Biocatalytic C-H Activation of the JAK Inhibitor Ruxolitinib

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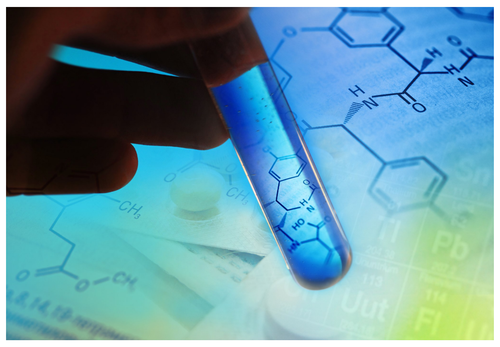
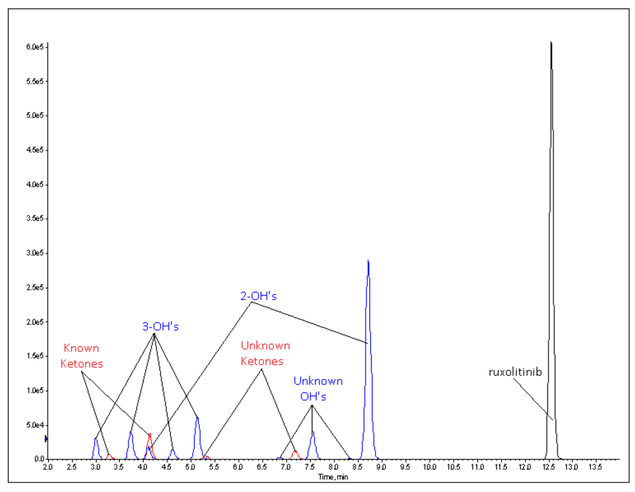


Summary: The first-in-class JAK inhibitor, ruxolitinib (trade names Jakafi®and Jakavi®), is primarily metabolized to a complex mixture of stereoisomeric cyclopentyl hydroxyl and keto metabolites. Application of Hypha's microbial-based biocatalytic C-H bond activation to ruxolitinib resulted in the production of an array of hydroxylated and further oxidized keto metabolites, many of which corresponded to circulating human metabolites. All possible oxidized isomers of the aliphatic cyclopentyl moiety were derived from a variety of microbial species which were readily scaled up, enabling efficient production of stereoisomer metabolite standards for structural characterization and bioanalytical monitoring.

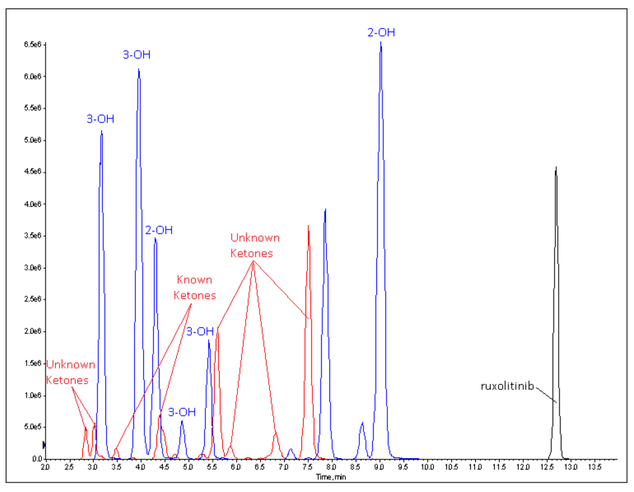
Introduction The activation of specific C-H bonds is one of the most challenging reactions in synthetic chemistry. However, nature is highly effective at selectively oxidizing C-H bonds enzymatically and Hypha's microbial-based process provides access to a platform delivering such aliphatic and aromatic hydroxylations. Application of this technology to hit and lead compounds can result in the production of an array of analogues, including human gut and circulating metabolites, as well as new microbial derivatives. Late stage functionalization using this route generates stereospecific derivatives providing unique SAR information complementary to that derived *via* chemical synthesis.

The majority of ruxolitinib metabolism in humans centers on the oxidation of the cyclopentane ring[1], which results in the introduction of 1 or 2 additional stereocenters in the ketone and hydroxylated metabolites, respectively. While positional assignment of oxidation was possible using NMR, multi mg quantities of metabolite standards were needed for stereochemical characterization via x-ray crystallography. Accordingly, the parent compound was screened against a specific panel of Hypha's hydroxylating strains to pinpoint a suitable strain for scale-up.

Process Ruxolitnib was dosed at 100mg/L to a panel of 23 bacterial and fungal strains in a 24 well microbioreactor. Analysis for the target mass of m/z 323 at 3 time points revealed the presence of multiple metabolites produced by a variety of strains. After an initial confirmation step, the strain delivering the best array of hydroxylated derivatives was scaled up to provide 10L of material. Purification of metabolites is usually undertaken by Hypha, however in this instance, Incyte required defatted extracts of broth and biomass to be sent for purification and testing of metabolites. All diastereomers generated by the selected strain from the scaled up fermentation were purified using achiral reversed-phase chromatography. A range between 50 to 120mg of desired metabolites were recovered. Crystal generation and crystallographic analysis is ongoing.



