

Evaluation of Human Plasma Protein Binding of Trabectedin (Yondelis™, ET-743)

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Abstract: Trabectedin (ET-743, Yondelis™) is a novel anticancer drug with impressive activity in soft tissue sarcoma with a manageable, non-cumulative toxicity profile. Protein binding can be a major determinant of unbound concentration, volume of distribution, renal and hepatic clearance, and the half-life of a drug. Human plasma protein binding of trabectedin has not previously been reported. Using ultrafiltration techniques, we determined the human plasma protein binding of trabectedin at a clinically relevant concentration. Experiments with a panel of co-medications representing all known protein-binding sites showed that the concentration of unbound trabectedin could be increased by high concentrations of phenytoin. The other tested co-medications, at concentrations covering their respective therapeutic ranges, did not displace trabectedin from its plasma protein binding. This suggests that trabectedin binds to albumin site I (total protein binding of 94.2 ± 0.6 %) displaying an association constant of 2.6 ± 0.2 10⁴ M⁻¹. Because trabectedin is an intermediate-to-high hepatic extraction drug, changes in unbound fraction will not have a major impact on elimination processes. The high protein binding may have implications for the interpretation of *in vitro* data, which are usually performed in the presence of low protein levels. We can conclude that the studied co-medications are unlikely to have clinically relevant effects on trabectedin binding to plasma proteins at therapeutic concentrations.

Key Words: Trabectedin, protein binding, human, displacement.

INTRODUCTION

Intravenous trabectedin (ET-743, Yondelis™, Fig. (1)) is approved as monotherapy in Europe for use in patients with advanced soft tissue sarcomas (STS) after failure of anthracyclines and ifosfamide, or who are unsuited to receive these agents [1]. Trabectedin has received orphan drug status in STS in the United States and in ovarian cancer in patients with recurrent ovarian cancer in both the United States and Europe [2]. The recommended dose for STS is 1.5 mg/m² administered over 24 h every 3 weeks, with i.v. 20 mg dexamethasone pre-treatment 30 min before trabectedin [1].

Trabectedin belongs to the Ecteinascidins, tetrahydroisoquinoline compounds isolated from the tunicate *Ecteinascidia turbinata* [3]. Several studies have investigated the pharmacokinetics of trabectedin [4]. Peak plasma concentrations after intravenous administration range from 0.3 to 17 ng/mL depending on the dosing schedule [4]. Trabectedin displays a volume of distribution of 1000 to 4000 L, suggesting extensive tissue binding [4]. In addition to being a major determinant of the volume of distribution, protein binding can also be a major influence on renal and hepatic clearance, and the half-life of a drug. It is the unbound concentration of a drug that drives tissue distribution and thereby effects therapeutic benefit and toxicity [5]. In addition, protein bind-

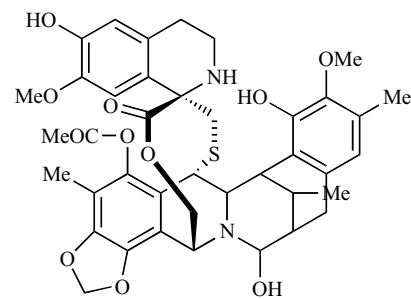


Fig. (1). Chemical structure of trabectedin (ET-743).

ing can be influenced by both co-medication and morbidity [6]. However, the plasma protein binding of trabectedin has not previously been reported.

The present investigation was aimed at determining trabectedin plasma binding and the influence of co-medications at therapeutically relevant concentrations.

EXPERIMENTAL

Plasma

Because storage of plasma is known to affect plasma protein binding of drugs [7], plasma was obtained by centrifugation of fresh, heparinized blood from healthy volunteers. To assure the quality of the plasma and to prevent possible bias, we determined standard clinical chemistry parameters in every plasma batch used for experiments. These parameters covered: alkaline phosphatase, lactate dehydro-

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genase, ALAT, ASAT, γ -glutamyltransferase, creatinine, urea, calcium, phosphatase, glucose, total and direct bilirubin, sodium, potassium, chloride, total proteins, albumin, creatin kinase, uric acid, cholesterol, triglycerides, amylase, iron, unsaturated and total iron binding capacity, IgG, IgA, IgM, C-reactive protein, lipase, α -1-acid glycoprotein, lipemic index, hemolytic index, and icteric index.

Chemicals and Reagents

Trabectedin and [^{14}C]-trabectedin were kindly provided by PharmaMar (Madrid, Spain). The panel of co-medications was selected based on their potential use along with trabectedin, or because they represented specific binding sites of human plasma proteins. Acetylsalicylic acid, diazepam, erythromycin, acetaminophen, phenytoin (sodium salt), valproic acid (sodium salt) and warfarin (sodium salt) were purchased from Bufa (Uitgeest, The Netherlands). Ceftazidime (pentahydrate) (Fortum[™]) was purchased from GlaxoSmithKline (Zeist, The Netherlands). Cloxacillin (sodium salt hydrate), diclofenac (sodium salt), digitoxin, propranolol (hydrochloride) and tamoxifen were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Centrifree Micropartition devices (Millipore, Bedford, MA) with a 30,000 a.m.u. molecular weight cut-off were used to generate ultrafiltrate. Methanol (Supra-Gradient grade) was purchased from Biosolve Ltd. (Amsterdam, The Netherlands). Ondansetron (hydrochloride dihydrate) was a generous gift from GlaxoSmithKline (Stevenage, UK). Ethanol was purchased from Merck (Darmstadt, Germany). Water was purified by the Milli-Q Plus[®] system (Millipore, Milford, USA).

