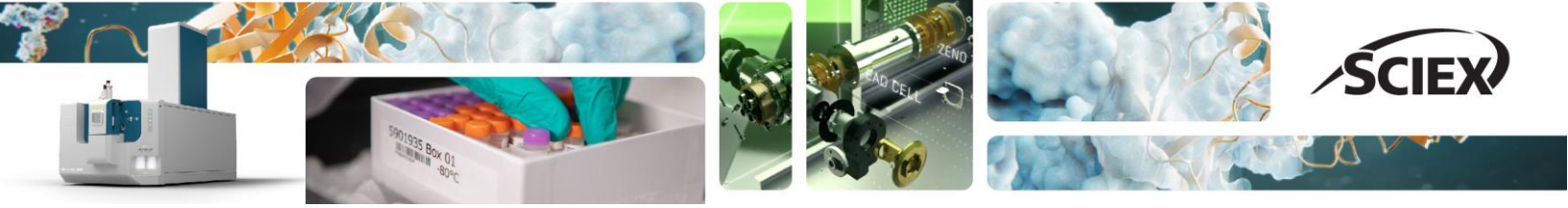

Automated workflow for targeted protein degrader (TPD) metabolite identification using collision-induced dissociation (CID) and electron-activated dissociation (EAD)

Ebru Selen, Rahul Baghla and Eshani Galermo
SCIEX, USA

This technical note demonstrates a software-assisted TPD metabolite identification workflow using the ZenoTOF 7600 system and Molecule Profiler software (**Figure 1**). This approach enables users to achieve increased confidence in metabolite structure assignments by utilizing the more information-rich EAD spectra generated using the ZenoTOF 7600 system.[1]

Drug metabolites contribute to efficacy, toxicity, and drug-drug interactions. Accurate identification and monitoring of drug metabolites is critical to ensure drug safety. LC-MS platforms are preferred in metabolite identification studies due to the selectivity and sensitivity they offer in the detection of unknown metabolites. An advanced and streamlined metabolite identification workflow with the ZenoTOF 7600 system and Molecule Profiler software enables the generation of informative data in a fast and intuitive manner, accelerating the early drug discovery process.

Key benefits for identification of TPD metabolites using the ZenoTOF 7600 system and Molecule Profiler software

- **Enhanced structure assignment:** EAD provides information-rich MS/MS spectra that enable the identification of possible TPD metabolites when compared to CID
- **Quick data processing:** Perform quick and efficient software-aided identification of drug metabolites using the Molecule Profiler software with the ZenoTOF 7600 system[1,2]
- **Streamlined workflow:** Develop confident structure-metabolic stability relationships for drugs utilizing an end-to-end workflow from data acquisition to analysis

