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ORIGINAL ARTICLE

Metabolism of trabectedin (ET-743, Yondelis™) in patients with advanced cancer

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Abstract
Purpose Trabectedin (ET-743, Yondelis™) is a novel anti-cancer drug currently undergoing phase II–III evaluation, that has shown remarkable activity in pretreated patients with soft tissue sarcoma. Despite extensive pharmacokinetic studies, the human disposition and metabolism of trabectedin remain largely unknown. We aimed to determine the metabolic profile of trabectedin and to identify its metabolites in humans.
Methods We analysed urine and faeces (the major excretory route) from eight cancer patients after a 3 or 24 h intravenous administration of [^{14}C]trabectedin. Using liquid chromatography with tandem quadrupole mass spectrometric detection (LC-MS/MS) and radiochromatography with off-line radioactivity detection by liquid scintillation counting (LC-LSC), we characterised the metabolic profile in 0–24 h urine and 0–120 h faeces.
Results By radiochromatography, a large number of trabectedin metabolites were detected. Incubation with β-glucuronidase indicated the presence of a glucuronide metabolite in urine. Trabectedin, ET-745, ET-759A, ETM-259, ETM-217 (all available as reference compounds) and a proposed new metabolite coined ET-731 were detected using LC-MS/MS. The inter-individual differences in radiochromatographic profiles were small and did not correlate with polymorphisms in drug-metabolising enzymes (CYP2C9, 2C19, 2D6, 2E1, 3A4, GST-M1, P1, T1 and UGT1A1 2B15) as determined by genotyping.
Conclusions Trabectedin is metabolically converted to a large number of compounds that are excreted in both urine and faeces. In urine and faeces we have confirmed the presence of trabectedin, ET-745, ET-759A, ETM-259, ETM-217 and ETM-204. In addition we have identified a putative new metabolite designated ET-731. Future studies should be aimed at further identification of possible metabolites and assessment of their activity.

Keywords Trabectedin · Metabolism · Excretion · Genotyping · Human

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Introduction

Trabectedin (ET-743, Yondelis™) belongs to the Ecteinascidins, compounds isolated from the tunicate

Ecteinascidia turbinata. Trabectedin is currently undergoing phase II–III investigation in patients with different tumour types [7, 28]. Phase II evaluation has shown activity in soft tissue sarcoma, ovarian and breast cancer [6]. Already, the activity in soft tissue sarcomas is evident from remarkably prolonged stable disease in, often heavily, pre-treated patients consistent in various phase I and phase II studies [28]. Structurally, trabectedin is composed of three tetrahydroisoquinoline subunits containing a central carbinolamine moiety (Fig. 1) [18]. Trabectedin displays sequence-specific, covalent binding to the minor groove of duplex DNA, and affects the transcription of several genes, including the unique abrogation of MDR1 gene transcriptional activation [12, 13, 17, 20]. Another remarkable characteristic of trabectedin is its high potency, as reflected in the low therapeutic doses applied [14]. Depending on the infusion schedule, recommended doses of trabectedin are 1.3 or 1.5 mg/m^2 every 3 weeks, or 0.58–0.61 mg/m^2 per week, corresponding to total doses of 1–3 mg by i.v. infusion over 1, 3 or 24 h. Trabectedin displays a large volume of distribution (V_{ss}) ranging from approximately 1,000–4,000 l [14]. This results in plasma concentrations in the pg/ml to low ng/ml range [14]. The reported terminal half-life ($t_{1/2}$) of trabectedin ranges from 26 to 89 h, corresponding to a mean residence time of 38–128 h. However, it is still largely unknown what occurs to trabectedin during this rather long retention in the body, and what metabolites are formed. The liver is the organ most likely involved in the elimination of trabectedin, because approximately 55% of a radioactive dose is excreted in faeces with less than 1% represented by unchanged trabectedin [1]. Less than 1% of a trabectedin dose is recovered in urine as unchanged trabectedin [25, 29], while nearly 6% of a radioactive dose is excreted in urine [1]. Therefore, the radioactivity excreted in faeces and urine after dosing of humans with [^{14}C]trabectedin predominantly consists of metabolites. A screening for metabolites in clinical plasma, urine and bile samples was performed but this did not result in identification of metabolites [25]. The low dose and plasma concentrations have been the most complicating factors in the elucidation of the metabolic fate of trabectedin so far. The use of radioactively labelled trabectedin in a mass balance study was considered to be a promising approach to gain more insight into the metabolism of this drug. (Fig. 2)

Our objective was to determine the metabolic profile of trabectedin and to identify its human metabolites. To achieve this aim, we analysed urine and faeces samples after intravenous administration of ^{14}C-labelled trabectedin to cancer patients.

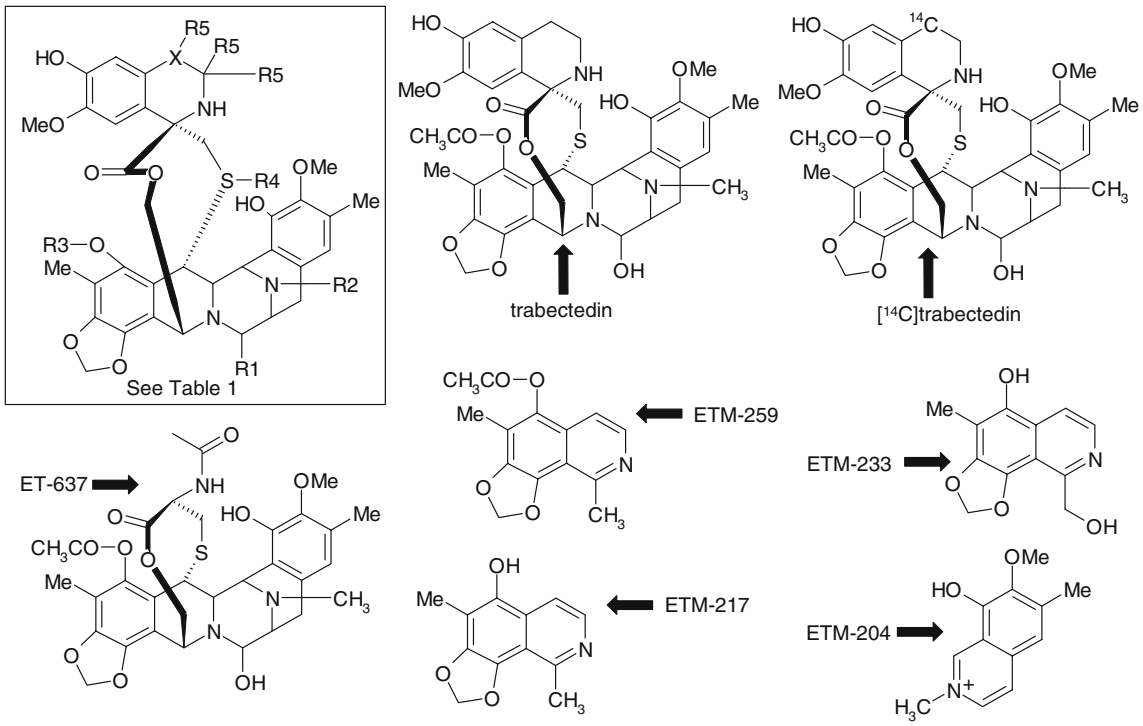

Fig. 1 Overall chemical structure of trabectedin and analogues (*upper left*, see Table 1 for substituents), and structures of trabectedin, [^{14}C]trabectedin, ET-637, ETM-259, ETM-233, ETM-217 and ETM-204

