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ORIGINAL ARTICLE

Mass balance, excretion and metabolism of [^{14}C] ASA404 in cancer patients in a phase I trial

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Abstract
Purpose To determine the mass balance, excretion and metabolism of the small molecule flavonoid tumour vascular disrupting agent ASA404 in patients with advanced cancer.
Methods Seven cancer patients were given a single dose of 3,000 mg [^{14}C] ASA404 by intravenous infusion over 20 min prior to collection of samples of plasma, urine and faeces. Pharmacokinetic samples were analysed by HPLC, liquid scintillation counting, mass spectrometry, glusulase treatment and comparison to authentic standards. Descriptive pharmacokinetic parameters were generated by non-compartmental analysis.
Results Mass balance was achieved (mean recovery of radioactivity in excreta = 86.9% of the dose) with balanced excretion between urine (mean recovery of radioactivity in urine = 53.9% of dose) and faeces (mean recovery of radioactivity in faeces = 33.3% of dose). ASA404 was eliminated as parent drug, three known metabolites (6-methylhydroxy-ASA404, ASA404 acyl glucuronide and 6-methylhydroxy-ASA404 acyl glucuronide) and two novel metabolites (an ASA404 dimer and an ASA404 dimer glucuronide conjugate). Unchanged ASA404 was the major radioactivity component detected in plasma within the first 24 h after dosing. At later time points, irreversibly protein bound ASA404 and all of the metabolites that had been detected in excreta contributed to total plasma radioactivity.
Conclusion This study defined the substantial excretion of ASA404, mainly as metabolites, in both urine (over half of the dose) and faeces (about one-third of the dose) after intravenous administration. Two novel metabolites were identified that were not reported by previous studies using nonradioactive techniques.

Keywords Excretion · Radiolabel study · Metabolism · Antivascular

Abbreviation
ASA404 5,6-dimethylxanthenone-4-acetic acid

Introduction

New treatment approaches to cancer are needed urgently as, globally, almost seven million people die from the disease each year despite currently available therapies [1]. One promising treatment strategy is the selective disruption of established tumour blood vessels by tumour vascular disrupting agents, whose action culminates in inhibition of blood flow and extensive necrosis within solid tumours [2]. Several tumour vascular disrupting agents are currently in clinical development and are categorised according to whether they are related to flavone acetic acid or tubulin-binding agents. Their mode of action differs from that of

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antiangiogeneic agents, which primarily act by inhibiting new blood vessel formation rather than against the established tumour vasculature.

ASA404 (5,6-dimethylxanthenone-4-acetic acid, vadimezan, DMXAA) is a small molecule flavonoid tumour vascular disrupting agent that acts upon the existing tumour vasculature thereby inhibiting blood flow and causing necrosis in solid tumours [3]. ASA404 has been evaluated in clinical trials as a single agent [4–6] and in combination with standard chemotherapy [7–10] in patients with advanced cancer. In this context, the phase I study we now report evaluated a single radiolabeled dose of [^{14}C] ASA404 given to assess its mass balance, excretion and metabolism in patients with advanced cancer, who were subsequently given therapeutic dosing of nonradioactive drug.

Several previous studies have investigated the disposition and metabolism of ASA404 in cancer patients but using nonradioactive techniques. ASA404 is known to undergo oxidation at its 5- and 6-methyl groups mediated by CYP1A2 and flavin monooxygenases [11, 12], and glucuronidation catalysed by UGT1A9 and UGT2B7 [13] to form acyl and other conjugates that are chemically unstable and may form adducts with plasma proteins [14]. The plasma pharmacokinetics of unchanged ASA404 were well-defined and are characterised by saturable elimination and plasma protein binding [6, 15, 16]. However, studies of the plasma metabolite profiles of ASA404 have been limited to date. Also, previous human studies of ASA404 excretion were semi-quantitative and exploratory as they were limited to urine only (not faeces), small patient cohorts and short collection periods following sub-therapeutic doses of ASA404 [14, 15].

Further investigation using radiolabeled drug was considered necessary to quantitate the urinary and faecal excretion of the parent drug and its metabolites, investigate its plasma metabolite profiles and determine whether metabolites other than those previously identified were formed during clinical treatment. To do so, we gave a single dose of 3,000 mg [^{14}C] ASA404 prior to pharmacokinetic sampling to characterise its mass balance, excretion and metabolism in patients with advanced solid tumours, who were then given extended therapeutic treatment with nonradioactive ASA404 either alone or combined with docetaxel, carboplatin and/or paclitaxel.

Materials and methods

Patients

Eligible patients had histologically proven advanced or metastatic solid tumours refractory to standard therapy or appropriate for treatment with an investigational agent alone or combined with docetaxel, carboplatin or paclitaxel. They had a WHO performance status of $\leq$1, age $\geq$18 years, life expectancy $\geq$3 months, adequate bone marrow, renal and liver functions, and were willing and able to comply with study procedures and to give written informed consent. A minimum of 4 weeks must have relapsed after prior treatment that was extended to 6 weeks for nitrosoureas or mitomycin C. Patients were excluded for central nervous system and/or leptomeningeal disease that was symptomatic or requiring corticosteroids, significant cardiovascular disease (including long QT syndrome, baseline 12-lead ECG QTc of >450 ms, congestive heart failure, myocardial infarction within 12 months, poorly controlled angina pectoris, labile hypertension, history of ventricular tachycardia, right bundle or bifascicular branch block, or bradycardia), uncontrolled infection, diagnosis of HIV or Hepatitis B or C, major surgery within 8 weeks or minor surgery or radiotherapy within 2 weeks. They were excluded for recent treatment with other investigational drugs, hematopoietic growth factors, co-medication that was known or suspected to induce, inhibit or be a substrate for CYP1A2 or UGTs, raise serotonin levels or cause Torsade de pointes. Patients were excluded if they were recent or current smokers, were pregnant, were breast feeding or had a positive pregnancy test or systolic blood pressure >160 mmHg or diastolic blood pressure >90 mmHg. Those with concurrent significant, severe or uncontrolled medical, neurologic or psychiatric disorders that could compromise trial compliance, safety or participation were excluded from the study.

