Metabolite profiling of $^{14}$C-omacetaxine mepesuccinate in plasma and excreta of cancer patients

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Abstract

1. Omacetaxine mepesuccinate (hereafter referred to as omacetaxine) is a protein translation inhibitor approved by the US Food and Drug Administration for adult patients with chronic myeloid leukemia with resistance and/or intolerance to two or more tyrosine kinase inhibitors.

2. The objective was to investigate the metabolite profile of omacetaxine in plasma, urine and faeces samples collected up to 72 h after a single 1.25-mg/m² subcutaneous dose of $^{14}$C-omacetaxine in cancer patients.

3. High-performance liquid chromatography mass spectrometry (MS) (high resolution) in combination with off-line radioactivity detection was used for metabolite identification.

4. In total, six metabolites of omacetaxine were detected. The reactions represented were mepesuccinate ester hydrolysis, methyl ester hydrolysis, pyrocatechol conversion from the 1,3-dioxole ring. Unchanged omacetaxine was the most prominent omacetaxine-related compound in plasma. In urine, unchanged omacetaxine was also dominant, together with 4'-DMHHT. In feces very little unchanged omacetaxine was found and the pyrocatechol metabolite of omacetaxine, M534 and 4'-desmethyl homoharringtonine (4'-DMHHT) was the most abundant metabolites.

5. Omacetaxine was extensively metabolized, with subsequent renal and hepatic elimination of the metabolites. The low levels of the metabolites found in plasma indicate that the metabolites are unlikely to contribute materially to the efficacy and/or toxicity of omacetaxine.

Keywords

Disposition, homoharringtonine, mepesuccinate, metabolism, metabolites, metabolite profiling, omacetaxine, pharmacokinetics

History

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Introduction

In 2012, omacetaxine mepesuccinate (SYNRIBO®, Teva Oncology, West Chester, PA), hereafter referred to as omacetaxine, was approved by the US Food and Drug Administration (FDA) for adult patients with chronic myeloid leukemia (CML) with resistance and/or intolerance to two or more tyrosine kinase inhibitors (Cortes et al., 2013a,b, 2015; Nicolini et al., 2013). Omacetaxine is the first protein translation inhibitor approved by the FDA. The mechanism of action begins with binding to the A-side cleft of free ribosomes, which causes inhibition of protein elongation (Fresno et al., 1977; Huang, 1975), and thus reducing formation of proteins such as the Bcr-Abl fusion protein, which is the main driver of CML (Deininger et al., 2000; Kantarjian et al., 2006; Quintas-Cardama & Cortes, 2009). Since the Bcr-Abl protein has a short half-life and a fast turn over, this tyrosine kinase is very vulnerable to the inhibition of protein translocation (Branford et al., 2003; Shah et al., 2002; Soverini et al., 2006).

Minimal metabolism results for omacetaxine have been published. Figure 1 shows the chemical structure of omacetaxine. In 1986, Savaraj et al. studied the clinical pharmacokinetics of $^3$H-homoharringtonine (HHT), they found that through
72 h after administration, 28.2% of the administered $^3$H-HHT was excreted in urine, with 10.8% of the dose excreted as unchanged HHT (Savaraj et al., 1986). However, no further metabolite identification was performed and therefore the full metabolite profile of omacetaxine remains not fully elucidated. The pharmacokinetics of $^3$H-HHT were also studied in dogs; over a time period of 72 h after administration, 40.1% of the administered dose was recovered in urine, of which 17.8% as unchanged drug (Lu et al., 1988). In mice, 29% of the administered dose was excreted as unchanged HHT and 20% as 4′-DMHHT (Ni et al., 2003). In previous studies, the metabolites cephalotaxine and 4′-desmethyl homoharringtontine (4′-DMHHT) have been described and quantified (Nemunaitis et al., 2013; Ni et al., 2003). Hydrolysis of the mepesuccinate ester of omacetaxine leads to the formation of cephalotaxine and 4′-DMHHT is formed by methyl ester hydrolysis of omacetaxine. The steady-state area under the curve (AUC) in plasma of 4′-DMHHT was approximately 13% of that of omacetaxine. Cephalotaxine was undetectable in most patients (Nemunaitis et al., 2013). In urine, the excretion of omacetaxine, 4′-DMHHT and cephalotaxine represented, respectively, 12%, 4% and 0.07% of the administered dose on day 1. Both cephalotaxine and 4′-DMHHT are known to be inactive (Nemunaitis et al., 2013).

