UNCOVERING GUT-DERIVED DRUG METABOLITES THROUGH HUMAN FAECAL INCUBATIONS

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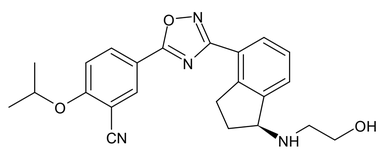
Abstract: **The human gut can contribute substantially to drug metabolism, generating metabolites through microbial enzymatic activity or spontaneous chemical reactions within the complex intestinal environment. These processes may produce metabolites that are absorbed into systemic circulation and can influence drug exposure, pharmacology and inter-individual response. Here, we highlight two case studies demonstrating the application of anaerobic human faecal incubations for the identification and characterisation of previously unknown or clinically relevant gut-derived metabolites.**

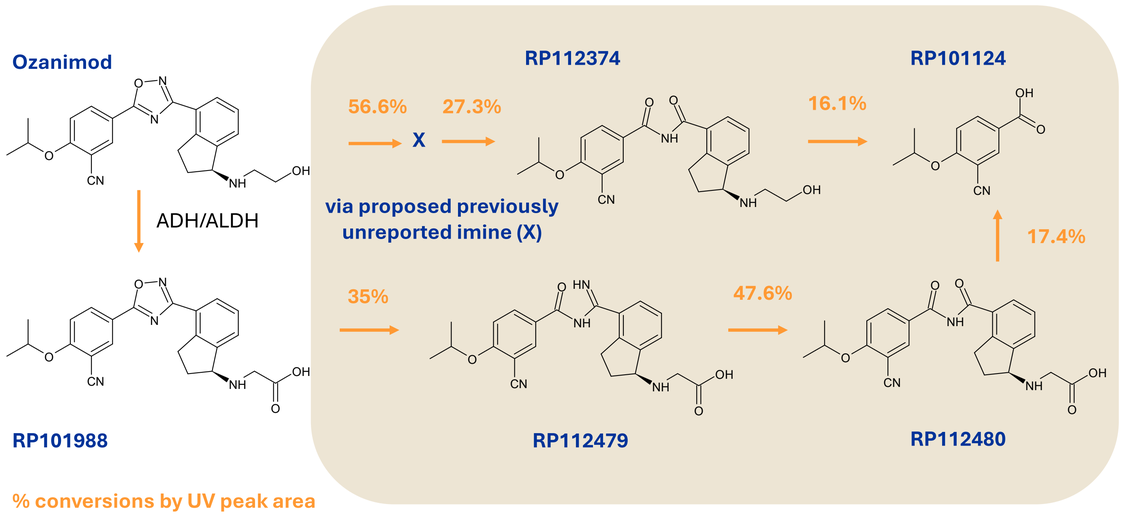
Gut microbiome metabolism of ozanimod

- BMS's ozanimod is the first sphingosine-1-phosphate receptor modulator approved for ulcerative colitis.



Ozanimod

- The drug undergoes extensive metabolism via multiple pathways including biotransformations mediated by anaerobic bacteria in the gut [1].
- Ozanimod and RP101988 (an ADH/ALDH- derived metabolite of ozanimod) were incubated with pooled human faecal material under anaerobic conditions for 48 hours and extracted samples analysed by LC-MS.
- All reported metabolites associated with gut-mediated metabolism were observed, including RP101124, a major circulating metabolite observed in humans. A previously unreported related metabolite (X) was also observed.



Reductive cleavage followed by hydrolysis resulting in oxadiazole ring scission

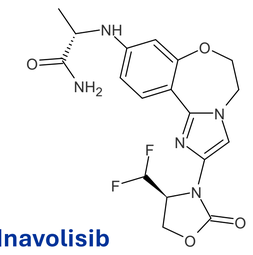
- Both ozanimod and RP101988 were completely metabolised during the faecal incubations. Mechanisms involve reductive cleavage followed by hydrolysis resulting in oxadiazole ring scission.
- The findings demonstrate that human faecal incubations can provide a physiologically relevant system for reproducing complex microbiota-mediated metabolic pathways and generating material for subsequent metabolite identification by LC-MS/MS.
- Mass spectrometry indicated X could be an imine. X's identity could be confirmed by scaling up the faecal incubations with subsequent purification for structure elucidation by NMR spectroscopy.

