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Characterisation and identification of the human N$^+$-glucuronide metabolite of cediranib

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ABSTRACT

Cediranib (4-[(4-fluoro-2-methyl-1*H*-indol-5-yl)oxy]-6-methoxy-7-[3-(1-pyrrolidinyl)propoxy]quinazoline; RECENTIN^{TM}), a vascular endothelial growth factor (VEGF) tyrosine kinase inhibitor (TKI) of all three VEGF receptors, is currently in Phase III clinical trials for the first-line treatment of colorectal cancer and the treatment of recurrent glioblastoma.

During its clinical development a unique human metabolite, an N$^+$-glucuronide, was identified as a major circulating metabolite and one of the major metabolites excreted into faeces. Given the possibility of four sites for the conjugation of the glucuronic acid moiety, determination of the location of the conjugation site on cediranib was warranted. A small quantity of the N$^+$-glucuronide metabolite of cediranib was initially generated using recombinant human uridine glucuronosyltransferase 1A4 (UGT1A4) enzymes. The metabolite generated was characterised by HPLC-UV and mass spectrometric (HPLC–MS^n) detection and confirmed by ^{1}H NMR spectroscopy. However, the exact site of conjugation could not be determined without generating more of the metabolite. Hence a subsequent biosynthetic scale-up experiment was devised to generate a sufficiently large quantity for full structural characterisation by ^{1}H NMR spectroscopy.

The identity of the N$^+$-glucuronide metabolite generated in the UGT1A4 scale-up experiment was confirmed by HPLC–MS^n and displayed the same retention time, molecular mass and mass fragmentation data as the metabolite generated in previous human liver microsomal and hepatocyte incubations.

^{1}H NMR spectroscopy clearly showed the characteristic anomeric doublet at approximately 4.7 ppm, which, following irradiation during selective Rotating frame Overhauser Effect Spectroscopy (ROESY) experiments, enabled the site of glucuronidation to be confirmed on the pyrrolidine nitrogen.

With the exception of the N$^+$-glucuronide metabolite, all other human metabolites of cediranib were observed following incubation with hepatocytes from rat and cynomolgus monkey, the species used for toxicology testing of the drug [6]. As the N$^+$-glucuronide was not detected in the preclinical species, it is suggested that its formation is more likely in human and higher primates (great apes), a finding widely supported in the literature.

1. Introduction

Cediranib (Recentin^{TM}) (Fig. 1) is currently in Phase III clinical trials for the treatment of colorectal cancer (CRC) and recurrent glioblastoma. It is a highly potent vascular endothelial growth factor (VEGF) inhibitor, known to show activity against all three VEGF receptor tyrosine kinases, and stem cell factor receptor (KIT). VEGF is involved in the regulation of key processes throughout the angiogenic cascade, and over-production of VEGF in tumours facilitates tumour progression by stimulating angiogenesis and increasing vascular permeability. Inhibition of VEGF receptor tyrosine kinases therefore stabilizes the progression of tumours by disrupting tumour-induced angiogenesis [1]. Preclinical studies have shown that cediranib prevents VEGF-induced angiogenesis *in vivo* and inhibits the growth of established human tumour xenografts in athymic mice in a dose-dependent manner [2]. In the clinic, cediranib has exhibited activity in patients with advanced CRC and recurrent glioblastoma [3–5].

Abbreviations: HLM, human liver microsomes; HPLC–MS, high performance liquid chromatography–mass spectrometry; UGT, uridine glucuronosyl transferase; UDPGA, uridine diphosphate glucuronic acid; NMR, Nuclear Magnetic Resonance Spectroscopy; NOE, nuclear Overhauser effect; ROESY, Rotating frame Overhauser Effect.

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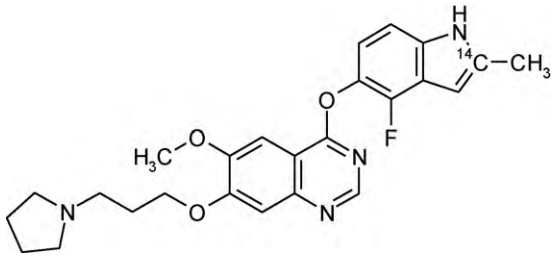


Fig. 1. The chemical structure of [^{14}C]-cediranib.

In preliminary preclinical studies, the routes of metabolism of cediranib were investigated in liver preparations from several species, including man. The *in vitro* metabolic fates were assessed and the identity of the hepatic human enzymes involved in the oxidation and conjugation of cediranib were confirmed and discussed in detail by Schulz-Utermoehl et al. [6].

In clinical studies, following an oral administration of [^{14}C]-cediranib, the N^+-glucuronide metabolite was identified as the most abundant metabolite in faeces, as well as being a major circulating metabolite in plasma. It was also detected as one of the minor metabolites in the metabolite envelope excreted into human urine (unpublished data).

Given the possibility of four sites for the conjugation of the glucuronic acid moiety, localising the exact conjugation site on cediranib was required. Initial attempts to fully identify the metabolite, isolated from an incubation with microsomes prepared from cell lines expressing a single UGT isoform, proved unsuccessful by ^{1}H NMR spectroscopy due to the small amount of metabolite generated (approx. 40 μg). Hence, formation of a sufficiently large amount of the metabolite was required using a biosynthetic *in vitro* system.

This paper describes the detection and initial characterisation of the N^+-glucuronide metabolite by HPLC–MS, its subsequent biosynthetic generation, and its subsequent full structural elucidation by ^{1}H NMR spectroscopy.

2. Materials and methods

2.1. Chemicals and materials

[^{14}C]-cediranib maleate (4-[(4-fluoro-[2-^{14}C]-2-methyl-1*H*-indol-5-yl)oxy]-6-methoxy-7-[3-(1-pyrrolidinyl)propoxy]quinazoline maleate) was synthesised in 2 batches (1R4, specific activity of 26.9 mCi/mmol and 1R6, specific activity of 59 mCi/mmol) by AstraZeneca UK Ltd., Macclesfield, UK and Selcia Ltd., Ongar, UK, respectively. The initial radiochemical purities of batches 1R4 and 1R6 were >98 and 99%, respectively.

Fisher Scientific supplied acetonitrile (ACN) and methanol (MeOH). All other chemicals were purchased from commercial suppliers and were of analytical grade or the best equivalent.

2.2. Characterisation of [^{14}C]-cediranib

Stock solutions of [^{14}C]-cediranib were diluted in either water:ACN 95:5% (v/v, both acidified with formic acid, 0.2%, v/v) or water:ACN 80:20% (v/v) to concentrations in the range of 74 to 100 μM (dependent on specific activity of the batch used for each experiment), before the radiochemical purity was assessed by HPLC with radiochemical detection (HPLC-RAD).

2.3. Hepatocytes

Viable fresh hepatocytes were isolated from male Han-Wistar rats (AstraZeneca Alderley Park; Macclesfield, UK) and freshly isolated human hepatocytes from 2 male and 3 female donors were purchased from the United Kingdom Human Tissue Bank (UKHTB; Leicester, UK). All human liver-derived samples were obtained with permission from the relevant local ethical committees.