The study was reviewed and approved by an ethics committee and was conducted according to the ethical principles of the Declaration of Helsinki. Informed consent was obtained from each subject in writing before conducting any study-specific procedures.

Study design

This was an open-label, single-centre, phase I study consisting of two phases, a core phase that used a single intravenous dose of 3,000 mg [^{14}C] ASA404 in advanced or metastatic solid tumour patients to characterise its mass balance, metabolism and elimination, and an extension phase following the completion of the core phase where patients were eligible to receive up to six cycles 3-weekly of nonradioactive ASA404 given either alone or in combination with docetaxel, carboplatin and/or paclitaxel.

Treatment

For the core phase of the study, an intravenous concentrate containing 3,000 mg ASA404 with 60 μCi of [^{14}C] for dilution

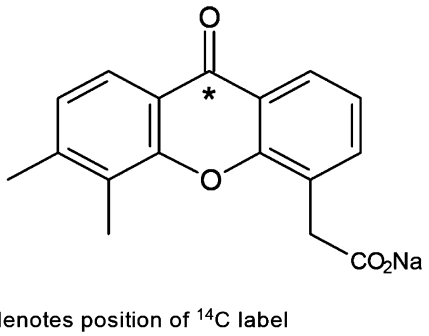

Fig. 1 Chemical structure of [^{14}C] ASA404

with 50 mL of 5% glucose solution was manufactured by the Isotope Lab and Pharmaceutical and Analytical Development, Novartis Pharmaceuticals. The chemical structure of [^{14}C] ASA404 is shown in Fig. 1. Chemical purity of at least ≥97% was verified by high-performance liquid chromatography–ultraviolet detection (HPLC–UV) and radiochemical purity of at least ≥95% was confirmed by HPLC radio-monitoring of the precursor batch of undiluted radiolabeled substance. The whole body committed effective dose was estimated to be 0.63 mSv, which is below the recommended limited for the public of 1 mSv per year.

Sample collection

Serial blood samples were collected during the core phase of the study to assess ASA404 and total radioactivity pharmacokinetics over a 7-day period at pre-dose, 10 and 20 min after the start of the infusion then at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144 and 168 h following the end of the infusion. Blood samples were taken in lithium-heparin tubes from the arm contralateral to the ASA404 infusion for at least the first 24 h of sampling, and plasma was prepared by centrifugation within 30 min of blood collection. Urine was obtained at the following collection periods, −8 to 0 h (pre-dose), 0–4, 4–8, 8–12, 12–16, 16–20, 20–24 h and every 24 h post-dose for 7 consecutive days. Faecal samples were collected pre-dose and in 24 h intervals after administration of [^{14}C] ASA404 including used toilet paper for up to 7 days. All samples for radioactivity counting and pharmacokinetic and metabolite analyses were kept in storage at approximately −20 or −80°C respectively, and protected from light during collection, handling and storage.

Measurement of ASA404 concentrations in plasma after radiolabeled dose

Sample preparation was performed using protein precipitation extraction with a Quadra 96 TomTec system (Hamden, CT). A 50-μL aliquot of each sample was pipetted into the appropriate well in a 96-well polypropylene plate or into 1.4-mL tube inserts in a 96-well format. A 100-μL aliquot of the internal standard solution (containing 2.50 μg/mL of internal standard) was added to all wells except for the blanks, to which a 50-μL aliquot of blank plasma was added. A 100-μL aliquot of 1% formic acid in methanol was added to each well. The plate was sealed with a cap mat and vortex mixed on a Glas-Col multi-pulse vortexer (Terre Haute, IN, USA) followed by centrifugation at approximately 3,000 RPM (∼1,000×*g*) for about 5 min at room temperature. The sample supernatant was transferred to a clean 96-well plate, evaporated to dryness at approximately 45°C under a flow of nitrogen and the sample residue reconstituted in 100 μL of 10% aqueous acetonitrile (containing 0.2% formic acid). A 20-μL aliquot was injected onto the HPLC system connected to a Sciex API 3,000 mass spectrometer. The mass spectrometer was operated in the positive electrospray ionisation mode.

ASA404 was monitored by the 283.1 *m/z* to 237.1 *m/z* transition, while internal standards [M + 6] ASA404 was monitored by the *m/z* 289.1 to *m/z* 243.1 transition. The calibration curves were fit to the instrument response × concentration using a quadratic regression with $1/x^2$ weighing. The lower limit of quantification (LLOQ) for ASA404 in plasma was 100 ng/mL using 50 μL samples. Concentrations below the LLOQ were reported as 0 ng/mL.

Measurement of total radioactivity in plasma, blood, faeces and urine

Measurement of total radioactivity in plasma, blood, faeces and urine were carried out using liquid scintillation counting. For total radioactivity in the blood, 2 mL of blood at each time point was transferred into a separate screw-cap polypropylene vial. Duplicate (0.1 mL) aliquots were taken for whole blood total radioactivity analysis. For total radioactivity in plasma, 3 mL of blood at each time point was transferred into a separate screw-cap polypropylene vial. A minimum of 1 mL of plasma was isolated from each 3 mL of whole blood sample collected, before transfer of two aliquots of plasma (0.2 mL each) from each plasma sample into separate scintillation vials containing 10 mL of cocktail. For total radioactivity in the urine, duplicate (0.2 mL) aliquots of urine were placed into separate scintillation vials containing 10 mL of cocktail. For total radioactivity in faeces, a pre-dose faecal sample was collected from each patient during baseline or screening period. Total radioactivity in the faeces was analysed daily by duplicate (0.2 mL) aliquots being taken for faeces total radioactivity analysis. Samples were solubilised and decolorised prior to radio assay by liquid scintillation counting. Radioactivity was determined in all faecal samples up to the first 8 days of collection.

An aliquot of water was added to the faeces to prepare a 1:1 v/w homogenate. A ~100 mg portion of each faeces homogenate (in duplicate) was pipetted into scintillation vials, and the exact weight was recorded. To each vial, 1 mL Solvable® was added to each sample, followed by incubation for approximately 18 h in a shaking water bath at 50°C. At the end of the incubation period, 100 μL of 100 mM disodium EDTA was added as an antifoaming agent, and 400 μL of 30% hydrogen peroxide was added to decolorise the samples. The vials were then slightly capped and returned to the water bath for an additional 3 h. Liquid scintillation cocktail (10 mL of Formula-989®) was added to each vial, and the samples were placed in the dark overnight to reduce chemiluminescence prior to counting.

