HYDROXYLATED HUMAN METABOLITES OF TIVANTINIB PRODUCED BY AN ENZYME IN HYPHA'S POLYCYPS™ CYTOCHROME P450 KIT

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Abstract: A cell-free kit of cytochrome P450 enzymes and ferredoxin/ferredoxin reductase redox partners, termed PolyCYPs™, is being developed for generating scalable quantities of oxidised metabolites. P450 cytochromes in the kit have been derived from some of Hypha's most talented biotransforming bacteria and are capable of generating human and other mammalian metabolites of drug compounds. The hydroxylating abilities of one P450 enzyme from the kit, PolyCYP™6.1, which has been cloned from an actinomycete species into *E.coli* together with redox partners, is illustrated using tivantinib. Tivantinib is an experimental anti-cancer drug which is extensively metabolised in humans, with two major circulating metabolites M4 and M5[1]. M4 and M5 are epimers formed by hydroxylation at the benzyl position of the tricyclic ring of tivantinib and comprise 19% and 12% of the total area under the concentration-time curve (AUC) respectively[2], implicating these metabolites under the FDA MIST guideline. Recombinant enzyme PolyCYP™6.1 was able to produce all four of the hydroxylated human metabolites originally observed in the wild-type microbial biotransformation, from which the cytochrome was derived. As well as utility for providing sufficient material for MetID, reactions can be scaled to produce milligram to gram quantities of metabolites and novel derivatives for further evaluation.

Background

The experimental anti-cancer drug tivantinib, is a MET tyrosine kinase inhibitor which exerts a cytotoxic effect through interfering with tubulin polymerization[1]. The drug is extensively metabolized in humans, in which the primary CYP isoforms involved are CYP2C19 and CYP3A4/5. Of the oxidation products, M4, M5, M7, M8 and M9 are observed in humans with M4 and M5 being major metabolites over the 10% AUC threshold[2], implicating such metabolites under the FDA MIST guideline.

Tivantinib was initially screened as part of a larger set of substrates against 6 of Hypha's prolific oxidative biotranformation strains - all of these strains produced M4, M5, M7 and M9 to varying extents, with 2 strains also producing a novel microbe-specific *N*-methyl metabolite. One actinomycete strain was selected for scale-up to purify and characterize oxidized metabolites by NMR spectroscopy. One of the hydroxylated metabolites could only be putatively assigned as M9, since previous researchers did not have sufficient material for definitive Met ID of this metabolite.

Process summary

PolyCYP™6.1 was mined from one of Hypha's actinomycete strains. Genome sequencing and bioinformatics revealed 34 cytochrome P450s, 19 ferredoxin proteins (Fd) and 9 ferredoxin reductase enzymes (FdR). A selection of the P450s were successfully cloned into *E.coli* and co-expressed with selected redox partners.

Tivantinib was incubated with reconstituted lyophilized enzyme material from one of these, PolyCYP™6.1, together with the usual NADPH regenerating system(NADPHrs), as shown in the adjacent process flow. Products were analyzed by LC-UV-MS (conditions detailed below) and matched against standards obtained from the original whole cell biotransformation of tivantinib by the wild type actinomycete strain from which PolyCYP™6.1 originated.

Results

- PolyCYP™6.1 was able to biotransform tivantinib into hydroxylated metabolites with M4/M5 and M7 predominating, and a total of 33% of parent converted to these 3 metabolites in an unoptimised reaction.
- 25% of tivantinib was converted to M4/M5 and 8% to M7. Only a trace of M9 was detected by LC-MS. This matches well with the ratio of metabolites seen from whole cell biocatalysis of tivantinib by the actinomycete from which PolyCYP™6.1 originated.

Chromatography details

Analysis gradient: Column: Waters Acquity UPLC BEH Shield RP18 1.7µm 2.1mm i.d. 50mm length, Solvents: A: H_2O, B: Acetonitrile (both containing 0.1% formic acid), Gradient (A%/B%): t=0mins: 98/2 to 2/98 at t=2.4mins and held for a further 0.4mins (2.8mins total), return to 98/2 over 0.05mins and re-equilibrated for 0.15mins (t=3mins) at a flow-rate of 1.0 ml/min. System: Pumps and autosampler: Waters Acquity UPLC QSM and Waters Acquity UPLC FTN, Detection: Waters Acquity UPLC PDA (UV-Vis detection), Detection (MS): Waters Acquity UPLC QDA.

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 human and other mammalian metabolites, as well as specialising in lead-diversification, production of microbially-derived chemicals and provision of natural product libraries derived from higher fungi. Clients routinely access our biocatalysis technology to generate phase I and II metabolites for MetID, stability testing, use as analytical standards and for producing larger amounts for further DMPK testing.

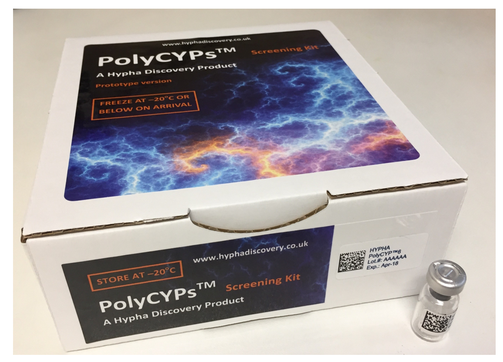
Process flow for biotransformation of tivantinib by PolyCYP™ enzyme 6.1 contained in Hypha's developmental PolyCYPs™ kit.