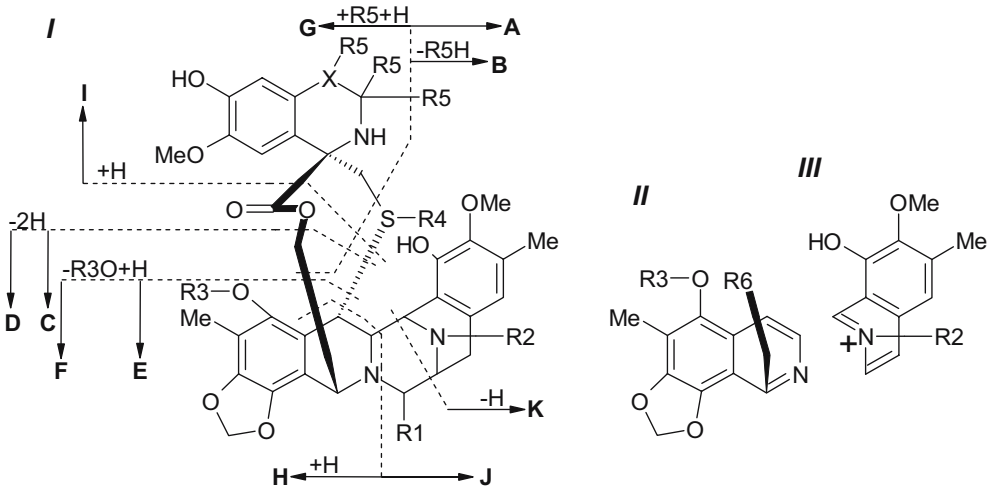

Fig. 2 Structures and putative fragmentation pathways of trabectedin (ET-743), isotopically labelled trabectedin and metabolite reference compounds

Materials and methods

Chemicals

[^{14}C]trabectedin (2.48 MBq/mg), provided by PharmaMar (Madrid, Spain), originated from BioDynamics Radiochemicals (Billingham, UK) and was purified by HPLC to >97.5% radiochemical purity. Reference compounds trabectedin (ET-743), [D_3] ET-743, [D_6]ET-743, ET-729, [D_6]ET-729, ET-775, ET-759A, ET-759B, ET-745, ET-701, ET-637, ETM-259, ETM-233, ETM-217 and ETM-204 originated from Pharma Mar (Madrid, Spain). See Fig. 1 for chemical structures. The reference compounds were selected to characterise the fragmentation pattern of the trabectedin structure (deuterated analogues) or because they were observed in degradation or metabolism studies. All other chemicals were of analytical grade.

Sample collection

We obtained urine and faeces samples from a mass balance study [1] involving intravenous administration of [^{14}C]trabectedin to eight cancer patients with advanced disease. The Medical Ethics Committee of the hospital approved the study protocol, and all patients had to give written informed consent. Trabectedin was administered to the first two patients as a 24-h infusion. The other six patients received trabectedin as a 3-h infusion. The administered dose was 1-mg trabectedin labelled with approximately 2.5 MBq [^{14}C]trabectedin (70 μCi), irrespective of body surface area.

The urine voided was collected in portions of 24 h up to day 10 after drug administration. Bovine Serum Albumin Fraction V (Roche Diagnostics, Mannheim, Germany) was added (1%, g/v) to the urine samples followed by thorough mixing, to prevent any adsorption to the container walls. Aliquots were stored at −70°C until analysis. Faeces were also collected in 24-h portions up to at least day 10. Faeces were homogenised with Milli-Q Plus® purified water (Millipore, Milford, MA, USA) in a 1:10 ratio and 50 ml samples were stored at −70°C until analysis.

Pooled 0–24 h urine samples were prepared per patient and a grand pool was also prepared from urine of patients 3–8. Similarly 0–120 h faecal pools were prepared.

The stability of trabectedin and its metabolites in biological samples other than plasma is unknown. Therefore, samples (urine and faeces) were stored at −70°C. As urine and faeces originate from an environment at 37°C, stability at −70°C was not expected to be an issue. To prevent any degradation of analytes in the matrices after excretion, urine was kept refrigerated until the end of the collection interval whereupon it was immediately frozen, and faeces were frozen immediately after excretion.

Sample preparation

Urine samples were injected without further sample preparation. Various organic solvents were tested to extract radioactivity from patient faecal samples. This resulted in an extraction of 1 ml of faecal homogenate with 1 ml of methanol. After vortexing and shaking for 5 min, the sample was centrifuged before removal of

the supernatant. The supernatant was evaporated to dryness under a vacuum. The dry residue was stored at −20°C until further analysis.

Mass spectrometric detection

Approximately 80% of the trabectedin administered to the patients in the mass balance study consisted of [^{14}C]trabectedin, while all the reference compounds were ^{12}C-based. Because the metabolites in the biological samples were ^{14}C-labelled at a specific position, the fragmentation of available (non-labelled ^{12}C) reference compounds was studied to determine which masses should be monitored in the biological samples. Reference compounds were dissolved in methanol, and mass spectra were obtained during continuous infusion into the mass spectrometer. Using the knowledge of the fragmentation of trabectedin and analogues, the MS/MS masses of the reference compounds were adapted for detection of the ^{14}C-variants of these compounds.

Bioanalysis

The chromatographic system used for radiochromatographic profiling of trabectedin metabolites consisted of a hand injector (Rheodyne, Cotati, CA, USA), a 500 µl sample loop, a programmable HPLC gradient pump (model 480, Separations Analytical Instruments, Hendrik Ido Ambacht, The Netherlands) and a Kromasil C-18 column (5 µm, 4.6 mm×250 mm, Alltech, Deerfield, IL, USA). Eluent A consisted of 10 mM ammonium acetate pH 6.0, and eluent B consisted of acetonitrile–water (90:10, v/v). The gradient system started with eluent B applied at 5% from 0–3 min, followed by a linear gradient with eluent B content increasing from 5 to 100% in 57 min followed by a 5 min wash-out period at 100% eluent B. The flow rate was 1 ml/min. Samples were injected in a volume of 500 µl, either directly (urine) or after reconstitution of the methanolic extract (faeces) using 50 µl methanol and 550 µl of mobile phase A, followed by sonication (5 min). Sixty-second fractions (1 ml) of the eluate were collected.