2.4. Human hepatocyte incubations

Human hepatocyte suspensions (donors 1, 2 and 3; approximately 1×10^6 cells/mL) and hepatocyte cultures (donors 4 and 5) were prepared and incubated with [^{14}C]-cediranib (10 μM final concentration) as described in detail in [6].

2.5. Liver microsomes

Pooled human liver microsomes (28 male and 5 female donors; lot 63103) were obtained from BD Biosciences (San Jose, CA). Pooled liver microsomes from cynomolgus monkey (8 male; lot 3), Dunkin-Hartley guinea-pig (8 male; lot 12635), Göttingen mini-pig (3 male; lot 71911), rhesus monkey (4 male; lot 1), New Zealand White rabbit (3 male; lot 98358), CD-1 mouse (150 male; lot 80662), beagle dog (20 male; lot 11969) and Han-Wistar rat (30 male; lot 37975) were purchased from BD Biosciences (San Jose, CA).

2.6. Recombinant enzymes

Samples of microsomal fractions prepared from insect cells transfected with plasmid vectors expressing human UGT1A1 (lot 13), UGT1A3 (lot 13), UGT1A4 (lots 9, 10, 11, 34582 and 59840), UGT1A6 (lot 10), UGT1A7 (lot 41931), UGT1A8 (lot 36867), UGT1A9 (lot 8), UGT1A10 (lot 51067), UGT2B4 (lot 4), UGT2B7 (lot 55297), UGT2B15 (lot 8) and UGT2B17 (lot 4) were purchased from BD Biosciences (San Jose, CA).

2.7. Phase II hepatic microsomal and recombinant UGT enzyme incubations

Incubations (1 mL) of liver microsomes from human, cynomolgus monkey, Dunkin-Hartley guinea-pig, Göttingen mini-pig, rhesus monkey, New Zealand White rabbit, CD-1 mouse, beagle dog and Han-Wistar rat (all at 1 mg protein/mL) with [^{14}C]-cediranib (10 μM final concentration), as well as the incubations with individual recombinant UGT enzymes, were conducted as described in detail in [6].

2.8. Metabolite profiling by HPLC-RAD

[^{14}C]-cediranib and its metabolites were separated on an ACE AQ column (4.6 × 150 mm, 5 μm; Advanced Chromatography Technologies (ACT), Aberdeen, UK) utilising Perkin Elmer 200 Series pumps (Beaconsfield, UK) and radiochemical detection *via* a Packard Radiomatic Flo-One Beta 500 TR series detector (Packard Instruments, Pangbourne, UK). The detailed experimental conditions are described in [6]. The recovery of radioactivity from the column was evaluated by comparing the radioactive content (determined by liquid scintillation counting) of the post-column eluent to the pre-column eluent. No loss of radioactivity on the column was found for any of the incubated samples. For the UGT phenotyping experiments and Phase II microsomal incubate samples, a shorter HPLC-gradient was employed and different HPLC systems were used as detailed in [6].

2.9. Metabolite identification by HPLC–MS^n

[^{14}C]-Cediranib and its metabolites were separated on a Polaris C18 column (4.6 × 150 mm, 5 μm, Varian, Yarnton, UK) using Agilent HP1200SL binary pumps (Agilent Technologies UK Ltd.,

Stockport, UK) with a flow rate of 1 mL/min. The mobile phase consisted of 0.2% formic acid in deionised water (solvent A) and 0.2% formic acid in ACN (solvent B). Mass spectrometry data were collected either using Xcalibur version 2.0 (Thermo Fisher Scientific, Waltham, MA) on a Thermo Finnigan LTQ ion trap instrument (Thermo Fisher Scientific, Waltham, MA) fitted with an electrospray ionisation (ESI) source operating in positive mode under optimised conditions (source voltage 3.5 kV; sheath gas, 60: auxillary gas, 80; capillary temperature 350 °C), or using the MassLynx v4.0 (Waters) on a Waters Micromass Quattro Micro™ API instrument (Waters, Elstree, UK) fitted with an electrospray ionisation (ESI) source operating in positive mode under optimised conditions (cone voltage 20 V; collision energy 20 eV).

2.10. Data analysis

The assessment of the metabolism of [^{14}C]-cediranib was made by both qualitative and quantitative analysis of the chromatographic patterns. Radiolabelled components were assumed to have the same specific activity as parent molecule.

MS^n data was obtained for the metabolites identified in the analysis by HPLC–MS^n with radiometric detection. Components were identified as being derived from [^{14}C]-cediranib if they demonstrated elements of the characteristic isotopic and fragmentation patterns observed with the parent molecule.

2.11. N^+-glucuronide metabolite biosynthesis

The N^+-glucuronide metabolite was generated *via* a biosynthetic route by Novacta Biosystems (Welwyn Garden City, UK), using recombinant UGT1A4 (BD Biosciences, Woburn, MA). Novacta Biosystems also performed the metabolite scale-up (100 mL reactions) and the isolation and purification of the N^+-glucuronide metabolite of cediranib.

Cediranib was incubated with UGT1A4 at a range of protein, cediranib and cofactor (UDPGA) concentrations for 18 h. The effects of temperature, buffer pH and the presence of BSA (bovine serum albumin, 3%, w/v) and the β-glucuronidase inhibitor, saccharolactone (5 mM) on metabolite formation were investigated (conditions summarised in Tables 2 and 3).

Optimum yield was achieved with incubations carried out at a UGT1A4 protein concentration of 0.25 mg/mL in 50 mM Tris HCl buffer, pH 7.5 containing 10 mM $MgCl_2$, 100 μg alamethicin/mg protein and 2 mM UDPGA. The total incubation volume was 100 mL. Cediranib (prepared as a 15.9 mM solution in DMSO) was diluted 1 in 200 with the microsomal suspension to achieve a final concentration of 79 μM. Incubations were maintained at 37 °C overnight in a shaking water bath and terminated by the addition of 100 mL of ACN.

2.12. Metabolite isolation

The N^+-glucuronide metabolite was isolated from the UGT1A4 bulk incubation mixture by solid phase extraction (SPE). To prevent significant degradation of the metabolite, the entire process was carried out on ice, wherever possible. The residual substrate (cediranib) in the terminated incubation mixture was removed into ACN (50 mL) using sodium chloride (20 g) to induce the phase separation, followed by continuous mixing for 1 h. The organic layer was then removed and discarded. An additional 150 mL ACN was added to the mixture, mixed for 1 h, before the organic layer was aspirated off once again. Any residual solvent was removed from the aqueous layer by rotary evaporation at room temperature. The aqueous sample was subsequently loaded onto a conditioned Biotage (Uppsala, Sweden) Isolute SPE cartridge (200 mg ENV+) and washed with water (2 mL). The sample was eluted into fresh vials with increasing amounts of ACN. HPLC-UV analysis was performed on representative fractions to determine the presence and purity of the N^+-glucuronide metabolite in each of the fractions. Fractions containing the metabolite of interest were finally combined and dried in a speed-vac with regular addition of toluene to azeotrope off any remaining water.