Ultrafiltration

Trabectedin was added to plasma to produce a concentration of 8 nM (6 ng/mL). Drug (co-medication) solutions were prepared in human plasma at a minimum of 3 concentrations, covering the therapeutic range [8-10] of each drug, respectively. The trabectedin containing plasma was mixed with an equal volume of a drug plasma solution. The final trabectedin concentration (4 nM, 3 ng/mL) was in the clinically relevant range [4]. Final drug concentrations were: 10, 100, 1000 μM acetylsalicylic acid; 10, 100, 1000 μM ceftazidime; 2.5, 25, 250 μM cloxacillin; 10, 100, 1000 nM diazepam; 0.10, 1.0, 10 μM diclofenac; 1, 10, 100 nM digitoxin; 1.5, 15, 150 μM erythromycin; 10, 100, 1000 nM ondansetron; 10, 100, 1000 μM acetaminophen; 4, 40, 400 μM phenytoin; 50, 500, 5000 nM propranolol; 10, 100, 1000 nM tamoxifen; 7, 35, 70, 700 μM valproic acid and 0.25, 2.5, 25 μM warfarin. The final solutions contained a maximum of 1% water or 0.5% ethanol. To evaluate the influence of the latter solvent, the effect of ethanol at 0.1, 0.5 and 1.0% on the protein binding of trabectedin was also evaluated.

Aliquots of 1 mL of the mixtures were added to micropartition devices (typically N=4) followed by incubation at 37 °C for 1 hour. Pilot experiments showed that protein binding at this time did not significantly differ from protein binding after a 2-h incubation. Preliminary tests showed that handling of trabectedin in plasma ultrafiltrate (protein-free) resulted in recoveries of less than 10%, probably because of non-specific adsorption [11]. From the literature, treatment with benzalkonium chloride or tween is known to reduce

non-specific binding [12]. However, we did not want to add non-physiological compounds to our system. Therefore, a 550 μL aliquot of blank plasma was added to the collection cups before ultrafiltration, reducing adsorption of trabectedin from the ultrafiltrate to plastic surfaces. To assess trabectedin adsorption to the membrane, a 25 ng/mL [^{14}C]-trabectedin plasma solution was incubated in a micropartition device for 1 h. After ultracentrifugation of approximately 20% of the volume (1500 x g, 37 °C), only 2% of radioactivity was recovered in the filter, probably due to residual protein precipitated on the membrane. This level of adsorption was considered acceptable.

Following incubation, the micropartition devices were centrifuged for 15 min, 1500 x g at 37 °C. Using the weights of the collection cups prior to and after centrifugation, we calculated the volume of ultrafiltrate collected. A 600 μL aliquot was taken from the collection cup and stored at -20 °C until analysis of trabectedin concentration. With each series of experiments (typically 2 drugs at 3 concentration levels), we also determined the protein binding of trabectedin without added drug. This was done by mixing an 8 nM trabectedin solution in plasma with an equal volume of blank plasma. From this solution, aliquots were taken for analysis of total trabectedin content and aliquots were used for ultrafiltration.

Trabectedin Bioanalysis and Calculation of Protein Binding

Often, protein binding of drugs is assessed using radioactively labelled drug. However, the specific radioactivity of the available [^{14}C]trabectedin (approximately 2.5 MBq/mg, corresponding to 80% of the trabectedin molecules containing the ^{14}C isotope) was not high enough to allow quantitation at the expected clinically relevant unbound plasma concentration of trabectedin. This necessitated the use of an ultra-sensitive LC-MS/MS assay for trabectedin quantification [13]. This assay has a dynamic range of 0.025 to 2.5 ng/mL, with a precision of <10% and accuracy within 10% of nominal values. The trabectedin concentration of the original ultrafiltrate (unbound trabectedin) was calculated using Eq. (1).

$$C_u = C_m * \frac{(550 + (W_a - W_b))}{(W_a - W_b)}$$

Equation 1. Calculation of the trabectedin concentration in ultrafiltrate (C_u) from the concentration measured (C_m) using the weights (mg) of the collection cup before (W_b) and after (W_a) ultrafiltration.

Statistical Analysis

A simplified power-test showed that at a protein binding of approximately 95%, a maximal relative standard deviation of 27% and statistical errors of $\alpha=0.05$ and $\beta=0.10$, we would be able to observe a 50% increase of unbound trabectedin.