Radioactivity was quantified using liquid scintillation counting (LSC) for 30 min per fraction after addition of 4 ml of Ultima Gold scintillation liquid (Packard, Groningen, The Netherlands). Counting was performed using a Tricarb Liquid Scintillation Analyzer 2300 TR (Packard, Meriden, CT, USA) with a ^{14}C counting protocol and automatic quench correction.

For LC-MS/MS analysis, a similar chromatographic system was used as described above, consisting of an HP 1100 liquid chromatograph (Agilent technologies, Palo Alto, CA, USA) with a binary pump, autosampler and degasser. Detection was performed using an API 3000 triple quadrupole MS equipped with an electrospray ion source (Sciex, Thornhill, ON, Canada). The quadrupoles were operated in the positive ionisation mode. Settings for the individual reference compounds were optimised during method development (data not shown). Because the injector of the LC-MS/MS system could handle a maximum volume of 100, 500 µl samples of urine were concentrated to 120 µl under nitrogen, and dried faecal residues were reconstituted in 120 µl solvent A–methanol (12:1, v/v) prior to injection of a 100 µl aliquot. The available reference compounds were injected to establish retention times and responses. Signals in the correct MS/MS trace at the correct retention time with a signal to noise ratio >3 confirmed the presence of the corresponding analyte in samples.

β-Glucuronidase incubation

To assess the presence of glucuronide metabolites of trabectedin, the faeces and urine grand pools were incubated with β-glucuronidase (type X-A, *E. coli* 9,000,000 U/g, Sigma-Aldrich Chemie, Steinheim, Germany). The pH of the urine and faeces samples was approximately 7 and required no adjustment to optimise the enzyme activity. Urine (500 µl) was incubated with 75 µl 25 mM potassium phosphate buffer pH 6.8 containing approximately 3,000 U of β-glucuronidase, at 37°C for 20 h. Faeces (1 ml) were incubated with 225 µl 25 mM potassium phosphate buffer pH 6.8 containing approximately 9,000 U of β-glucuronidase, at 37°C for 20 h.

Identification of polymorphisms in drug metabolising enzymes

We screened patients for genetic polymorphisms of drug metabolising enzymes. Genotyping for single nucleotide polymorphisms (SNPs) in the following genes was performed: CYP2C9 (*2 and *3), CYP2C19 (*2), CYP2D6 (*3 and *4), CYP2E1 (*5 and *6), CYP3A4 (*1B, *2, *3 and *12), CYP3A5 (*2 and *3) and GST M1, P1 and T1, UGT1A1 (*6, *7, *27, *28 and *29) and UGT2B15 (*2 and *3). The method of Boom [3] was used to isolate genomic DNA from EDTA-anticoagulated blood. SNPs were determined with a polymerase chain reaction assay followed by restriction fragment length polymorphism or sequencing (CYP3A4 and UGT1A1) [8, 10, 11, 16, 22–24, 26, 27, 30].

Results

Sample preparation

Overall recovery of radioactivity (as percentage of total radioactivity in the untreated faeces homogenate) in the methanolic extraction (1 ml faeces homogenate with 1 ml methanol) was consistently ±35% (after loss of approximately 10% during evaporation). Extractions using acetonitrile, acetone, as well as liquid–liquid extractions with chloroform, diethyl ether and ethyl acetate under neutral, basic and acidic conditions were performed. None of these procedures yielded a recovery higher than 35%. The recovery could be increased to about 45% (after loss of approximately 30% during evaporation) by using 0.5 M hydrochloric acid in methanol. However, the use of hydrochloric acid did not have an impact on the appearance of the resulting radiochromatogram, while it increased the risk of acid-catalysed chemical degradation.

To avoid possible degradation of metabolites in faeces by acid or an undesired selective extraction by liquid–liquid extraction, we selected the described milder and non-selective methanolic extraction as sample preparation for faeces samples.

Characterisation of trabectedin, analogues and reference compounds

Fragmentation of reference compounds

The mass spectrometric fragmentation of the reference compounds is displayed in (Fig. 2) with the corresponding masses in Table 1. The presented pathways explain the major fragments observed in the product ion scans of the (pseudo)molecular ions of the reference compounds. The fragmentation of these compounds displayed some common characteristics. Most parent ions were pseudo-molecular ions resulting from loss of the R1 moiety, specifically when R1 was a hydroxyl moiety. Replacement of this hydroxyl moiety

Table 1 Structures and putative fragmentation pathways of trabectedin (ET-743), isotopically labelled trabectedin and metabolite reference compounds

Code	Fig. 2			Substituent							*m/z* Masses observed in fragmentation pathways[b]										
		Mwt	*M/z*[a]	R1	R2	R3	R4	R5	R6	X	A	B	C	D	E	F	G	H	I	J	K
Trabectedin	*I*	761	744	OH	CH_3	CH_3CO	–	H	–	^{12}C	495	493	477	475	463	405	250	246	224	218	204
[^{14}C] Trabectedin	*I*	763	746	OH	CH_3	CH_3CO	–	H	–	^{14}C	495	493	477	475	463	405	252	246	226	218	204
[D_3] Trabectedin	*I*	764	747	OH	CH_3	CH_3CO	–	D	–	^{12}C	496	493	477	475	463	–	253	246	227	218	204
[D_6] Trabectedin	*I*	767	750	OH	CH_3	CD_3CO	–	D	–	^{12}C	499	496	480	478	466	–	253	249	227	218	204
ET-729	*I*	747	730	OH	H	CH_3CO	–	H	–	^{12}C	481	479	463	461	449	391	250	246	224	204	190
[D_6]ET-729	*I*	753	736	OH	H	CD_3CO	–	D	–	^{12}C	485	482	466	464	452	–	253	249	227	204	190
ET-759B	*I*	777	760	OH	CH_3	CH_3CO	O	H	–	^{12}C	511	509	477	475	463	–	–	246	–	218	204
ET-701	*I*	719	702	OH	CH_3	H	–	H	–	^{12}C	453	451	435	433	421	405	250	246	224	218	204
ET-637	*I*[c]	655	638	OH	CH_3	CH_3CO	–	H	–	^{12}C	495	493	477	475	463	–	–	–	–	218	204
ET-745	*I*	745	746	H	CH_3	CH_3CO	–	H	–	^{12}C	–	495[d]	–	477[d]	465[d]	–	–	–	–	220[d]	204
ET-759A	*I*	759	760[e]	=O	CH_3	CH_3CO	–	H	–	^{12}C	–	509[d]	–	491[d,e]	–	–	–	–	224	232[d]	204
ET-775	*I*	775	776	OCH_3	CH_3	CH_3CO	–	H	–	^{12}C	–	525[d]	509[d]	507[d]	–	–	250	–	–	250[d]	204
ETM-259[f]	*II*	259	260	–	–	CH_3CO	–	–	H	–	–	–	–	–	–	–	–	–	–	–	–
ETM-233[f]	*II*	233	234	–	–	H	–	–	OH	–	–	–	–	–	–	–	–	–	–	–	–
ETM-217[f]	*II*	217	218	–	–	H	–	–	H	–	–	–	–	–	–	–	–	–	–	–	–
ETM-204[f]	*III*	204	204	–	CH_3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] Pseudomolecular ions are $[M+H-H_2O]^+$, except ET-745 $[M+H]^+$, ET-759A $[M+H]^+$, ET-775 $[M+H]^+$, ETM-259 $[M+H]^+$, ETM-233 $[M+H]^+$, ETM-217 $[M+H]^+$ and ETM-204 $[M^+]$