The HPLC system comprised an Agilent pump (1100 series quarternary gradient pump) equipped with a Agilent 1100 autosampler and Agilent 1050 DAD detector (λ-range: 220-270 nm), operated *via* Chemstation 6.0. The characterisation of the N^+-glucuronide metabolite was achieved by solubilisation of the isolated SPE fraction (100 μL) in 200 μL of 50% (v/v) ACN. The HPLC separation was carried out on a Zorbax SB-18 column (5 mm, 150 × 4.6 mm) at a flow rate of 1 mL/min. The mobile phase consisted of solvent A (10:90% ACN:water, v/v) and solvent B (90:10% ACN:water, v/v), both solvents acidified with 0.1% trifluoroacetic acid. A linear gradient was employed increasing solvent B from 0 to 100% for 10 min, held for 1 min at 100% B, before returning to 0% B over 5 min.

In total, 1.5 mg of the N^+-glucuronide metabolite of cediranib with a purity of >99% (as determined by HPLC-UV) was successfully prepared using recombinant UGT1A4.

2.13. Metabolite characterisation by HPLC–MS

The biosynthetic N^+-glucuronide metabolite generated in the scale up experiment with UGT1A4 was characterised by HPLC (Polaris C_{18} 5 μm 150 × 4.6 mm) with UV (Jasco 2070) and Mass Spectrometric (ThermoFinnigan LTQ) detection, as described previously.

2.14. Metabolite identification/characterisation by ^{1}H NMR spectroscopy

^{1}H NMR spectra were acquired on a Bruker AV700 NMR spectrometer operating at 700.13 MHz resonance frequency, equipped with a TCI-cryoprobe (^{1}H, ^{13}C and ^{15}N detection). ^{1}H NMR data were collected and analysed using Topspin software (version 2.0).

Cediranib and the isolated [^{14}C]-N^+-glucuronide metabolite (ca. 40 μg) were initially reconstituted in 300 μL MeOH-d_4 prior to analysis by ^{1}H NMR spectroscopy. Due to partial overlap problems of the water signal at 4.7 ppm and signals of interest, DMSO-d_6 was considered a more suitable choice of solvents. Subsequently, for full structural characterisation, both, cediranib standard and 300 μg of the biosynthetic unlabelled N^+-glucuronide metabolite of cediranib were dissolved in 300 μL of DMSO-d_6.

Initially, cediranib, the isolated [^{14}C]-N^+-glucuronide metabolite and the biosynthetic N^+-glucuronide metabolite were characterised by 1D ^{1}H NMR spectroscopy. Spectra were acquired into 65536 data points with a spectral width of 14423 Hz (approximately 20 ppm), resulting in an acquisition time of 2.27 s. A relaxation delay of 3 s between scans ensured T1 relaxation. Spectra of parent and metabolite were acquired with between 32 scans and 512 scans, depending on concentration.

For full structural elucidation of the N^+-glucuronide metabolite sample, selective 1D ROESY (Rotating frame Overhauser Effect SpectroscopY) experiments were acquired into 32768 data points over a spectral width of 14097 Hz, resulting in an acquisition time of 1.16 s. A relaxation delay of 5 s was employed with a spin-lock of 200 ms duration. Spectra were acquired with 512 scans, selectively exciting the doublet at 4.7 ppm from the anomeric proton of the glucuronide conjugate.

Finally, an absolute mode 2D COSY experiment was acquired with the biosynthetic N^+-glucuronide metabolite, collecting data into 2048 data points over a spectral width of 9803 Hz, resulting in an acquisition time of 0.12 s. A relaxation delay of 1.5 s was employed between successive scans. 256 increments were

acquired in F1 consisting of 64 scans each. The data were zero-filled in F1, and a window function (sine) was employed prior to Fourier Transformation.

3. Results and discussion

3.1. Metabolism profile of [^{14}C]-cediranib in hepatocytes

The HPLC-RAD metabolic profiles of [^{14}C]-cediranib in human hepatocyte suspension and hepatocyte culture are shown in Fig. 2.

One major metabolite (metabolite M3) was detected in the human hepatocyte suspension incubations, which accounted for approximately 9.8% of total chromatogram radioactivity, (Fig. 2A). Further minor metabolites were present, including metabolites M1, M2 and M4, which accounted for approximately 0–1.6% of the total chromatogram radioactivity [6].

In human hepatocyte culture, [^{14}C]-cediranib was also metabolised to these 4 components, a finding consistent with the HPLC-RAD profiles of the human hepatocyte suspensions, although additional minor metabolites (namely M5 and M7) were detected (Fig. 2B and [6]).

In vitro cross-species investigations showed that all the metabolites detected in human cultures were also observed in the hepatocyte cultures of either rat and/or cynomolgus monkey, with the exception of metabolite M3, which was unique to human hepatocytes [6].

In rat and cynomolgus hepatocyte incubations, metabolite M4 was reported to be the major metabolite accounting for 30 and 9% of total chromatogram radioactivity, respectively. Metabolites M8, M10 and M11 were detected in rat and cynomolgus monkey hepatocyte samples but not in human hepatocytes, whilst metabolites M6 and M10 were only detected in cynomolgus monkey hepatocytes [6]. The mass spectral data for cediranib and its metabolites in all the species investigated are summarised in Table 1 .

3.2. Metabolite identification in hepatocytes

[^{14}C]-cediranib was shown to be metabolised involving mainly oxygenation reactions, with very few conjugation products generated [6]. A summary of the proposed metabolic structures of [^{14}C]-cediranib following incubation with hepatocytes from human and pre-clinical species is provided in Table 1.

Metabolite M3 was of particular interest as it was not detected in the hepatocyte cultures of rat and cynomolgus monkey. It appeared to be uniquely formed by human hepatocytes.

Furthermore, it was found to be a major excretory metabolite in human faeces, as well as being a major circulating metabolite in human plasma (unpublished data).

Metabolite M3 was identified by mass spectrometry to have a $[M+H]^+$ ion of m/z 629 representing a gain of 176 atomic mass units from [^{14}C]-cediranib (m/z 453) (Table 1), which is characteristic of the addition of a glucuronic acid moiety to the molecule. Considering the absence of hydroxyl or carboxyl groups in [^{14}C]-cediranib, the structure was suggested to be an N^+-glucuronide. Investigations by HPLC–MS^n to identify the site of conjugation were inconclusive as fragmentation produced a major molecular ion of m/z 453, representative of the [^{14}C]-parent, itself. Hence, the exact site of glucuronidation, was yet to be confirmed by ^{1}H NMR spectroscopy.

