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ORIGINAL ARTICLE

Phase I study of orally administered ^{14}Carbon-isotope labelled-vistusertib (AZD2014), a dual TORC1/2 kinase inhibitor, to assess the absorption, metabolism, excretion, and pharmacokinetics in patients with advanced solid malignancies

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Abstract

Purpose Vistusertib is an orally bioavailable dual target of rapamycin complex (TORC) 1/2 kinase inhibitor currently under clinical investigation in various solid tumour and haematological malignancy settings. The pharmacokinetic, metabolic and excretion profiles of ^{14}Carbon-isotope (^{14}C)-labelled vistusertib were characterised in this open-label phase I patient study.

Methods Four patients with advanced solid malignancies received a single oral solution dose of ^{14}C-labelled vistusertib. Blood, urine, faeces, and saliva samples were collected at various time points during the 8-day in-patient period of the study. Safety and preliminary efficacy were also assessed.

Results ^{14}C-labelled vistusertib was rapidly absorbed following administration (time to maximum concentration (T_{max}) < 1.2 h in all subjects). Overall, > 90% of radioactivity was recovered with the majority recovered as metabolites in faeces (on average 80% vs. 12% recovered in urine). The majority of circulating radioactivity (~ 78%) is unchanged vistusertib. Various morpholine-ring oxidation metabolites and an *N*-methylamide circulate at low concentrations [each < 10% area under the concentration–time curve from zero to infinity ($AUC_{0-\infty}$)]. No new or unexpected safety findings were observed; the most common adverse events were nausea and stomatitis.

Conclusions The pharmacokinetic (PK) profile of vistusertib is similar to previous studies using the same dosing regimen in solid malignancy patients. The majority of vistusertib elimination occurred via hepatic metabolic routes.

Keywords Vistusertib · AZD2014 · ADME · Solid malignancies · Phase I · Metabolism

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Introduction

Vistusertib (formerly designated AZD2014) is a highly selective, orally bioavailable inhibitor of the kinase activity of mammalian target of rapamycin (mTOR) [1, 2], a serine/threonine kinase belonging to the phosphatidylinositol 3 kinase (PI3K) phosphatidyl inositol 3′ kinase-related kinases (PIKK) superfamily of kinases. The mTOR is an essential component of the PI3K-AKT(Ak strain transforming)-mTOR signalling pathway [3, 4], where it functions as a sensor of mitogen, energy, and nutrient levels and is a central controller of cell growth. Although mTOR is mutated only in some human cancers, the PI3K–AKT–mTOR pathway in general is one of the most frequently activated pathways in human tumours. Everolimus and temsirolimus (so-called rapalogs) are potent, allosteric inhibitors [5] of the

rapamycin-sensitive mTOR complex (TORC1) and have been shown to be clinically effective in certain cancer types including renal cell carcinoma, estrogen receptor positive (ER+) breast cancer and endometrial cancers, and mantle cell lymphoma [6–8]. However, several resistance mechanisms are suggested to limit the clinical effectiveness of rapalogs including upregulation of AKT signalling via TORC2, due to negative feedback caused by inhibition of TORC1 [9]. Vistusertib, as an mTOR kinase inhibitor, inhibits the activity of both mTOR complexes, and may overcome this limitation. Vistusertib is currently undergoing clinical trials in a number of solid and haematological tumour types.

In an exploratory Phase I setting, orally administered vistusertib has an acceptable tolerability, pharmacokinetic and pharmacodynamic profile in patients with advanced solid malignancies [10]. However, to date there is no information on the metabolic conversion of vistusertib in humans, or the routes and rates of excretion of vistusertib or any metabolites. The overall objectives of this study were to characterise the PK, metabolic and excretion profiles of ^{14}C-labelled vistusertib and circulating metabolites at a therapeutic dose in the same patient population (ClinicalTrials.gov identifier: NCT02640755).