[b] Masses were observed in MS spectra [product ion scan from (pseudo)molecular ions] of the reference compounds. All reported *m/z* values for pathways B, C, D, E, F and J are after loss of R1, unless indicated otherwise

[c] In ET-637 the upper tetrahydroisoquinoline ring is substituted by "–$CH(NHCOCH_3)$–" representing a ring opening of this part of the original trabectedin structure

[d] Substituent R1 is not eliminated

[e] Loss of CO from *m/z* 760 to *m/z* 732 and *m/z* 491 to *m/z* 463, respectively, was also observed

[f] Fragmentation of these compounds resulted in losses of (–CH_2CO–) 42, (H_2CO) 30, (CO) 28, (H_2O) 18 and (H_2) 2

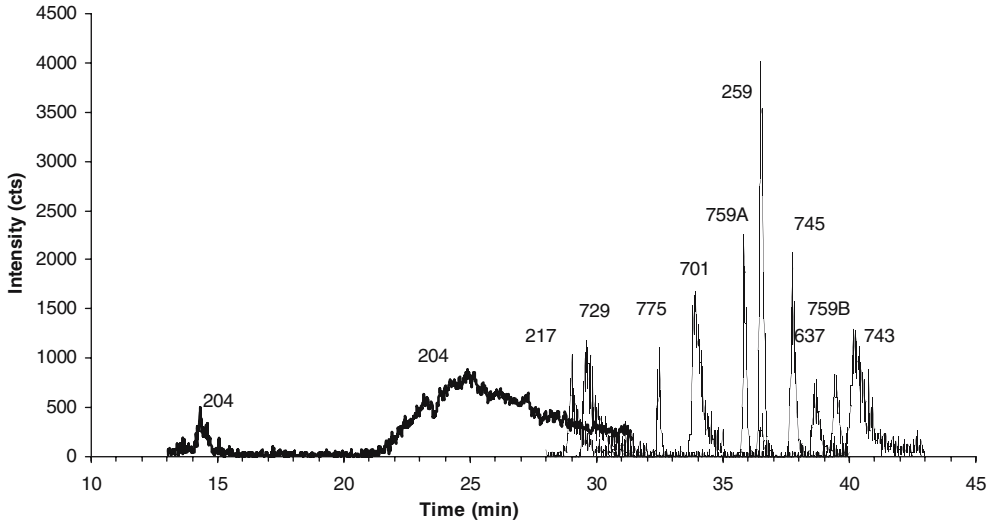

Fig. 3 Composite chromatogram showing MS/MS traces of available reference compounds injected onto the LC-MS/MS system with trabectedin (ET-743) eluting last. Reference compounds used are ETM-204 (204: *m*/*z* 204.1–160.1), ETM-217 (217: *m*/*z* 218.0–190.0), ET-729 (729: *m*/*z* 730.2–481.1), ET-775 (775: *m*/*z* 776.3–204.0), ET-701 (701: *m*/*z* 702.2–433.1), ET-759A (759A: *m*/*z* 760.2–224.0), ETM-259 (259: *m*/*z* 260.0–217.9), ET-745 (745: *m*/*z* 746.2–477.1), ET-637 (637: *m*/*z* 638.2–463.0), ET-759B (759B: *m*/*z* 760.2–224.0) and trabectedin (743: *m*/*z* 744.2–494.9)

by a different functional group (ET-745, ET759A and ET-775) drastically changed the fragmentation of these compounds. Furthermore, fragmentation of the reference compounds was characterised by a frequent loss of the single isoquinoline moiety by cleavage at the ester and thio-ether bonds. The fragmentation of the four smaller reference compounds (consisting of single isoquinoline structures) indicated consecutive losses of small fragments.

Chromatography

Injection of a mix of the available reference compounds resulted in a compound chromatogram as depicted in Fig. 3. Corresponding MS/MS masses, retention times, amounts (ng) injected into the LC-MS/MS system, responses (counts) and ^{14}C-adapted MS/MS masses (for analysis of patient samples) are tabulated in Table 2. Injection of ETM-233 did not result in a distinct peak, but in a steadily rising baseline in the respective MS/MS channel (not included in the compound chromatogram). ETM-204 showed a peak at approximately 14 min, followed by a broad peak with extensive tailing at approximately 25 min.

Analysis of patient samples

To characterise the human metabolites of trabectedin, we followed two approaches. Our first approach was radiochromatography consisting of liquid chromatography with collection of fractions (1 min each). These fractions were quantitated for their radioactivity content.

Radiochromatographic detection of metabolite fractions in urine and faeces samples

Figure 4 displays representative radiochromatograms of a urine (a) and faeces (b; methanolic extract) sample with all designated metabolite fractions (U for urine fractions, F for faeces fractions). The urine radiochromatographic profile is characterised by several fractions containing more than average amounts of radioactivity, with a background of radioactivity continuously eluting between 10 and 40 min. Even more so, the radiochromatographic profile of faeces is characterised by large amounts of radioactivity eluting between 20 and 30 min. This complicated the designation of single fractions containing the metabolic products of trabectedin. Therefore, we only selected and numbered a fraction when it contained large amounts of radioactivity relative to adjacent eluting fractions. Table 3 shows the fractions occurring in urine and faeces, in parallel with the retention times of the available reference compounds. Typically 107–146% and 78–88% of injected radioactivity was recovered in chromatograms from urine and faeces samples, respectively.