The metabolism of omacetaxine was further investigated in this study as part of a regulatory phase IV post marketing commitment to the FDA. The objective of this study was to further investigate the metabolism of 14C-omacetaxine after subcutaneous injection in cancer patients. Samples obtained from a human mass balance study with 14C-omacetaxine were selected, and metabolites were identified using high-performance liquid chromatography (HPLC), followed by off-line liquid scintillation counter (LSC) and ion-trap mass spectrometry (MS) characterization (high-resolution). The plasma exposure of the metabolites and the percentages of the individual metabolites that were excreted in urine and feces were determined up to 72 h after administration.

Materials and methods
Reference standards
Reference standards of omacetaxine ($C_{29}H_{39}NO_{9}$, $m/z$ 316), 4′-DMHHT ($C_{28}H_{36}NO_{9}$, demethylated omacetaxine, $m/z$ 532) and cephalotaxine ($C_{18}H_{21}NO_{4}$, $m/z$ 546) were provided by Teva Pharmaceuticals (North Wales, PA).

Chemicals
Labeled 14C-omacetaxine (chemical purity 99%, radiochemical purity 98% and chiral purity 100%) was prepared by Selcia Limited (Ongar, Essex, UK) and supplied by Teva Pharmaceuticals (North Wales, PA). Methanol (Supra-Gradient grade), acetonitrile (Supra-Gradient grade) and water (Supra-Gradient grade) were purchased from Biosolve Ltd (Amsterdam, the Netherlands). Formic acid (98%) and hydrogen peroxide were obtained from Merck (Darmstadt, Germany). Water (used for sample preparation) was obtained from B Braun (Melsungen, Germany), 2-propanol from Fluka (Buchs, Switzerland) and ammonium acetate (LC-MS grade) from Fluka (Zwijndrecht, the Netherlands). Solvable$^9$ and Ultima Gold liquid scintillation cocktail were purchased from PerkinElmer (Waltham, MA).

Ethics
The protocol for the human study was approved by the institutional ethics committee, and all human patients provided written informed consent before participating in this study. The human study was conducted in compliance with the principles set forth in the Declaration of Helsinki, International Conference on Harmonization Guideline for Good Clinical Practice and Food and Drug Administration Good Clinical Practice regulations.

Sample collection and storage
The samples used for the metabolite profiling were collected during a single-center, open-label, mass balance study of 14C-omacetaxine in cancer patients at the Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute. Enrollment was open to patients with hematologic malignancy or solid tumors in order to ensure faster inclusion of patients. In total, six patients with solid tumors (three with colorectal, two with lung and one with ovarian) received a single 1.25-mg/m$^2$ subcutaneous dose of 14C-omacetaxine. Mean total radioactivity (TRA) administered to each patient was 109 ± 7 μCi (range, 97.7–117 μCi). All six patients were Caucasian, five were female and one was male, with a mean age of 56.7 years (range 43–69).

Pre-dose and post-dose blood samples were collected at specific time points in K$_2$EDTA tubes and stored as plasma after centrifugation (4°C, 10 min, 2000 g). The samples were stored at nominally −80°C pending pooling and analysis.

For urine and feces, a pre-dose sample was collected, and after administration, the urine and feces samples were collected as voided until the TRA in each 24-h collection period was less than 1% of the total administered dose (range from 144 to 312 h post-dose). Urine samples were aliquoted and stored at nominally −80°C pending pooling and analysis. The fecal portions were weighed, stored refrigerated and homogenized after addition of water (1:3 w/v, depending on the consistency of the stool). The homogenized feces samples were aliquoted and stored at nominally −80°C pending pooling and analysis.

Selection of samples
Samples were collected up to 312 h post-dose for individual patients, but for the metabolite profiling samples up to 72 h...
post-dose were analyzed because the majority of the radioactivity was excreted at this point.

The metabolite profiling approach consisted of two parts: (1) metabolite screening and identification and (2) metabolite quantification.

Inter-patient pooled plasma, urine and feces samples were analyzed during the metabolite screening and identification using LC-LSC-MS\(^n\). For the plasma and urine screening, samples from all six patients were used for pooling. For feces, the samples of three patients were pooled instead of six patients, because the recovery in feces samples collected through 72 h was too low in the other three patients (range 0.39–5.71%). Plasma samples collected up to 4 h after administration were pooled across patients. From each patient sample, 100 \(\mu\)L was used to obtain 600 \(\mu\)L in total, and this was done for each time point (pre-dose, 15 min, 30 min, 45 min, 1 h, 2 h and 4 h). For TRA analysis (to determine the pooling accuracy), 200 \(\mu\)L was used and 300 \(\mu\)L aliquoted for LTQ-analysis. Since urine and feces samples were collected as voided, pools of 24-h intervals were made (intra-patient pools and subsequently inter-patient pools). Urine and feces samples were pooled in proportion to the weight of the excreta collected (Penner et al., 2009). For all matrices, a pooled blank sample was analyzed and the pooling accuracy was determined in all matrices by the assessment of the radioactivity using a LSC Model TRI-CARB 2800 TR (Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA), equipped with a quenching correction system.

