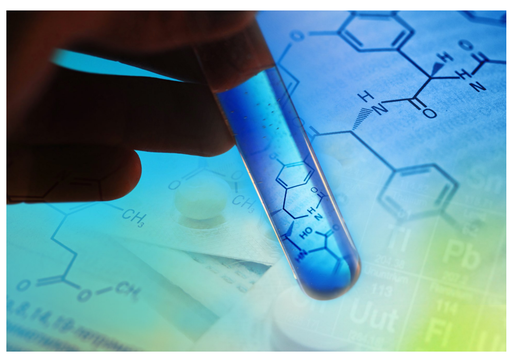
Scale-up Supply of a Major Human Metabolite of a Dermal Drug Candidate for Structure Determination, Biological Quantification and *in vitro* Biological Tests

Liam Evans[1], Headley Williams[1], Richard Phipps[1], Bethlehem Kebede[1], Steven Harte[1], Julia Shanu-Wilson[1], Jonathan Steele[1], Morten Funch Carlsen[2], Martin Stahlhut[2], Lars Dencker Nielsen[2] and Grethe Aagaard[2]

[1]Hypha Discovery Ltd., Brunel Science Park, Uxbridge, UB8 3PQ, UK

[2]LEO Pharma, Ballerup, Denmark

Correspondence: liam.evans@hyphadiscovery.co.uk



Summary: Ingenol disoxate is a chemically-stable drug developed by LEO Pharma, effective at treating actinic keratosis topically and currently in Phase 3 clinical trials. Profiling of ingenol disoxate against multiple species of hepatocytes revealed M27 as a predominant metabolite, particularly in human hepatocytes. Although accurate mass spectrometry indicated the metabolite was mono-hydroxylated in the ingenol moiety, the precise location of the hydroxyl group could not be identified. Consequently, chemical synthesis was not feasible, nor biological quantification and further biological testing possible.

Screening ingenol disoxate against Hypha's Phase I microbial panel highlighted several strains that produced oxidized metabolites, with one strain selected for scale-up to provide 100s of milligrams of M27 at >99% purity for unambiguous MetID and further biological studies. This poster illustrates the process undertaken at Hypha to obtain M27, together with information on the studies performed with the material by LEO Pharma for compliance with metabolite safety testing (MIST) and drug-drug interaction (DDI) guidelines.

Metabolite screening and scale-up process for M27 (Hypha)

Ingenol disoxate screened at 100mg/L against 23 strain Phase I microbial panel.

Oxidized metabolites formed by 14 strains with 2 strains in particular showing good conversion (strains 1c and strain 59). Less onward metabolism of M27 observed by strain 1c.

LCMS/MS confirmation of M27 metabolite with sample from *in vitro* metabolite profiling study (LEO).

4L scale up of strain 1c targeting ca. 50mg for structure elucidation studies and standard reference material, generated 86.8mg at 99% purity by ELSD and ^{1}H NMR. Early harvest was found to be key to minimize onward metabolism of M27 to unwanted dihydroxylated derivatives, and thus maximize the yield of M27.

Further request by LEO Pharma

13L scale-up of strain 1c to provide at least 200 mg M27 for further biological testing yielded 415mg at 99% purity. Dosing was increased to 250mg/L for improved yield with formulation to improve solubility at this higher concentration.

Summary of *in vitro* and *in vivo* characterisation of M27 (LEO)

- **CYP450 studies**

No inhibition or induction potential at clinically relevant exposure.

Phenotyping: routes of metabolism similar to parent compound.

- **Plasma protein binding (PPB)**

% free fraction in animals ≥ in humans.

- ***In vitro* potency**

M27 qualitatively showed similar effects as the parent compound, but 5 to 20 fold lower potency.

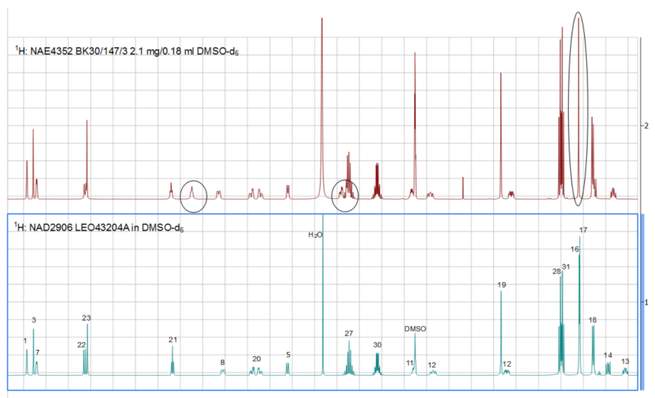
- **Human pharmacokinetics vs toxicokinetic data in animals**

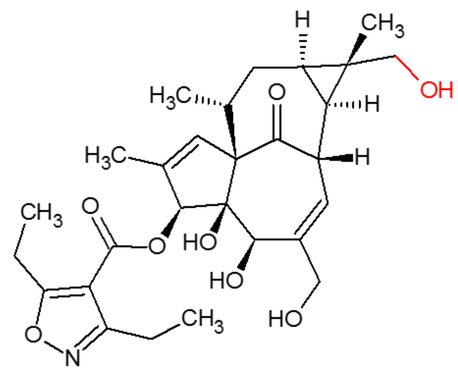
PK and TK data on the metabolite showed acceptable animal-to-human safety ratios.

Structure determination

Accurate mass spectroscopy revealed mono-hydroxylation somewhere in the ingenol moiety

^{1}H-NMR of ingenol disoxate (lower) and "M27" (upper) performed at LEO Pharma. In the 1.0 – 1.1 ppm region of the M27 spectrum only one CH_3 signal is observed, in the 3.0 – 3.2 ppm region two extra methylene proton signals are observed and there is an extra signal at 4.50 ppm (-OH).





M27 - 16-hydroxy-ingenol disoxate

Elucidation of the structure of the human metabolite M27 by NMR spectroscopy as 16-hydroxyingenol disoxate

Outcome and discussion

- Hypha's biocatalytic process was reproducibly able to generate 100s of milligrams of the human metabolite M27 to satisfy LEO Pharma's requirements for structure elucidation, *in vitro* potency assays and sufficient reference standard for bioanalysis.

- The lead time from project initiation until availability of 86.8 mg purified metabolite (step 1—4 above) was < 2 months.

- *In vitro* potency assays and human PK-data showed that M27 was an active major metabolite, and therefore further material was needed (step 5 in process above) to enable conduct of the *in vitro* DDI (CYP450) and PPB studies requested by the FDA.

- To improve robustness of bioanalytical methods, the process outlined above could advantageously have been used to generate stable isotope labelled M27 in parallel.

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.

Ref: Carlsen *et al*., 2016. Biosynthesis, structural identification and quantification of low pg/ml levels of a major human metabolite of a dermal drug candidate. Oral presentation at European Bioanalysis Forum Open Symposium, Barcelona, Nov. 2016.