For blood and serum, the measured radioactivity was converted to a concentration of drug-related material (ngEq/mL) using the measured volume of the aliquot and the known specific activity of the drug substance. For urine and faeces, the measured radioactivity was converted to a % recovery value using the weight of the aliquot, the total weight of the collected sample and the known radioactivity of the dose.

Determination of metabolite profiles and identification of metabolites

Plasma proteins were precipitated by the addition of 1,000 μL acetonitrile with 1% formic acid (v/v) to 400 μL of each plasma sample. Samples were vortex mixed, sonicated and centrifuged at 3,500 rpm (~1,000×*g*) for 5 min at 10°C. The supernatant was concentrated to dryness under nitrogen. The residue was reconstituted with 200 μL water : acetonitrile with 1% formic acid (50:50, v/v), vortex mixed and centrifuged. The supernatant was transferred into an HPLC autosampler vial for analysis. Measurements for recovery were performed on a Packard Tri-Carb 3170 TR liquid scintillation counter (Packard Instrument Co., Downer's Grove, IL). All other off-line measurements were performed on a Packard TopCount NXT microplate reader (Packard Instrument Co. Downer's Grove, IL). The radioactive recoveries were based on the initial plasma radioactivity and ranged from 72.6 to 96.2%.

A pooled urine sample was prepared for each patient by combining 2.5% by volume aliquots from each time interval. The time intervals used were as follows: Patient 1, 0–72; Patient 2, 0–120 h; Patient 3, 0–72 h; Patient 4, 0–72 h; Patient 5, 0–72 h; Patient 6, 0–96; Patient 7, 0–96 h. An aliquot was centrifuged at 3,500 rpm and 10°C for 10 min. An aliquot of ~20 μL of each was injected onto the HPLC system with off-line radioactivity detection. Aliquots of urine from before and after the pre-concentration step were mixed with Packard Flo-Scint II liquid scintillant and counted on a Packard Tri-Carb 3,170 TR liquid scintillation counter (Packard Instrument Co., Downer's Grove, IL). This analysis indicated that the recovery of radioactivity was approximately 90%.

A pooled faecal sample was prepared for each patient by combining equal per cent by weight aliquots from each homogenate. The time intervals used were as follows: Patient 1, 0–120 h; Patient 2, 0–120 h; Patient 3, 48–144 h; Patient 4, 0–96 h; Patient 5, 0–96 h; Patient 6, 0–72 and 168–216 h; Patient 7, 0–96 h. For all seven patients, these time intervals accounted for >90% of the total radioactivity excreted in the faeces. An aliquot from each faeces pool (~1 g) was extracted sequentially with 10 mL of 1:1 acetonitrile/methanol, vortex mixed, followed by centrifugation at 3,500 rpm and 10°C for 10 min. After combining the two extracts, the samples were concentrated on a Turbo-Vap LV to dryness. The residue was reconstituted in 5 mL of 1:1 water/acetonitrile, of which 50 μL aliquots were injected onto the HPLC system with off-line radioactivity detection. The recovery of radioactivity after sample processing was ~87% for all seven patients.

[^{14}C] ASA404 and its metabolites in the plasma and excreta were analysed by HPLC with off-line radioactivity detection. A Waters Acquity UPLC system (Milford, MA) was used. The chromatographic separations were performed on a Waters Xbridge column (150 × 4.6 mm, 5 μm) maintained at a temperature of 30°C. ASA404 and its metabolites were resolved with gradient elution consisting of solvent A (5 mM ammonium formate with 0.1% formic acid, pH ~3) and B (acetonitrile with 0.1% formic acid), at a flow rate of 1.0 mL/min. A linear gradient programme was used. Chromatograms were evaluated using the Laura 4 radiochromatography software version 4.0275 (LabLogic, Sheffield, England).

Metabolites were identified by liquid chromatography—mass spectrometry (LC–MS and LCMS/MS) on an LTQ-Orbitrap hybrid mass spectrometer (with high resolution and accurate mass capabilities) (Thermo Fisher, Waltham, MA) interfaced with a Waters Acquity Separation Module, which included an autosampler and solvent delivery system. Qualitative analyses (metabolite structure identification) were carried out using electrospray ionisation (ESI) in the positive ion mode. The HPLC separation method was identical to that used for metabolite profiling. The flow rate was 1.0 mL/min with a 1:4 split to the mass spectrometer ion source and 96-well Lumaplates. The MS/MS spectra were obtained through a higher-energy collisional dissociation (HCD) of [M + H]+.

Pharmacokinetic analyses and statistics

Pharmacokinetic parameters including Cmax, Tmax, $T_{1/2}$, AUClast, AUCinf, Cl, and Vz of parent ASA404 and total radioactivity were derived from blood and plasma concentration versus time data in each patient by non-compartmental analysis using WinNonLin (version 5.2).

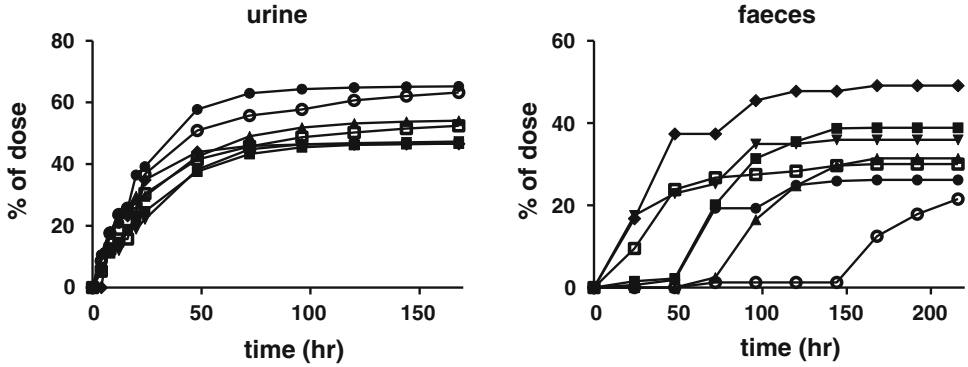

Fig. 2 Cumulative urinary and faecal excretion of radioactivity following a single IV infusion of 3,000 mg [^{14}C] ASA404

Results

Patient population

Seven patients were enrolled and completed the core phase of the study. Six of the patients were women and one was a man. Five were Caucasian and two were Asian ethnicity. Their mean age was 57 years (range, 45–68 years), and their mean body weight was 60 kg (range, 57–78 kg). All had ECOG performance status of 1. Three patients had nonsmall-cell lung cancer, two had ovarian cancer and one each had head and neck cancer or colon cancer.