F1 and U1 F1 and U1 both eluted at 4–5 min. The compounds eluting here may represent glucuronide metabolites as indicated from the β-glucuronidase experiment showing a decrease in radioactivity of U1

Table 2 Available ^{12}C-reference compounds and the response at a signal to noise ratio of approximately 10 (absolute amounts injected on column), and ^{14}C-adapted masses for detection of corresponding [^{14}C]metabolites in patient samples

Analyte	^{12}C-MS/MS masses (*m*/*z*)	Retention (~min)	Injected (ng)	Response (counts)	^{14}C-MS/MS masses (*m*/*z*)
Trabectedin	744.2/494.9	40.2	0.4	700	746.2/494.9
ET-729	730.2/481.1	29.6	2	400	732.2/481.1
ET-775	776.3/204.0	32.5	0.5	550	778.3/204.0
ET-759A	760.2/224.0	35.8	0.2	450	762.2/226.0
ET-759B	760.2/224.0	39.4	2	600	762.2/226.0
ET-745	746.2/477.1	37.8	0.09	650	748.2/477.1
ET-701	702.2/433.1	33.9	2	1,000	704.2/433.1
ET-637[a]	638.2/463.0	38.7	0.4	500	638.2/463.0 640.2/463.0
ETM-259	260.0/217.9	36.5	0.002	1,780	260.0/217.9
ETM-233[b]	234.0/188.1	–	–	–	234.0/188.1
ETM-217	218.0/190.0	29.0	0.09	420	218.0/190.0
ETM-204[c]	204.1/160.1	14+25	1	600	204.1/160.1
ET-731[d]	732.2/463.3	30.3	–	–	734.2/463.3

[a] The origin of the acetyl moiety of the acetamide part of ET-637 may be acetylation (non-radiolabelled resulting in 638.2/463.0), or oxidation of the original isoquinoline ring (radiolabelled resulting in 640.2/463.0). Therefore, both these mass transitions were monitored

[b] ETM-233 did not yield a peak using the described LC procedure

[c] ETM-204 showed a peak at approximately 14 min, followed by a broad peak with extensive tailing at approximately 25 min (not included in Fig. 3)

[d] The ET-731 masses were added to the list of monitored MS/MS masses. These masses correspond to the compound, which is the summation of ET-745 and ET-729: loss of the carbinolamine OH and loss of the N–CH_3. ET-731 was not available as reference compound, but after detection of this compound during the method development for faeces extraction, the corresponding masses were added

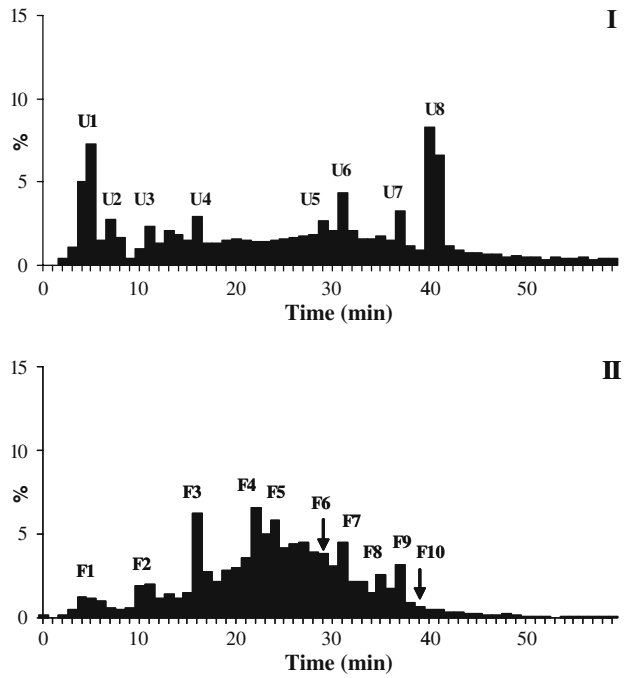

Fig. 4 Radiochromatograms (off-line LSC) of 0–24 h pooled urine of patient 3–8 (*I*; approximately 7 ng trabectedin equivalents injected: 5% corresponds to 0.35 ng) and an extract of 0–120 h pooled faeces of patient 3 (*II*; approximately 14 ng trabectedin equivalents injected: 5% corresponds to 0.7 ng) displaying the fractions typically containing relatively large amount of radioactivity with metabolite designations. Radioactivity as percentage of total is plotted versus elution time

after incubation (see below). The decrease in radioactivity eluting in U1 paralleled an increase in radioactivity eluting at the retention time of F4. We could not further characterise these fractions.

F2, U2 and U3 U2 eluted at 7–8 min. F2 and U3 both eluted at 10–11 min. No reference compound eluted at these times, and we could not further characterise its contents.

F3 and U4 F3 and U4 both eluted at 16 min. Although ETM-204 eluted at the same time, the radioactivity in F3 or U4 could not be ascribed to ETM-204. When [^{14}C]trabectedin is converted into ETM-204, it does not contain the ^{14}C-label and is thus undetectable by LSC.

F4 F4 eluted at 22 min. At this retention time, an increase in radioactivity was observed after incubation of urine with β-glucuronidase. Thus, the eluting compound may represent the aglycon of the glucuronide in U1. The presence of F4 in faeces with no corresponding fraction in urine could be explained by the presence of bacterial glucuronidase activity in faeces [9]. In faeces, bacterial glucuronidase may have converted the F1/U1-glucuronide metabolite into the F4-aglycon, while in urine this glucuronide metabolite is still in tact and present in U1. The

Table 3 Overview of designated urine and faeces fractions containing trabectedin metabolites (LC-LSC) with retention times of reference compounds and LC-MS/MS observations of these reference compounds in patient samples

LC-LSC			LC-MS/MS		Observed[a]	
Elution (min)	Urine	Faeces	Reference	Retention (min)	Urine	Faeces
4–5	U1	F1	–			
7–8	U2		–			
10–11	U3	F2	–			
16	U4	F3	ETM-204	14+20–25	y	
22		F4	–			
24		F5	ETM-204	14+20–25	y	
29	U5	F6	ETM-217	29.0		y[b]
			ET-729	29.6		y[c]
31	U6	F7[d]	ET-775	32.5		
35		F8	ET-701	33.9	y	y[c]
			ET-759A	35.8		
37	U7	F9	ETM-259	36.5	y	
			ET-745	37.8	y	y
39–40	U8	F10	ET-637	38.7	y	y
			ET-759B	39.4		
			Trabectedin	40.2		

[a] Signals in the correct MS/MS trace at the correct retention time with a signal to noise ratio above three confirmed the presence of the corresponding analyte

[b] Observed after acid and neutral extraction of 1 ml faeces homogenate with 5 ml of diethyl ether

[c] Only observed after extraction of patient faeces samples under strongly acidic conditions. These compounds were also observed after extraction of trabectedin-spiked faeces under acidic conditions, indicating that these metabolites can be formed by chemical degradation of trabectedin

[d] Using LC-MS/MS, we observed "ET-731" at the elution time of this fraction in faeces

structure of the aglycon or the position of the glucuronidation is conjecture because the structure of trabectedin provides numerous amines and hydroxyl moieties available for glucuronidation, either directly or after demethylation.