Reconstitute lyophilised buffered enzyme and NADPHrs in water and mix gently

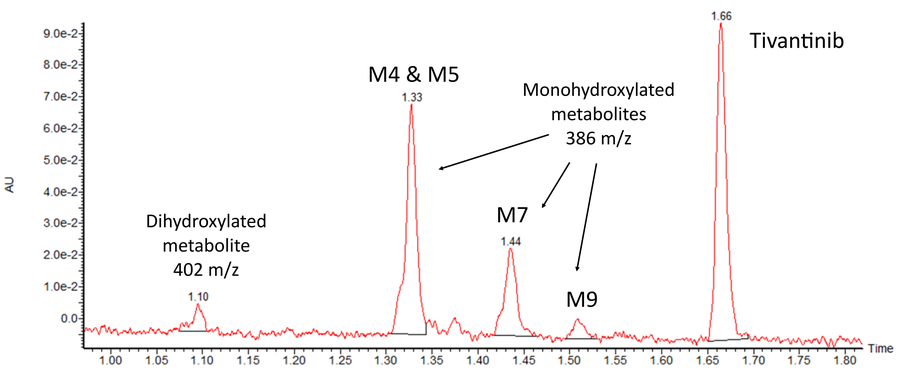
Add tivantinib to a final concentration of 0.1 mg/ml to the reconstituted enzyme and add cofactors

Shake in a microtitre plate at 200rpm for 21hrs (or in the supplied vial at 100 rpm for 16-22 hrs) at 27 °C

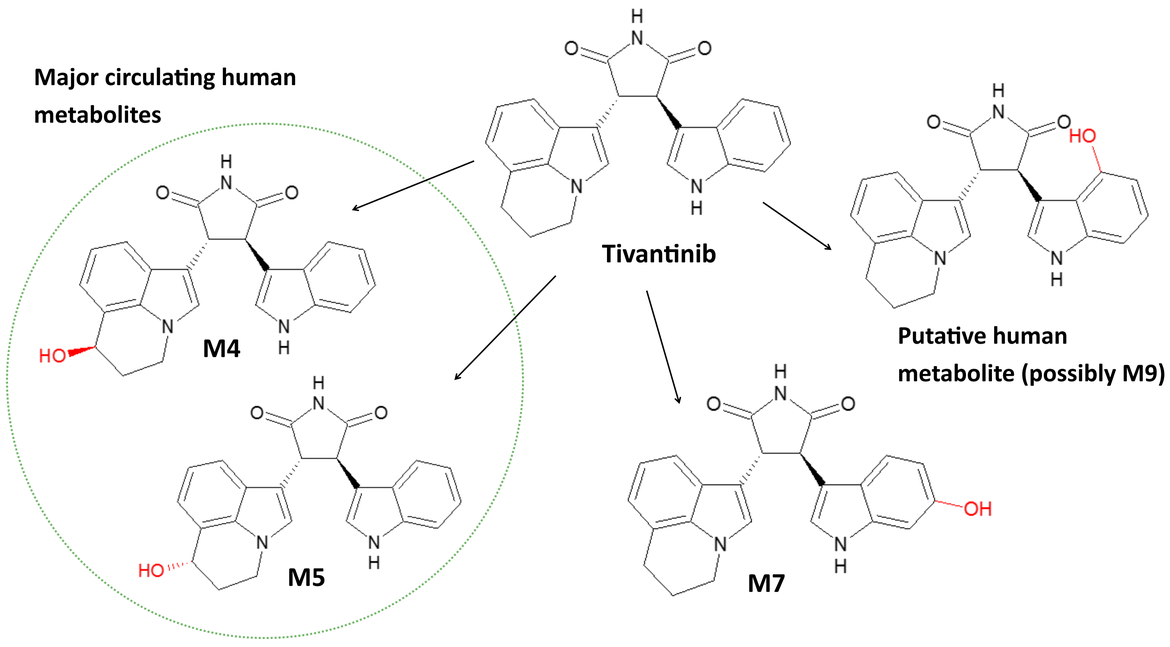
Stop the reactions with acetonitrile, centrifuge and analyse supernatant by LC-UV-MS



Enzyme PolyCYP™6.1 from Hypha's prototype PolyCYPs™ kit. The kit is simple to use and contains all reagents needed for conducting oxidation reactions.



Hydroxylated human metabolites of tivantinib generated by PolyCYP™ enzyme 6.1 derived from one of Hypha's actinomycete strains



Conclusions

- One of Hypha's talented biotransformation strains is a source of at least one functional CYP enzyme, exemplified by PolyCYP™ enzyme 6.1, which has utility for hydroxylating compounds to generate human drug metabolites.
- PolyCYP™ enzyme 6.1 is one of a suite of enzymes being developed for Hypha's forthcoming PolyCYPs™ metabolite kit. The kit will give scalable point-of-use access to hydroxylated human metabolites and novel derivatives useful for lead diversification and late stage functionalization.

References

[1]Munoz, 2017. Non-kinase targets of protein kinase inhibitors. Nature Reviews Drug Discovery (2017).

[2]Nishiya *et al*., 2016. Stereoselective hydroxylation by CYP2C19 and oxidation by ADH4 in the *in vitro* metabolism of tivantinib. Xenobiotica 46 (11), 967-976.

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