STRUCTURAL IDENTIFICATION AND SYNTHESIS OF THE ACYL GLUCURONIDE OF THE MACROLIDE MCL-1 INHIBITOR AZD5991

Liam Evans[1], Scott Martin[4], Chris Drake[1], Richard Phipps[1], Julia Shanu-Wilson[1], Jonathan Steele[1], Jim Horstick[2], Amber Balazs[2], Aixiang Xue[3], Graeme Scarfe[4], Alex Hird[2], Eric T Gangl[2]

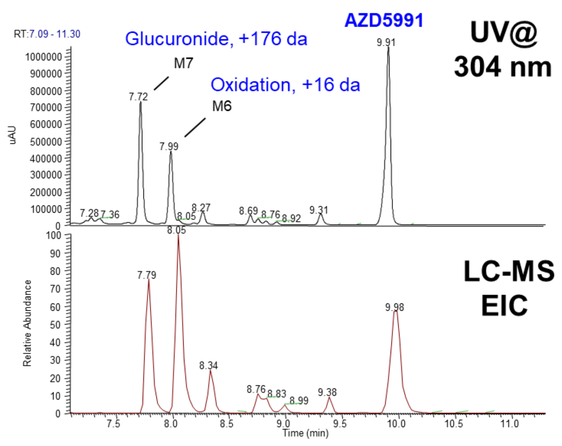
[1]Hypha Discovery, 154B Brook Drive, Milton Park, Abingdon, Oxfordshire, OX14 4SD, UK. [2]Research and Early Development, Oncology R&D, AstraZeneca, Waltham, MA, USA. [3]Research and Early Development, Biopharmaceuticals R&D, AstraZeneca, Waltham, MA. [4]Research and Early Development, Oncology R&D, AstraZeneca, Cambridge, UK.

Abstract: **AZD5991 is a potent and selective inhibitor of Mcl-1 which entered clinical development for treatment of haematological malignancies[1]. Preclinical metabolite profiling of AZD5991 in human hepatocytes revealed the presence of an abundant glucuronide metabolite which required further characterization. Unfortunately, efforts to chemically synthesize analytical quantities of material were unsuccessful. Separately, *in vivo* rat bile duct cannulated studies revealed abundant excretion of the glucuronide in bile enabling milligram quantities to be isolated. The material obtained from bile excreta was of sufficient quantity and purity to obtain an NMR spectrum that confidently revealed an acyl glucuronide structure. Material from rat was also sufficient to test in an acyl migration assay. To support future bioanalytical method development and validation needs for clinical studies, significantly larger quantities were required. Toward that end, AZD5991 was screened against a panel of microbes at Hypha Discovery which revealed extensive metabolism via phase I and phase II pathways. As observed in pre-clinical studies, a prominent glucuronide was formed by several microbes. The glucuronide produced was demonstrated by LC-MS/MS and NMR to match that observed from human hepatocyte incubations and excreta in rat bile. A 10L scale up of one biotransformation reaction yielded 109.6 mg of the purified acyl glucuronide of AZD5991.**

Objectives

- Determine the structure of M7, an abundant glucuronide of AZD5991, by NMR spectroscopy
- Characterize acyl migration potential in an LC-MS assay.
- Reference standard scale-up synthesis for bioanalysis method development and quantitation.

Representative Rat Bile LC-UV-MS Chromatogram

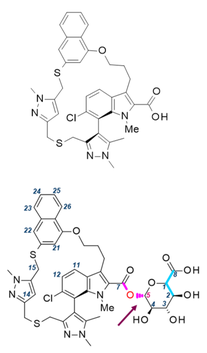


Purification of acyl glucuronide from biological samples (AZ)

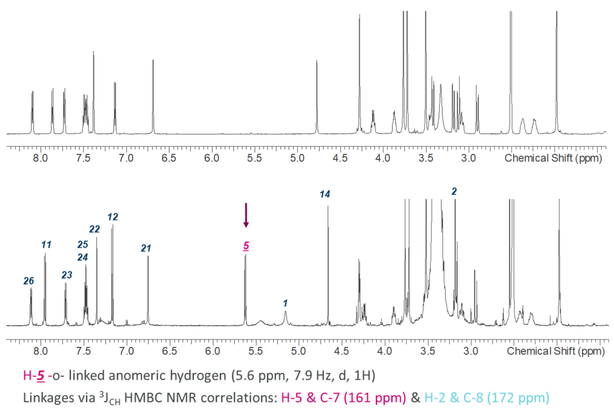
- While preliminary LC-MS data indicated that the metabolite did not undergo acyl migration under physiological conditions, more definitive characterization of structure and stability by NMR was needed. Attempts to chemically synthesize the acyl glucuronide (AG) were unsuccessful and precluded definitive NMR characterization. In order to obtain sufficient quantities of the AG for structure elucidation and stability studies, the metabolite was isolated from *in vivo* rat bile excreta.
- A single rat was dosed at 100 mpk PO and bile was collected. The AG was purified by liquid-liquid extraction of the biological sample followed by separation and fraction collection on a reversed-phase C18 LC column.
- UV signal and NMR purity assessment estimated that, after administering 22.4 mg to a single rat, 5.6 mg of the metabolite was purified from bile with ~85% purity.

