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Running title: Analysis of BT1718 DM1 in human plasma

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Development of a LC-MS/MS method for the quantification of toxic payload DM1 cleaved from its peptide conjugate BT1718 in a Phase I clinical trial setting

Abstract

Background: BT1718 is a novel bicyclic peptide anticancer drug targeting MT1-MMP to release its toxic payload DM1. A LC-MS/MS method was validated to quantify DM1 generated from BT1718 in a Phase I/IIa clinical trial.

Methods & Results: Plasma samples underwent a reduction reaction to artificially cleave BT1718 into DM1 and its bicycle components. An alkylation step was carried out to stabilise the reaction products, and plasma proteins extracted using acetonitrile. LC-MS/MS analysis utilised a C_{18} column and Agilent 6460 triple quadrupole mass spectrometer. The method was fully validated over a linear range of 200-50,000 ng/ml BT1718, with overall precision ≤10% and accuracy 89-102%.

Conclusion: A novel method for quantifying DM1 yielded from BT1718 has been validated and is now being utilised clinically.

Keywords: BT1718, DM1, MT1-MMP, peptide-drug conjugate, LC-MS/MS, pharmacokinetics, cancer

Introduction

Membrane type I matrix metalloproteinase (MT1-MMP) is the founding member of the matrix metalloproteinase (MMP) family, first identified in 1962 by Gross and Lapiere, and now containing 23 human MMPs [1, 2]. MMPs are involved in tissue remodelling, through the proteolysis of collagen and other extracellular matrix components [3]. MMPs are upregulated in a range of diseases including rheumatoid arthritis and cancer, with high MMP levels often correlated with poor disease prognosis [4]. In humans, six MT-MMPs form a subgroup within the MMP family. They share a common domain structure, are cell surface expressed and have the same basic amino acid motif of signal peptide, pro-domain, catalytic domain, hinge, hemopexin-like domain and stalk region [5]. There is a complex relationship between MMPs and disease which is not fully understood, but overexpression of MT1-MMP in many solid tumours and surrounding stroma, is linked to cell invasion and migration [6]. Poor prognosis and shorter survival in non-small cell lung cancer [7, 8], breast cancer [9, 10] and other solid malignancies [11-13] is associated with MT1-MMP overexpression, which is observed in a wide range of cancers [3].

Over fifty MMP inhibitors have been investigated, the majority targeting the highly conserved catalytic domain of the MMP family, but their successful development failed in part due to reasons relating to poor pharmacology, metabolic stability, bioavailabilty and/or dose limiting toxicity [14]. BT1718 is a Bicycle Toxin Conjugate (BTC) developed by Bicycle Therapeutics Ltd, consisting of a novel bicyclic peptide (Bicycle) which binds to the hemopexin domain of MT1-MMP with high affinity and selectivity, connected through a molecular spacer and a cleavable disulfide linker to the potent cytotoxic tubulin inhibitor, DM1 (N2'-deacetyl-N2'-(3-mercapto-1-oxopropyl)-maytansine) (Figure 1) [15]. BT1718 was optimised for affinity to MT1-MMP, selectivity over related MMP proteins and improved *in vitro* human plasma stability [16].

BT1718 is not an MMP inhibitor and instead exploits MT1-MMP to target tumours by binding to cell surface MT1-MMP overexpressed on tumour cells, which facilitates the delivery of the cytotoxic payload, DM1. Once released by tumour-localised cleavage of the linker, active unconjugated DM1 is then able to bind to microtubules and block tumour cell division, ultimately leading to cell death and reduction of tumour size.

Preclinical xenograft models have shown that following BT1718 dosing, high levels of DM1 can be found in the tumour site with the small peptidic conjugate eliminated via the kidneys, thus reducing the potential for liver toxicity [15]. Following completion of successful preclinical development studies, a Phase I/IIa clinical trial is currently ongoing to determine the safety and tolerability of BT1718 in a solid tumour patient population. To understand the clinical pharmacokinetics and drug disposition

properties of BT1718, two LC-MS/MS plasma assays have been developed and validated, one for parent compound, BT1718 and one that measures total-DM1 related material (DM1-VP). Bearing in mind the inherent instability and reactivity of free DM1 once free from the parent drug, this was felt to be the most reliable way to generate informative exposure data in an early phase clinical trial setting. Such an approach is well established in the clinical development of drug-toxin conjugates using a DM1 payload [17] and is in line with the regulatory expectations of the supportive analytical methods. Here we describe the method for measuring plasma concentrations of total-DM1. Due to inherent challenges associated with the reactivity of DM1, the method involves an initial reduction step using TCEP (tris(2-carboxyethyl)phosphine) followed by stabilisation with VP (4-vinylpyridine). Therefore, DM-1 from BT1718, any peptidyl-DM1 metabolites of BT1718 and any other DM1-containing mixed disulfides, in addition to free DM1, will be converted to DM1-VP, and the concentration of DM1-VP measured to quantify the total DM1 content.

Experimental

Materials

To avoid binding of BT1718 parent material, reduction and alkylation reactions were carried out in low bind centrifuge tubes (Thermo Scientific, UK) and samples were prepared for HPLC injection in glass inserts (Jaytee, UK).

Standards and chemicals

Analytical standard BT1718 (molecular weight: 3511.41 g/mol) was provided by Bicycle Therapeutics (Cambridge, UK). DM3 (molecular weight: 765.46 g/mol) was purchased from Almac (Edinburgh, UK). LC-MS-MS grade acetonitrile, formic acid and DMSO were purchased from Fisher Scientific (Loughborough, UK). 95% pure 4-Vinylpyridine (4-VP), 99.5% pure ammonium bicarbonate, and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) were purchased from Merck (Hertfordshire, UK). Control human sodium citrate plasma for the daily preparation of standard calibration curves and quality control samples was obtained from the Blood Transfusion Centre (Newcastle, UK).