Excretion and mass balance of radioactivity in urine and faeces

The excretion of radioactivity in urine and faeces over a 210-h period following the administration of a single 3,000 mg dose of radiolabeled ASA404 over 20 min is shown in Fig. 2. Mass balance was achieved with cumulative recovery of radioactivity from both urine and faeces accounting for 86.9% of the administered dose (range, 82.4–95.8%). The mean contributions of the urinary and faecal routes to the overall elimination of ASA404 were 53.9% (range, 46.7–65.2%) and 33.3% (range, 21.5–49.1%) of the administered dose, respectively. Urinary contributions to the excretion of radioactivity were greater than faecal in 6 of 7 patients with faecal being slightly dominant over urinary in one subject only. Most of the urinary excretion occurred within 48 h of dosing. In contrast, the time course of faecal excretion was more variable and delayed relative to the time of dosing.

Characterisation of radiolabeled components in urine and faeces

Urine and faeces samples from each patient were pooled for analysis by radiochromatography, mass spectrometry, glusulase treatment and by comparison to authentic standards when these were available. Six radiolabelled components were detected on radiochromatograms (Fig. 3) that included parent ASA404 (Fig. 4a), three known metabolites (Fig. 4b–d) and two novel metabolites (Fig. 4e, f). Parent ASA404 (Rt, 33.6 min) (Fig. 3) had a molecular ion at *m*/*z* 283 and fragment ion at *m*/*z* 237 due to alpha cleavage of its carbonyl carbon (Fig. 4a). M1 (Rt, 21.1 min) (Fig. 3a) had a molecular ion at *m*/*z* 299 that was 16 *m*/*z* greater than ASA404, and major fragment ions at *m*/*z* 281, 253 and 223 corresponding to alpha cleavage of its 6-methylhydroxy and carbonyl carbons (Fig. 4b). On this basis, M1 was identified as the known 6-methylhydroxy metabolite of ASA404 [12, 17].

M2 (Rt, 24.5 min) (Fig. 3a) had a molecular ion at *m*/*z* 459 that was 176 *m*/*z* greater than parent ASA404. Its major fragment ions at *m*/*z* 283 and 237 corresponded with cleavage of a C–O bond between a glucuronide conjugated to ASA404 and alpha to the carbonyl carbon (Fig. 4c). It was converted to ASA404 by glusulase treatment of urine samples (Fig. 3b). On this basis, it was identified as the known acyl glucuronide metabolite of ASA404 [14]. This was further supported by the presence of 3 isomeric peaks in the radiochromatogram due to acyl migration around the glucuronic moiety (Fig. 3a). M4 (Rt, 17.5 min) (Fig. 3a) had a molecular ion at *m*/*z* 475 (Fig. 4d) that was 92 *m*/*z* greater than parent ASA404. Its major fragment ions at *m*/z 299, 253 and 235 corresponded with cleavage sites previously identified for parent ASA404, M1 and M2 (Fig. 4d). M4 disappeared from urine samples after treatment with glusulase (Fig. 3b). On this basis, it was identified as the known glucuronide conjugate of 6-methylhydroxy ASA404 [14].

M7 was a novel metabolite (Rt, 40.9 min) (Fig. 3c) with a molecular ion at m/z 563 that is approximately double the mass of parent ASA404. Its major fragment ions at m/z 517 and 237 corresponded with alpha cleavage of the carbonyl carbon of ASA404 (Fig. 4e). On this basis, it was identified as a novel dimeric (esterified) ASA404 metabolite where a C–O bond had formed between the 6-methyl group of one and the acyl group of another ASA404 molecule.

M10 was a novel metabolite (Rt, 36.5 min) (Fig. 3c) and a molecule ion at m/z 723 that was more than double the mass of parent ASA404. Its major fragment ions at m/x