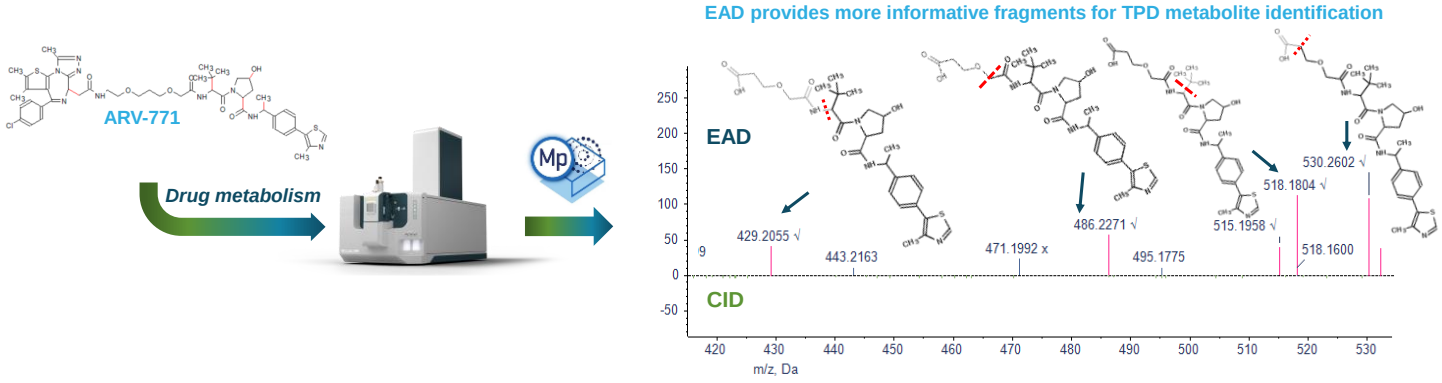

Figure 1: Metabolite identification workflow using ARV 771. ARV 771 was incubated in hepatocytes for in vitro drug metabolism. Metabolites were detected from EAD and CID data collected on the ZenoTOF 7600 system. The Molecule Profiler software aided in the identification of the biotransformation of ARV 771. An automated structure assignment provides high-confidence detection of drug metabolites and a quick and easy-to-use workflow experience for the users. EAD spectra provided more informative and unique fragments for the identification of metabolites from ARV 771.

Introduction

In the early stages of drug discovery, in vitro assays are utilized to estimate metabolic clearance rates and identify metabolic soft spots. LC-MS tools are frequently employed to conduct these studies because they can provide quantitative and qualitative data with ample sensitivity, especially when detecting unknown metabolites.

Drug metabolite analysis is commonly performed using CID. However, CID is unable to capture information from labile modifications, which can be critical. The ZenoTOF 7600 system offers an alternative fragmentation method called EAD, which provides complementary sequence information to CID and preserves labile modifications that undergo neutral loss in a CID experiment.[3-4]

Molecule Profiler software was used to identify biotransformations in therapeutic compounds after in vitro incubation, demonstrating a streamlined workflow. The structure assignments for biotransformation are prioritized and scored based on EAD and CID data in the Molecule Profiler software. The single result file feature for EAD and CID in Molecule Profiler software makes it ideal for comparing MS/MS spectra and identifying exclusive fragments.

Methods

Sample preparation: ARV 771 at 5µM starting concentration was incubated in rat hepatocytes at 37°C for 30 minutes. Samples were removed from incubation and quenched with methanol at a ratio of 2:1 (v/v). Samples were vortexed for 30 seconds and centrifuged at 12000 rcf for 12 minutes at room temperature. The supernatant was transferred to a vial.

Chromatography: Analytes were separated using a Phenomenex Kinetex™ Polar C18 column (2.1 x 100 mm, 2.6 µm, 100 Å) column. The ExionLC AD system was operated at a 0.5 mL/min flow rate. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in methanol. An injection of 5 µL was subjected to analysis. The chromatographic gradient conditions are summarized in **Table 1**.

Table 1: LC gradient.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	95	5
0.5	95	5
9	5	95
10.9	5	95
11	95	5
12	95	5

Mass spectrometry: The samples were analyzed using the data-dependent acquisition (DDA) method with Zeno CID DDA and Zeno EAD DDA on the ZenoTOF 7600 system (2 separate experiments). Table 2 summarizes the source and gas conditions and Table 3 summarizes the method conditions.

Table 2: Source and gas parameters.

Parameter	Value
Polarity	Positive
Ion source gas 1	50 psi
Ion source gas 2	60 psi
Curtain gas	45 psi
Source temperature	500°C
Ion spray voltage	5500 V
CAD gas	9

Table 3: Zeno DDA parameters

Parameter	Value
Polarity	Positive
Method duration	10 minutes
TOF MS start-stop mass	100 - 1000 Da
TOF MS Accumulation time	0.05 s
Maximum candidate ions	5
TOF MS/MS start-stop mass	100 – 1500 Da
TOF MS/MS Accumulation time	0.1 s
Collision energy (CID)	55V
Collision energy spread (CID)	15V
Electron kinetic energy (EAD)	12 eV
Electron beam current (EAD)	6000 nA

Data processing: The SCIEX OS software was used for data acquisition. The Molecule Profiler software was used to identify biotransformation sites using Zeno CID DDA and Zeno EAD DDA data.

