Role of 4-methyl sterols in post-squalene metabolic diseases and cancers Hedgeogh protein pathway depending

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The aims of this project are to characterize the enzymes of the steroid-C4-demethylase complex and to evaluate the role of 4-methyl-sterols in the Hedgehog (*Hh*) protein pathway, a signalling cascade involved in the embryogenesis, in post-squalene metabolic and cancer diseases.
Firstly, a fermentative method producing 4-methylzymosterone, substrate of an enzyme (HSD17B7) of the demethylase complex and probably correlated to a defect of the Hh signalling pathway activation, was developed starting from yeast strains engineered in the *ERG27* (3-ketosteroid reductase) gene. Next, compound was fully characterized by spectroscopic (NMR) analysis, and a second product, 4-methyl fecosterone was isolated and characterized. The two 4-methylsterones were tested on two different cell lines and the acquired preliminary data showed an anti-proliferative activity of the two molecules Therefore, in collaboration with the Turku Center for Disease Modeling in Finland, we tested the molecules activity *in vivo* on pregnant murine models to verify the supposed direct association between 4-methylzymosterone accumulation and embryo malformations [Barsh et al., 2011]. The preliminary results acquired show some interesting morphological and histological differences between control and treated groups.
A method to produce a mixture (named Keto 1 fraction) of the above 4-methyl sterones in the radioactive form was also developed, in order to use them as substrates in enzymatic characterization of C4-demethylase complex. To better characterize the mammalian HSD17B7 (the 3-ketosteroid reductase belonging to the C4-demethylase complex), human enzyme was expressed in *E. coli* cells. Comparative experiments were carried out with the yeast orthologue ERG27 enzyme expressed in *E. coli* cells too.
Experiments with human and yeast recombinant enzymes incubated with radioactive 4-methyl-zymosterone, as sterol substrate, revealed that both are highly active, thus demonstrating that they do not need the presence of the other proteins of the complex. Assays with radioactive estrone, as steroid substrate, showed that the human enzyme possesses an intrinsically high estrogenic activity outside of its cellular/tissue environment, contrary to the yeast enzyme in which estrogenic activity is completely absent.
Once we obtained these results using the radioactive substrates, we started to investigate deeper into the intrinsic double reduction activity of HSD17B7 in two different ways. (i) Using the non-radioactive substrates, more available than the radioactive one, we tested, on both the activities, different inhibitors, already proved to be active on the estrone reduction activity [Bellavance et al.,2009]. Surprisingly, at the same experimental conditions, they inhibited all the steroid reduction activities contrary to the sterol one, which was not affected even by high inhibitors concentrations (100 μM). These evidences posed more interesting questions about the enzyme structure-activity relationship. (ii) Therefore, a period was spent abroad, at the King's College of London, to design and produce different HSD17B7 point-mutated versions. Some mutants showed differences between the steroid reduction activity end the sterol one. Moreover, in the same period, we undertake different strategies to study and increase the solubility of this membrane enzyme, for a crystallization purpose; a promising result was obtained using an oxidative refolding process.
To enlarge the collection of intermediates to be used as substrates of C4-demethylase complex and/or inhibitors of the Hedgehog proteins, radiolabelled and non-radiolabelled 4-methylsterones were treated with $NaBH_4$ to produce 3-hydroxy derivatives. The two radioactive isomers (3-beta, 3-alfa) produced by chemical reduction were isolated and used as substrates in preliminary assays with cell homogenates from wild-type *S. cerevisiae* strain (SCY876). Results showed that the yeast demethylase complex had a marked preference for the beta-isomer (68% vs. 13% transformation). This evidence points out a high stereoselectivity for the enzymes of C-4 demethylase complex suggesting that the "bad substrate" alpha isomer could be used as inhibitor.