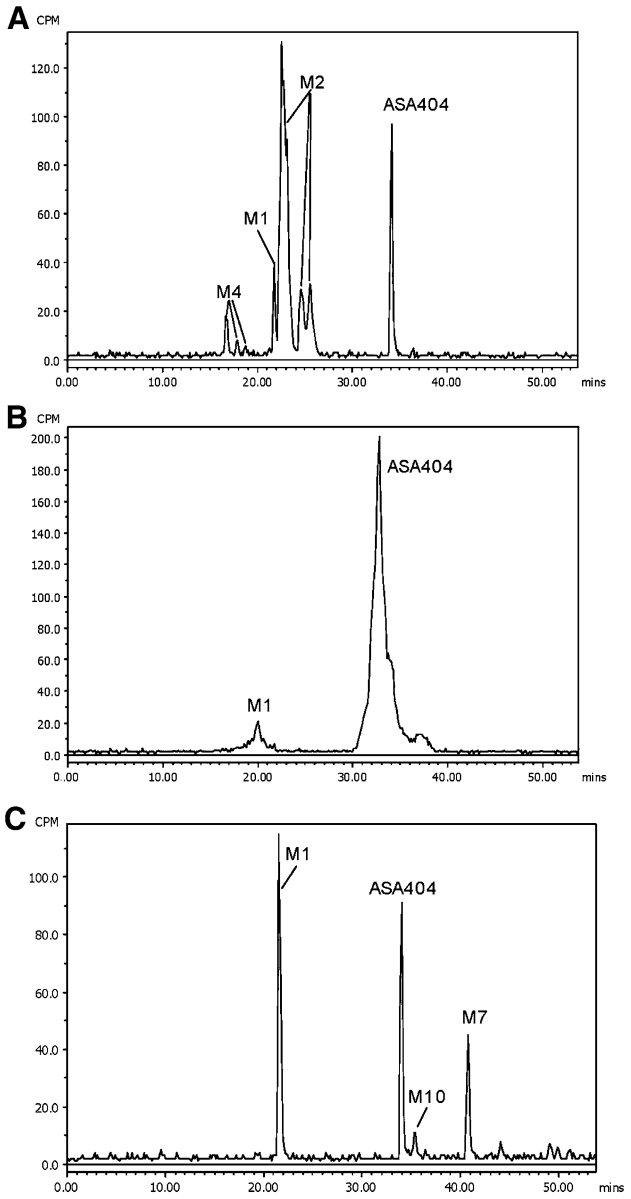

Fig. 3 Representative radiochromatograms of pooled samples of human urine, b human urine treated with glusulase and c human faeces, after a single intravenous dose of 3,000 mg [^{14}C] ASA404 (All samples from subject 1)

441, 423, 283 and 237 corresponded with cleavage sites of other ASA404 glucuronide conjugates, and like these, it disappeared from the sample after treatment with glusulase. On this basis, it was identified as a novel ASA404 dimer conjugated through their respective acyl groups by a single glucuronide molecule. The structure of this metabolite was suggested solely on the basis of mass spectral analysis.

Recovery of radiolabeled components in urine and faeces

In urine, recovery of unchanged ASA404 accounted for between 2.30 and 10.2% of the dose (Table 1). The major urinary component was the acyl glucuronide metabolite (M2) whose excretion in urine accounted for between 31.5 and 48.2% of dose. Other metabolites detected as radioactivity in urine included the 6-methylhydroxy metabolite (M1) and the 6-methylhydroxy acyl glucuronide metabolite (M4) whose urinary excretion accounted for between 3.02 and 11.2% and 0 and 5.18% of dose, respectively.

Table 1 Recovery of radiolabeled constituents in human urine and faeces as percent of dose (values are the median (range). ND, not detected)

	Urine	Faeces
M1	4.47 (3.02–11.2)	18.8 (7.25–26.5)
M2	38.5 (31.5–48.2)	0 (0–3.63)
M4	4.31 (0–5.88)	ND
M7	ND	3.06 (trace–4.28)
M10	ND	Trace (trace–4.93)
ASA404	4.73 (2.3–10.2)	11.3 (2.64–19.7)

In faeces, recovery of parent ASA404 accounted for between 2.64 and 19.7% of dose (Table 1). The major faecal component was the 6-methylhydroxy metabolite (M1) whose excretion in faeces accounted for 7.25–26.5% of dose. Other metabolites detected as radioactivity in the faeces included an ASA404 dimer (M7) and the ASA404 dimeric glucuronide conjugate (M10), which accounted for up to 4.38 and 4.93% of dose, respectively.

Plasma pharmacokinetics of total radioactivity and ASA404

The mean concentration–time profiles and pharmacokinetic parameters for total radioactivity in blood and plasma, and for parent ASA404 measured in plasma by LC–MS/MS, are shown in Fig. 5 and Table 2. Tmax values coincided with the end of the 20-min ASA404 infusion. Elimination half-lives were longest for total radioactivity in blood (mean, 157 h) followed by total radioactivity in plasma (mean, 122 h) and shortest for parent ASA404 in plasma (mean, 27.2 h). Clearance for parent ASA404 in plasma was faster (mean, 2.53 L/h) than for total radioactivity in blood (mean, 1.27 L/h) or plasma (mean, 1.03 L/h). The ratio of mean AUCinf values for total radioactivity in plasma versus blood was 1.33, suggesting incomplete distribution of radioactivity to blood cells. The ratio of mean plasma AUCinf values for parent ASA404 and total radioactivity was 0.41, indicating the presence of radiolabeled components other than parent ASA404 contributing to the total radioactivity in plasma possibly due to formation of metabolites or irreversible plasma protein binding of ASA404.

Radiochromatograms showed that unchanged ASA404 was the major unbound drug component detected in plasma