The remaining metabolites detected in the human hepatocyte incubations (M4, M1, M2, M7, M5, Fig. 2) are briefly summarised below, although a detailed account is provided in [6]. Thus, metabolite M4 was identified as the cediranib pyrrolidine *N*-oxide (based on a $[M+H]^+$ ion of m/z 469), and although a minor metabolite in human hepatocyte culture, it was abundant in cynomolgus monkey and rat [6]. The identity of metabolite M7 was confirmed as a carboxylic acid derivative, based on the $[M+H]^+$ ion of m/z 483, 30 atomic mass units higher than that of protonated [^{14}C]-cediranib. M7 was present as a very minor metabolite in human hepatocyte culture (<1% of total chromatogram radioactivity [6]). Of the remaining metabolites, M1, M2 and M5 had mass spectral profile characteristics of di- and tri-oxygenations, with m/z 485, 485 and

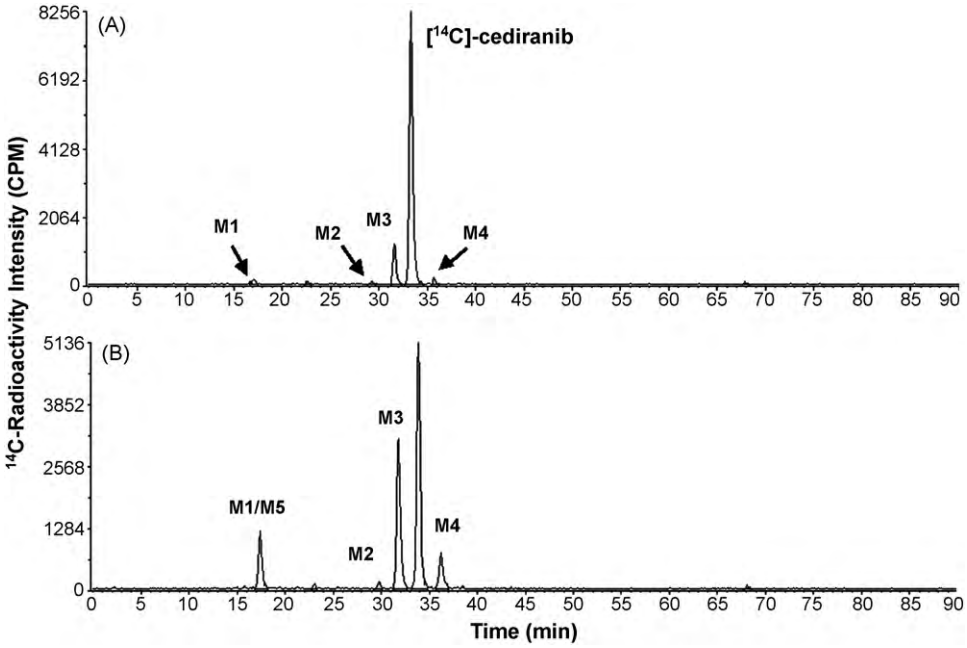


Fig. 2. Representative HPLC-RAD metabolite profiles following incubation of [^{14}C]-cediranib (10 μM) with (A) human hepatocyte suspensions (donor 2; 1×10^6 cells/mL, at 37 °C for 240 min) and with (B) human hepatocyte cultures (donor 5, 132,000 viable cells/cm^2, at 37 °C for 72 h; 5% CO_2). The exact proportions of radioactivity attributed to each metabolite are provided in [6].

Table 1
A summary of collision-induced dissociation mass spectra of [^{14}C]-cediranib and its metabolites, in order of elution, from all species investigated (data collected from [6]).

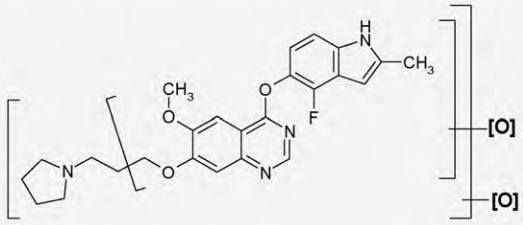
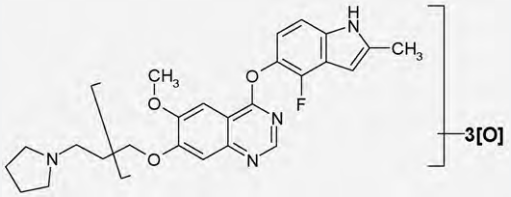
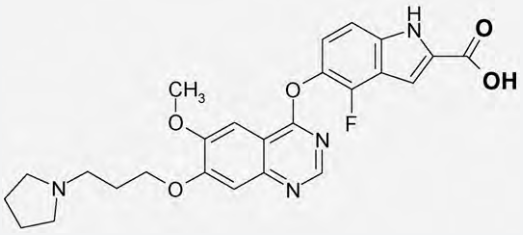
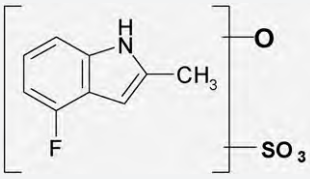
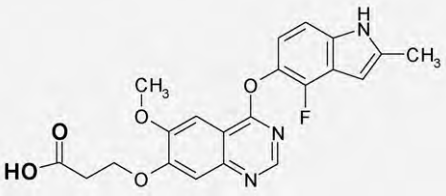
Parent/ metabolite	Species	Approx. RT/min	$[M+H]^+$ (m/z)[a]	Diagnostic fragment ions (m/z)	Metabolite identity	Proposed structure
M1	H, R, M	17.8	485	467, 358, 342	Di-oxygenated	
M5	H, R, M	18.2	501	483, 389, 374	Tri-oxygenated	
M6	M	22.0	–	–	*Not identified*	*n/a*
M7	H, R, M	24.0	483	465, 439, 372, 356	Fluoro-indole carboxylic acid	
M2	H, R, M	30.5	485	342	Di-oxygenated	
M8	R, M	31.5	248	168	Fluoro-indole, monooxy-genated sulfate	
M3	H	32.0	629	453, 342, 326	N^+-glucuronide	See Fig. 6
Parent	H, R, M	34.0	453	342, 326, 112	[^{14}C]-Cediranib	See Fig. 1
M9	R	35.0	–	–	*Not identified*	*n/a*
M4	H, R, M	36.5	469	382, 342, 128, 84	*N*-oxide	
M10	M	62.5	–	–	*Not identified*	*n/a*

Table 1 (*Continued*)

Parent/ metabolite	Species	Approx. RT/min	$[M+H]^+$ (m/z)[a]	Diagnostic fragment ions (m/z)	Metabolite identity	Proposed structure
M11	R, M	67.3	414	342	Propanoic acid	
M12	R, M	68.2	–	–	*Not identified*	*n/a*

Species: detected in H = human, R = rat, M = cynomolgus monkey.
n/a = not available, unassigned.
[a] ^{14}C isotope is the predominant ion where present.

501, respectively. These metabolites were present in all 3 species investigated [6].

Any further metabolites and the enzymes catalysing their formation are described in [6].

3.3. Understanding the formation of metabolite M3 in human in vitro systems

As it was not possible to determine the structure using MS-based approaches, further investigations were required regarding the formation of metabolite M3, especially in view of producing sufficiently high quantities of this metabolite for structural analysis by ^1H NMR spectroscopy.

Thus, a single metabolite, consistent with M3, was observed following human liver microsomal incubations containing [^{14}C]-cediranib and UDPGA, with this metabolite accounting for 15.2% of the total chromatogram radioactivity [6], as shown in Fig. 3. This metabolite displayed the same chromatographic retention characteristics, the $[M+H]^+$ ion of m/z 629 and identical mass spectral fragmentation patterns as the M3-metabolite identified previously in the human hepatocyte incubations.