Figure 2: Results panel in the Molecule Profiler software. The panel shows potential metabolites with modifications identified by Molecule Profiler software (I), details with a drop-down menu that includes scoring and structure (II), options to view chromatograms including TIC and XIC (III), TOF MS (IV) and MS/MS data (V).

Streamlined metabolite identification workflow on ZenoTOF 7600 system using Molecular Profiler software

Zeno CID and Zeno EAD data were collected on the ZenoTOF 7600 system. The Molecule Profiler software, integrated into SCIEX OS software, was used to process and analyze both data sets in a single result file. Figure 2 shows the overview of the results panel, where users can view the list of potential metabolites and an overview of assigned structures and scoring information. Data can be presented with TOF MS or MS/MS and XIC views. The software also displays the mass defect and isotope pattern of the metabolites (**Figure 2A-E**).[1,2]

The software used an automated process to assign structures based on the weighting of Zeno EAD and Zeno CID MS/MS spectra on a scale of 0-100%. Additionally, the software provided the ability to assign structures in the interpretation pane and generate a total score for the assigned structures.

Several phase 1 metabolites were identified following a 30-minute incubation of ARV 771 in rat hepatocytes (**Figure 2A**). **Figure 3** and **Figure 4** illustrate the MS/MS spectra overlay from the interpretation pane for phase 1 metabolites from ARV 771, indicating carboxylation (following demethylation) where a loss of the target protein binding site (**Figure 3**) and proteasomal recognition site (**Figure 4**) are observed.

Figure 3 displays the identification of carboxylation (following demethylation) at the linker site of the ARV 771 where a loss of the target protein binding site is observed. Unique EAD fragments at m/z 530.2602, 518.1804, 486.2271, 429.2055 and 357.1622 were used to trace the possible carboxylation sites. Furthermore, a unique fragment at m/z 113.0229 in the CID spectrum supported the possible carboxylation on the proteasomal recognition moiety (**Figure 3**). In **Figure 4**, automated structure assignment using Molecule Profiler software detected carboxylation (following demethylation) where a loss of the proteasomal recognition site was observed. The unique EAD fragments at m/z 471.1475, 444.1251, 413.1060 and 382.0643 were used to identify possible localization of the carboxylation site on the aliphatic chain (**Figure 4**).