Preparation of Solutions

Two separate working stock solutions of BT1718 for standards and QC samples were prepared in dimethyl sulfoxide (DMSO) at a concentration of 5 mg/ml (aliquoted and stored at -80°C). DM3 powder was dissolved in dimethylacetamide at 1.0 mg/ml and stored at -20°C. Calibration curves and QCs were prepared by serially diluting 5 mg/ml BT1718 in control human citrate plasma containing 1% DMSO to yield standard concentrations of 200, 300, 500, 1000, 2500, 5,000, 10,000 and 50,000 ng/ml, and QC samples at concentrations of 400 (LQC), 4000 (MQC), and 40,000 ng/ml (HQC). Standards were prepared from working stock daily, whereas QCs were prepared in advance in plasma and stored as aliquots in low bind tubes at -80°C. Each assay included a blank (citrate plasma alone + 1% DMSO) and an internal standard (IS) blank (citrate plasma + 1% DMSO and DM3). Ammonium bicarbonate was prepared at a concentration of 50 mM in DI water, TCEP was prepared in 50mM ammonium bicarbonate at a concentration of 52 mM, and 25% 4-VP was prepared in acetonitrile and stored under nitrogen using a Dundee StoragePod™ (Roylan, Surrey, UK). DM3 was diluted to 0.5 µg/ml in ammonium bicarbonate on the day of analysis.

Processing samples

All plasma aliquots were thawed in the fridge and vortex mixed prior to use. On ice, plasma aliquots (25 µl) of study samples, standards or QC samples were transferred into low bind 1.5 ml centrifuge tubes containing 150 µl of 50 mM ammonium bicarbonate / 0.5 µg/ml DM3 (AB/DM3) solution. TCEP (10 µl) was added to each tube and all tubes were vortexed before being transferred to a 50°C water

bath to incubate for 30 min. Following incubation, 10 µl of a 25% 4-VP solution was added to each tube, all tubes were vortexed and incubated at room temperature for 30 min. An illustration of the reduction alkylation reaction can be seen in Figure 2. Following incubation, acetonitrile (1ml) was added to each tube containing the reaction products on ice, vortexed and centrifuged at 4°C for 5 min at 17,000 *g*. Aliquots of supernatant (900 µl) were transferred into fresh low bind 1.5 ml tubes and evaporated to residue under a stream of nitrogen. Resuspension was with 50/50 (v/v) purified water/acetonitrile (200 µl) with repeated and thorough inversion and vortex. Centrifugation for 5 min at 17,000 *g* was followed by transfer of supernatant into LC-MS autosampler vial inserts for analysis.

Chromatography conditions

A 1260 series Infinity II HPLC (Agilent, Cheshire, UK) coupled with an Agilent Eclipse Plus RRHD C18 (3mm x 50 mm, 1.8µm) column with C18 Phenomenex Security Guard (4 x 3mm) cartridge precolumn was utilised for sample separation. Mobile phase A was 0.1% formic acid and mobile phase B was 100% acetonitrile. Needle wash solution was 50/50 (v/v) water/acetonitrile. A gradient elution method was employed, starting at 100% A, changing to 100% B over 5 min before returning to starting conditions over 0.5 min. A re-conditioning time of 4.5 min was used, giving a total run time of 10 min. The flow rate was 0.3 ml/min with an injection volume of 5 µl.

Mass spectrometry conditions

An Agilent 6460 triple quad mass spectrometer was utilised for this assay. MS parameters were determined by injection-based optimisation. Positive ion mode (MS1) was used to screen for multiple charge states and adducts (Na^+, K^+, NH_3^+) of intact BT1718, Bic-VP and DM1-VP (Figure 3). The three most prominent charge states were then optimised for product ion spectra (MS2). Neither Bic-VP or intact BT1718 produced reliable mass transitions so were analysed with identical MS1 and MS2 masses. Source temperature, gas and voltage optimisation was performed by repeat injection testing with a range of settings. The instrument incorporated a JetStream source with Nitrogen for all gas supplied and utilised the following settings: Gas Temp 300°C; Gas Flow 5 L/min; Nebuliser 40psi; Sheath Gas Temp 250°C; Sheath Gas Flow 11 L/min; Capillary 3500V, VCharging 2000V.

After testing in blank matrix, under final chromatographic conditions, the best performing transition by S/N comparison was chosen as the final transition for each component. Quantification was carried out in MRM mode using the transitions of 1170.9 – 1170.9 [Intact BT1718], 1441 – 1441 [Bic-VP], 843.2 – 547.2 [DM1-VP] and 871.6 – 547.2 [DM3-VP] internal standard (Figure 4). Data acquisition was with MassHunter Acq 10 (Agilent) and data processing was carried out using MassHunter Quant 8 (Agilent).

Method optimisation

Due to the nature of the analyte, being yielded from a chemical reaction and used as a surrogate for the parent BT1718, the following parameters were investigated: BT1718 pH stability, TCEP concentration during BT1718 reduction, 4-VP concentration during alkylation, BT1718 storage solution, plastic and glass binding.

BT1718 pH stability

Intact BT1718 was prepared at a concentration of 50 µg/ml in ammonium bicarbonate buffer and subsequently diluted 1:6 in water previously adjusted with NaOH or HCl to yield the following pH values: 10, 9, neutral, 5, 4 and 3. The resulting solutions were analysed by LC-MS/MS to determine the amount of BT1718 present.

TCEP reduction optimisation

Intact BT1718 was reduced with TCEP in at the following concentrations in triplicate; 26, 52, 104, 208, 260 and 650 mM and reduced with 25% VP. The yield of DM1-VP and loss of intact BT1718 signal measured by LC-MS/MS response was used to determine the concentration of TCEP that yielded the most complete and reproducible reduction of BT1718.