For the metabolite quantification, individual plasma samples and intra-patient pooled urine and feces samples were analyzed by LC-LSC-MS\(^n\). The plasma samples were selected up to 24 h after administration because the TRA was too low after 24 h to construct radiochromatograms (15 min, 30 min, 1 h, 2 h, 8 h and 24 h after administration). Since urine and feces samples were collected as voided, pools of 24-h intervals were made for each patient individually up to 72 h (intra-patient pooling). For feces, intra-patient pooled samples from three patients were analyzed instead of samples from all six patients, because the recovery in feces samples collected through 72 h was too low in three patients. For all matrices, a blank sample was analyzed for each patient.

**Preparation of biological samples**

**Plasma**

Plasma samples were pretreated by adding 900 \(\mu\)L of acetonitrile/methanol (50:50, v/v) to 300 \(\mu\)L of plasma, followed by mixing (10 s), shaking (10 min at 1250 rpm) and centrifuging (10 min at 23, 100 g) to precipitate proteins. The clear supernatant was evaporated to dryness using nitrogen at 40 °C and reconstituted with 100 \(\mu\)L of acetonitrile/water/formic acid (100:900:1, v/v/v). For the recovery calculations, 20 \(\mu\)L of the final extract was used for LSC analysis and the remaining extract was transferred to an auto sampler vial and stored at −70 °C until further testing.

**Urine**

The urine samples were vortex mixed and directly injected onto the HPLC system. The recovery was not determined in these samples.

**Feces**

Feces samples were pretreated by adding 300 \(\mu\)L of acetonitrile/methanol (50:50, v/v) to 300 \(\mu\)L of feces homogenate, followed by mixing (10 s), shaking (10 min at 1250 rpm) and centrifuging (1 min at 23 100 g). The clear supernatant was transferred to another tube and the extraction was repeated. The combined supernatant was then evaporated to dryness using nitrogen at 40 °C, and the residue was reconstituted with 300 \(\mu\)L of acetonitrile/water/formic acid (100:900:1, v/v/v). After mixing and centrifugation, 20 \(\mu\)L of the final extract was used for LSC analysis for recovery calculations and the remaining extract was transferred to an auto sampler vial and stored at −70 °C pending further analysis.

**LC-MS systems**

In this study, two chromatographic methods were used, each with a run time of 60 min. One method was used to obtain radiochromatographic data and normal resolution MS data (LC-LSC-MS\(^n\)) (this method will be referred to as method 1) and the other method was used to obtain high-resolution data (LC-MS\(^n\)) (this method will be referred to as method 2).

The LC-LSC-MS setup for method 1 included a Shimadzu LC-20AD pump and SIL-HTc autosampler (4 °C) (Shimadzu, Kyoto, Japan). A Synergi Hydro RP 80 Å column (150 × 4.6 mm ID, 4 μm particles; Phenomenex, Torrance, CA) was preceded by a 0.2-μm inlet filter (Upchurch Scientific, Oak Harbor, WA), and the column heater was set to 35 °C. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile, with a gradient starting at 5% B for 5 min, increasing to 18% B over 40 min, increasing to 40% B over 5 min, increasing to 90% B in 6 min, where it stayed to 55 min, after which it was re-equilibrated at 5% B for 5 min. The 1-mL/min flow was split post-column using an accurate splitter (LC packings, Sunnyvale, CA), directing ¼ of the flow to the mass spectrometer and ¾ of the flow to a fraction collector (LKBFrac-100, Amersham Biosciences AB, Uppsala, Sweden), collecting 1-min fractions in liquid scintillation vials. After the addition of 4 mL of scintillation cocktail, the TRA of each fraction was determined on the Tri-carb 2800TR LSC, using a counting time of 20 min. The resulting values were used to construct the radiochromatograms. When the peak in the radiochromatogram was above the lower limit of quantitation (LLOQ) in a pooled sample, this indicated a potential metabolite and was further quantified.

A linear ion-trap mass spectrometer (LTQ XL, Thermo Electron, Waltham, MA) was used for the LC-LSC-MS analysis (method 1), applying positive ionization and using a scan range of 100–1100 amu. The sheath gas, aux gas and sweep gas were 40, 5 and 0 arbitrary units, respectively. The spray voltage was 4.5 kV and the normalized collision energy was 35 V. Data-dependent acquisition using a predefined parent list was used to collect the MS data. Total ion current (TIC) chromatograms were obtained and from each m/z signal above a threshold of 750 cps, MS/MS spectra were generated when a match was found with a (predicted) parent list.