Representative LC-MS/MS Chromatogram of Ruxolitinib Clinical Plasma Analysis



LC-MS/MS Analysis of Hypha-Generated Ruxolitinib Metabolites

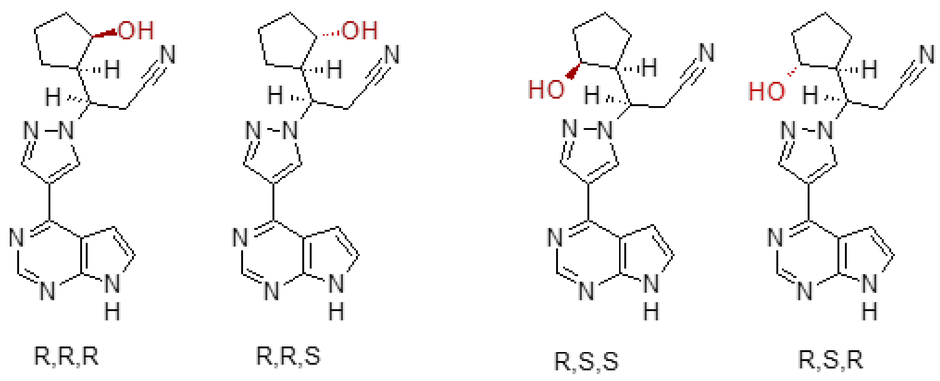
LC-MS/MS Analysis (Incyte) Samples were assayed using positive electrospray ionization on a Sciex API4000 mass spectrometer with multiple reaction monitoring (MRM) transitions specific for ruxolitinib and metabolites of interest. Chromatographic separation of ruxolitinib and its metabolites was achieved using a Waters Atlantis T3 C18 column (3µm, 2.1x100 mm) with a 16 minute step gradient and mobile phase consisting of 100% methanol and 10mM ammonium formate pH 3.0 aqueous solution.

References

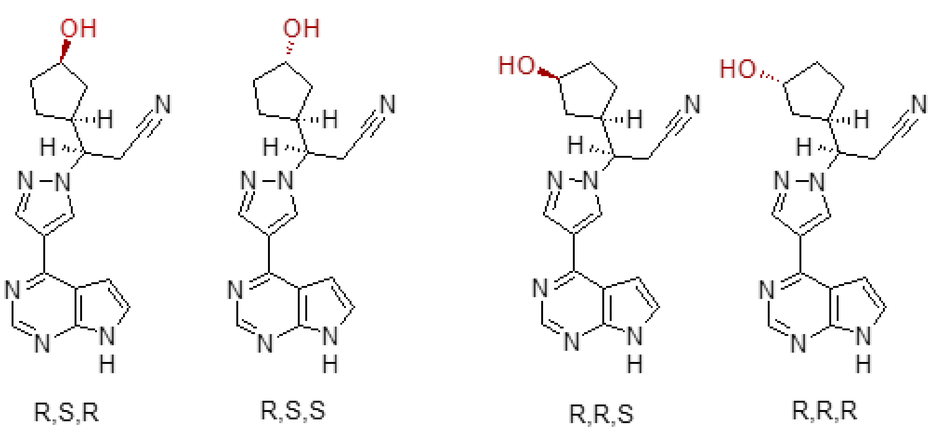
1. Shilling AD, et al. (2010) Metabolism, Excretion, and Pharmacokinetics of [14C]INCB018424, a Selective Janus Tyrosine Kinase 1/2 Inhibitor, in Humans. *Drug Metab Dispos 38:2023–2031.*

Putative 2– and 3-cyclopentyl hydroxylated stereoisomeric metabolites of ruxolitnib produced via aliphatic methylene hydroxylation by a selected bacterial strain from Hypha's biotransformation panel. Corresponding cyclopentyl ketone derivatives of metabolites were also produced and identified.

2-Hydroxylation of Cyclopentane Moiety



3-Hydroxylation of Cyclopentane Moiety



Outcome and Discussion

- Hypha's biocatalytic process used enantiomerically pure parent starting material, to generate metabolites possessing the appropriate stereochemistry of the parent stereocenter. Thus, all metabolites generated were diastereomers and readily resolved using reversed-phase chromatography for purification.
- This project exemplifies a route to producing metabolites that are difficult to synthesize chemically, at such scale to enable unambiguous MetID.
- In addition to the production of phase I metabolites and conjugates such as glucuronides and sulphates, this platform catalyzes diverse oxidations. These reactions may be outside the scope of routine chemical synthesis, thereby complementing hit-to-lead chemistry and lead optimization, and providing additional SAR. The resulting analogues may themselves have superior properties to the parent, or be useful as late stage functionalized intermediates for further modification.

About Hypha Discovery

Hypha Discovery Ltd is a UK-based microbial biotechnology company helping partners in pharmaceutical and agrochemical R&D worldwide succeed through the production of mammalian and microbial metabolites, as well as specialising in microbially-derived chemicals and provision of natural product libraries derived from higher fungi. In addition to our lead diversification capabilities, clients routinely use our biotransformation technology to generate Phase I & II metabolites for MetID, Stability testing, use as Analytical standards and for producing larger amounts for further DMPK testing.