4-VP alkylation optimisation

Intact BT1718 was reduced with TCEP at 52 mM and alkylated in triplicate at 10, 25, 50 and 75% 4-VP. The yield of DM1-VP and loss of intact BT1718 and free DM1 signal measured by LC-MS/MS response was used to determine the concentration of 4-VP that yielded the most complete alkylation of cleaved DM1.

BT1718 and DM1-VP solvent stability

Intact BT1718 was prepared at concentrations of 5, 50 and 500 µg/ml dissolved in the following solvents: 100% DMSO; 1:1(*v/v*) DMSO/acetonitrile; 1:1:1(*v/v*) DMSO/acetonitrile/water; 1:1(*v/v*) acetonitrile/water. The yield of DM1-VP after carrying out the assay from each of these solvent mixtures was determined by LC-MS/MS.

Binding

Six replicates of intact BT1718 were placed in 2 ml glass HPLC vials or 1.5 ml plastic Eppendorf tubes and then analysed by LC-MS/MS to determine loss through binding. Six replicates of reaction mixture containing DM1-VP and Bic-VP were placed in 2ml glass HPLC vials or 1.5ml plastic Eppendorf tubes and then analysed by LC-MS/MS to determine loss through binding.

Method validation

Validation of the method was carried out to European Medicine Agency (EMA) and US Food and Drug Administration (FDA) guidelines [18, 19]. Validation was only carried out for reaction components DM1-VP with IS DM3-VP, although other reaction components were evaluated when assessing the efficiency of the reduction/alkylation reaction. Parameters validated included recovery, selectivity, matrix effect, anticoagulant comparison, lower limit of quantitation (LLOQ), linearity and range, intra- and inter-day precision and accuracy, dilutional integrity, carryover, short- and long-term stability, freeze-thaw stability and a partial investigation into incurred sample reproducibility (ISR).

Recovery

The percentage recovery of DM1-VP was determined using four QC concentrations (200, 400, 4000, and 40,000 ng/ml in triplicate), of BT1718 in human plasma reacted to yield DM1-VP, containing 500 ng/ml DM3 internal standard. The peak area for DM1-VP extracted from human plasma was compared with the peak area of DM1-VP yielded from BT1718 in buffer at matching concentrations in triplicate. From this data the absolute recovery was calculated. The coefficient of variation (CV) was required to be within 15%.

Selectivity and matrix effect

Six independent sources of blank plasma matrix were analysed to detect any response with similar retention time to the analyte and IS. Any response with similar retention time to the analyte was required to be ≤ 20% of the response for the lowest calibration standard. Any response with a similar retention time to the IS was required to be ≤ 5% of the response for the IS peak. Six independent sources of blank plasma were investigated for DM1-VP and DM3-VP internal standard by calculating the ratio of the peak area of DM1-VP in the presence of matrix (measured by analysing blank matrix spiked after extraction with analyte) to the peak area in absence of matrix (pure solution of the analyte) at LQC and HQC concentrations of BT1718. The CV of the IS-normalized matrix effect was required to be within 15%.

Anticoagulant comparison

The effect of different anticoagulants was determined by analysing three replicates at LQC and HQC concentrations of DM1-VP. QC samples were prepared using blank plasma generated using three different anticoagulants: sodium citrate, potassium EDTA (K2 EDTA) and lithium heparin. The CV was required to be ≤ 15% and accuracy was required to be within 85-115%.

Lower limit of quantification

The LLOQ was determined by establishing the lowest concentration of BT1718 which yielded a DM1-VP peak height with a S/N ratio ≥ 10, with precision of ≤20% and accuracy within 80-120% of the

nominal value. This was carried out by analysing five replicates of BT1718 reacted to form DM1-VP at the defined concentration.

Linearity, Range and Weighting

The range was investigated by spiking plasma samples with BT1718 and reacting to yield DM1-VP, in duplicate during four separate analytical runs. The ratio of DM1-VP peak area to IS was calculated for each standard and plotted against the nominal standard of drug. Linearity was determined by regression analysis. A comparison of different mathematical curve weightings was made; $1/y$, $1/y^2$, $1/x$, $1/x^2$ and no weighting. Goodness of fit was determined with Pearson's determination coefficient R^2 with a requirement of an R^2 value of >0.99. A comparison of true and back calculated concentrations of standards was carried out, where back calculated values were required to be within 80-120% of the theoretical concentration. At least 75% of standards were required to meet these criteria.

Intra/Inter-assay precision and accuracy

BT1718 was spiked into blank human plasma at 4 concentrations (200, 400, 4000, and 40,000 ng/ml) along with 1000 ng/ml DM3 (IS) to produce 5 replicates of each. Each replicate was reacted to yield DM1-VP. The ratio of DM1-VP peak area to IS was calculated for each replicate. Accuracy was determined by expressing the mean calculated concentration as a percentage of the nominal concentration. Precision was determined by calculating the CV of the five replicates. The CV was required to be ≤ 15% for all concentrations (≤ 20% for the LLOQ) and the accuracy was required to be within 85-115% of the nominal value (80-120% for the LLOQ). Four separate analytical runs containing triplicates of QC samples reacted to yield DM1-VP at three concentrations on different days, were assayed to assess the inter-day precision in control human plasma. Individual measured values were required to have a precision of ≤ 15%, except the LLOQ which was permitted ≤ 20%, and an accuracy within 80-120% of the nominal value. The inter-day accuracy was calculated by the mean values for each concentration, across the five analytical runs. The inter-day precision was calculated by the CV of the mean values for each concentration, across the four analytical runs.