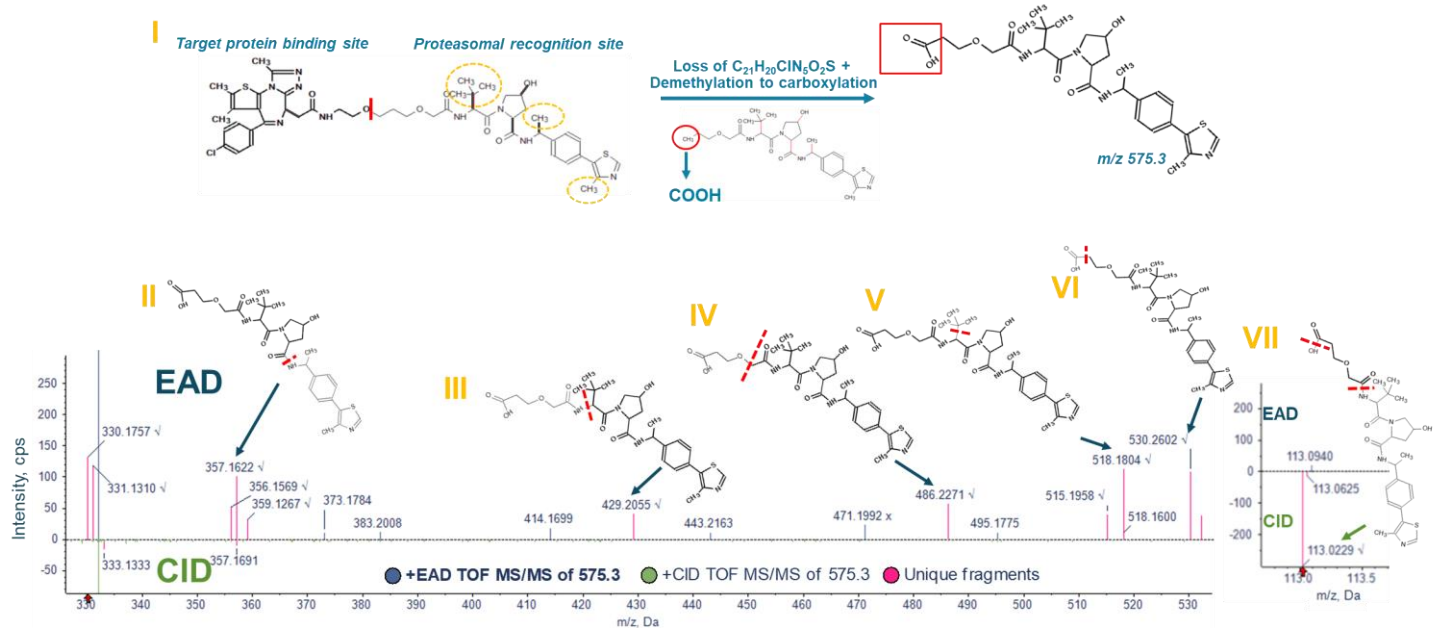

Figure 3: Automated structure assignment for demethylation to carboxylation of the proteasomal recognition site on ARV 771. Automated structure assignment detected demethylation to carboxylation changes following the loss of the target protein binding site. The solid red line indicates fragmentation. The potential methyl groups that could be transformed to carboxylation in the molecule are highlighted in yellow circles to assess and localize the carboxylation site (I). The unique EAD fragments at 530.2602 (EAD), 518.1804 (EAD), 486.2271 (EAD), 429.2055 (EAD) and 357.1622 (EAD) (II-VII) indicate possible demethylation to carboxylation. Unique fragment at m/z 113.0229 in the CID spectrum supports the possible site of the biotransformation on the proteasomal recognition moiety (VII). Fragments in pink are unique fragments when comparing EAD and CID spectra.