The LC-MS setup for method 2 was an Ultimate 3000 Standard LC System ( Dionex, Sunnyvale, CA), with the autosampler set on room temperature. This method used a
Synergi Hydro RP 80 Å (150 × 0.5 mm ID, 4 μm particles; Phenomenex) with the same mobile phases as method 1. The gradient was slightly different, starting with 3% B for 5 min, increasing to 10% B over 40 min, increasing to 40% B over 5 min, increasing to 90% B in 6 min, where it stayed to 55 min, after which the column was re-equilibrated to 3% B for 5 min.

Two samples were selected on the basis of the results from the LTQ XL data. A plasma and a urine sample containing all metabolites with low noise levels were chosen and were additionally analyzed with a high-resolution mass spectrometer, an LTQ Orbitrap (Thermo Electron). All settings were similar to the settings of the LTQ XL. Relative retention times were used to identify the metabolites and high-resolution MS² spectra of ions were obtained using a full data scan and a parent scan.

**Metabolite screening and identification**

LC-MSⁿ was used as primary tool to elucidate the molecular structures of the metabolites. The metabolites were identified by comparing the HPLC retention times and fragmentation patterns to the results from the available reference standards. If reference standards were not available, the mass of the molecular ions (high-resolution, if available) and fragmentation patterns were used to identify the metabolite. In addition, the ratio of non-labeled omacetaxine and ¹⁴C-omacetaxine in the formulation given to the patients was approximately 60:40, which showed a distinct isotope pattern in the full MS spectrum of two ions 2 Da apart (from ¹⁴C and ¹²C) with a proportion of 6:4.

The metabolites were named after their non-labeled, protonated m/z values (for instance, M520), except for omacetaxine (6), 4'-DMHHT (5) and cephalotaxine (1), because these metabolites were already known and named. In previous studies (no published data), a letter (for instance, M548A) was added to the metabolite name if there was already a metabolite with the same m/z value. In this study, this nomenclature was also used. In addition, the metabolites were assigned an ID number, which was assigned according to the retention time of the metabolite.

**Calculations**

The limit of detection (LOD) of the TRA measurements was calculated with formula (1) (Zhu et al., 2005)

\[
\text{LOD} = \frac{2.71}{TE} + 4.65 \sqrt{\frac{B}{TE}}.
\]  

(1)

With a counting time (T) of 20 min, an observed background (B) of 11.0 DPM (disintegrations per minute) and a counting efficiency (E) of 93% the LOD is 3.7 DPM, which was rounded to 4 DPM.

At the lower limit of quantification (LLOQ), a maximum precision error of 20% was considered acceptable, therefore, formula (2) was used to calculate the LLOQ above background (Dubbelman et al., 2011)

\[
\text{LLOQ} = \frac{50}{TE} \left( 1 + \sqrt{1 + \frac{2TEB}{25}} \right).
\]  

(2)

Using the same values for T, E and B as before, the calculated LLOQ was 14 DPM.

The area under the concentration–time curve from 0 to 24 h after drug administration (AUC₀–₂₄h) was calculated with the trapezoidal rule (3), using time since administration and concentration at data point i (tᵢ and Cᵢ, respectively) and n as the total number of data points

\[
\text{AUC}₀–₂₄h = \sum_{i=0}^{n-1} \frac{tᵢ₁₊₁ - tᵢ}{2} × (Cᵢ + Cᵢ₊₁).
\]  

(3)

**Results**

**Metabolites in plasma**

The mean recovery after protein precipitation of all samples (including inter-patient pooled plasma samples and individual patient samples) was 92.6%, which was considered to be acceptable. The pooling accuracy of the analyzed inter-patient pooled samples was between −3.8% and −1.5%.

For the metabolite profiling of plasma, samples after 24 h were not analyzed due to low radioactivity. Radiochromatograms could therefore not be constructed for the samples collected after 24 h. The compound names are derived from the unit mass results, and the ID numbers were assigned in the order of elution (retention time). The profile in all selected samples was similar: omacetaxine (6) is the main peak in plasma with a mean AUC₀–₂₄h of 66.1% of the TRA AUC₀–₂₄h. The mean omacetaxine (6) concentration showed a decrease in time both in absolute and relative terms, representing a mean of 86.9% of the TRA at 15 min after administration and 60.4% at 24 h after administration. Figure 2 shows the concentration–time profiles of radioactive components in plasma.

4'-DMHHT (5) was also a major peak in plasma with a mean AUC₀–₂₄h of 13.7% of the TRA AUC₀–₂₄h. The mean 4'-DMHHT (5) concentration showed an increase over time up to 8 h and then the concentration decreased. At 15 min, 8 h and 24 h after administration, the concentration was 1.5%, 9.1% and 2.2% of TRA, respectively.