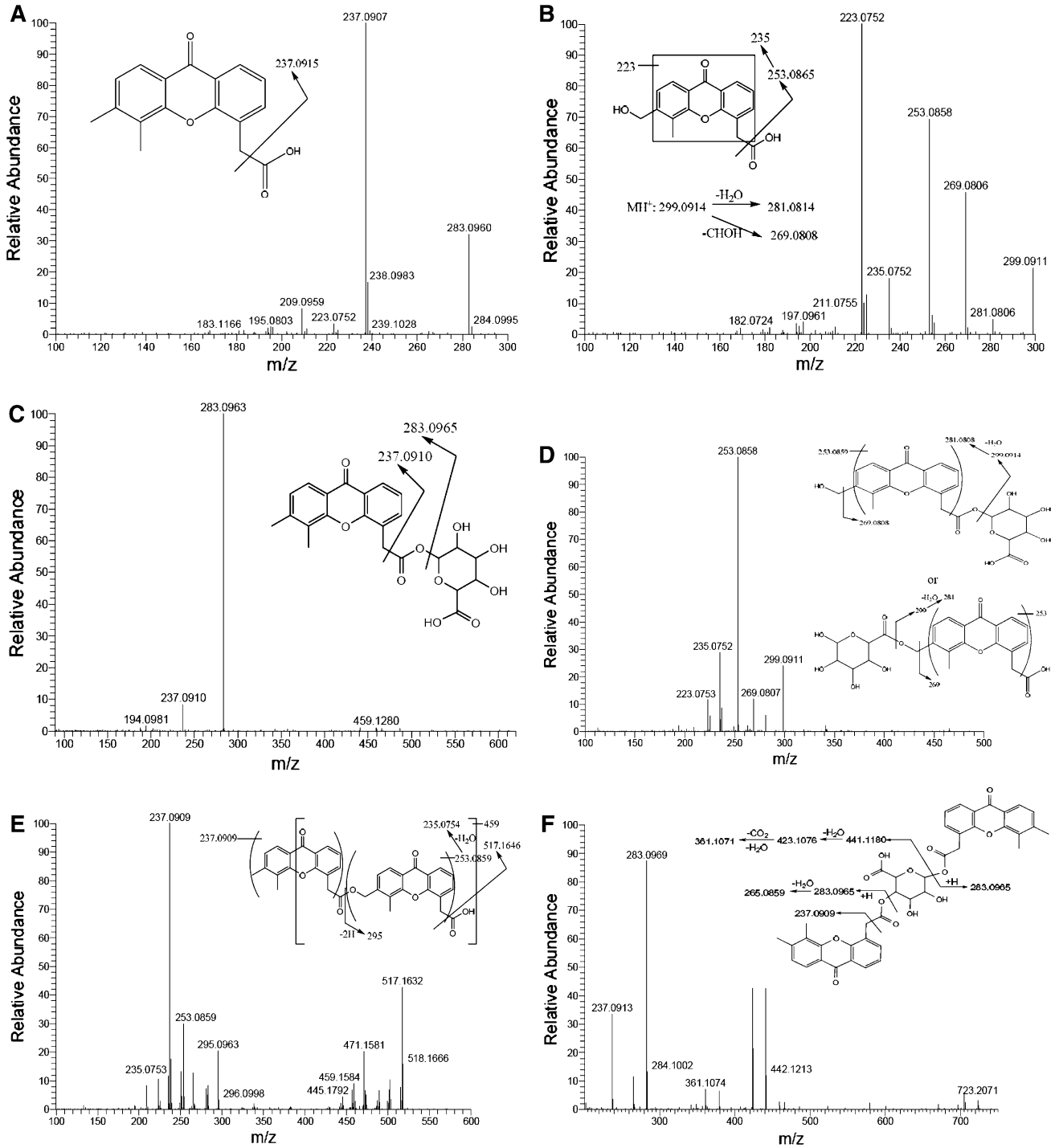

Fig. 4 Product ion mass spectra and assigned chemical structures for parent ASA404 (a) and metabolites M1 (b), M2 (c), M4 (d), M7 (e) and M10 (f)

(Fig. 6), but did not detect any ASA404 metabolites. Mass spectrometry analysis of plasma, however, detected all of the ASA404 metabolites that had been identified in excreta (M1, M2, M4, M7 and M10). Due to their differing ionisation efficiencies, limited amount of plasma sample available and/or low levels of metabolite in plasma, these plasma metabolites could not be quantified as a percentage of the AUC in plasma.

In preliminary experiments, plasma samples were processed by protein precipitation, extensive washing, solvent solubilisation of the protein pellet and filtration to detect drug–protein adducts. Residual radioactivity was detected

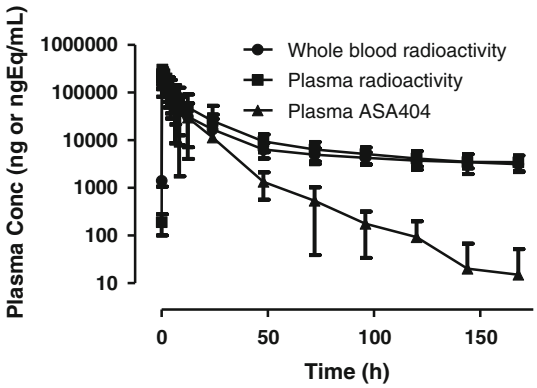

Fig. 5 Mean concentration–time profiles for total radioactivity in blood and plasma, and parent ASA404 in plasma, following a single IV infusion of 3,000 mg [^{14}C] ASA404

Table 2 Pharmacokinetic parameters for total radioactivity in blood and plasma, and for parent ASA404 in plasma, following a iv infusion of 3,000 mg [^{14}C] ASA404

Pharmacokinetic parameters	Total radioactivity in blood	Total radioactivity in plasma	ASA404 in plasma
Tmax (h)	0.33 (0.33–0.83)	0.33 (0.33–0.83)	0.33 (0.33)
Cmax (μg or μgEq/mL)	259 ± 63	308 ± 52	281 ± 42
AUClast (μg or μgEq*h/mL)	2,007 ± 1,049	2,934 ± 1,531	1,459 ± 873
AUCinf (μg or μgEq*h/mL)	2,725 ± 1,354	3,612 ± 2,136	1,466 ± 870
$T_{1/2}$ (h)	157 ± 48	122 ± 61.5	27.2 ± 19.8
CL (L/h)	1.27 ± 0.44	1.03 ± 0.44	2.53 ± 0.9
Vz (L)	1,283 ± 140	158 ± 59	103 ± 79

* median (range)

in the plasma protein pellet consistent with the covalent plasma protein binding of ASA404.

Discussion

We report the first in vivo study of radiolabeled ASA404 and the insights it provided into the human disposition and metabolism of this tumour vascular disrupting agent. The study quantified the mass balance of ASA404, and the routes, time course, extent and contribution of metabolites to its excretion in cancer patients. In addition, it identified two novel metabolites that had not been found in previous studies of ASA404 using nonradioactive techniques.

Our study found that almost the entire radioactivity administered as a short infusion of [^{14}C] ASA404 was subsequently recovered in the excreta within 7 days (≥83%). Between 26 and 49% of the radioactive dose was recovered in the faeces, and between 47 and 65% was recovered in the urine. Urinary excretion was complete within 48 h, whereas faecal excretion was more delayed. The balanced elimination of ASA404 in both urine and faeces is likely to reduce any potential impact of impairment of either liver or renal function on the total clearance of ASA404, as the impaired organ system will contribute only fractionally to ASA404 excretion.

ASA404 was eliminated primarily as metabolites. The acyl glucuronide ASA404 metabolite was the major metabolite excreted in urine and accounted for the elimination of between 32 and 48% of the dose. The 6-methylhydroxy metabolite of ASA404 was the major metabolite excreted in the faeces and accounted for the elimination of between 7 and 27% of the dose. Urinary and faecal excretion of parent ASA404 accounted for only 10 and 20% or less of the dose, respectively. In contrast, parent ASA404 was the major radioactive drug-derived material present in the systemic circulation during the first 24 h after dosing, which is when the tumour vascular disrupting activity occurs [3]. At later time points, other radioactive components appeared to contribute to the total radioactivity in the plasma, either in the form of ASA404 metabolites or irreversibly bound plasma protein–drug adducts.