Dilution integrity and carryover

A 40,000 ng/ml solution of BT1718 was prepared in human plasma and diluted 1 in 100 with control plasma to give a 400 ng/ml sample. Five replicates of each concentration were reacted to form DM1-VP. The peak area for DM1-VP at 400 ng/mL was multiplied by 100 and this value was required to be within ±15% of the value obtained from analysis of the 40,000 ng/ml DM1-VP peak area. Carryover of the analyte and IS was evaluated by placing a control plasma sample without analyte or IS directly after the highest calibration standard. Response for analyte in the carry over sample was required to

be ≤20% of the response observed for the LLOQ. The response for the IS in the carry over sample was required to be ≤5% of the response for the control matrix + IS.

Stability

The short term stability of DM1-VP yielded from BT1718 was assessed by analysing QC samples of BT1718 at 400, 4000, and 40,000 ng/ml in triplicate that had been reacted to form DM1-VP, with measurements repeated after 24, 48 and 108 hours following storage of samples at 8°C in the HPLC autosampler during this period. The observed responses were required to be within 15% of the nominal concentrations. The long term stability of BT1718 in plasma was assessed by analysing QC samples in triplicate following storage at -80°C over a period of 10 months. In all cases, QC samples were analysed against a calibration curve generated from freshly spiked standards, reacted to form DM1-VP and concentrations determined against nominal standard concentrations. Precision was determined by calculating the CV of the replicates and was required to be ≤ 15% for all concentrations. The accuracy was required to be within 85-115% (80-120% LLOQ) of the nominal value.

Freeze thaw stability

Three freeze thaw cycles from -80°C to room temperature (with 24 hours between each cycle) of parent stock BT1718 at 5mg/ml in DMSO were carried out before reaction to yield DM1-VP and the response of DM1-VP measured. The accuracy was required to be within 85-115% of the nominal value. QC samples at 400, 4000, and 40,000 ng/ml in triplicate of BT1718 in human plasma were taken through three freeze thaw cycles from -80°C to room temperature. Aliquots were thawed at 8°C and there was a minimum of 24 hours between each cycle. Reaction to yield DM1-VP was then performed and the response of DM1-VP measured for all aliquots. QC samples were analysed against a calibration curve generated from freshly spiked standards, reacted to form DM1-VP and concentrations determined against nominal standard concentrations. Precision was determined by calculating the CV of the replicates and was required to be ≤ 15% for all concentrations. The accuracy was required to be within 85-115% of the nominal value.

Incurred sample reproducibility (ISR)

The ISR was investigated by repeat analysis of clinical samples, but this was limited by sample numbers accessible during an early phase clinical trial. Re-analysis of patient samples on separate runs, with the longest gap between runs of 10 months was used to determine analytical variability. The percentage difference was calculated according to EMA guidelines on bioanalytical method validation[15]. The percentage difference between the concentrations measured for ISR was required to be no greater than 20% of their mean, for at least 67% of the repeats.

Patient Sample Analysis

Blood samples for pharmacokinetic analysis were obtained from patients receiving BT1718 as part of an ongoing Phase I/IIa clinical trial (ClinicalTrials.gov identifier: NCT03486730). Blood samples were collected at the following time points: pre-dose, 20 and 50 mins post start of BT1718 infusion, end of infusion, 10, 20, and 40 mins post end of infusion and 1, 2, 3, 6, and 24 hours post end of infusion (for cycle 1). Samples were collected in EDTA tubes, immediately centrifuged at 1200 *g* for 5 minutes at 4°C and the plasma obtained stored at -80°C prior to analysis. Results were generated using Agilent Mass Hunter Software and further analysed using Phoenix WinNonlin version 8.1 (Certara, St Louis, USA).

Results

BT1718 pH stability

BT1718 stability in solutions of pH 10, 9, neutral, 5, 4 and 3 was assessed. Analysis by LC-MS/MS indicated that BT1718 was most stable at pH 10, with pH 9 resulting in a 20% loss compared to pH 10. Loss of BT1718 followed in a linear fashion with decreasing pH, with 75% of BT1718 lost at pH 5, and 95% lost at pH 3 relative to the signal at pH 10.

TCEP reduction optimisation

TCEP concentrations of 26, 52, 104, 208, 260 and 650 mM were assessed to determine optimal conditions for BT1718 reduction. Optimal TCEP concentration to yield maximal reduction of BT1718 to DM1 was determined at 208 mM, but there was minimal variation between 52 mM and 260 mM concentrations. Concentrations of 26 mM and 650 mM TCEP both produced DM1 with less than 50% efficiency of the production achieved by concentrations ranging from 52-260 mM.

4-VP alkylation optimisation

Optimal 4-VP concentration was determined by comparing alkylation solutions of 10, 25, 50 and 75% 4-VP. A 25% solution of 4-VP produced the largest yield of DM1-VP, but there was no significant difference between the concentrations tested. A 25% 4-VP solution was selected for use in the assay as reduced variability was observed at this concentration.

BT1718 and DM1-VP solvent stability

Intact BT1718 was prepared at concentrations of 5, 50 and 500 µg/mL dissolved in various solvents as described. The yield of DM1-VP was highest and most reproducible for all concentrations using a 1:1(*v/v*) DMSO/acetonitrile solution for BT1718. The lowest response by LC-MS/MS was with 1:1(*v/v*) acetonitrile/water.

Binding

The potential loss of parent BT1718 and the reaction products through physical binding was investigated using glass HPLC vials and plastic Eppendorf tubes. For both intact BT1718 and the yielded reaction products, plastic produced the lowest loss through binding and therefore Eppendorf tubes were used for all assay validation work.

Recovery

Recovery was determined in triplicate using four QC concentrations by the comparison of peak areas of DM1-VP in spiked plasma following reaction and extraction, with peak areas of DM1-VP in buffer and reacted but unextracted. Recovery percentages were 96, 98, 99 and 92%, at concentrations of

200, 400, 4000 and 40,000 ng/ml respectively, with a recovery of 112% for the IS. All data were generated in an expected analytical range and with no significant variation in peak areas (1-7%).