Materials and methods

Patients

Key eligibility criteria for all patients were as follows: male or females aged ≥ 18 years with a solid malignant tumour refractory or resistant to standard treatments; body mass index (BMI) ≥ 18 and ≤ 35 kg/m2 and weight at least 50 kg; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 2 with no deterioration over the previous 2 weeks and minimum life expectancy of 12 weeks; absolute neutrophil count $\geq 1.5 \times 10^9$/L; platelet count $\geq 100 \times 10^9$/L; haemoglobin ≥ 90 g/L; alanine aminotransferase (ALT) or aspartate aminotransferase (AST) ≤ 2.5 times upper limit of normal (ULN) if no demonstrable liver metastases or ≤ 5 × ULN in the presence of liver metastases; total bilirubin ≤ 1.5 × ULN if no demonstrable liver metastases or ≤ 3 × ULN in the presence of liver metastases; serum creatinine ≤ 1.5 × ULN concurrent with creatinine clearance ≥ 50 mL/min; normal echocardiogram at baseline (left ventricular ejection fraction ≥ 55% and shortening fraction ≥ 15%); mean resting QT interval corrected for heart rate (QTc) using Fridericia's formula (QTcF) < 470 ms and history of regular bowel movements (≥ 1 stool per day). Patients with history of the following (within 12 months) were ineligible: drug or alcohol abuse; coronary artery bypass graft; vascular stent; myocardial infarction; angina pectoris; congestive heart failure (New York Heart Association Grade 2); ventricular arrhythmias requiring continuous therapy; supraventricular arrhythmias including atrial fibrillation, which were uncontrolled; haemorrhagic or thrombotic stroke, including transient ischaemic attacks or any other central nervous system bleeding. In addition; patients with the following were also ineligible: pre-existing renal disease; abnormal fasting glucose [> 126 mg/dL (> 7 mmol/L)]; diabetes Type 1 or uncontrolled Type 2 (glycosylated haemoglobin > 8% (64 mmol/mol)]; refractory nausea and vomiting at screening, chronic gastrointestinal disease or inability to swallow the investigational medicinal product (IMP) or previous significant bowel resection that could preclude adequate absorption of the IMP.

Study design

This was a Phase I, open-label, single centre, non-randomised, non-controlled, fixed sequence study. The study consisted of two periods, a single dose period and a multiple dose period. The study was conducted at the Clinical Trials Unit, The Christie NHS Foundation Trust, Manchester, UK.

Following a fast of at least 2 h, patients received a single dose 125 mg ^{14}C-labelled vistusertib (100 µCi) on day 1 administered as 100 mL oral water solution. The dosing vessel was rinsed twice with 70 mL of water, and the contents were also swallowed. The dosing vessel was retained for residual radioactivity analysis. Subjects remained as inpatients in the trial unit until discharge on day 8. Based on pre-clinical studies it was anticipated that > 90% of total radioactivity would have been recovered in this period. The study was originally designed so that the total radioactivity of the first patient was assessed following release post-day 8, i.e., the first patient was discharged before the radioactivity assessment was conducted, but enabling re-adjustment of the in-patient period for subsequent patients. Subsequent to the analysis of total radioactivity of the first patient, the protocol was amended to allow daily monitoring of the radioactivity of each patient. Release from the trial unit was dependent on recovery of > 90% radioactivity. From day 8 onwards patients were able to continue to receive vistusertib, either as monotherapy or, dependent on tumour type, in combination with fulvestrant or paclitaxel until disease progression or any other discontinuation criteria were met. The 125 mg vistusertib dose was the recommended phase II dose (administered twice daily 2 days on/5 days off weekly intermittent regimen) previously identified in the Phase I/II dose-escalation study conducted in advanced solid tumour patients [8]. The amount of radioactivity (100 µCi or 3.7 MBq) administered was considered to be the minimum required to achieve the study objectives.

Compliance with ethical standards

Informed consent was obtained from all individual participants included in the study. The trial was approved by all relevant institutional ethical committees or review bodies and was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonization/Good Clinical Practice and the AstraZeneca policy on Bioethics.

Study objectives

The primary objective of the study was to characterise the absorption, metabolism, excretion and PK of a single oral dose of 125 mg ^{14}C-vistusertib in patients with advanced solid malignancies. Secondary objectives were to assess the safety and tolerability of vistusertib and to investigate the anti-tumour activity of vistusertib.

Assessments

Samples of blood, urine, faeces and saliva were collected prior to study treatment and at various time points during the in-patient period days 1–8.

Blood samples were collected pre-dose and 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 32, 48, 72, 96, 120, 144 and 168-h post-dose for radioactivity and vistusertib pharmacokinetic analysis and metabolite profiling. Saliva was collected at 1,2 4, 6, 8, 10, 12 and 24 h post-dose. Urine was collected for the same analysis over 0–6, 6–12, 12–24 h post-dose and then at 24 hourly intervals for the remaining in-patient period. All faecal material over the in-patient period was collected for radioactivity and metabolite analysis. Any additional biofluids such vomitus or drained ascites, would be kept for radioactivity analysis as required.

