Confident transformation site localization of PROTAC drug metabolites facilitated by multi-stage fragmentation LC-HRAM-MS

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Abstract

Purpose: Confident characterization of PROTAC drug metabolites for transformation site localization and soft-spot analysis.

Methods: Drug metabolites were analyzed using MS^n fragmentation data acquisition on a Thermo Scientific™ Orbitrap™ Ascend Biopharma Tribrid™ mass spectrometer and data interpretation in Thermo Scientific™ Compound Discoverer™ 3.3 SP3 software.

Results: PROTAC drug metabolism was found to be extensive for the investigated compounds. Among the detected metabolites, several isomeric compounds were found where MS^n fragmentation data could be used to reduce ambiguity in the transformation site localization, especially for metabolites with higher molecular weights.

Introduction

The study of a drug's metabolism is an integral part of drug discovery and development, from improving drug exposure to flagging potential toxicity from its metabolites. The identification of drug metabolites routinely relies on LC/MS experiments, with fragmentation data enabling their structure elucidation. In recent years, hetero-bifunctional small molecule drugs, acting on a given protein of interest (POI) through recruitment of inherent protein degradation pathways rather than inhibition, have gained significant interest as new modalities, with multiple such proteolysis targeting chimera (PROTAC™) drug candidates being investigated in clinical trials.[1] However, due to PROTAC compounds' unique properties, the identification of their metabolites can be challenging.[2]

Here, we describe the utility of multi-stage fragmentation (MS^n) for the elucidation of transformation sites on PROTAC drug metabolites, based on analysis of the representative compounds shown in Figure 2.

Materials and methods

Two commercially available PROTAC compounds, namely MZ1 and dBET1 (obtained from MedChemExpress), were dissolved in DMSO and incubated at 5 µM with Human liver S9 fraction (1 mg/mL in 100 mM phosphate buffer) in the presence of NADPH (1 mM) for 0 and 4 hrs at 37 °C, before terminating enzymatic reactions with the addition of 1 equivalent of ice-cold acetonitrile. All metabolism samples and matrix blank incubations were then centrifuged at 5,000 rpm for 5 minutes before transferring the supernatant into glass autosampler vials for analysis using LC/MS.

LC separation was carried out using a Thermo Scientific™ Vanquish™ Horizon UHPLC system and a Thermo Scientific™ Accucore™ C18 column (2.1 x 100 mm, 2.6 µm) at a flow rate of 0.4 mL/min. The eluents used were (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile, employing the gradient separation detailed in Table 1. Mass spectral data were acquired in positive mode, with fragmentation data acquired using the Thermo Scientific™ AcquireX™ Background Exclusion workflow on a Orbitrap Ascend BioPharma Tribrid mass spectrometer. The MS method parameters and the MS^n decision tree are detailed in Table 2 and Figure 3.

The LC/MS data were subsequently analyzed using the Metabolite Identification workflow in Thermo Scientific Compound Discoverer 3.3 SP3 software using combined targeted and untargeted peak detection.

Table 1. LC gradient conditions

Time (min)	Mobile Phase B (%)
0.0	5
1.0	5
10.0	95
15.0	95
15.1	5
20.0	5

Figure 1. UHPLC-HRAM-MS setup comprised of a Vanquish Horizon UHPLC system and Orbitrap Ascend Biopharma Tribrid MS

Results

PROTAC drugs - chemical structure considerations for metabolism

Among the many different PROTAC drug candidates that have been developed and disclosed to date, a common theme is their general structure containing two protein-binding motifs for the protein of interest and a ligase for the given degradation pathway (e.g., E3 ubiquitin ligase) as well as a linker. Figure 2 shows the structure of the two compounds investigated here.

As in the case of MZ1 and dBET1, PROTAC compounds' molecular weight typically exceeds 500 Da, which can create challenges for delivery and bioavailability. As highlighted by Goracci *et al.*, their metabolism is also not readily predicted from their substructures and the choice of linker can have a profound impact on the extend of metabolites formed.[2]

Figure 2. Chemical structures of the two investigated PROTAC compounds

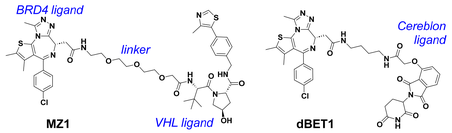
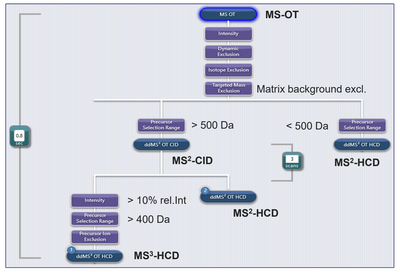


Table 2. MS Method parameter overview

ESI(+) ddMS^n TopSpeed experiment	
MS1 mass range	*m/z* 150–1500
Lock mass correction	RunStart Easy-IC™
MS^1/MS^2 resolution	60,000/15,000 @ *m/z* 200
TopSpeed cycle time	0.8 s
Precursor filters	>2e5 Intensity 4 s dynamic Exclusion **AcquireX** Background Exclusion Precursor mass selection
MS^n decision tree setup	<500 Da: MS^2-HCD only >500 Da: MS^2-CID + MS^3-HCD / MS^2-HCD MS^3 criteria: >400 Da and >10% (up to 3)

Figure 3. Experiment decision tree used for MetID on the Orbitrap Ascend MS



Flexible MS data acquisition method for elucidation of drug metabolites

The analysis of the metabolism of MZ1 and dBET1 from incubations with Human liver S9 fractions was carried out using an Orbitrap Ascend MS with data-dependent acquisition that followed the decision tree structure detailed in Table 2 and Figure 3. Data acquisition was thus adapted on-the-fly depending on the precursor masses and obtained fragmentation spectra at a given moment, to maximize the relevant data and enable structure elucidation. Figure 4 shows an example of this based on the parent MZ1 and one of its metabolites detected in the 4h timepoint.