M562A (7) was less abundant and showed a large variation between the patients. The mean AUC₀–₂₄h was 6.2% (range 0.0–16.9%) of the TRA AUC₀–₂₄h. The mean M562A (7) concentration also showed an increase over time up to 2 h, followed by a decrease, representing a mean of 1.3%, 9.1% and 2.2% of TRA at 15 min, 2 h and 24 h after administration, respectively. However, the AUC₀–₂₄h and concentrations of M562A (7) might be slightly overestimated because M562A (7) co-elutes with other, lower concentration radioactive compounds at the end of the gradient profile (high percentage of strong eluent). For the quantification, the fractions that corresponded with the MS data were used, but these fractions may have contained other low-abundance radioactive metabolites as well.

The sum of these three compounds (i.e. omacetaxine, 4'-DMHHT and M562A) account for 86% of the TRA AUC₀–₂₄h. Table 1 summarizes the relative AUC₀–₂₄h for each metabolite.

Figure 3 displays the radiochromatograms showing the metabolite profile of omacetaxine in plasma over time from a representative patient. The peaks were designated based on
the LC-MS data. The metabolite profiles in plasma show the conversion of omacetaxine into the more polar metabolite, 4′-DMHHT (5), formed by hydrolysis of the methyl ester which resulted in demethylation, and small amounts of M562A (7), which is presumably formed by oxidation.

**Metabolites in urine**

Urine was analyzed without sample pretreatment; so recovery was not determined. The pooling accuracy for the inter-patient pooled samples (used for metabolite screening and identification) was between −0.8% and 1.4%. The pooling accuracy for the intra-patient pooled samples (used for the metabolite quantification) was between −5.7% and 2.7%. The results show that a mean of 35.2% of the administered radioactivity was recovered in urine through 72 h after administration. Unchanged 14C-omacetaxine was the largest labeled component in urine, accounting for a mean of 19.0% of the total dose administered. 4′-DMHHT (5) and M534 (4) were the main metabolites in urine accounting for a mean of 7.8% and 3.4% of the administered radioactivity, respectively. M520 (2), M548A (3) and M562A (7) accounted for small portions of the radioactivity, i.e., means of 1.3%, 0.6% and 0.9% of the administered radioactivity, respectively. A mean of 2.2% of the administered radioactivity found in urine was not accounted for in the radiochromatograms.

Table 1. Summary of omacetaxine metabolite profiling.

| Compound (ID) | RT (min) | Proposed metabolic pathway(s) | Plasma exposure\(^a\) (% of AUC\(_{0–24h}\) from total 14C)\(^b\) | Amount excreted\(^a\) |
|--------------|----------|--------------------------------|-------------------------------------------------|---------------------|
| Cephalotaxine (1) | 11.1 | Mepesuccinate ester hydrolysis | ND | ND |
| M520 (2) | 21.2 | Methyl ester hydrolysis + 1,3-dioxole ring opening to pyrocatechol | ND | 1.3% 1.2% 2.5% |
| M548A (3) | 25.8 | Methyl ester hydrolysis + oxidation of the cephalotaxine ring system | ND | 0.6% 0.3% 0.9% |
| M534 (4) | 28.3 | 1,3-Dioxole ring opening to pyrocatechol | ND | 3.4% 11.8% 15.2% |
| 4′-DMHHT (5) | 30.3 | Methyl ester hydrolysis | 13.7% | 7.8% 11.6% 19.4% |
| Omacetaxine (6) | 40.9 | Parent drug | 66.1% | 19.0% 2.0% 21.0% |
| M562A (7) | 43.9 | Oxidation of the cephalotaxine ring system | 6.2%\(^d\) | 0.9% 0.8% 1.7% |
| Radioactivity in matrix | | | 100% | 35.2% 37.7% 72.9% |
| Supernatant after sample preparation | | | 92.0%\(^e\) | NA\(^f\) 30.4% NA |
| Total accounted for via radiochromatograms | | | 86.0% | 33.0% 27.9% NA |
| Unaccounted for (sample pretreatment loss + not detected in radiochromatogram) | | | 14.3% | 2.2% 9.8% NA |

\(^a\)Factions < LOD (4 DPM) were regarded as containing 0 DPM.
\(^b\)Calculated from plasma concentrations at 15 min, 30 min, 1 h, 2 h, 8 h and 24 h after administration.
\(^c\)The amount excreted for feces was determined for three patients instead of six patients.
\(^d\)The AUC\(_{0–24h}\) might be slightly overestimated because M562A co-elutes with other, lower concentration radioactive compounds at the end of the gradient profile.
\(^e\)Mean plasma sample recovery from plasma concentrations at 15 min, 30 min, 1 h, 2 h, 8 h and 24 h after administration from six patients.
\(^f\)Urine samples were analyzed without sample pretreatment.