It is noteworthy, that no metabolic biotransformations were observed, following incubation of [^{14}C]-cediranib in microsomes in the presence of UDPGA, from several species, such as cynomolgus monkey, Göttingen mini-pig, rhesus monkey, CD-1 mouse, beagle dog and Han Wister rat (data not shown). The metabolite was also not formed in the microsomal incubations of New Zealand White rabbit, a non-primate species with known potential of forming quaternary N^+-glucuronides [7,8] and the Dunkin-Hartley guinea-pig which has also been shown to form the N^+-glucuronide of lamotrigine [9].

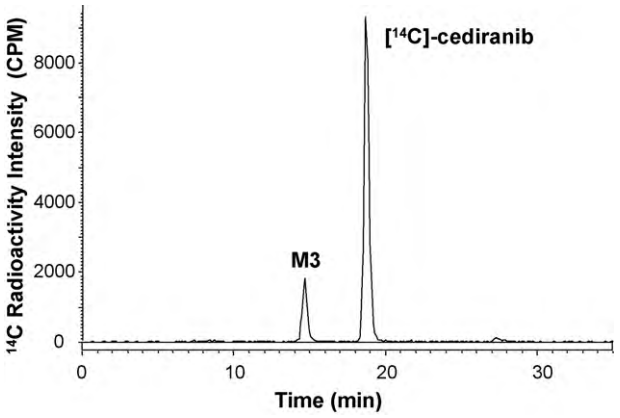


Fig. 3. Formation of [^{14}C]-cediranib N^+-glucuronide (M3) in human liver microsomes in the presence of UDPGA (10 μM [^{14}C]-cediranib at 37 °C for 60 min; 1 mg/mL protein).

However, distinct species differences in the formation of N-glucuronides are well documented in the literature. The glucuronidation of tertiary amines, in particular, appears to occur predominantly in humans and higher primates [8,10–13].

Furthermore, it was of interest to identify the specific UGT enzymes involved in the glucuronidation of [^{14}C]-cediranib, using recombinant UGT enzymes. Hence, amongst the individually expressed UGT enzymes (UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17) it was demonstrated that the metabolism of [^{14}C]-cediranib was exclusive to UGT1A4. The amount of chromatogram radioactivity represented by [^{14}C]-cediranib decreased from 94.9 to 78.2% whilst one major component, metabolite M3, was formed representing 16.2% of total chromatogram radioactivity [6].

So, a representative sample of metabolite M3 was generated from the incubation with heterologously expressed UGT1A4, which was characterised and confirmed by HPLC-UV and HPLC–MS^n as the N^+-glucuronide metabolite. A sample of approximately 40 μg was generated following this approach for ^1H NMR spectroscopy. However, whilst additional signals characteristic of the presence of a glucuronide moiety were detected in the ^1H NMR spectrum, it became apparent that the amount of metabolite generated and isolated was insufficient for full structural confirmation, i.e. the determination of the exact site of conjugation (data not shown).

3.4. Biosynthesis of human metabolite M3

As the yield of the N^+-glucuronide metabolite in human liver microsomes (HLM) was low (ca. 15%), this methodology was therefore not a feasible source to generate sufficient quantities for analysis. However, this study demonstrated that recombinant human UGT1A4 enzymes could generate the N^+-glucuronide metabolite of cediranib on a small scale sufficient for further structural analysis by ^1H NMR spectroscopy.

Hence, a biosynthetic approach was sought in order to scale up metabolite production.

Parameters initially investigated included cediranib and cofactor (UDPGA) concentrations, buffer pH, and presence and absence of bovine serum albumin and saccharolactone – as detailed in Tables 2 and 3. Optimum results were achieved with incubations carried out at a UGT1A4 protein concentration of 0.25 mg/mL in 50 mM Tris HCl buffer, pH 7.5 containing 10 mM $MgCl_2$,

Table 2
Effect of buffer pH, incubation temperature and the presence of BSA and saccharolactone on the formation rate of the cediranib–glucuronide metabolite in recombinant UGT1A4.

Buffer pH	Incubation temperature (°C)	BSA (3%, w/v)/saccharolactone (5 mM)	AZD2171-glucuronide metabolite as a proportion of total chromatogram (%)
7.0	RT	–	7.9
7.0	RT	+	3.3
7.0	37	–	37.0
7.0	37	+	19.8
7.5	RT	–	9.1
7.5	RT	+	8.5
7.5	37	–	43.0
7.5	37	+	30.3
8.0	RT	–	10.5
8.0	RT	+	7.5
8.0	37	–	44.1
8.0	37	+	36.0

Incubation conditions: 100 μM AZD2171; 0.25 mg/mL UGT1A4, 100 μg alamethicin/mg protein and 2 mM UDPGA in 50 mM Tris HCl containing 10 mM $MgCl_2$ for 18 h.

100 μg alamethicin/mg protein and 2 mM UDPGA, yielding a pure product (>99%, as determined by HPLC-UV) of approximately 1.5 mg total weight.

During the ^{1}H NMR spectroscopic investigation of the biosynthetic N^+-glucuronide, ethanol was identified as an impurity, most likely a contaminant introduced during sample processing or a by-product of fermentation. Whilst being UV–transparent, it accounted for ca. 7% of the weight of the biosynthetic product. Its presence, however, did not perturb the structural identification of the N^+-glucuronide.

Characterisation of the biosynthetic product was again conducted by HPLC and HPLC–MS^n analyses. The biosynthetic metabolite exhibited the same retention-time, molecular mass and mass fragmentation data (shown in Fig. 4) as the metabolite, M3, generated in the human liver microsomal incubation (HLM) of [^{14}C]-cediranib in the presence of UDPGA, and the human hepatocyte incubations.

3.5. Structural characterisation of human metabolite M3 by ^{1}H NMR spectroscopy

Although the evidence provided by HPLC–MS^n confirmed the formation of the N^+-glucuronide conjugate, full structural characterisation was still required to determine the exact site of conjugation.

As mentioned earlier, initially, a sample generated and isolated from expressed UGT1A4 (approximately 40 μg) was investigated by ^{1}H NMR spectroscopy. The spectrum clearly showed the presence of a doublet at 4.68 ppm with a coupling constant (J_{HH}) of 8.8 Hz, indicative of the presence of a β-glucuronic acid moiety, with the residual protons from the cediranib-moiety being preserved (data not shown). However, the sample was too weak for further detailed structural confirmation *via* through space interaction NMR experiments.

Identification of the site of conjugation typically require NOE (nuclear Overhauser effect) – experiments, irradiating protons close to the site of glucuronidation, whilst observing changes in the intensity of signals in the vicinity (up to approximately 4 Å). However, these signal enhancements are generally weak and initial attempts with the isolated metabolite failed due to lack of sufficient compound (data not shown). Hence, 300 μg of the biosynthetic metabolite were provided for the structural analysis of metabolite M3.

The ^{1}H NMR spectrum of the scaled-up biosynthetic metabolite was identical to the ^{1}H NMR spectrum of the isolated metabolite from expressed UGT1A4, as described above.