Selectivity and matrix effect

Selectivity was investigated with six different sources of human plasma. Responses with similar retention times were less than 5% of the signal for DM1-VP generated by the LLOQ and less than 0.5% of the signal generated for DM3-VP IS across all six batches. The effect of matrix was evaluated with six different batches of human plasma at LQC and HQC concentrations by calculating the ratio of peak area response of DM1-VP in the presence and absence of matrix. IS normalised matrix effect was within acceptable limits at 13% and 6% for LQC and HQC respectively.

Anticoagulant comparison

The potential effect of the anticoagulant used to generate plasma for analysis was investigated using three replicates at both LQC and HQC concentrations. The anticoagulants evaluated were sodium citrate, potassium EDTA and lithium heparin. No effect of anticoagulant used was observed, with a CV of ≤15% and accuracy of 95-111%.

Lower limit of quantification

The LLOQ was defined at 200ng/ml with precision of 1.2% and accuracy of 107% after analysing five replicates of BT1718 reacted to form DM1-VP. The S/N of the response was ≥ 10 for all replicates. Figure 5 shows a chromatogram obtained following analysis of the LLOQ, alongside a blank sample, a standard concentration of 2,500 ng/ml and a patient sample.

Linearity, Range and Weighting

Linearity was evaluated with concentration range of BT1718 from 200 – 50,000 ng/ml with a linear correlation of ≥0.98. The calibration curve was typically described by the linear equation y=0.069x-0.005661 with a weighting of $1/x^2$ yielding the best linear response. Table 1 shows linearity and range data determined over seven separate analytical runs.

Intraday and Interday precision and accuracy

The intra-assay study at four concentrations of BT1718 reacted to form DM1-VP showed precision of ≤5% and accuracy ranging from 91-107% (n=4). The inter-assay study, carried out over four separate analytical days, showed precision of ≤10.2% and accuracy ranging from 81-107% as shown in Table 2.

Dilution integrity and carryover

A 40,000 ng/ml solution of BT1718 was prepared in five replicates in human plasma and diluted 1 in 100 with plasma control to give a 400 ng/ml sample. The dilution accounted value matched the

original undiluted response with an accuracy of 92%. Carryover response in blank matrix following injection of highest calibration standard was 0.03%.

Stability

BT1718 stock solutions in DMSO were determined to be stable over three freeze thaw cycles with an accuracy of 92-100%. Triplicate samples of BT1718 in human plasma at LQC, MQC and HQC concentrations were subjected to three freeze thaw cycles before being reacted to form DM1-VP. Stability was demonstrated in these samples with an accuracy of 88-115%. The short-term stability of DM1-VP yielded from BT1718 was assessed by analysing QC samples of BT1718 at 400, 4000 and 40,000 ng/ml in triplicate. Stability was demonstrated in these samples over 24 hours at 8°C with an accuracy of 98-113%, over 48 hours at 8°C with an accuracy of 92-105%. And over 108 hours with an accuracy of 95-105%. Long-term stability of BT1718 in human plasma at LQC, MQC and HQC concentrations at -80°C was assessed over a period of 14 months and demonstrated an accuracy of 105-112% (LLOQ 118%).

Incurred sample reproducibility

The ISR was investigated by re-analysis of patient samples on separate runs to determine any analytical variability. The calculated percentage difference between initial concentration and repeat measured concentration was ≤17% for 100% of repeated patient samples.

Patient Sample Analysis

Analysis of plasma samples collected from patients receiving BT1718 once weekly indicated that the validated assay could be utilised to quantify DM1 derived from BT1718 in clinical trial samples. Figure 6 shows plasma concentration versus time curves for two patients receiving doses of 9.6mg/m^2 and 20mg/m^2. Total DM1 generated from BT1718 was quantified over a 24 h period following intravenous drug administration over 60 min.

Discussion and Conclusion

A quantitative plasma assay for DM1 derived from BT1718, has been validated for use in a clinically relevant concentration range in patients currently participating in an early-phase trial of this novel agent. The method developed involved the generation of DM1-VP from parent drug in a reproducible and robust manner. The nature of bicyclic peptides makes their direct analysis challenging, and a more extensive programme of work was required than would be required of a small-molecule bioanalytical method. The assay required careful preparation of samples, with samples thawed in a fridge and prepared on ice to ensure the required level of reproducibility. Through mixing at all steps using vortex and inversion was also found to be essential.

Investigation of potential co-administered drugs was not carried out as part of this validation. Due to the novel nature of the parent compound and the reaction required to yield the analytical component DM1-VP, it was felt unlikely that a compound of similar chemical structure and MS profile would interfere with the assay. Future experiments to investigate specific co-administered drugs may be appropriate, but the large number of possible compounds co-administered to late stage cancer patients entering a phase I clinical trial makes selection of representative concomitant medications difficult at this stage.

The first in human study of BT1718 is currently ongoing. The trial includes adult patients with advanced solid tumours refractory to all other appropriate standard care options, comprising dose escalation and expansion stages to determine the recommended phase 2 regimen and dose and preliminary efficacy of BT1718. The assay described is in use to generate pharmacokinetic data for total DM1 generated from BT1718 in this study, and will allow determination of circulating concentrations of DM1-related material for comparison with parent BT1718 concentrations. Results from the clinical trial will be published in due course.

Future perspective

The past decade has seen an increasing array of classes of novel anticancer drugs being developed, including small molecule inhibitors, monoclonal antibodies, microRNA drugs and antibody-drug conjugates. In light of these advances, the development of methods for the quantification of drug levels in early phase clinical trials becomes increasingly challenging and open to interpretation, in terms of the most appropriate moieties or species to be measured. Moving forwards it is important that novel method development continues apace, in order to ensure the generation of meaningful clinical pharmacology data from early phase clinical trials.

Executive Summary

Background

• a LC-MS/MS method was developed to quantify DM1 generated from BT1718, a novel bicyclic peptide anticancer drug, in human plasma.