Vistusertib plasma, saliva and urine concentrations were measured using high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS/MS) detection (Covance Bioanalytical Ltd, Harrogate, UK). The lower limits of quantitation (LLOQ) were 20, 20 and 10 ng/mL for plasma, saliva and urine, respectively. Each analytical run included a single calibration curve (with duplicate lower LOQ and upper LOQ calibration standards), a matrix blank, a control zero sample (matrix blank containing internal standard), a reagent blank and duplicate undiluted quality control samples at three concentrations within the calibration range. Radioactivity in weighed aliquots of plasma, saliva, urine, faeces, whole blood and vomitus were determined by liquid scintillation counting (LSC) (Covance Metabolism Ltd, Harrogate, UK). Blood, vomit and faeces samples were combusted in oxygen using a Sample Oxidiserd prior to LSC. Radioactivity in each sample was measured for 5 min using a Packard Tri-Carb liquid scintillation counter (Canberra Packard, Pangbourne, Berks) with the facilities for computing quench-corrected disintegrations per minute (dpm). Efficiency correlation curves were prepared and routinely checked by the use of 14C-toluene or Ultima Gold™ quenched standards (Perkin Elmer). For metabolite profiling, plasma samples were time proportionally pooled for each patient. Urine, faeces and saliva samples were pooled across all time points, respectively, to create single samples for each patient and matrix. Following extraction, where appropriate, samples were profiled and quantified by HPLC-RAM-MS. with quantification conducted via fraction collection and offline 96-well microplate scintillation analysis (PerkinElmer) (Unilab Bioanalytical Solutions, York, UK). The HPLC post-column [Synergi Max-RP C12, 4 µm, 100×4.6 mm (Phenomenex)] eluent, was split (~9:1) with the majority of the flow (1 mL/min) directed toward the fraction collector [222XL liquid handler (Gilson)] for offline radio-detection [MicroBeta (PerkinElmer)] and the remainder to the mass spectrometer (ThermoScientific LTQ Orbitrap XL linear ion-trap mass). As a result of splitting the HPLC eluent between the fraction collector and the MS, there was a difference in the recorded retention time between the offline MicroBeta and MS data for individual components eluting from the HPLC columns. Due to the co-elution of a number of metabolites during the preliminary analysis selected samples were subsequently re-analysed using the same analytical approach, but with an extended gradient. The additional resolving power of the extended gradient allowed more robust quantitative data to be generated for a number of the metabolites which had co-eluted during the initial analysis. Additional mass spectrometric analysis was performed on selected samples using the same analytical approach but utilising a Q-Tof Premier mass spectrometer with Acquity LC system (Waters).The recovery of radioactivity from the HPLC column was determined for each sample matrix. Reconstructed radio-chromatograms were produced following import of 96-well microplate count data, collected from the Microbeta instrument, into Laura 4.1.7.70 (LabLogic Systems,UK). Data from the ThermoScientific LTQ Orbitrap XL mass spectrometer were acquired and processed using Xcalibur v2.0.7 software. Data from the Waters Q-Tof Premier mass spectrometer were acquired and processed using MassLynx 4.1 software. Metabolite numbers were assigned on the basis of HPLC retention time determined during this study (i.e. M1 corresponds to the metabolite with the shortest retention time, etc.).

Antitumor activity was assessed by evaluation of tumour response at baseline, and at 8 weekly intervals using Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1. Vital signs, standard safety laboratory assessments including haematology, clinical chemistry and urinalysis were assessed regularly during the in-patient period and throughout the study. Adverse events were recorded according to the National Cancer Institute (NCI) Common Terminology Criteria (CTC) version 4.03.

Statistical analysis

No formal statistical hypothesis was investigated. Sample size was based on feasibility and was approved with the original aim to recruit 4–6 patients to ensure 4 evaluable patients. An evaluable patient was defined as a patient who received the whole dose of [^{14}C]-vistusertib, did not vomit within the first 2 h postdose, and completed the scheduled blood PK sampling. Non-evaluable patients would be replaced. Individual PK parameters for radioactivity and vistusertib were estimated using non-compartmental methods using Phoenix Winnonlin version 6.3 (Certara Inc, Ca, US). Descriptive statistic summaries were generated using SAS version 9.2. Plots were generated using R version 3.4.3.