F5 F5 eluted at 24 min. Although ETM-204 eluted at around the same time, the radioactivity in F5 could not be ascribed to ETM-204 as it no longer contains the ^{14}C-label and is thus undetectable by LSC.

F6 and U5 F6 and U5 eluted at 29 min. Both ETM-217 and ET-729 eluted at this time. The radioactivity in F6 or U5 cannot be ascribed to ETM-217 because this structure no longer contains the ^{14}C-label and is thus undetectable by LSC.

F7 and U6 F7 and U6 eluted at 31 min. ET-775 reference compound eluted at 32.5 min.

F8 F8 eluted at 35 min. Both ET-701 and ET-759A eluted with the same retention time.

F9 and U7 F9 and U7 eluted at 37 min near the retention time of ETM-259 and ET-745.

F10 and U8 F10 and U8 eluted at 39–40 min. Trabectedin, ET-637 and ET-759B eluted with similar retention times.

Inter-patient variability in radiochromatographic profile was present, but only moderate. We did not observe a difference in metabolic profiles between the 3 and 24 h administration schedule. Because of the low amounts of the compounds in the collected fractions, further characterisation by recording of mass spectra was not feasible.

LC-MS/MS detection of metabolites in urine and faeces samples

Our first approach was suitable to detect the presence of any metabolites of trabectedin containing the ^{14}C-label. However, this non-selectivity is a drawback if a number of compounds are co-eluting, because the lack of structural information of radiochromatography hinders positive identification of the eluting compounds. Therefore, we took our second approach to characterise these metabolites. Using an LC-MS/MS method optimised for the sensitive and selective detection of the available reference compounds, we screened clinical urine and faeces

samples to confirm the reference compounds as human metabolites of trabectedin. An additional advantage of this approach is that both metabolites that still contain the ^{14}C-label and those that have lost it, will be detected by the mass spectrometer. Table 3 shows the fractions designated by radiochromatographic profiling and whether or not a signal corresponding to a reference compound was observed in the LC-MS/MS assay.

There is the possibility that we detected ETM-204 in patient urine samples eluting with an unexpected double peak at 14 and 25 min. This twin signal was also observed with ETM-204 reference compound.

We detected ETM-217 in patient faeces (diethyl ether extract) eluting at 29 min. We also detected ET-729 after extraction of faeces samples under acidic conditions (6% acetic acid in methanol). However, ET-729 could also be detected in trabectedin-spiked faeces after extraction with acidified methanol, suggesting ET-729 is formed as a chemical degradation product during extraction under acid conditions.

We detected ET-731, the dehydroxylated and demethylated metabolite of trabectedin, in patient faeces samples eluting at 31 min (corresponding to F7 and U6). During the method development of the faeces extraction, ET-745 was ubiquitously observed in faeces extracts (also in neutral methanolic extracts). The detection of this de-hydroxylated trabectedin metabolite (ET-745) prompted us to search for de-hydroxylated analogues of the other reference compounds. In subsequent experiments, this led to detection of the compound corresponding to de-hydroxylated ET-729 (coined "ET-731") in methanolic extracts of faeces, eluting at 31 min. Thus, F7 and possibly U6 contain ET-731.

We detected ET-759A in patient urine samples and in trabectedin-spiked faeces eluting at 35 min (corresponding to F8). The detection in spiked faeces indicates that ET-759A in faeces may be formed by chemical degradation of trabectedin.

We detected ETM-259 and ET-745 in patient faeces and urine samples eluting at 37 min (corresponding to F9 and U7). ETM-259 in patient faeces samples was observed only after extraction with acidic methanol. ET-745 occurred ubiquitously in the extracts of patient faeces samples (detected by LC-MS/MS) and is the most likely candidate for F9.

We detected trabectedin in patient faeces and urine samples eluting at 39–40 min (corresponding to F10 and U8). However, while U8 represents approximately 14–28% of the radioactivity in the radiochromatograms of patients 1–8, LC-MS/MS quantification of trabectedin in urine [1, 21] showed that trabectedin constitutes less than 1% of radioactivity cumulatively excreted in urine after 24 h (data not shown). Therefore, besides trabectedin, U8 contains one or more additional metabolites. Neither ET-637 nor ET-759B have been detected by LC-MS/MS in urine samples. However, for the latter this may be a sensitivity problem as ET-759B is only detected if approximately 1–2 ng is injected (see Table 2), and this is more than actually elutes (in ng trabectedin equivalents) at 39–40 min.

Figure 5 displays the proposed metabolic fate of trabectedin as far as established in the present investigation.

β-Glucuronidase incubation to detect glucuronide conjugates

Incubation with β-glucuronidase can reveal the presence of glucuronide metabolites by shifts of radioactivity in the radiochromatogram. We did not observe such a shift in radioactivity after incubation of faeces with β-glucuronidase. However, incubation of urine resulted in a decrease of radioactivity eluting at approximately 5 min, with a concomitant appearance of a radioactive peak eluting at 23 min, see Fig. 6 (experiment was performed in duplicate with similar results). This suggests the conversion of the more polar and early eluting glucuronide metabolite into its less polar and later eluting aglycon. None of the available reference compounds displayed a retention time at 23 min. Thus, the aglycon eluting at 23 min represents an as yet unidentified phase I metabolite of trabectedin.

Polymorphisms in drug metabolising enzymes and their effect on the metabolic profile

Genotyping of the patients resulted in the detection of heterozygous and mutant types of virtually all-genotyped drug metabolising enzymes. On an opportunistic basis, it was attempted to match inter-individual differences in metabolic profiles (presence and relative amount of radioactivity of peaks in the radiochromatogram) with mutations in the genotyped metabolising enzymes. We did not observe a correlation between metabolic profile and genotypes of drug metabolising enzymes (data not shown).