Experimental

• The method incorporates a reduction reaction to cleave BT1718 into DM1 and its bicycle components, an alkylation step to stabilise the reaction products, and protein extraction with acetonitrile.

Results and discussion

• A novel assay was developed according to EMA and FDA guidelines for bioanalytical method validation, over a linear range of 200-50,000 ng/mL BT1718.

• The method is being used to successfully quantify DM1 yielded from BT1718 in clinical samples obtained from patients recruited to an ongoing phase I trial.

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TABLES

Table 1. Linearity and Range for calibration curves of DM1-VP yielded from BT1718 in human plasma.

	BT1718 plasma concentrations (ng/mL)						
Day	**200**	**300**	**500**	**1000**	**5000**	**10000**	**50000**
1	181	272	446	944	5401	11654	55465
2	171	258	462	1002	5526	11110	51702
3	174	296	517	1087	5485	10449	40627
4	177	361	462	1002	5526	11110	51785
5	197	284	526	1009	5384	10435	44721
6	220	296	472	1025	5219	10829	45281
7	188	260	424	911	5319	11606	60018
Mean (n=7)	186.9	289.6	472.7	997.1	5408.6	11027.6	49942.7
SD	17.1	35.1	36.8	56.7	114.0	494.3	6761.1
Accuracy (%)	93.4	96.5	94.5	99.7	108.2	110.3	99.9
Precision (%)	12.5	4.35	4.08	2.91	4.90	5.68	16.29

SD: Standard deviation

Table 2. Intra-/interday precision and accuracy of the analytical method of DM1-VP yielded from BT1718 in human plasma (LLOQ, LQC, MQC, HQC).

Sample	Day	BT1718 concentration (ng/mL)		Accuracy (%)	Precision (%)	BT1718 concentration (ng/mL)		Accuracy (%)	Precision (%)	RE (%)
		Mean	SD			Mean	SD			
LLOQ	1	214	2.5	107	1.2					
	2	208	19	104	9.1					
	3	194	79.7	97	10.2					
	4	203	7.1	101	3.5					
	1-4					204.8	8.5	102.4	4.1	2.4
LQC	1	381	18.9	95	5					
	2	360	13.5	90	3.7					
	3	326	1.5	81	0.5					
	4	361	13.5	90	3.7					
	1-4					357.0	22.8	89.3	6.4	-10.8
MQC	1	3642	102	91	2.8					
	2	3481	57.1	87	1.6					
	3	3753	23	94	0.6					
	4	3713	48.7	93	1.3					
	1-4					3647.3	120	91.2	3.3	-8.8
HQC	1	38172	981	95	2.6					
	2	36572	591	91	1.6					
	3	41387	959	104	2.3					
	4	33023	782	83	2.4					
	1-4					37289	3478	93.2	9.3	-6.8

% RE = ((measured value – nominal value)/nominal value)) X 100

Figure Legends

Figure 1. Structure of BT1718.

Figure 2. Reaction mechanism for BT1718 to its components.

Figure 3. Mass spectrum of BT1718 (top) and BT1718 components (bottom).

Figure 4. Chromatogram of Bic-VP (blue), DM1-VP (red) and internal standard DM3-VP (green).

Figure 5. Chromatograms obtained following analysis of a blank sample (A), a patient sample (B), the LLOQ (C) and a standard concentration of 2,500 ng/ml BT1718.

Figure 6. Plasma concentration-versus-time profile of DM1 derived from BT1718 in two patients following administration of BT1718 (9.6 mg/m^2 and 20 mg/m^2 once weekly dose levels).