Two novel ASA404 metabolites were identified that had not been found in previous studies using nonradioactive techniques. M7 was a dimeric metabolite in which a C–O bond had linked the acyl group of one with the 6-methyl group of another ASA404 molecule. M10 was another dimeric metabolite in which two ASA404 molecules were conjugated together through their respective acyl group by a single glucuronide molecule. Both M7 and M10 were detected in both plasma and faeces, and M10 was also detected in urine, by both mass spectrometry and radiochromatography. These findings indicate that M7 and M10 were genuine novel metabolites formed from ASA404 in vivo by metabolic routes of formation that are currently undefined.

Other metabolites were found including 6-methylhydroxy ASA404 (M2), an acyl glucuronide of ASA404 and an acyl glucuronide of 6-methylhydroxy-ASA404, confirming the importance of 6-methylhydroxylation and glucuronidation of the carboxylic acid moiety to the human disposition of ASA404, as reported in previous studies [12–14, 17]. The balanced metabolism of ASA404 by both glucuronidation and oxidation may tend to minimise any pharmacokinetic variability resulting from functional polymorphisms or drug–drug interactions that inhibit or induce ASA404-metabolising enzymes. Furthermore, the specific human enzymes involved in ASA404 6-methylhydroxylation (CYP1A2 and flavin monooxygenases) [12, 17] and glucuronidation (UGT1A9 and UGT2B7) [13, 14] are not known to be readily inhibited by clinically achievable concentrations of drugs expected to be given in combination with ASA404, such as carboplatin, paclitaxel and docetaxel.

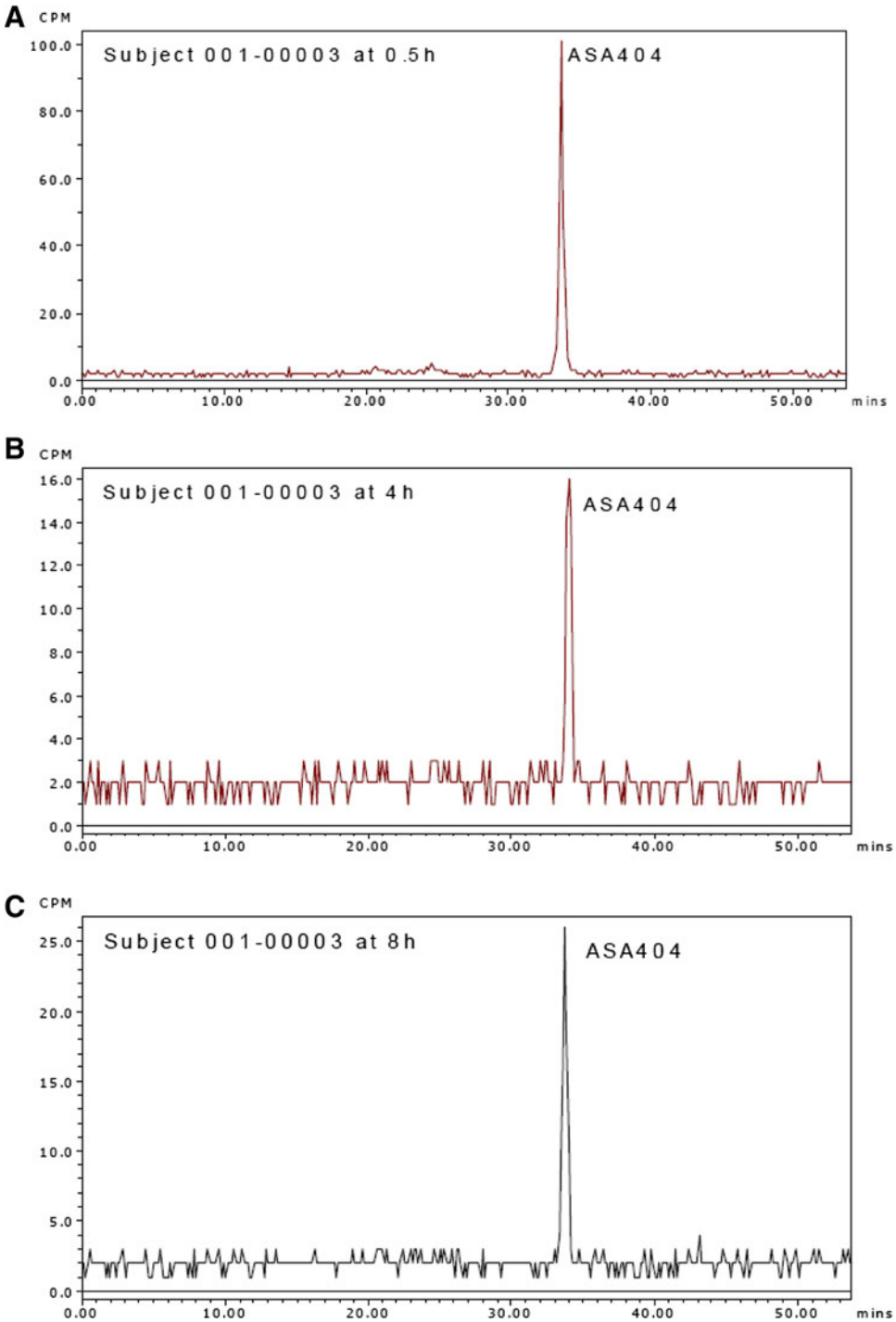

Fig. 6 Representative radiochromatograms of human plasma taken 0.5 (a), 4 (b) and 8 h (c) after a single intravenous dose of 3,000 mg [^{14}C] ASA404 (All samples from subject 3)

The future clinical development of ASA404 as an anticancer drug is currently uncertain due to a recent report of a negative phase III trial [10]. However, interest remains generally high in treatment approaches based on disrupting established tumour blood vessels with ASA404, other flavonoid compounds or tubulin-binding molecules. Pharmacological information like that reported here will remain important for the clinical evaluation of novel tumour vascular disrupting agents as they are likely to be used in clinical settings with significant risk of pharmacokinetic variability from interactions with concomitant medication or altered excretion from impaired organ function.