A comparison of the 1D ^{1}H NMR spectrum of the biosynthetic metabolite to that of the parent confirmed the presence of additional signals from protons H_1–H_5 of the glucuronide moiety (labelled G, as shown in Fig. 5). Furthermore, it highlighted that there was no change in chemical shift in the aromatic signals, whilst some of the aliphatic signals experienced a shift to higher frequency.

Finally, a series of selective ^{1}H NMR ROESY experiments were carried out with of the biosynthetic material (dissolved in DMSO-d_6 at a concentration of 1 mg/mL).

Considering the possibility of four nitrogens for conjugation, the anomeric proton of the glucuronide moiety itself proved the most suitable signal for irradiation. Hence, whilst selectively exciting the anomeric proton at 4.68 ppm (labelled G1 in Fig. 6) no enhancement of the signals in the aromatic region was observed, however, some signals in the aliphatic regions showed positive NOE's (nuclear Overhauser effects). The enhanced signals consisted of complex multiplets at 3.92 ppm, 3.85 ppm and 2.4 ppm from the cedinarib-

Table 3
Effect of protein, cediranib and UDPGA concentration on the formation rate of the cediranib–glucuronide metabolite in recombinant UGT1A4.

UGT1A4 protein concentration (mg/mL)	AZD2171 concentration (μM)	UDPGA cofactor concentration (mM)	AZD2171-glucuronide metabolite as a proportion of total chromatogram (%)
0.05	100	2	9
0.125	100	2	22[a]
0.125	200	2	13
0.125	400	2	12
0.25	100	2	38[b]
0.25	200	2	18
0.25	400	2	13
0.25	100	0.5	33
0.25	100	4	42

Incubation conditions: 100 μg alamethicin/mg protein in 50 mM Tris HCl pH 7.5 containing 10 mM $MgCl_2$ for 18 h at 37 °C.

[a] Incubations were carried out in duplicate, with values ranging from 21 to 23. The mean value is stated.

[b] Incubations were carried out in triplicate, with values ranging from 38 to 39. The mean value is stated.

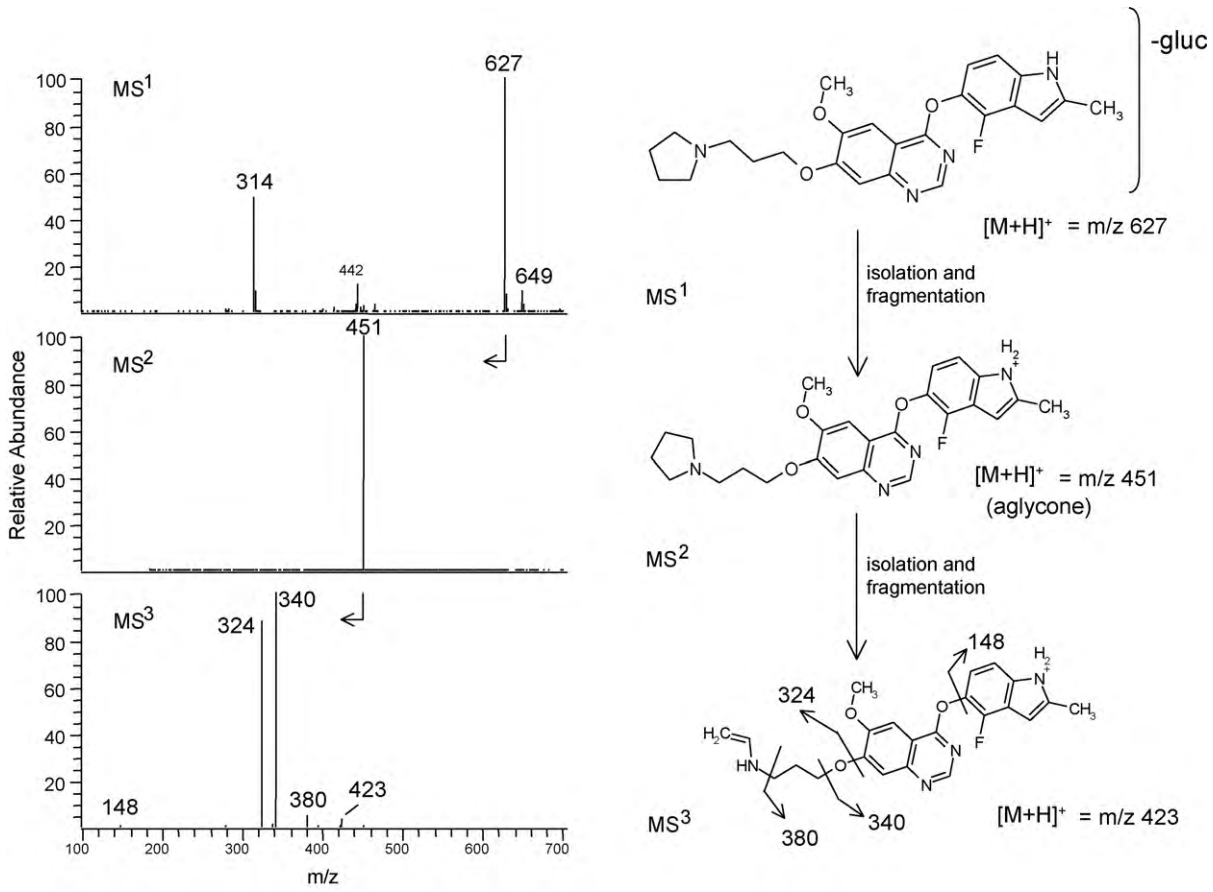


Fig. 4. HPLC–MS^n fragmentation pattern of the N$^+$-glucuronide conjugate of the unlabelled biosynthetic cediranib.

moiety, and a doublet at 3.45 ppm and a 'triplet' at 3.32 ppm from the glucuronide-moiety.

The identity of these signals was confirmed by a 2D ^{1}H-^{1}H-COSY (Fig. 7); displaying connectivities over 3 bonds within the molecule. This experiment confirmed that the multiplets at 3.92 and 3.85 ppm were connected to the multiplets at 2.21 and 2.18 ppm, which were assigned as the pyrrolidine protons (l, l′ and m, m′, respectively).

Likewise, the multiplet at 4.28 ppm (labelled i) showed connectivities to both, the multiplets at 3.71 and 3.62 ppm (k, k′) and the multiplet at 2.42 ppm (j), representative of the propyl side-chain link of the pyrrolidine ring. Similarly, the anomeric proton of the glucuronide moiety (4.68 ppm, G1) displayed a cross-peak to the doublet-of-doublets at 3.67 ppm (G2), which in turn showed further connectivities to the doublets-of-doublets at 3.32 ppm (G3) and 3.2 ppm (G4), and ultimately the doublet at 3.45 ppm (G5).