Results

Patients

Between February and December 2016 four patients were enrolled in the study. The baseline demographic and disease characteristics were representative of the intended patient population for this study (Table 1). All patients received study drug on day 1 and completed the 8 day inpatient period. All patients subsequently entered the multiple dose part of the study; the uterine clear cell carcinoma patient receiving vistusertib in combination with fulvestrant (500 mg/month, per I.M) with the remaining patients receiving vistusertib monotherapy.

Pharmacokinetics and recovery of radioactivity

Geometric mean concentration–time profiles for vistusertib and total radioactivity are presented in Fig. 1a. Corresponding summary PK parameters are shown in Table 2.

The radioactive vistusertib solution was rapidly absorbed with peak radioactivity and vistusertib concentrations in blood, plasma and saliva concentrations occurring within 1.2 h of administration in all patients. The highest peak concentrations of radioactivity and vistusertib were observed in saliva at 1 h post-dose. It is not known whether this is due to systemic excretion through the oral mucosa or residual solution left from incomplete ingestion of the radioactive drug solution. Radioactivity and vistusertib concentrations fell rapidly in all matrices over the first 24 h post-administration. Radioactivity was not detectable in whole blood or saliva samples beyond 12 h post-dose. The rate of decline in radioactivity in blood and plasma was similar over the 12 h post-dose period. There was evidence of an additional phase of decline of radioactivity in plasma from 24 h post-dose onwards. The highest exposure to radioactivity or vistusertib (as measured by $AUC_{0-\infty}$ or $AUC_{0-t_{last}}$) was in plasma.

Mean cumulative radioactivity recovery-time courses are shown in Fig. 1b. The target dose of ^{14}C-vistusertib was 100 μCi; the actual dose received ranged from 98 to 101 μCi. Overall, on average 92% of total radioactivity was recovered in three patients, with excretion essentially complete after 72 h post-dose. On average 80% of the radioactivity was recovered in faeces. One patient was excluded from the radioactivity summary analysis due to technical problems with faeces collection. This patient had a low total radioactive

Table 1 Patient demographics and baseline characteristics

Patient characteristic	Vistusertib 125 mg ($n=4$)
Median age (range) (years)	59 (49–65)
Male/female, *n*	1/3
Race, *n*	
White	4
Median body weight (range) (kg)	68 (60–105)
ECOG performance status, *n*	
Normal activity (0)	1
Restricted activity (1)	3
Primary tumour location [histology and stage (AJCC at diagnosis)]	Peritoneum (epithelioid) Unknown primary (adenocarcinoma[a], IV) Breast (invasive ductal, IV) Uterus (endometrial clear cell, IIIA)
Prior lines of treatment median (range)	3.5 (0–13)
Treatment assigned during multiple dose part of the study	All patients received vistusertib monotherapy dosed continuously (50 mg BD) in tablet formulation except the patient with uterine clear cell carcinoma who received vistusertib (125 mg BD 2 days on/5 days off) in combination with fulvestrant