Discussion

An earlier effort to identify potential trabectedin metabolites in plasma, urine and bile samples obtained from patients was not successful [25]. Results from the

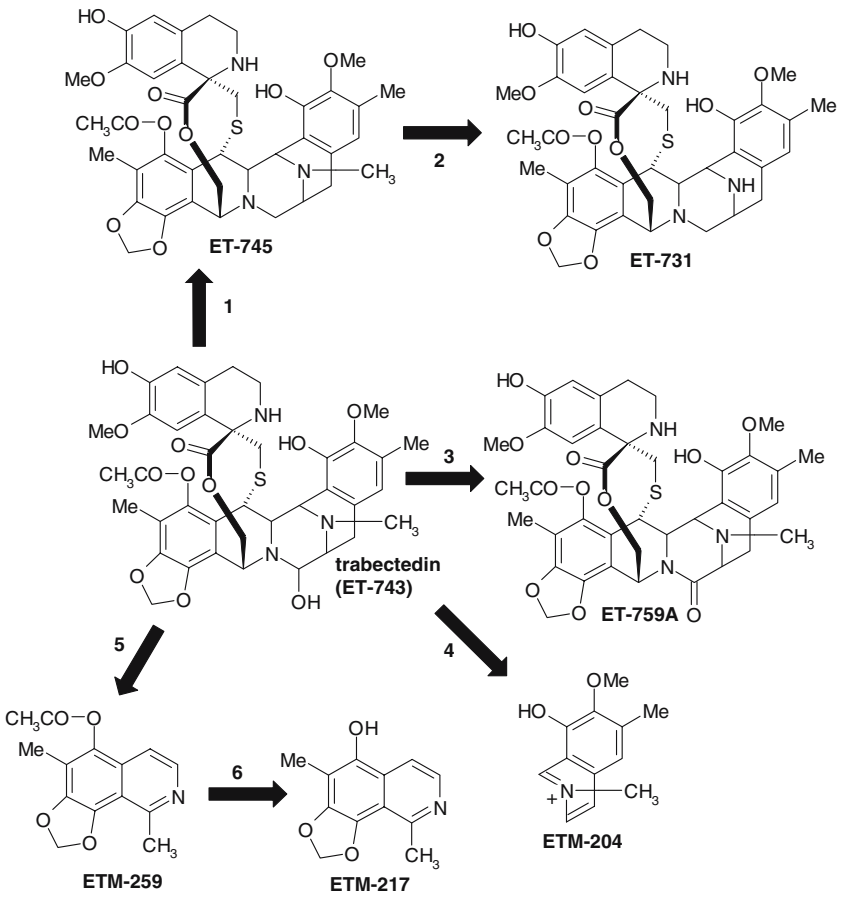

Fig. 5 Proposed metabolic fate of trabectedin in humans. Trabectedin undergoes: dehydroxylation of the carbinolamine moiety (*1*) followed by demethylation (*2*); oxidation of the carbinolamine moiety to an amide (*3*); and breaking up of the molecule to the individual tetrahydroisoquinoline subunits (*4* and *5*); and acetate ester hydrolysis (*6*)

recent mass balance study suggested that the liver is the principal eliminating organ and faecal excretion (55.5% of radioactivity administered versus 5.9% in urine) is the major excretory pathway [1]. Our objective was to determine the trabectedin metabolic profile and to identify its human metabolites. The low dose, high volume of distribution and long terminal half-life of trabectedin in humans, however, result in very low concentrations of trabectedin and metabolites in urine and faeces. This is a major hurdle for any structural elucidation of metabolites of trabectedin. Therefore we developed a sensitive LC-MS/MS assay for the detection of trabectedin and reference compounds as potential metabolites. The detection of a signal at the same retention time, and the same Q1 and Q3 masses as a reference compound confirms the presence of this compound in the sample. In parallel, we assayed samples using off-line radioactivity detection of fractions (with counting times of 30 min) to increase sensitivity. The yet unknown trabectedin metabolites eluted, however, at concentrations too low to allow recording of full mass spectra for structural elucidation.

Our results indicate that, after intravenous administration to humans, trabectedin is rapidly metabolised to a large number of compounds, without any one predominating metabolite. Using [^{14}C]trabectedin, all radioactive fractions eluted before the parent compound, suggesting a more hydrophilic nature for these metabolites. In both the urine and faeces chromatograms, discretely eluting peaks can be observed, but, especially in faeces, most of the radioactivity appears to consist of a large number of metabolites continuously eluting from the column. The large number of metabolites suggests that several metabolic pathways are involved in the human metabolism of trabectedin. This is consistent with the involvement of multiple human CYP450 enzymes (3A4, 2C9, 2C19, 2D6, 2E1) in the phase I metabolic degradation of trabectedin that has been reported using in vitro techniques [4]. Because the extraction recovery of radioactivity from faeces was about 35%, even more trabectedin metabolites may be present in faeces. However, these compounds appear to be tightly bound to the solid faeces particles. Treatment with acid could partly release these compounds from the faeces matrix, increasing recovery of radioactivity to approximately 70% but during evaporation of the extract to dryness, most of these compounds were lost. This may imply they are