In summary, selectively exciting the anomeric proton at 4.68 ppm (G1) caused enhancement of the pyrrolidine protons, labelled l and l′, as well as the propyl side-chain protons, labelled

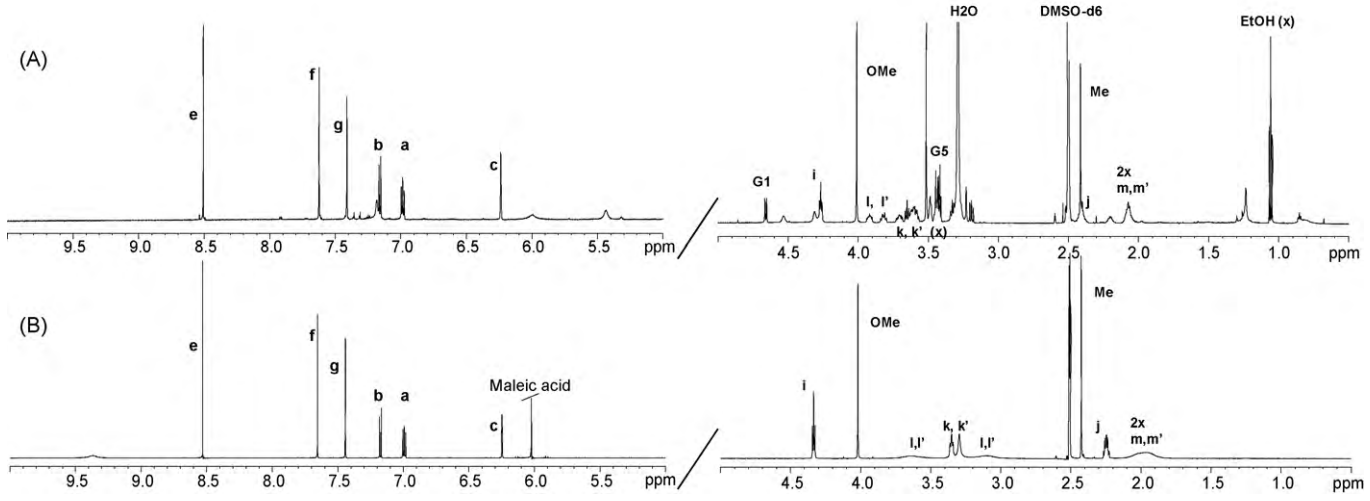


Fig. 5. ^{1}H NMR spectra of the biosynthetic cediranib N$^+$-glucuronide metabolite (A) and [^{14}C]-cediranib (B). Approximately 300 μg were dissolved in 300 μL of DMSO-d_6. The metabolite contained a single major contaminant, namely ethanol, labelled in the spectrum as EtOH (×).

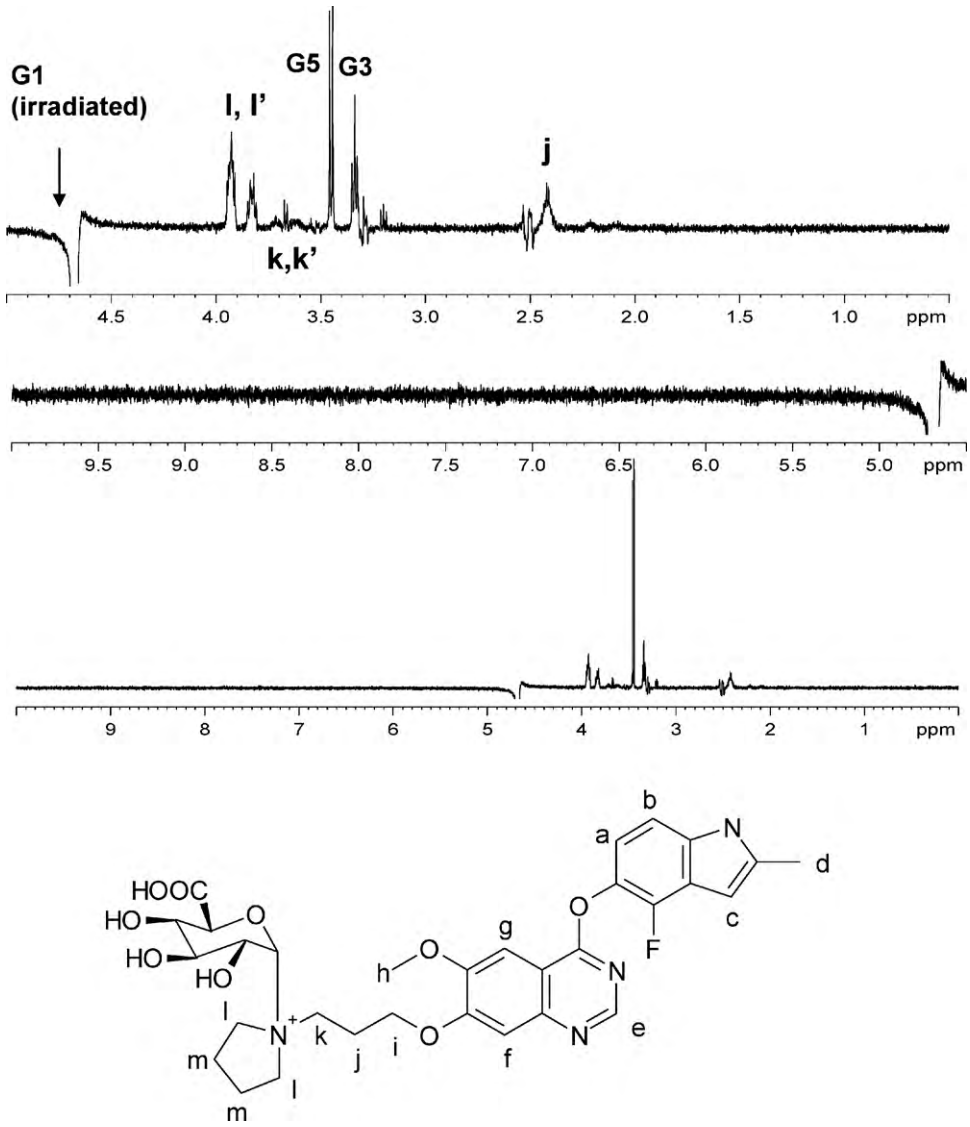


Fig. 6. ^{1}H NMR spectrum of the selective ROESY experiment of the biosynthetic N^{+}-glucuronide metabolite of cediranib, following selective excitation of the anomeric proton (labelled G1). Shown are the whole spectrum (bottom), the aromatic region (middle) and the aliphatic region (top), as well as the structure with individual proton labels.

j, and to a lesser extent k and k′, together with enhancement of the glucuronide protons G5 and G3. This finding provided conclusive evidence that glucuronidation had occurred on the pyrrolidine nitrogen.

Further supporting evidence was available from data gathered in the literature. It has been highlighted by Hawes [13] that whilst the anomeric protons of aromatic N^{+}-glucuronides resonated at approx. 5.3 to 5.9 ppm, those of aliphatic N^{+}-glucuronides resonated at 4.3 to 5.0 ppm, as observed in this study. Additional confirmation of site of conjugation was gained by the observation of chemical shift changes between the spectra of cediranib parent and N^{+}-glucuronide metabolite. Chemical shift changes were only experienced in the aliphatic region of the spectrum of the N^{+}-glucuronide metabolite, whilst the aromatic protons remained static, indicating that the site of glucuronidation occurred on the pyrrolidine nitrogen. Hence, the protons labelled l, l′, k, k′ and j experienced a shift to higher frequencies, described as diagnostic to the conjugation of their neighbouring pyrrolidine-nitrogen.