[a]Found in lymph node

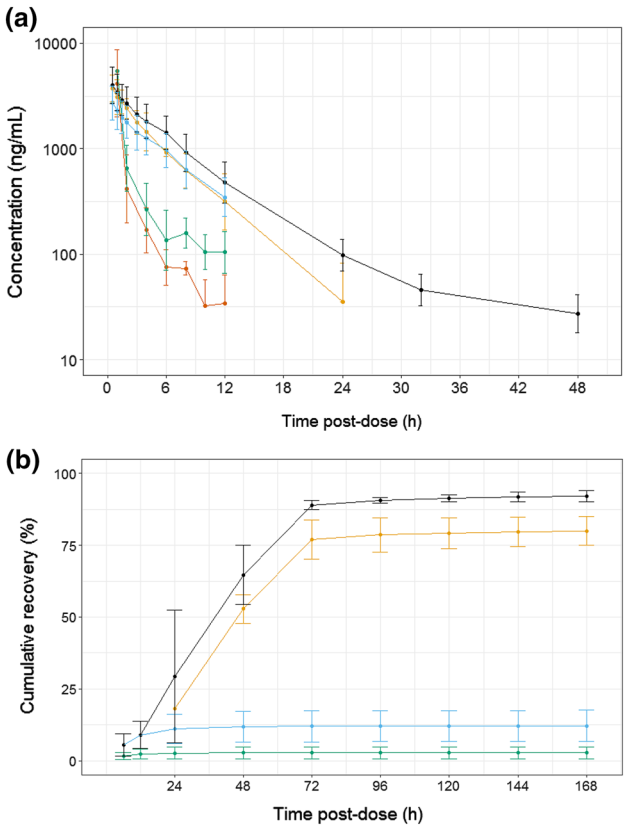

Fig. 1 **a** Geometric mean (±sd) pharmacokinetic time course of vistusertib and total radioactivity (ng or ng Equivalents/mL) in blood, plasma and saliva following a single oral dose of 125 mg ^{14}C-labelled vistusertib in solid tumour cancer patients ($n=4$). Total radioactivity: plasma (black), whole blood (blue) and saliva (green). Vistusertib: plasma (yellow) and saliva (orange). **b** Mean (±s.d) percentage cumulative radioactivity recovery-time course in total biofluids (black), faeces(yellow) and urine (blue) and urinary recovery of vistusertib (green)

Table 2 summary pharmacokinetic parameters for vistusertib and total radioactivity in plasma, whole blood and saliva following single oral dose of 125 mg ^{14}C-labelled vistusertib in solid tumour cancer patients

Parameter	Radioactivity			Vistusertib	
	Blood	Plasma	Saliva	Plasma	Saliva
C_{max} (ng/mL or ng Equivalents/mL)					
n	4	4	4	4	3
Geo. mean	3004	4379	6966	4254	5410
CV%	36.91	38.26	76.90	37.73	94.34
T_{max} (h)					
n	4	4	4	4	3
Median	0.53	0.53	1.00	0.53	1.00
Min/max	0.50–0.57	0.50–0.57	0.98–1.17	0.50–0.57	0.98–1.17
$AUC_{0-t_{last}}$ (ng h/mL or ng Equivalents h/mL)					
n	4	4	4	4	3
Geo. mean	14,280	23,290	8943	16,510	6025
CV%	48.55	37.95	56.67	29.31	78.70
t_{last} (h)					
n	4	4	3	4	4
Median	18.1	48.3	12.9	18.0	11.1
Min/max	12.0-32.1	32.1–120	12.1–24.0	12.0–24.0	8.0–12.9
$AUC_{0-\infty}$ (ng h/mL or ng Equivalents h/mL)					
n	4	4	–	4	–
Geo. mean	16,150	24,320	n.d.	17,170	n.d.
CV%	43.12	39.92	n.d.	29.80	n.d.
$t_{1/2}$ (h)					
n	4	4	–	4	–
Mean	6.8	29.0	n.d.	3.7	n.d.
sd	4.5	37.1	n.d.	1.2	n.d.

ng Equivalents are units of measurement for total radioactivity as comprises unchanged vistusertib and metabolites so assumes molecular weight of metabolites are equivalent to vistusertib

n.d. not determined

recovery of 41%. In general recovery of radioactivity in urine was low, approximately 10% of the total dose, and essentially complete in all patients after 48 h post-dose.

Metabolic profiling

Unchanged vistusertib was identified as the major circulating drug-related component and accounted for 78% of the plasma radioactivity AUC. Metabolites were observed at low levels in plasma (individually < 10% of the radioactivity $AUC_{0-\infty}$) and included products resulting from biotransformation of the morpholine moieties (M5, M8; M9, M10, M12 and M13) and the *N*-methylamide group (M11 and M15).

In contrast, unchanged vistusertib accounted for a small proportion of the eliminated dose (5%), indicating metabolism as the major route of elimination for the compound in patients. The most abundant excreted metabolite resulted from mono-oxidation of a morpholine moiety (M5; 41% of the dose) and this was mainly excreted in the faeces (38% of the dose). A number of additional metabolites (M1, M2, M4, M6, M7, M8, M9, M10 and M14) involving biotransformation of the morpholine moieties were identified and in combination with M5 accounted for 66% of the dose (no individual component other than M5 accounted for > 10% of dose). Biotransformation of the *N*-methylamide group was also a notable site of metabolism, with products resulting from carbinolamide (M11; 6%) and carboxylic acid (M15; ~ 1% and M14; 3%) formation identified in excreta. Metabolites were only observed at trace levels in saliva (data not shown). Radiochromatograms from pooled samples are presented in Fig. 2.