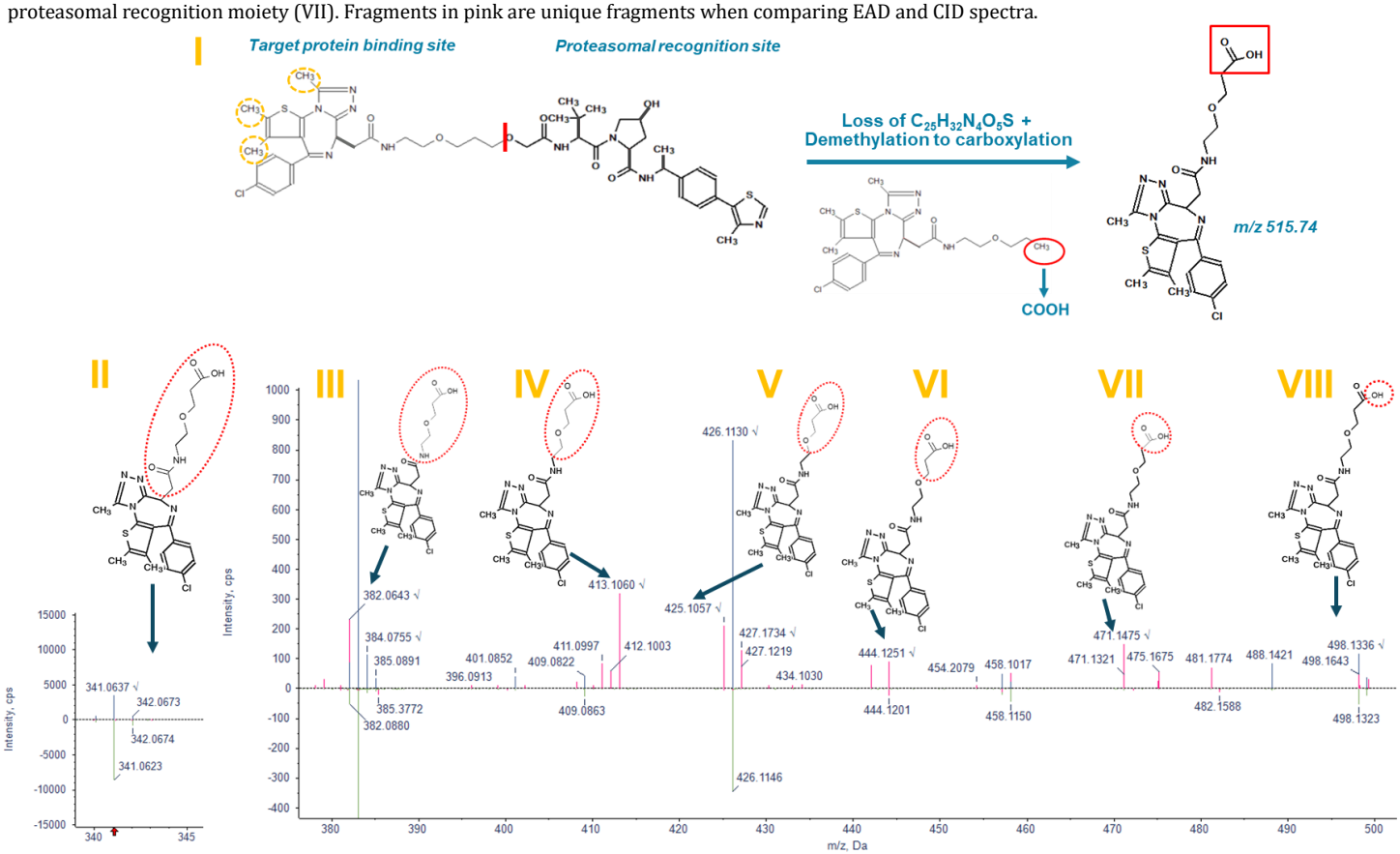

Figure 4: Mirror plot of EAD and CID MS/MS spectra for demethylation to carboxylation metabolite at the aliphatic linker of ARV 771. Automated structure assignment detected demethylation to carboxylation following the loss of the proteasomal recognition site. The solid red line indicates fragmentation. The potential methyl groups that could be transformed to carboxylation in the molecule are highlighted in yellow circles to assess and localize the carboxylation site (I). EAD and CID-derived fragment m/z 341.0637 show the possible site of carboxylation at the aliphatic chain (II-VIII). Unique fragments captured in the EAD spectrum show carboxylation on the aliphatic chain (IV- VII).

Conclusions

- Metabolite identification was performed on the ZenoTOF 7600 system using unique fragments from EAD spectra, CID spectra, or a combination of both spectra. More distinctive and informative fragments were observed from EAD spectra aiding in the identification of TPD metabolites.
- Molecule Profiler software enables users to identify unique fragments from EAD and CID spectra in a single result file to achieve more accurate structure assignment of metabolites and to determine the possible location of the modifications
- The demonstrated workflow can be easily adapted for in vivo metabolism studies to detect low-level metabolites with the enhanced sensitivity provided by the Zeno trap
- Informative data was generated using a quick and easy-to-use workflow on a streamlined platform, accelerating the early drug discovery process

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701
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