Figure 1

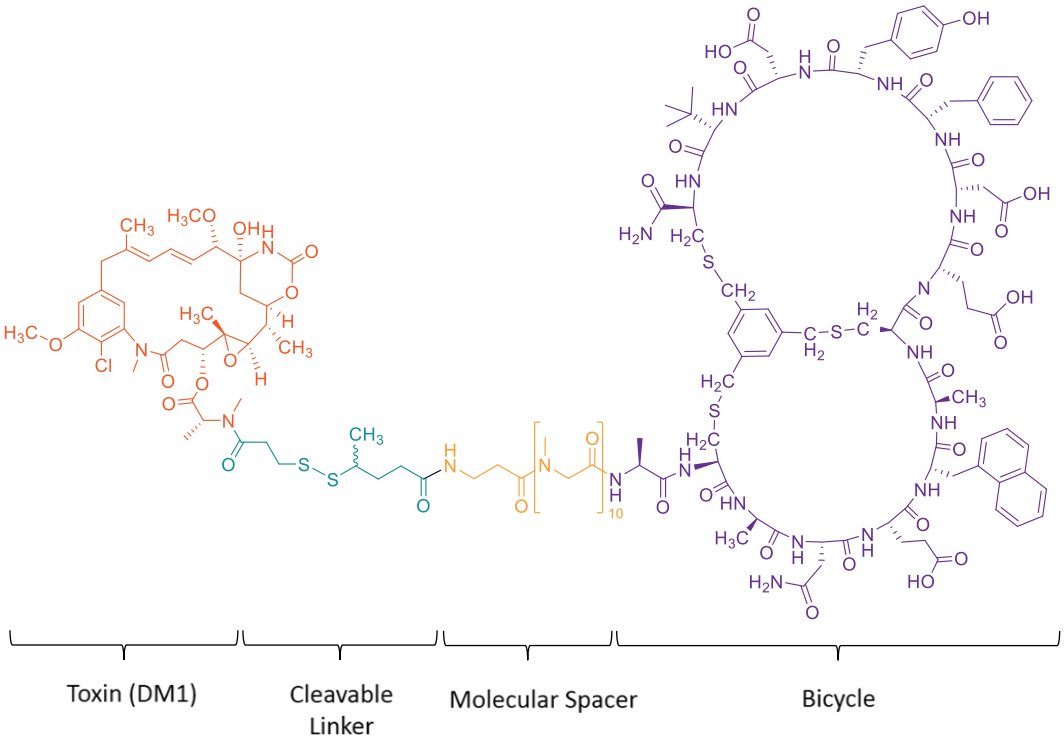


Figure 2

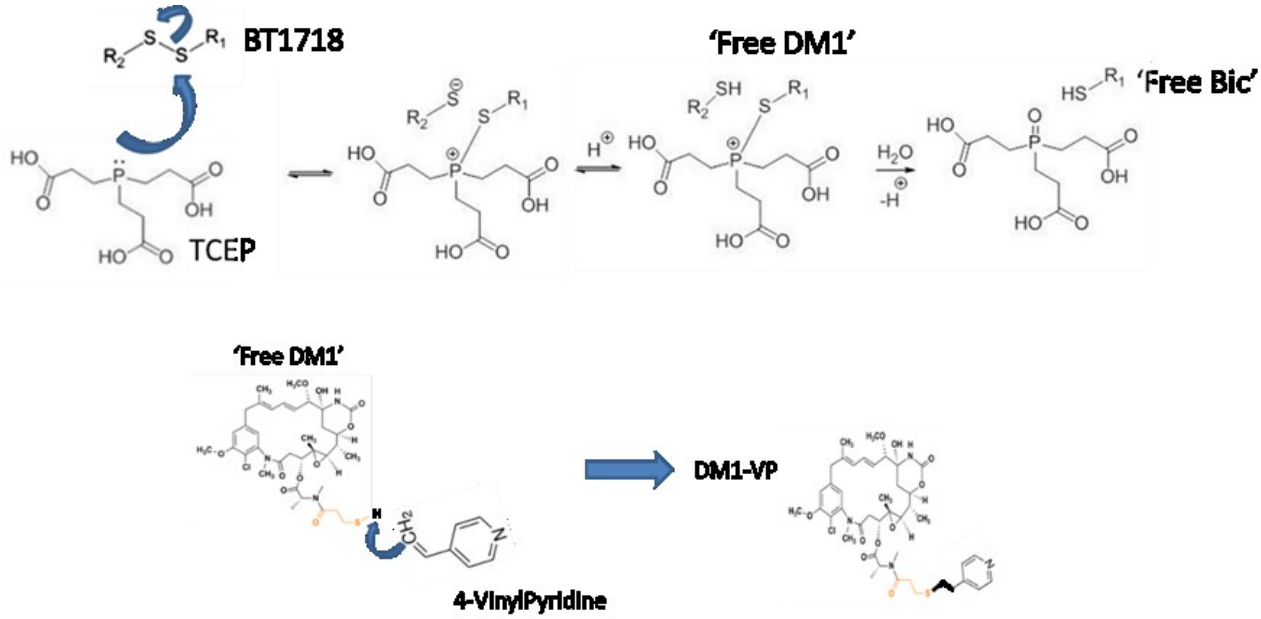

BT1718
TCEP
'Free DM1'
'Free Bic'
'Free DM1'
4-VinylPyridine
DM1-VP

Figure 3

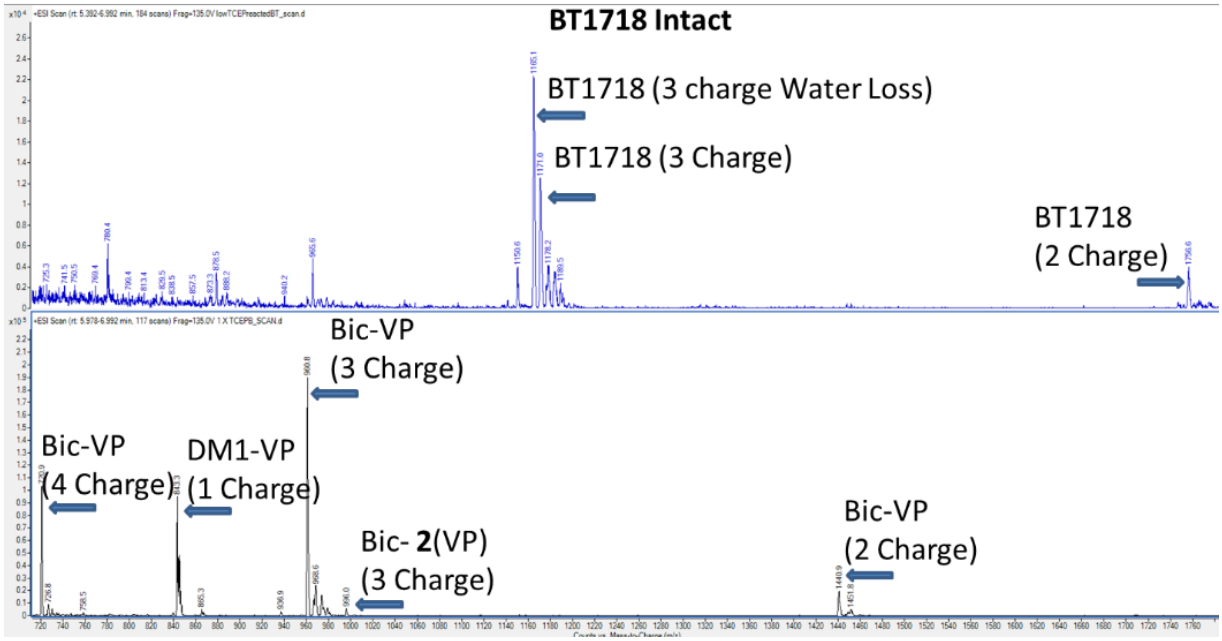

BT1718 Intact
BT1718 (3 charge Water Loss)
BT1718 (3 Charge)
BT1718
(2 Charge)
Bic-VP
(3 Charge)
Bic-VP
(4 Charge)
DM1-VP
(1 Charge)
Bic- 2(VP)
(3 Charge)
Bic-VP
(2 Charge)

