
<tr>
<td>FE&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Accurate mass [M+H]&lt;sup&gt;+&lt;/sup&gt;</td>
<td>No NMR</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------</td>
<td>--------</td>
</tr>
</tbody>
</table>
| ![M12](image)  | Observed: 573.2511
Calculated: 573.2511
391, 309, 223, 209 |        |

<table>
<thead>
<tr>
<th>PL&lt;sup&gt;1&lt;/sup&gt;, FE</th>
<th>Accurate mass [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Remaining resonances obscured</th>
</tr>
</thead>
</table>
| ![M14](image)     | Observed: 527.2634
Calculated: 527.2634 | 8.12 (s, 1H), 7.85 (d, 2H), 7.79 (d, 2H), 7.78 (d, 2H), 7.44 (d, 2H), 4.96 (s, 2H), 4.71, (s, 2H) |

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<table>
<thead>
<tr>
<th>Molecule</th>
<th>Formula</th>
<th>Accurate Mass $[M+H]^+$</th>
<th>Observed</th>
<th>Calculated</th>
<th>Mass Difference</th>
<th>Spectral Data</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>M16</td>
<td>FE</td>
<td>Observed: 655.2363</td>
<td>655.2366</td>
<td>637, 529, 484, 363, 323, 315, 235, 207</td>
<td>7.79 (d, 2H), 7.67 (d, 2H), 7.51 (d, 2H), 7.37 (d, 2H), 7.29 (dd, 2H), 6.90 (t, 2H), 5.16 (dd, 1H), 4.80 (s, 2H), 4.71 (s, 2H)</td>
<td>Mixture of rotamers, major rotamer assigned, remaining signals obscured</td>
<td></td>
</tr>
<tr>
<td>M19</td>
<td>FE</td>
<td>Observed: 683.2712</td>
<td>683.2679</td>
<td>594</td>
<td>7.78 (d, 2H), 7.68 (d, 2H), 7.53 (d, 2H), 7.40 (d, 2H), 7.29 (dd, 2H), 6.89 (t, 2H), 4.79 (s, 2H), 4.65 (s, 2H), 3.21 (t, 2H)</td>
<td>Mixture of rotamers, major rotamer assigned, remaining signals obscured</td>
<td></td>
</tr>
</tbody>
</table>
| FE³ | **M21** | Accurate mass \([M+H]^+\)  
Observed: 529.2424  
Calculated: 529.2427 | No NMR |
|---|---|---|---|
| FE³ | **M22** | Accurate mass \([M+H]^+\)  
Observed: 683.2683  
Calculated: 683.2679  
391,351,333,235 | No NMR |

PL = seen in plasma  
FE = seen in feces  
PL¹/FE¹ = only seen in oral matrix
Figure 1

- Darapladib (SB-480848)
- M4 (SB-553253)
- M10 (SB-554008)
- M3 (SB-823094)
- M11 (GSK219147)
- M7 (SB-735258)

* position of radiolabel
Figure 2

a  Single dose IV infusion (n = 8)

b  Single Dose Oral (n = 8)

c  Repeat Dose Oral (n = 7)

d  Total Plasma Radioactivity
Figure 5

M10 (SB-554008) Plasma Single, Repeat Faeces IV, Single, Repeat
M7 (SB-735258) Plasma Single Faeces IV, Single, Repeat
M11 (GSK219147) Plasma Single, Repeat Faeces IV, Single, Repeat
M19 (N-oxide) Faeces IV, Single, Repeat
M3 (SB-823094) Plasma Single, Repeat Faeces IV, Single, Repeat
M4 (SB-553253) Plasma IV, Single, Repeat Faeces IV, Single, Repeat
M16 (hydroxy SB-553253) Plasma Single Faeces IV, Single, Repeat

IV = Intravenous dose
Single = Single oral dose
Repeat = Repeat oral dose

M8 (loss of 32 amu)