Figure 4. a) Total ion chromatogram overlays of 0h and 4h timepoints for MZ1 and matrix blank, showing the decrease in parent and appearance of multiple metabolite peaks; b) MS1 spectra of MZ1 and one of its metabolites (M10), as well as the fragmentation data acquired based on the respective precursor *m/z* and observed MS^2 fragments.

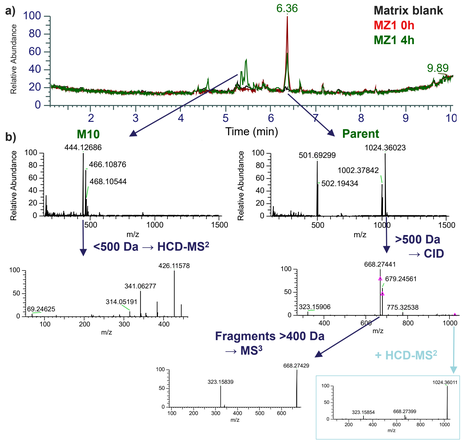
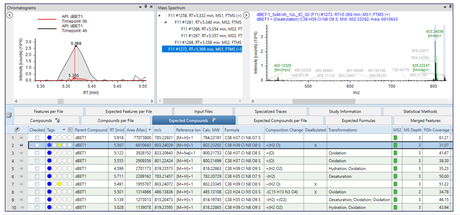


Figure 5. Identification of dBET1 transformation products (based on expected Phase I+II metabolism and dealkylation reactions) in Compound Discoverer software

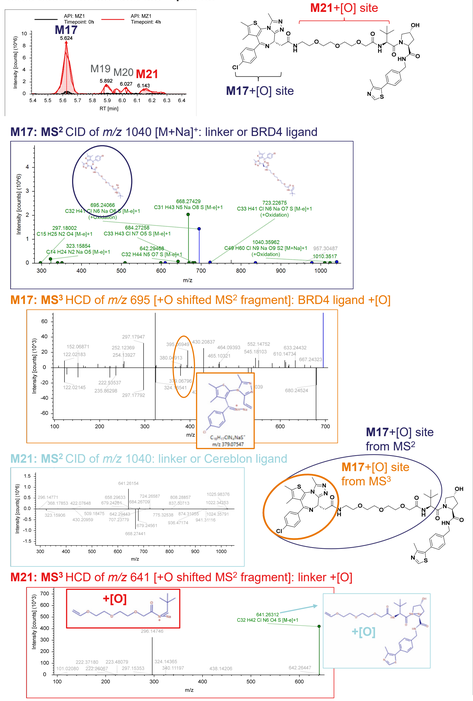


Using MS^n data for precise localization of oxidative transformation products

The 'Expected Compounds' node in Compound Discoverer software could be used to reveal and identify metabolites from MZ1 and dBET1 incubations based on known Phase I+II transformations as well as dealkylation reactions (Figure 5).

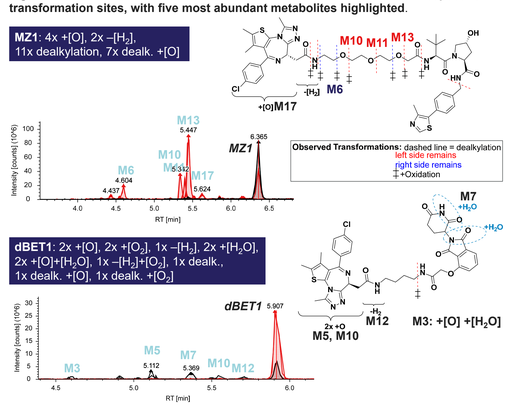
In addition to general information on which transformations occurred, information on transformation sites is important for optimization of lead candidate structures. Figure 6 below details the investigation of two isomeric oxidation metabolites detected for MZ1. The automatically acquired MS^3 fragmentation spectra more precisely localize the respective oxidation sites than what was possible just from the MS^2 spectra.

Figure 6. Structure elucidation of two [+O] metabolites of MZ1 detected in the liver S9 incubation at 4h to allow determination of their transformation sites based on MS^2 and MS^3 spectra.



In total, 24 and 13 metabolites were found and identified above a threshold of 1% relative MS peak area (compared to the parent compounds MZ1 and dBET1, respectively, at t=0h). In the case of the ethyleneglycol linker containing MZ1, metabolism leading to cleavage across the linker was very abundant, while the linker in dBET1 was found to be more resistant to metabolism in liver S9 fraction. Figure 7 gives an overview of all metabolites and their transformation site localization (confirmed with their fragmentation spectra).

Figure 7. Overview of detected metabolites for MZ1 and dBET1 and their respective transformation sites, with five most abundant metabolites highlighted.



Conclusions

- The utility of flexible MS data acquisition methods for metabolite identification could be demonstrated with two representative PROTAC compounds, MZ1 and dBET1, which exhibit extensive metabolism *in vitro*.
- MS^n fragmentation data helped to enhance the precision of transformation site localization, particularly for isomeric metabolites and those with high molecular weights.

References

1. Z. Hu, *et al.* "Recent Developments in PROTAC-mediated Protein Degradation: From Bench to Clinic" *Chembiochem* **2022**, *23*, e202100270. doi:10.1002/cbic.202100270
2. L. Goracci, *et al.* "Understanding the Metabolism of Proteolysis Targeting Chimeras (PROTACs): The Next Step toward Pharmaceutical Applications" *J. Med. Chem.* **2020**, *63*, 11615. doi: 10.1021/acs.jmedchem.0c00793

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