The LC-MS data. The metabolite profiles in plasma show the conversion of omacetaxine into the more polar metabolite, 4′-DMHHT (5), formed by hydrolysis of the methyl ester which resulted in demethylation, and small amounts of M562A (7), which is presumably formed by oxidation.

**Metabolites in urine**

Urine was analyzed without sample pretreatment; so recovery was not determined. The pooling accuracy for the inter-patient pooled samples (used for metabolite screening and identification) was between −0.8% and 1.4%. The pooling accuracy for the intra-patient pooled samples (used for the metabolite quantification) was between −5.7% and 2.7%.
Figure 4 displays the radiochromatograms showing the metabolite profile of omacetaxine in urine over time from a representative patient. The peaks were designated based on the LC-MS^n data. The profiles show conversion of omacetaxine into more polar metabolites. Even though omacetaxine is converted into metabolites, unchanged omacetaxine stays predominant in urine. Metabolites formed over time are cephalotaxine (1) formed by ester hydrolysis, M534 (4) formed by transformation of the 1,3-dioxole ring to pyrocatechol, M520 (2) formed by demethylation of M534 (4), 4'-DMHHT (5) which is a demethylated form of omacetaxine, M548A (3) formed by methyl ester hydrolysis and oxidation of the cephalotaxine ring system and M562A (7) which is formed also by oxidation of the cephalotaxine ring system. Cephalotaxine (1) was detected in several urine samples, but the measured radioactivity in the collected fractions was not above the LLOQ. Hence, cephalotaxine (1) was therefore not further quantified in urine.

**Metabolites in feces**

The mean sample pretreatment recovery of all feces samples (including inter-patient pooled feces samples and intra-patient pooled samples) was 80.8%, which was considered to be acceptable. The pooling accuracy of the inter-patient pooled
samples (used for metabolite screening and identification) was between $-3.9\%$ and $0.8\%$. The pooling accuracy of the intra-patient pooled samples (used for the metabolite quantification) was between $-5.1\%$ and $2.7\%$.

The results show that a mean of $37.7\%$ of the administered radioactivity was recovered in feces through 72 h after administration. Unchanged $^{14}$C-omacetaxine excreted in feces accounted only for $2.0\%$ of the total dose administered. M534 (4) and 4'-DMHHT (5) were the main metabolites found in feces, representing means of $11.8\%$ and $11.6\%$ of the administered radioactivity, respectively. M520 (2), M548A (3) and M562A (7) accounted for means of $1.2\%$, $0.3\%$ and $0.8\%$ of the administered radioactivity, respectively. A mean of $9.8\%$ of the administered radioactivity recovered in feces was not accounted for as individual components.

Figure 4 displays the radiochromatograms showing the metabolite profile of omacetaxine in feces over time from a representative patient. The peaks were designated based on LC-MS$^n$ data. Unlike in urine, unchanged omacetaxine concentrations in feces are very low through 72 h after administration. The metabolites M520 (2), M548 (3), M534 (4), 4'-DMHHT (5) and M562A (7) were detected and quantified in human feces.

Identification of metabolites

Table 2 summarizes the accurate mass data (high-resolution) and structural information for omacetaxine and its metabolites in human plasma, urine and feces samples. Supplementary
Table 2. Summary of accurate mass data and structural information for omacetaxine and its metabolites in human plasma, urine and feces samples.

| Compound (ID number) | Proposed metabolic pathways(s) | RT (min) | Formula | Calculated m/z | Observed m/z | D/C ppm | MS data | Matrix | Structure characterization |
|----------------------|---------------------------------|----------|---------|---------------|-------------|---------|---------|--------|--------------------------|
| Omacetaxine (6)      | Parent drug                      | 40.9     | C16H20NO9 | 546.2698      | 546.2672    | 4.8     | NA      | P, U, F | Confirmed by authentic standard |
| M520 (2)             | Methyl ester hydrolysis          | 21.2     | C27H38NO10 | 520.2541      | 520.2541    | NA      | MS2, Spectrum found during LC-LTQ analysis. ND, Metabolite was not detected during the Orbitrap measurements; NA, Metabolite was not detected during the Orbitrap measurements so therefore no D ppm could be calculated. Matrix: U, urine; F, feces; P, plasma. |
| M534A (3)            | Methyl ester hydrolysis + hydroxylation of the cephalotaxine ring system | 25.8     | C28H40NO9  | 534.2698      | 534.2673    | 4.7     | NA      | P, U, F | Proposed |
| M534 (4)             | 1,3-Dioxole ring opening to pyrocatechol | 28.3     | C28H40NO9  | 534.2698      | 534.2698    | 4.8     | NA      | P, U, F | Proposed |
| M548A (3)            | Methyl ester hydrolysis + hydroxylation of the cephalotaxine ring system | 30.3     | C28H40NO9  | 534.2698      | 534.2698    | 4.8     | NA      | P, U, F | Proposed |
| DMHHT (5)            | Methyl ester hydrolysis          | 43.9     | C28H40NO9  | 546.2698      | 546.2697    | 4.3     | NA      | P, U, F | Proposed |

The bold values indicate most abundant product ions. 