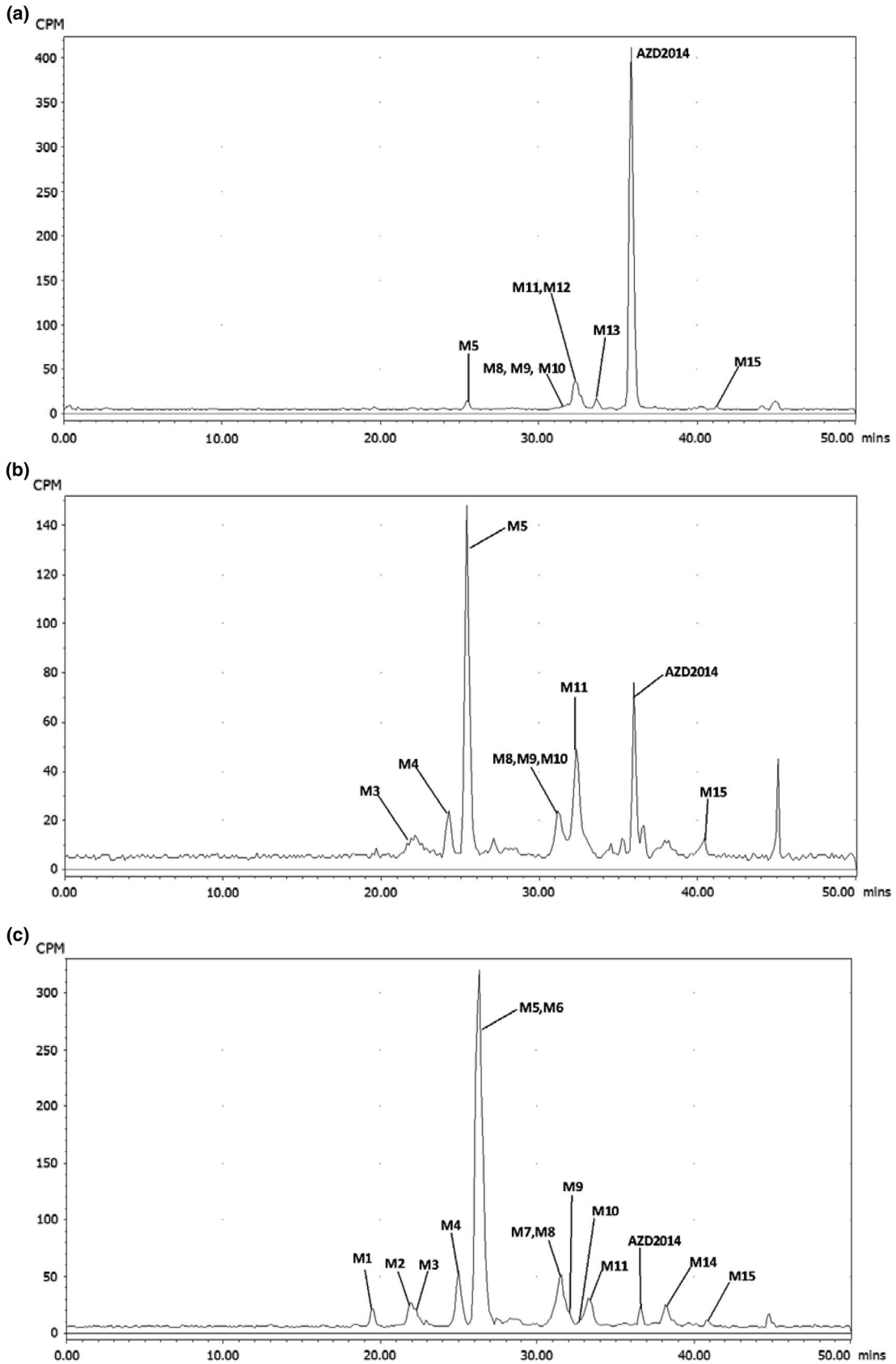

Fig. 2 Radio-chromatograms from pooled **a** plasma, **b** urine and **c** faeces respectively following single oral dose of 125 mg ^{14}C-labelled vistusertib in solid tumour cancer patients ($n = 3$). Structures of metabolites denoted by M number can be seen in Fig. 3