To determine statistically significant differences ($p<0.05$) in unbound trabectedin concentration, we performed ANOVA

with a post-hoc Dunnett test using the drug-free unbound concentration of trabectedin as control set. Statistically significant differences were confirmed by a repeat experiment. The association constant of trabectedin and albumin (K_a) was calculated using Eq. (2) assuming a single binding site.

$$K_a = \frac{DP}{D * P} = \frac{(C_{tot} - C_u)}{C_u * P}$$

Equation 2. Calculation of the association constant (K_a) of the binding of trabectedin (D) to albumin (P) using the total plasma trabectedin concentration (C_{tot}), the concentration of unbound trabectedin and the albumin concentration all in molar concentrations [19].

RESULTS

The clinical chemistry parameters of the plasma batches were within normal limits. A concentration of 1.0% ethanol increased unbound trabectedin by 47% ($p=0.049$), whereas 0.5% and 0.1% of ethanol did not.

The drug-free trabectedin protein binding determined in 9 separate runs is displayed in Table 1. Trabectedin was 94.2 \pm 0.6 % protein bound. Statistically significant differences in the concentration of unbound trabectedin were observed after incubation with 400 μ M phenytoin (28% increase in unbound trabectedin, $p=0.01$) (Table 2). We repeated the experiment with 400 μ M phenytoin using $n=8$ for both phenytoin spiked plasma and the controls. This confirmed the earlier results with a 50% increase in the concentration of unbound trabectedin ($p=0.002$).

Table 1. *In Vitro* Protein Binding of Trabectedin at Approximately 3 ng/mL Plasma

Run	Protein Binding (%)
1	94.9
2	95.2
3	94.4
4	93.8
5	94.5
6	93.7
7	93.5
8	93.7
9	94.4
Mean	94.2
SD	0.62

The displacement caused by phenytoin suggests that trabectedin may be mainly bound to albumin at the warfarin and azapropazone binding site (albumin site I) [6, 14]. For the binding to albumin, the observed free and unbound concentrations correspond to an association constant (K_a) of

2.56 \pm 0.23 $\cdot 10^4$ M^{-1} , assuming a molecular weight of 67,000 g/mol for human albumin and a single binding site.

DISCUSSION

The present investigation was aimed at supplementing current information on the distribution characteristics of trabectedin.

At clinically relevant trabectedin plasma concentrations in the low nanomolar region [4], unbound trabectedin concentrations range in the lower picomolar range. Trabectedin is extensively bound to human plasma at 94%. From our panel of co-medication, only a high level of phenytoin (400 μ M) was capable of displacing trabectedin and increasing the concentration of unbound trabectedin. This suggests binding of trabectedin at site I of albumin. The binding of trabectedin to albumin together with its large volume of distribution implies that it is highly unlikely that clinically relevant interactions will occur based on displacement of trabectedin from plasma proteins. Because of the high association constant of trabectedin to albumin and the low trabectedin concentration relative to albumin, moderate changes in albumin concentration, due to e.g. morbidity, will also not change the fraction of bound trabectedin or elimination processes [6, 14].

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Table 2. Effect of the Highest Concentrations of Drugs on the *In Vitro* Plasma Protein Binding of Trabectedin

Drug	Binding Site	Concentration (μM)	Change in Unbound Trabectedin Concentration (%)	p-Value
acetylsalicylic acid	albumin I ^a	1000	4.2	0.83
ceftazidime		1000	7.1	0.95
cloxacillin	albumin II ^a	250	-22	0.26
diazepam	albumin II ^a	1	8.3	0.92
diclofenac	albumin I and II ^b	10	-4.1	0.97
digitoxin	albumin ^c	0.1	3.8	0.82
erythromycin	α1-acid glycoprotein ^a	150	29	0.08
ethanol		1% (v/v)	47	0.049*
ondansetron		1	24	0.34
acetaminophen		1000	7.6	0.94
phenytoin (1)	albumin I ^a	400	28	0.01*
phenytoin (2)	albumin I ^a	400	50	0.002*
Propranolol	α1-acid glycoprotein ^a	5	32	0.27
tamoxifen	albumin ^d	1	-24	0.20
valproic acid	albumin I ^a	700	28	0.12
warfarin	albumin I ^a	25	26	0.09

*P<0.05

^a [14]^b [20]^c [21]^d [18]

using purified human serum albumin will be needed to confirm our hypothesis and explain our observations more mechanistically.

The clinical relevance of the trabectedin displacement is limited. A phenytoin concentration of 400 μM is well over the upper limit of its therapeutic range and would be associated with severe toxicity [9]. Because trabectedin is an intermediate to high extraction drug, changes in unbound fraction will not have a major impact on elimination processes either [4, 14]. We can thus conclude that within the therapeutic ranges of trabectedin and the tested co-medication, no clinically relevant effects on trabectedin binding to plasma proteins are expected.

The high protein binding may have implications for the interpretation of *in vitro* data, which are usually performed in the presence of only 10% serum. A 10-fold decrease in protein content at equal total concentration of trabectedin results in a 6.5-fold increase in free trabectedin concentrations. This effect needs to be taken into account when judging the clinical relevance of *in vitro* experiments, such as those aimed at defining the mechanism of action of trabectedin [4].

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