Metabolite Characterization

- Rat *in vivo* collected material was characterized by high resolution MS and NMR spectroscopy to definitively establish the metabolite structure.
- Peak 5 in the lower NMR spectrum revealed the characteristic signal of the anomeric hydrogen at the *O*-linked position of AZD5991 acyl glucuronide.
- Given early attempts to synthesize the acyl glucuronide were unsuccessful, microbial biotransformation was evaluated as a technique to make larger quantities for bioanalytical method development and validation.

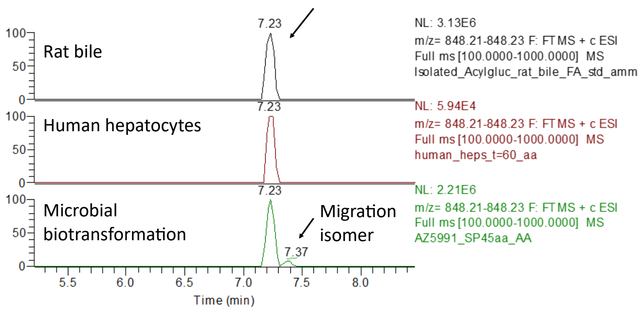


Proton NMR Assessment



H-**5** -o- linked anomeric hydrogen (5.6 ppm, 7.9 Hz, d, 1H)
Linkages via $^{3}J_{CH}$ HMBC NMR correlations: H-5 & C-7 (161 ppm) & H-2 & C-8 (172 ppm)

LC-MS/MS Assessment AZD5991 acyl glucuronide



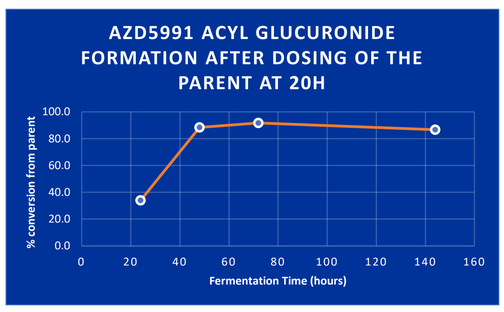
Extracted ion mass chromatogram (*m/z* 848)

Process for microbial biotransformation of AZD5991

AZD5991 screened against microbial panel
↓
Analysis by LC-MS at Hypha and matching at AZ
↓
10L Scale-up biotransformation (Sp45)
↓
Purification and Supply

Scale-up of AZD5991 acyl glucuronide (Hypha)

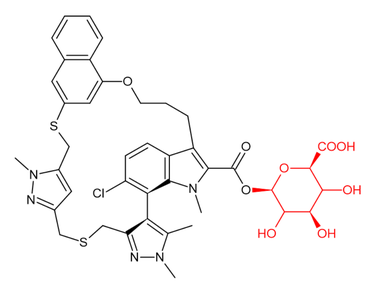
- AZD5991 was extensively biotransformed by 6 microbes into a varying number and intensity of metabolites with the following mass changes: 1 x -14da, 5 x +2da, 5x +16da, 5 x +18da, 1 x +34da, 1 x +148da and 1 x +176da.
- 2 microbes produced a glucuronide (*m/z* 848, +176 da) in good yield with strain Sp45 providing the highest yield. The putative acyl glucuronide was matched to that produced in human hepatocytes by LC-MS/MS profiling at AZ.
- Experiments pointed to earlier dosing resulting in greater stability, with over 90% conversion of AZD5991 to the acyl glucuronide.
- A 2 x 5L scale-up of Sp45 with dosing of AZD5991 at 100 mg/L at 20h and harvesting at 48h, resulted in good production of the acyl glucuronide.



- Purification of the acyl glucuronide from harvested material proceeded through acidification, centrifugation, extraction of the biomass with MeCN, partitioning in a biphasic system, SPE fractionation, followed by 2 rounds of orthogonal prep HPLC (Waters C18 X-Bridge and Waters C8 Symmetry Shield).
- 109.6 mg of AZD5991 glucuronide was isolated at >93% purity by LC-UV and >98% by LC-ELSD. ^{1}H NMR analysis confirmed the purity observed in UV-ELSD analyses. A certificate of analysis was issued.

Conclusions

- Glucuronidation is a major route of metabolism for AZD5991 in pre-clinical *in vitro* and *in vivo* studies .
- Collection of animal excreta can yield sufficient material for NMR characterization.
- Microbial biotransformation proved a successful route to obtain larger quantities of the acyl glucuronide metabolite for further studies.



Reference: [1]**Tron, A.E., Belmonte, M.A., Adam, A. *et al.* Discovery of Mcl-1-specific inhibitor AZD5991 and preclinical activity in multiple myeloma and acute myeloid leukemia. Nat Commun 9, 5341 (2018). https://doi.org/10.1038/s41467-018-07551-w**

About Hypha: **Hypha Discovery is a specialist CRO with expertise in the scalable synthesis, purification and identification of drug metabolites. Using our biocatalytic and organic chemistry capabilities, we also make oxidized derivatives of lead compounds and API degradation products. Hypha also has a wealth of experience in the production, purification and structure elucidation of natural products. We work with pharmaceutical and agrochemical companies globally. Contact enquiries@hyphadiscovery.com**

www.hyphadiscovery.com