HUMAN METABOLITES OF BOSENTAN PRODUCED BY ENZYMES 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 under development for generating scalable quantities of oxidised metabolites. Cytochrome P450s in the kit have been derived from Hypha's talented biotransforming actinomycetes and are capable of generating human and other mammalian metabolites of drug compounds. The catalytic abilities of two P450 enzymes in the kit, which have been cloned from two different actinomycete species into *E. coli*, are illustrated using bosentan. Bosentan has one major active metabolite (Ro 48-5033), formed by hydroxylation at the t-butyl position[1], and two other minor metabolites (Ro 47-8634 and Ro 64-1056) produced via *O*-demethylation of bosentan and Ro 48-5033[1]. Recombinant enzymes PolyCYP™6.1 and PolyCYP™14.1 were each able to produce one of the reported human metabolites. Furthermore, the third minor metabolite could be produced through use of a combination of PolyCYP™6.1 and PolyCYP™14.1. 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 bosentan is an endothelin receptor antagonist used for treatment of pulmonary hypertension. Bosentan has one major active metabolite (Ro 48-5033), which is formed by hydroxylation at the t-butyl position[1], and which accounts for 10-20% of the total pharmacological activity on administration of the drug. Two other minor inactive metabolites (Ro 47-8634 and Ro 64-1056) are produced via *O*-demethylation of bosentan and Ro 48-5033[1].

Bosentan was screened as part of a larger set of substrates against 6 of Hypha's prolific oxidative biotranformation strains - all of these strains produced a hydroxylated metabolite with one strain also producing a demethylated metabolite. Selected cytochrome P450 enzymes mined from these six strains were then tested for their ability to produce the reported major and minor human metabolites of bosentan.

Process summary

PolyCYPs™6.1 and 14.1 were mined from two of Hypha's actinomycete strains (strains 43 and 48 respectively). A selection of the P450s were successfully cloned into *E. coli* and co-expressed with selected redox partners.

Bosentan was incubated with reconstituted lyophilized enzyme material from the two enzymes termed PolyCYP™6.1 and PolyCYP™14.1, together with the NADPH regenerating system (NADPHrs) as shown in the adjacent process flow. Additionally the potential for sequential reactivity was assessed by incubating purified Ro 47-8634 with PolyCYP™6.1. All samples were analyzed by LC-UV-MS.

Results

- PolyCYP™6.1 was able to biotransform bosentan into the major active human metabolites Ro 48-5033 which is hydroxylated at the t-butyl position.
- PolyCYP™14.1 catalyzed *O*-demethylation of bosentan to yield the minor inactive human metabolite Ro 47-8634.
- The third minor metabolite Ro 64-1056 was formed by incubation of purified Ro 47 -8634. with PolyCYP™6.1

Process flow for biotransformation of bosentan by Hypha's PolyCYPs™ enzymes

Reconstitute lyophilised enzyme and mix with bosentan at a final concentration of 0.125 mg/ml

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Add NADPHrs and shake in a microtitre plate at 200rpm for 16hrs (or in the supplied vial at 100 rpm for 16 hrs) at 27 °C

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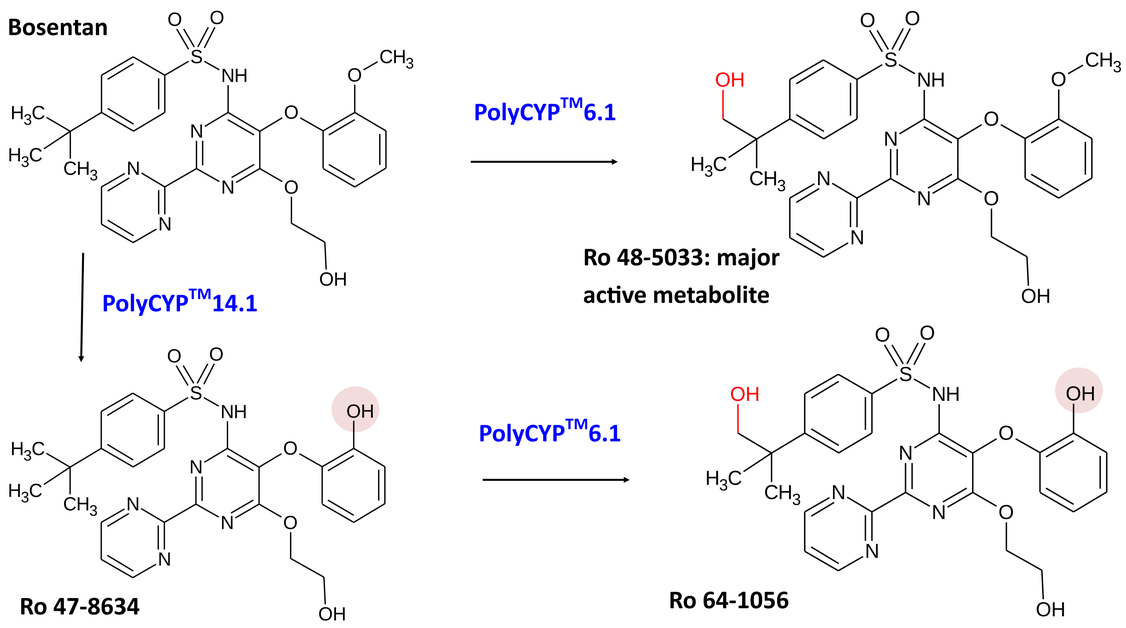
Stop the reaction with acetonitrile, centrifuge and analyse supernatant by LC-UV-MS

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

Major human metabolites of bosentan generated by PolyCYP™ enzymes 6.1 and 14.1 derived from two different actinomycete strains, as confirmed by LC-MS



Conclusions

- Two of Hypha's talented biotransformation strains are each a source of at least one functional CYP enzyme, exemplified by PolyCYPs™ 6.1 and 14.1, which have utility for hydroxylating and demethylating drugs to generate an array of human metabolites. Enzymes can be used singly or in sequential or multiple formats to create secondary products.
- The kit will give scalable point-of-use access to hydroxylated human metabolites and other novel derivatives useful for lead diversification and late stage functionalization.

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 and production of microbially-derived chemicals. 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 pharmacological testing.

References

[1]Weber *et al*., 1999. Absorption, excretion and metabolism of the endothelin receptor antagonist bosentan in healthy male subjects. DMD 27(7), 810-815.

[2]Tracleer® (bosentan) product information. Actelion Pharmaceuticals US, Inc. October 2016.

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