The identities of compounds were confirmed by authentic standards and their reference standards were used for the establishment of the structural characterization.

Figure 1 shows the proposed metabolite structure and fragmentation and the MS² and MS³ spectra of the proposed metabolites. The proposed metabolic pathway in human is illustrated in Figure 5. The rationale for the structural characterization is described below.

Omacetaxine (6)

The identity of omacetaxine (6) was confirmed with the reference standard of omacetaxine (6). The protonated molecular ion at m/z 546 gave product ions at m/z 316 and 298 (loss of mepesuccinate side chain). The MS, MS/MS spectrum and retention time of 14C-omacetaxine (6) in the samples corresponded to the reference standard of omacetaxine (6).

Cephalotaxine (1)

The identity of cephalotaxine (1) was confirmed with the reference standard of cephalotaxine (1). The protonated molecular ion at m/z 284 gave a product ion at m/z 266. The MS, MS/MS spectrum and retention time of 14C-cephalotaxine (1) in the samples corresponded to the reference standard of cephalotaxine (1). Metabolite cephalotaxine (1), which has been previously described (Nemunaitis et al., 2013), was also detected in several urine samples. The measured radioactivity in the collected fractions was, however, not above the LLOQ and cephalotaxine (1) was therefore not further quantified in urine.

M520 (2)

M520 (2) had a protonated molecular ion at m/z 520 which is a loss of 26 Da relative to m/z 546, the mass of omacetaxine (6). The major product ion was m/z 286 (loss of mespecuccinate side chain) and the MS³ spectrum of m/z 286 showed a fragment at m/z 254 (loss of methyl alcohol). MS/MS fragments of M520 (2) are similar to the fragments of M534 (4), which indicates that M520 (2) is also a pyrocatechol metabolite of omacetaxine (6). The additional loss of 14 Da to m/z 534 (4), the mass of M534, indicates a potential demethylation due to hydrolysis of the methyl ester on the mepesuccinate side chain. Therefore, it is proposed that M520 (2) is the methyl ester hydrolysis product of the pyrocatechol metabolite, which is supported by the accurate mass data (Table 2).

M548A (3)

M548A (3) had a protonated molecular ion at m/z 548, which is an additional mass of 16 Da relative to m/z 534, the mass of mepesuccinate (4). The MS³ spectrum of m/z 548 did not show a fragment at m/z 254 (loss of methyl alcohol). MS/MS fragments of M548A (3) also show an additional mass of 16 Da relative to the corresponding fragments of 4'-DMHHT (5). Hence, M548A (3) represents a metabolite that has undergone hydrolysis of the methyl ester and hydroxylation of the cephalotaxine ring system.

M534 (4)

M534 (4) has a protonated molecular ion at m/z 534 which is a loss of 12 Da relative to m/z 546, the mass of omacetaxine (6).
(6). The major product ion was $m/z$ 286 (loss of mepesuccinate side chain) and the MS$^3$ spectrum of $m/z$ 286 showed a fragment at $m/z$ 254 (loss of methyl alcohol). The MS/MS fragments of M534 (4) also show a loss of 12 Da compared with the corresponding fragments of omacetaxine (6). This indicates transformation to a pyrocatechol of the 1,3-dioxole ring. M534 (4) is therefore proposed to be the pyrocatechol metabolite of omacetaxine (6), which is supported by the accurate mass data (Table 2).

$4'$-DMHHT (5)

The identity of $4'$-DMHHT (5) was confirmed with the reference standard of $4'$-DMHHT (5). The protonated molecular ion at $m/z$ 532 gave product ions at $m/z$ 316 and 298. The MS, MS/MS spectrum and retention time of $^{14}$C-$4'$-DMHHT (5) in the samples corresponded to the reference standard of $4'$-DMHHT (5). The loss of 14 Da relative to $m/z$ 546, the mass of omacetaxine, and the similar MS/MS fragments to omacetaxine (6) indicates that $4'$-DMHHT (5) represents a metabolite that has undergone hydrolysis of the methyl ester.