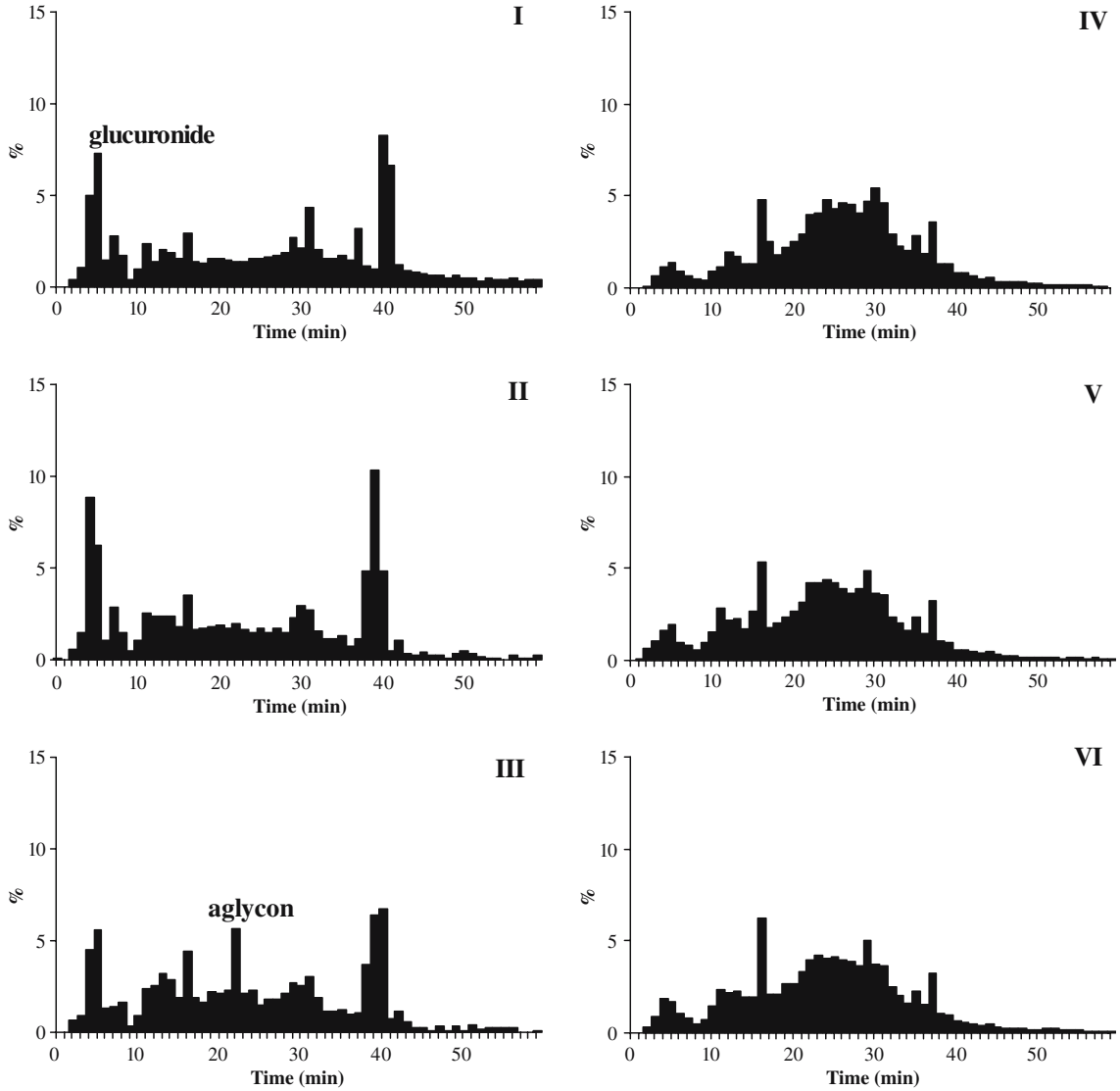

Fig. 6 Radiochromatograms of 0–24 h pooled urine (*I–III*) and 0–120 h pooled faeces (*IV–VI*) samples of patients 3–8 after a 3 h intravenous administration of [^{14}C]trabectedin. Samples were analysed without incubation (*I*, *IV*), after incubation with buffer (*II*, *V*), or after incubation with β-glucuronidase (*III*, *VI*)

small, non-polar, volatile compounds carrying the radioactive ^{14}C-label.

We selected eight fractions in urine and ten fractions in faeces, that contained separately eluting radioactive metabolites. For urine and faeces samples, the typical amounts of radioactivity injected on the LC system corresponded to only 3–8 and 13–30 ng trabectedin-equivalents, respectively. Consequently, the level of radioactivity in the resulting fractions eluting from the column was not high enough to allow recording of full mass spectra of the analytes. Nonetheless, LC-MS/MS analysis of complete extracts of the same urine and faeces samples resulted in the detection of the following compounds (IC_{50} in murine L1210 leukaemia cells, ng/ml [19]) trabectedin (0.5), ET-745 (88), ET-759A (8.5), ETM-259, ETM-217 (all were available as reference compounds) and a proposed new metabolite coined ET-731. The smaller metabolites are not expected to have great activity because they lack the active carbinolamine moiety. Possibly, ETM-204 was also detected in urine. A potential explanation for the observed double elution of ETM-204 might be that it is unstable and is partly converted to an isomer with an identical mass (a rearrangement rather than any other degradation), yet a different polarity and thus elution time. In addition, the mass of 204 found may be a fragment of trabectedin or a metabolite generated during fragmentation

in the mass spectrometer, rather than ETM-204 itself, as supported by the isolation in urine of radioactive fractions (thus not corresponding to ETM-204) with this molecular mass. The observation of the same double elution pattern in both the biological samples and the reference compound supports the hypothesis of a rearrangement, but this requires confirmation. The presence of ET-745 and ET-731 suggests a dehydroxylation of the original trabectedin structure, a rather unusual metabolic conversion. Similar dehydroxylation reactions have been observed with bile acids under the influence of intestinal bacterial flora [5] and these compounds may thus have been formed in the faeces. Although a demethylation process was indicated by in vitro incubation experiments with trabectedin in human hepatic microsomes and ET-729 (*N*-desmethyl trabectedin, IC_{50} of 0.05 ng/ml [19]) was found in human CYP isoform incubations, the actual presence of ET-729 (possibly involved in trabectedin mediated elevations of liver enzymes in plasma [2]) has never been confirmed in humans [15, 21, 25]. We did observe ET-729 in faeces extracts, but only after extraction under acidic conditions. In extracts of patient samples, ET-729 may merely be a chemical degradation product of trabectedin, because ET-729 was also observed after acidic extraction of trabectedin-spiked blank faeces. However, this does not exclude ET-729 as a potential metabolite of trabectedin.

Incubation of urine with β-glucuronidase indicated the presence of a glucuronide metabolite in urine. No glucuronides were found in faeces. Possibly, any glucuronide metabolites excreted in faeces had already been de-conjugated by bacterial glucuronidase activity which would also explain the relative absence of radioactivity eluting in the first 5 min of the radiochromatogram of faeces extracts. The potential glucuronide found in urine appears to be a derivative from a metabolite rather than from trabectedin itself. In addition, in vitro experiments have shown little [25] or no glucuronidation of the unchanged drug (data on file, PharmaMar). Thus, direct glucuronidation of trabectedin is probably not an important elimination route. The concentrations were too low to determine the chemical structure of the glucuronide or the respective aglycon.

The metabolism of trabectedin as judged from the radiochromatographic profiles of urine and methanolic faeces extracts did not show large inter-individual differences. We could not match observed differences in metabolic profile with polymorphisms of drug metabolising enzymes. Given the presumed complexity of trabectedin metabolism with multiple metabolic pathways, it is unlikely that a single polymorphism would be reflected by a change in the metabolic profile.

The chemical structure of trabectedin provides numerous sites for metabolic conversion, as indeed reflected in the plethora of compounds that were observed in the radiochromatograms. In addition, concentrations of these metabolites were extremely low, and did not allow direct recording of complete mass spectra. However, a parallel approach of in vitro metabolism experiments (at higher concentrations) producing reference compounds and screening for these compounds in patient samples using the developed LC-MS/MS assay was successful and led to the identification of several trabectedin metabolites in humans.

In conclusion, trabectedin is metabolically converted to a large number of compounds that are excreted in both urine and faeces. In urine and faeces we have confirmed the presence of trabectedin, ET-745, ET-759A, ETM-259, ETM-217 and ETM-204. In addition we have identified a putative new metabolite encoded ET-731. Given the presumed complexity of trabectedin metabolism with multiple metabolic pathways, it is unlikely that a single polymorphism is clearly reflected by a change in the metabolic profile or that inhibition of a single pathway by an interacting drug causes a significant reduction in clearance, although this needs confirmation. Further research is needed to elucidate the complete metabolic fate of trabectedin in humans and to establish the activity of the identified metabolites.

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