About Hypha's faecal incubation service

- Available for **human and rat** studies (other species on request)
- **16S DNA profiling** of human mixed sex faecal pool confirms representative gut-associated bacterial communities
- **Anaerobic incubation** under controlled conditions
- **Time coursing** and analysis by LC-MS/MS
- **Scale-up and purification** of metabolites of interest
- **Cryoprobe NMR spectroscopy** for definitive MetID
- **Stability testing** to assess formation / disappearance of metabolites

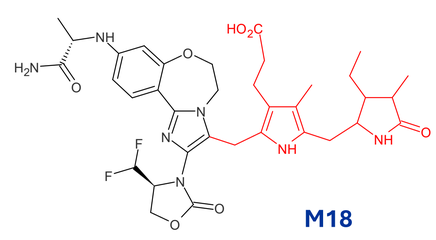
Discovery of unprecedented gut human stercobilin conjugates of inavolisib

- Inavolisib is an oral targeted anti-cancer medication developed by Genentech to treat specific types of advanced breast cancer.
- The parent drug comprises the main circulating component in humans with hydrolysis of the amide moiety to form metabolite M8.

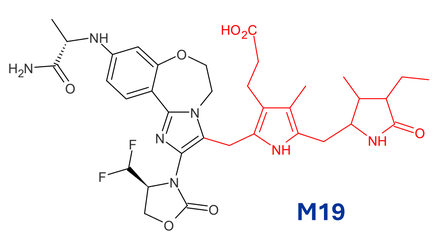


Inavolisib

- Several unusual conjugates were observed in faeces which possessed a mass change of parent +302 or +304 Da, with or without amide hydrolysis [2]. Together the 9 metabolites observed with these mass changes comprised nearly 25% of drug related material in faeces.
- Inavolisib was incubated in pooled human faeces under anaerobic conditions for 48 h and the reaction scaled up to 1 L to generate purified material of the 2 main non-hydrolysed +304 Da metabolites M18 (2.4 mg) and M19 (1.74 mg) for NMR.
- Interpretation of NMR spectra obtained on the two non-hydrolysed +304 Da, metabolites M18 and M19, revealed novel structures arising from conjugation of the imidazole ring of inavolisib with either half of stercobilin.



M18



M19

Conjugation of inavolisib to half structures of stercobilin in faeces. M18 and M19 differ only in the positions of the methyl and ethyl groups on the stercobilin pyrrolidin-2-one moieties

- Conjugation likely occurs by nucleophilic attack from the imidazole ring of inavolisib to the bridging carbon of stercobilin [2].
- The conjugation of inavolisib with stercobilin occurs in both live and heat-treated faecal incubations indicating a chemical reaction, despite conjugation occurring to a much higher extent in live faeces.
- Although stercobilin-derived drug metabolites have not previously been published, other drugs with electron-rich imidazoles or similar ring systems could potentially undergo reactions with stercobilin in the gut.

Key NMR Data

- **Both M18 & M19 lacked the imidazole C4 methine proton signal present in inavolisib.**
- **The oxazolidinone and fused oxazepine–aniline ring systems of inavolisib remained unchanged. Both contained pyrrole-linked structures connected to inavolisib.**
- **Data showed clear presence of a 4-ethyl-3-methylpyrrolidin-2-one moiety for M18 and a 3-ethyl-4-methylpyrrolidin-2-one moiety for M19.**
- **Database searching identified stercobilin as the only matching structure.**
- **HMBC correlations confirmed the proposed structures supported by database matching.**

Take-home message

Human faecal incubations, combined with analytical detection, metabolite isolation and structural elucidation, provides a powerful approach for investigating microbiome- and gut environment-derived biotransformations, revealing metabolites and pathways that may not be readily predicted using conventional hepatic in vitro metabolic systems.

References:

1. Surapaneni et al. Absorption, Metabolism, and Excretion, In Vitro Pharmacology, and Clinical Pharmacokinetics of Ozanimod, a Novel Sphingosine 1-Phosphate Receptor Modulator. Drug Metab Dispos. 2021 May;49(5):405-419. doi: 10.1124/dmd.120.000220.
2. Cho et al. Discovery of Unprecedented Human Stercobilin Conjugates. Drug Metab Dispos. 2024 Aug 14;52(9):981-987. doi: 10.1124/dmd.124.001725. PMID: 38991780.

Hypha Discovery is a specialist CRO with expertise in LC-MS/MS and NMR based MetID together with scalable synthesis and purification of metabolites.

We work with pharmaceutical and agrochemical companies globally to support drug discovery and development projects for clients.

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