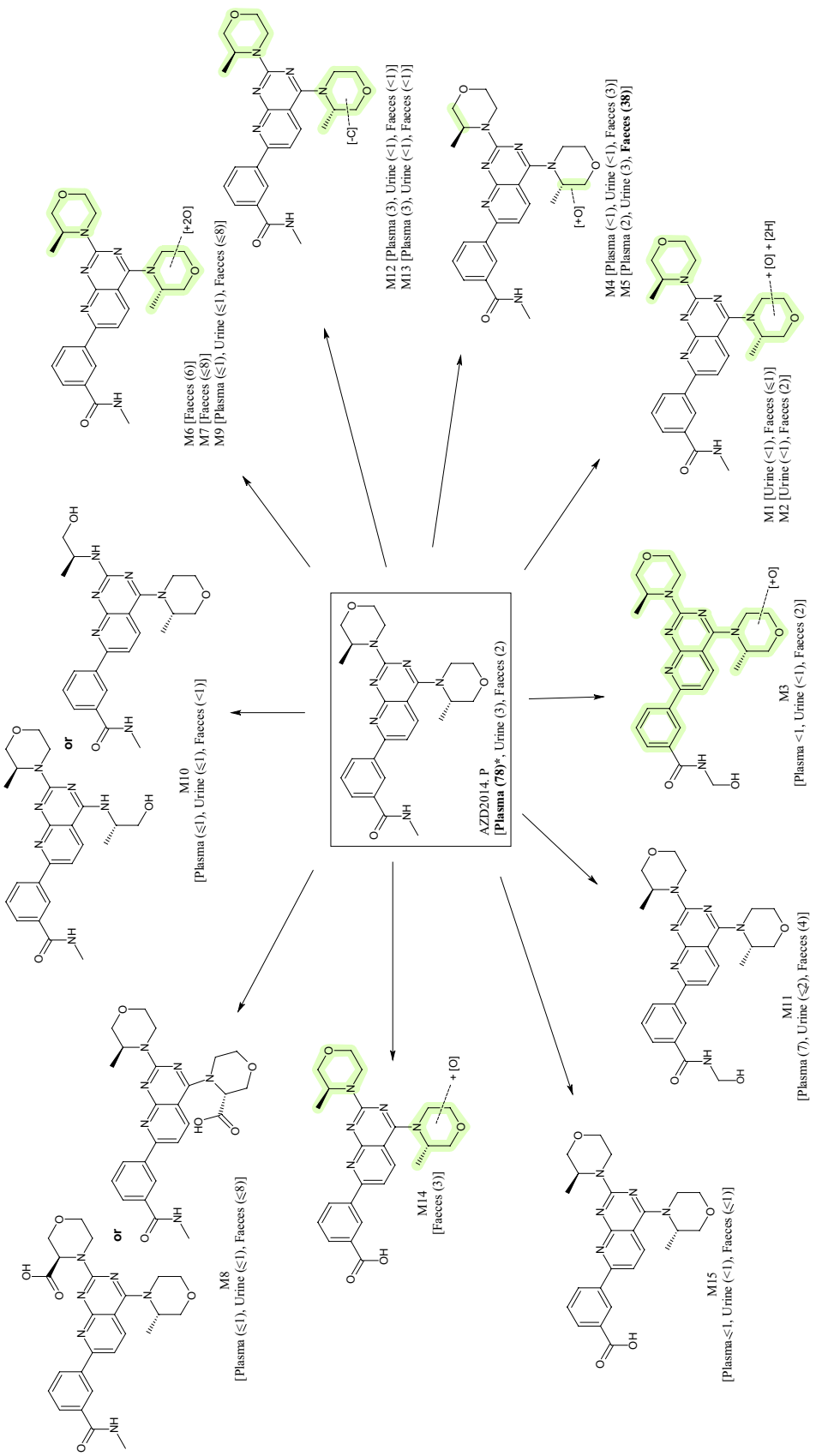

Fig. 3 Putative metabolic pathway of vistusertib following oral administration in advanced solid malignancy patients. Chemical structures marked in green are yet to be fully elucidated. Square brackets indicate matrices in which metabolite was detected. Values in parentheses indicate mean percentage dose excreted as each metabolite. *Plasma values indicate percentage chromatogram and are equivalent to % of the plasma AUC for total radioactivity. Bold type denotes > 10% dose excreted as this metabolite in this matrix or > 10% of the radioactivity in the plasma chromatogram. Molecular masses of vistusertib and M5 are 464.5 and 479(p + 16) g/mol, respectively