Figure 4

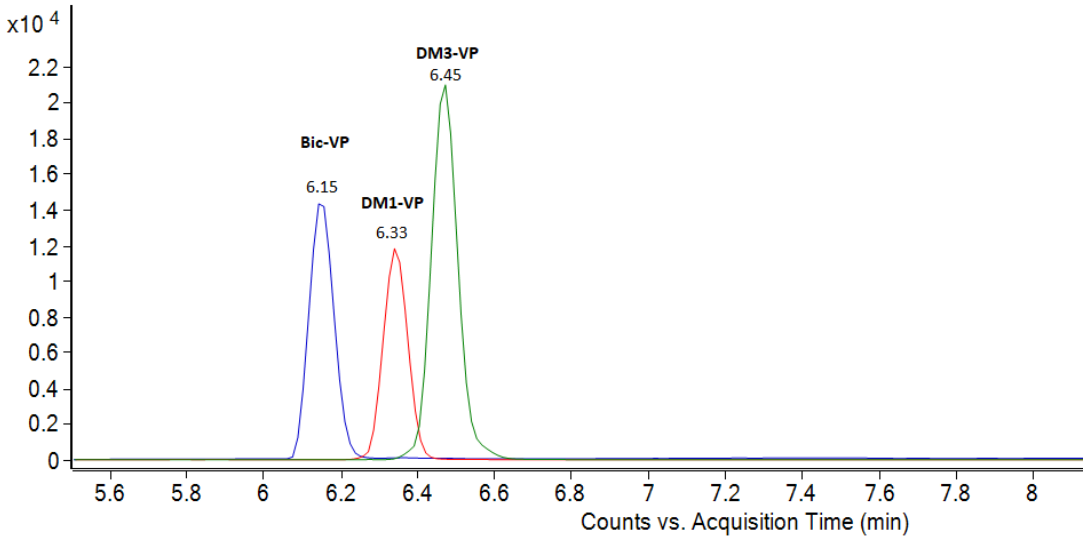
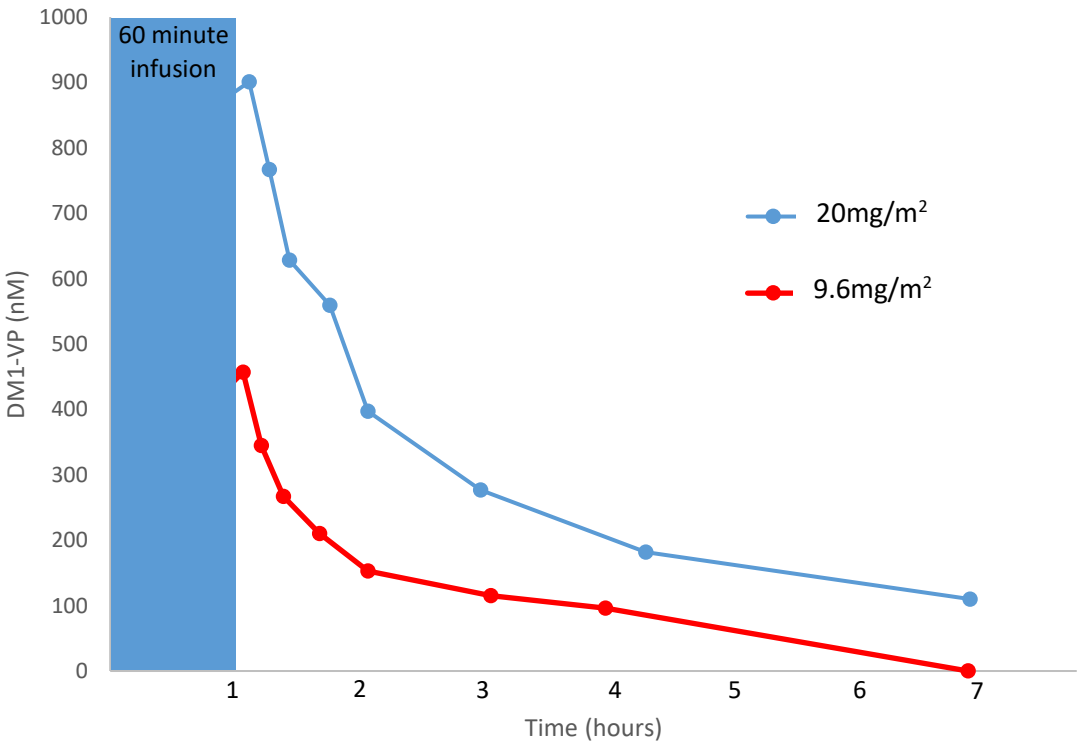

x10 4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Bic-VP
6.15
DM1-VP
6.33
DM3-VP
6.45
5.6
5.8
6
6.2
6.4
6.6
6.8
7
7.2
7.4
7.6
7.8
8
Counts vs. Acquisition Time (min)

Figure 5


DM1-VP: +ESI MRM Frag=200.0V CID@32.0 (843.2 -> 547.2) 190402 Blank a.d
A
DM1-VP: +ESI MRM Frag=200.0V CID@32.0 (843.2 -> 547.2) 190402 Pt 16,024 C1St2 20 min post start.d
B
DM1-VP: +ESI MRM Frag=200.0V CID@32.0 (843.2 -> 547.2) 190402 LLOQ 0.2 old a.d
C
DM1-VP: +ESI MRM Frag=200.0V CID@32.0 (843.2 -> 547.2) 190402 standard 2.5 a.d
D
Counts vs. Acquisition Time (min)

Figure 6


60 minute infusion
1000
900
800
700
600
500
400
300
200
100
0
DM1-VP (nM)
1
2
3
4
5
6
7
Time (hours)
20mg/m²
9.6mg/m²