M562A (7)

M562A (7) had a protonated molecular ion at $m/z$ 562, which is an additional mass of 16 Da relative to $m/z$ 546, the mass of omacetaxine (6). The major product ion was $m/z$ 314 (loss of mepesuccinate side chain) and the MS$^3$ spectrum of $m/z$ 314 showed a fragment at $m/z$ 314. The additional mass indicates the presence of an oxygen atom (or hydroxyl group). It was not possible to elucidate the exact chemical structure of M562A (7).

**Discussion**

This study describes the metabolite profile of $^{14}$C-omacetaxine in plasma, urine and feces samples of adult patients.
suffering from cancer. In total, 6 omacetaxine-related compounds were detected in addition to unchanged omacetaxine. Metabolic conversions on the mepesuccinate side chain were complete ester hydrolysis and hydrolysis of the methyl ester of the side chain. In addition, the opening of the 1,3-dioxole ring to form a pyrocatechol partial structure was observed, as was oxidation of the cephalotaxine ring system. Combinations of methyl ester hydrolysis and 1,3-dioxole ring opening or oxidation of the cephalotaxine ring system were also observed. Figure 5 displays the proposed metabolite pathways of omacetaxine.

In plasma, only unchanged omacetaxine (6), 4'-DMHHHT (5) and M562A (7) were detected. Unchanged omacetaxine was the most prominent omacetaxine-related compound in plasma. The relative AUC_{0-24h} of metabolite 4'-DMHHHT (5) was 13.7%, which indicates that 4'-DMHHHT (5) is a major metabolite (FDA, 2008; ICH, 2008). However, 4'-DMHHHT (5) is far less toxic than omacetaxine, as was shown previously in mice. The lethal dose of omacetaxine affecting 50% (LD50) of mice was 6.7 mg/kg, but 4'-DMHHHT (5) produced no apparent toxic effects at doses up to 280 mg/kg (Ni et al., 2003).

So far, the structure of M562A (7) remains unknown. The metabolite could not be elucidated via high-resolution MS, but the unit resolution MS indicated an addition of 16 Da; therefore, this metabolite was identified as omacetaxine + O. Since the relative AUC_{0-24h} of M562A (7) is 6.2%, the metabolite is considered a minor metabolite (FDA, 2008; ICH, 2008). The calculated relative AUC_{0-24h} might also be overestimated because M562A (7) co-eluted with other, lower-concentration radioactive compounds at the end of the gradient profile.

Overall, the same metabolites were found in urine and feces. The only exception was cephalotaxine (1), which was detected only in urine, although at concentrations too low for quantitation. Unchanged omacetaxine (6) was the largest single component in urine, but it was a minor component in feces. However, recovery of parent drug was predominantly within the first 24 h after administration. The pyrocatechol metabolite of omacetaxine, M534 (4) and 4'-DMHHHT (5) were the most abundant metabolites in feces. 4'-DMHHHT (5) was also the second most abundant omacetaxine-related compound in urine. M520 (2), the demethylated pyrocatechol metabolite of omacetaxine, M548A (3), another demethylated metabolite of omacetaxine which is oxidized on the cephalotaxine moiety, and M562A (7) (omacetaxine + O) were detectable, but at low levels, in both urine and feces.

Omacetaxine has been of interest for its anti-tumor effects since the 1970s, and since that time several metabolism studies have been performed. The excretion of unchanged omacetaxine and its main metabolite, 4'-DMHHHT, in urine in the current study were generally consistent with the previously reported results. In this study, 35.2% of the administered dose was recovered in urine 72 h after administration, with 19.0% found to be unchanged omacetaxine and 7.8% found to be 4'-DMHHHT. In previous studies, they did not test for other metabolites, but the similarities between the amount of omacetaxine-related compounds excreted in urine and the amount of unchanged omacetaxine do suggest that the processes involved in the metabolic elimination of omacetaxine are similar in humans, dogs and mice (Lu et al., 1988; Ni et al., 2003; Savaraj et al., 1986).

Conclusion

Omacetaxine was extensively metabolized, with subsequent renal and hepatic elimination of the metabolites. Renal excretion of unchanged omacetaxine is a prominent elimination pathway. The major metabolite, 4'-DMHHHT is known to be inactive and non-toxic (Ni et al., 2003). The other metabolites circulate and are excreted in low quantities. The low levels of the metabolites found in plasma compared with the relatively high levels of unchanged omacetaxine in plasma indicate that the metabolites are unlikely to contribute materially to the efficacy of the compound, also suggesting that they have limited contribution to its toxicity.

Declaration of interest

P. Robertson Jr and E.T. Hellriegel are employees of Teva Branded Pharmaceuticals R&D, Inc. The other authors report no conflicts of interest.

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**Supplementary material available online**

**Supplementary Figure 1**