Safety

There were no deaths reported during the study. No dose reductions occurred during the study and there were no dose interruptions during the inpatient period. All four patients experienced adverse events. The most commonly reported AEs were nausea, vomiting, anaemia, stomatitis, dry skin, pruritus, mucosal inflammation, and reductions in white blood cell count. No treatment-related serious adverse events were reported. The highest grade adverse event experienced was CTC grade 3 [infection ($n=1$), lymphopaenia ($n=2$), and mucositis ($n=3$)]. No additional haematological abnormalities were observed and there were no clinically relevant changes in clinical chemistry, vital signs, ECGs or physical findings.

Efficacy

No patient achieved an objective anti-tumour response as assessed by RECIST. Three patients had stable disease lasting ≥ 8 weeks and the total time on treatment for each patient was 318 days (peritoneal mesothelioma), 120 days (unknown primary tumour), 64 days (breast) and 55 days (uterine) respectively. All patients had disease progression by the end the study.

Discussion

This small Phase I study has defined the PK, metabolic and excretion profiles of orally administered ^{14}C-vistusertib in patients with advanced solid malignancies. We do not consider that the interpretation of our results is substantially affected by the sample size ($n=4$) or the exclusion of one subject from the total and faecal radioactivity assessment. The radioactivity recovery of this patient was low at 41% in comparison to the other patients which ranged from 90 to 94%. In addition to the technical problems resulting in probable loss of radioactive faecal material other factors may have also contributed to the low radioactivity recovery. This patient had abdominal disease (peritoneal mesothelioma), and although with a recent history of regular bowel movements entering the study, was unable to produce a stool sample until 48 h after the ^{14}C-vistusertib dose. In contrast to the other patients, there was still ~4% dose of radioactivity in the last stool sample from the patient before discharge on day 8. Of note, the patient was concomitantly taking buprenorphine and bisacodyl. It is possible that these or other factors resulted in delayed faecal excretion contributing to lower faecal radioactivity collection, relative to the other patients, during the in-patient collection period. Notwithstanding, the blood and plasma PK of vistusertib and radioactivity, urinary excretion and the metabolic profile of this subject were consistent with the other patients.

The results of the pharmacokinetic analysis of vistusertib are consistent with those previously reported for advanced solid malignancy patients [10, 11]. Vistusertib is rapidly absorbed in solution form and is eliminated mainly via oxidative metabolic conversion and subsequent excretion into both faeces and urine as various metabolites. The short terminal half-life observed for parent drug (~4 h) is consistent with the twice daily regimens currently being investigated in clinical trials. The similar rate of decline of radioactivity in blood and plasma suggests rapid equilibration between plasma and blood cell components. The ratio of radioactivity in blood to plasma ranged from 0.62 to 0.77 over the 12 h post-dose period, demonstrating preferential distribution of vistusertib and metabolites in the plasma compartment.. Urinary excretion of vistusertib and metabolites was low (~3% and ~12% total, respectively) suggesting changes in renal function e.g. renal impairment, are unlikely to have a clinically relevant impact on the pharmacokinetics of vistusertib.

This is the first clinical study to characterise the metabolic pathway of vistusertib in humans. The majority of circulating vistusertib-related material is parent drug and individual metabolites circulate at low levels (< 10% of AUC). While the pharmacological activity of hydroxylation product M11 (highest circulating metabolite by AUC, ~7% plasma) has yet to be characterised, even assuming similar potency to vistusertib (~3 nM IC_{50} in kinase binding assay [1]), it is unlikely to contribute to the pharmacological effect in humans in vivo. Based on a comparison with data from non-clinical species and human systems in vitro (unpublished), no metabolites unique to human metabolism in vivo were observed. Quantitatively, based on the in vitro data, the metabolism of vistusertib in humans appears to almost exclusively via cytochrome P450 3A (both 3A4 and 3A5). While clinical drug interaction studies have not yet been conducted, it is anticipated that clinically relevant pharmacokinetic drug–drug interactions with CYP3A inhibitors and inducers may occur. The safety and tolerability profile was consistent with previous clinical investigations [10, 11], and while there were no objective responses observed in this setting, three out of four patients had some disease stabilisation.

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Compliance with ethical standards

Conflict of interest All authors are current employees of AstraZeneca.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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