A detailed account of N-glucuronide conjugates is provided in the literature [8,13], examining and summarising their formation, detection and stability. N^{+}-glucuronides are described as relatively stable in neutral and acidic conditions, hence, many studies have shown them to be present in the urines from human subjects.

It has also been stressed that relatively little information is available on N-glucuronide metabolites in animal urine in general, partly due to their instability (especially in view of glucuronides of primary and secondary amines, which are acid-labile), but possibly also due to their elimination in bile, or indeed, lack of formation.

Most of all, however, distinct species differences have been reported with different classes of substrates, which appear to determine the type of N-glucuronidation. It has been highlighted that the preferential glucuronidation of tertiary amines occurs solely in higher primates (chimpanzees) and man.

Furthermore, in line with the findings in this study, investigations with [^{14}C]-cediranib *in vivo* examining the metabolic differences in several preclinical species (rat, dog, cynomolgus monkey), compared to humans, have also highlighted the N^{+}-glucuronide to be a uniquely human metabolite. Although the majority of radioactivity, following administration of [^{14}C]-cediranib, was predominantly recovered in the faeces in all species, the N^{+}-glucuronide metabolite was only observed in humans, where it was a major excretory product in faeces, whilst being a minor component in urine. Additionally, it was identified as

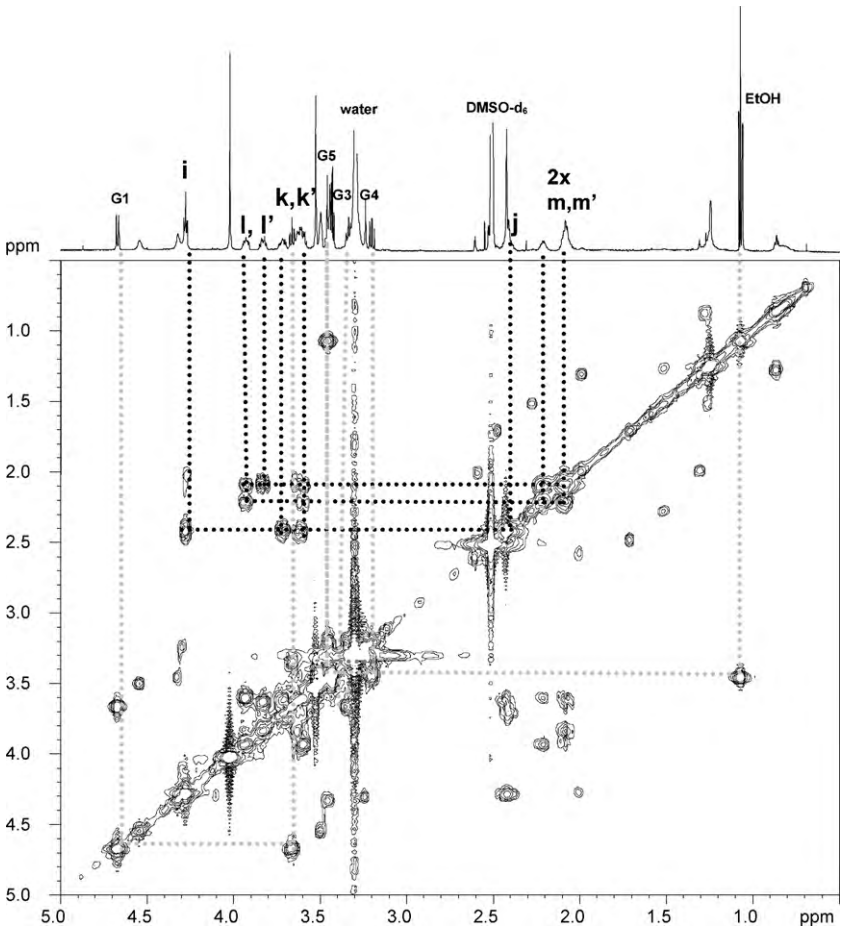


Fig. 7. 2D ^{1}H-^{1}H COSY NMR spectrum of the biosynthetic N^{+}-glucuronide metabolite of cediranib. The connectivities supporting the identification of the signals detected in the ROESY experiment are highlighted.

the major circulating metabolite in human plasma (unpublished data).

The enzyme catalysing the N^{+}-glucuronidation of cediranib was identified to be the human UGT isoform, UGT1A4 [6], an observation consistent with published data confirming the involvement of UGT1A4 in the N^{+}-glucuronidations of cyclic tertiary amines (e.g. [14]). Although information on the expression levels of this enzyme in different species appears to be limited, it might provide an explanation for the species differences observed in the formation of the quaternary N^{+}-glucuronide of cediranib.

N-glucuronidation reactions are generally regarded as detoxification reactions, although some compounds are known to form labile conjugates (as in the case of arylamine glucuronides which have been linked to bladder cancers). Other reports have described adverse reactions to the N^{+}-glucuronide metabolite of the antidepressant, amitriptyline, when administered intravenously [15].

The largest body of data describing the presence of quarternary glucuronides has been reported with tricyclic antidepressants and antipsychotics, as well as antihistamine drugs, where N^{+}-glucuronides have been almost exclusively observed in humans [8,16,17]. Despite the widespread occurrence of the N^{+}-glucuronides in humans, based on the ubiquitous use of these agents, there are however, few reports of their pharmacology and toxicity. It appears that, with the exception of the data provided in [15], the assessment of their potential biological activity has been generally compromised by the presence of the active pharmacological agents themselves. Hence, the biosynthetic method presented in this paper shows a viable alternative to isolation from a biological matrix for the generation of an N^{+}-glucuronide, should further pharmacological assessments be required. Recent literature has also demonstrated advances in purely synthetic approaches to the production of quarternary N^{+}-glucuronides [18].

4. Conclusion

Schulz-Utermoehl et al. [6] have demonstrated that the VEGF inhibitor, cediranib, was metabolised in hepatocyte preparations to a series of mainly oxidative products.

A single glucuronide conjugate was identified to be the N^{+}-glucuronide metabolite formed in hepatocyte cultures and suspensions, as well as in HLM in the presence of UDPGA.

Furthermore, this N^{+}-glucuronide metabolite was identified as a unique human metabolite, not formed in the hepatocyte incubations from, either, rat or cynomolgus monkey, the toxicological species in this study.

Microsomal incubations from other species in the presence of UDPGA also failed to form the N^{+}-glucuronide metabolite.

In an attempt to identify the specific UGT enzyme responsible for the formation of the N^{+}-glucuronide metabolite, it was found that its formation was mediated selectively by the human UGT1A4 enzyme. Employing recombinant UGT1A4 enzymes for the generation of this metabolite yielded a small amount of the N^{+}-glucuronide metabolite, which could be identified by HPLC–MS. However, the amount was insufficient for its full characterisation by ^{1}H NMR spectroscopy. Hence, a biosynthetic route for the scaling up of this metabolite was perfected, producing sufficient material for its full characterisation by 1D and 2D NMR experiments. In view of 4 possible sites for the conjugation of glucuronide moiety on the

molecule, the exact site of glucuronidation was confirmed to be on the pyrrolidine nitrogen.

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