In conclusion, this clinical study of [^{14}C] ASA404 defined the substantial excretion of ASA404 and its metabolites in both urine (over half of the dose) and faeces (about one-third of the dose) after its intravenous administration, and identified two novel metabolites not reported in previous studies that used nonradioactive techniques.

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References

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. CA Cancer J Clin 55(2):74–108
2. McKeage MJ, Baguley BC (2010) Disrupting established tumor blood vessels: an emerging therapeutic strategy for cancer. Cancer 116(8):1859–1871. doi:10.1002/cncr.24975
3. Baguley BC (2003) Antivascular therapy of cancer: DMXAA. Lancet Oncol 4(3):141–148. doi:S1470204503010180
4. Jameson MB, Thompson PI, Baguley BC, Evans BD, Harvey VJ, Porter DJ, McCrystal MR, Small M, Bellenger K, Gumbrell L, Halbert GW, Kestell P (2003) Clinical aspects of a phase I trial of 5, 6-dimethylxanthenone-4-acetic acid (DMXAA), a novel antivascular agent. Br J Cancer 88(12):1844–1850. doi:10.1038/sj.bjc.6600992
5. Rustin GJ, Bradley C, Galbraith S, Stratford M, Loadman P, Waller S, Bellenger K, Gumbrell L, Folkes L, Halbert G (2003) 5, 6-dimethylxanthenone-4-acetic acid (DMXAA), a novel antivascular agent: phase I clinical and pharmacokinetic study. Br J Cancer 88(8):1160–1167. doi:10.1038/sj.bjc.6600885
6. McKeage MJ, Fong P, Jeffery M, Baguley BC, Kestell P, Ravic M, Jameson MB (2006) 5, 6-Dimethylxanthenone-4-acetic acid in the treatment of refractory tumors: a phase I safety study of a vascular disrupting agent. Clin Cancer Res 12(6):1776–1784. doi:10.1158/1078-0432.CCR-05-1939
7. McKeage MJ, Reck M, Jameson MB, Rosenthal MA, Gibbs D, Mainwaring PN, Freitag L, Sullivan R, Von Pawel J (2009) Phase II study of ASA404 (vadimezan, 5, 6-dimethylxanthenone-4-acetic acid/DMXAA) 1800 mg/m(2) combined with carboplatin and paclitaxel in previously untreated advanced non-small cell lung cancer. Lung Cancer 65:192–197. doi:10.1016/j.lungcan.2009.03.027
8. McKeage MJ, Von Pawel J, Reck M, Jameson MB, Rosenthal MA, Sullivan R, Gibbs D, Mainwaring PN, Serke M, Lafitte JJ, Chouaid C, Freitag L, Quoix E (2008) Randomised phase II study of ASA404 combined with carboplatin and paclitaxel in previously untreated advanced non-small cell lung cancer. Br J Cancer 99(12):2006–2012. doi:10.1038/sj.bjc.6604808
9. Pili R, Rosenthal MA, Mainwaring PN, Van Hazel G, Srinivas S, Dreicer R, Goel S, Leach J, Wong S, Clingan P (2010) Phase II study on the addition of ASA404 (vadimezan; 5, 6-dimethylxanthenone-4-acetic acid) to docetaxel in CRMPC. Clin Cancer Res 16(10):2906–2914. doi:10.1158/1078-0432.CCR-09-3026
10. Lara PN Jr, Douillard JY, Nakagawa K, von Pawel J, McKeage MJ, Albert I, Losonczy G, Reck M, Heo DS, Fan X, Fandi A, Scagliotti G (2011) Randomized phase III placebo-controlled trial of carboplatin and paclitaxel with or without the vascular disrupting agent vadimezan (ASA404) in advanced non-small-cell lung cancer. J Clin Oncol 29(22):2965–2971. doi:10.1200/JCO.2011.35.0660
11. Zhou S, Kestell P, Paxton JW (2002) 6-methylhydroxylation of the anti-cancer agent 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) by flavin-containing monooxygenase 3. Eur J Drug Metab Pharmacokinet 27(3):179–183
12. Zhou SF, Paxton JW, Tingle MD, Kestell P (2000) Identification of the human liver cytochrome P450 isoenzyme responsible for the 6-methylhydroxylation of the novel anticancer drug 5, 6-dimethylxanthenone-4-acetic acid. Drug Metab Dispos 28(12):1449–1456
13. Miners JO, Valente L, Lillywhite KJ, Mackenzie PI, Burchell B, Baguley BC, Kestell P (1997) Preclinical prediction of factors influencing the elimination of 5, 6-dimethylxanthenone-4-acetic acid, a new anticancer drug. Cancer Res 57(2):284–289
14. Zhou SF, Paxton JW, Tingle MD, Kestell P, Jameson MB, Thompson PI, Baguley BC (2001) Identification and reactivity of the major metabolite (beta-1-glucuronide) of the anti-tumour agent 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) in humans. Xenobiotica 31(5):277–293. doi:10.1080/00498250110043544
15. Jameson MB, Baguley BC, Kestell P, Zhao L, Paxton JW, Thompson PI, Waller S (2007) Pharmacokinetics of 5, 6-dimethylxanthenone-4-acetic acid (AS1404), a novel vascular disrupting agent, in phase I clinical trial. Cancer Chemother Pharmacol 59(5):681–687. doi:10.1007/s00280-006-0322-6
16. Li J, Jameson MB, Baguley BC, Pili R, Baker SD (2008) Population pharmacokinetic-pharmacodynamic model of the vascular-disrupting agent 5, 6-dimethylxanthenone-4-acetic acid in cancer patients. Clin Cancer Res 14(7):2102–2110. doi:10.1158/1078-0432.CCR-07-1475
17. Zhou SF, Kestell P, Paxton JW (2002) 6-Methylhydroxylation of the anti-cancer agent 5, 6-dimethylxanthenone-4-acetic acid (DMXAA) by flavin-containing monooxygenase 3. Eur J Drug Metab Pharmacokinet 27(3):179–183