Cannabinoids and zebrafish

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SUMMARY

Cannabinoids are a group of terpenophenolic compounds containing a C_1, C_3 or C_5 side chain. They are found in the cannabis plant (*Cannabis sativa L*). Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the psychoactive cannabinoid. It is an approved stimulant of appetite and antiemesis in patients under chemotherapy and HIV therapy regimes and is used to treat a number of disease states including pain, anxiety, asthma, glaucoma and hypertension. There is a growing interest in developing new derivatives of Δ^9-THC with high medicinal value. The high lipophilicity of Δ^9-THC is hindering the further development of this compound into a pharmaceutical product. The human body contains high lipid contents, which are not only the body fats but also present in brain and cell membranes. Δ^9-THC binds strongly to plasma protein and other fatty tissues, which prolongs its release from the body. So, there is a need to structurally transform the compound to increase its polarity and its rapid release from the body. This thesis is focused on investigating the biotransformation potential of alkane degrading bacterial strains and *Catharanthus roseus* plant cell culture to produce more polar derivatives of Δ^9-THC to facilitate rapid release of the drug from the body.

In **chapter 2** we reviewed a large library of Δ^9-THC metabolites developed through bioconversions with mammalian, fungal, bacterial and plant cell cultures. These organisms have unique enzymes which catalyzed the conversion of Δ^9-THC at different positions and thus provide a source for a variety of derivatives. These compounds can be used to obtain a wealth of information about the pharmacodynamics and pharmacokinetics of Δ^9-THC and thus pave the way to the discovery of new compounds, with improved therapeutic properties.

In an effort to generate highly polar derivatives of Δ^9-THC, we screened 206 alkane degrading bacterial strains. The alkyl side chain of Δ^9-THC was found

the specific target site for alkane degrading bacteria to convert it into more polar derivatives. Gram-positive strains from the genera *Rhodococcus*, *Mycobacterium*, *Gordonia*, and *Dietzia* were found to be most efficient in transformation of Δ^9-THC. In total, eight derivatives were produced on a mg scale. All of these transformants possessed modified alkyl chains, with hydroxy, carboxy and ester functionalities **(Chapter 3).**

Besides microorganisms, plant cell cultures also act as suitable biocatalysts to perform complex biochemical reactions. The enzymatic system of plant cells can successfully convert the exogenously applied substrates into novel and valuable products with improved properties regarding stability, bio-activity and solubility. We investigated the potential of cell suspension culture of *Catharanthus roseus* to transform Δ^9-THC. Unlike bacterial strains, *Catharanthus roseus* cells transformed Δ^9-THC to its glycosylated and additionally hydroxylated derivatives. Δ^9-THC was found to be degraded into cannabinol (CBN) which was also converted to its glycosylated derivative. Our findings suggest that microorganisms and plant cell cultures have different Cytochrome P450 enzymes and consequently results into different metabolic pathways for Δ^9-THC **(Chapter 4)**.

The zebrafish embryo holds great promise for high-throughput screening of new drug candidates. It could be helpful for a rapid, high-throughput and low-cost assays, e.g. in the early (pre-regulatory) stages of drug testing and also for behavioral testing. The behavioral effect of Δ^9-THC is mediated by the central CB1 receptor in rats. The discovery of CB receptors in zebrafish embryos can be helpful to gain further insight in the pharmacology of cannabinoids and it might also be helpful to resolve some unclear features of the cannabinoids mode of action, like the phenomenon of tolerance and dependence caused by cannabinoid based drugs. We used whole mount staining, visual motor response

test and ^{1}HNMR based metabolomic approach in order to study the cannabinoid associated phenotypic, behavioral and metabolic effects in zebrafish embryos.

Chapter 5 reports the effects of the cannabinoids Δ^{9}-THC, WIN 55,212-2 and CP 55,940, and the cannabinoid antagonist (Am 251) on zebrafish embryo locomotor activity. We found a significant similarity between physiological responses of rodents and zebrafish embryos to cannabinoids. The zebrafish embryo seems to be a reliable behavioral model and could provide new opportunities for the preliminary screening of psychoactive compounds.

High-resolution proton nuclear magnetic resonance (^{1}H NMR) is an ideal tool for the metabolite profiling of biofluids, tissue extracts and intact tissues. It has been used to investigate the biochemical composition of different tissues in an organism and drug toxicity assessment in blood serum, liver and testis of rodents. We have successfully applied ^{1}H NMR in combination with Multivariate data analyses for the metabolite profiling of 5 days old fertilization (5dpf) zebrafish embryos. Embryos exposure to Δ^{9}-THC and AM251 showed a pronounced effect on the metabolites which are directly involved in neurotransmission. The opposed effects of Δ^{9}-THC and AM251 indicate the involvement of CB_1 receptors in Δ^{9}-THC regulated metabolites in zebrafish embryos. Our findings suggest that zebrafish embryos can be used as a model organism to study the metabolic signatures of certain drugs **(Chapter 6).**

The bioassays are based on the solvents to solubilize the hydrophobic experimental drugs. Water-soluble compounds can simply be dissolved in the aqueous medium used to culture different experimental cells or organisms. It is important to know whether the solvent itself – including the type of buffer - has an effect on the organisms, cells or assay. For this reason, we assessed the metabolic effects of organic solvents (dimethyl sulfoxide, Ethanol) and two

commonly used aqueous buffers (HBSS and EW) in which zebrafish embryos are grown. Dimethyl sulfoxide (DMSO) significantly decreased the level of many primary metabolites. Whereas, ethanol did not alter the embryos metabolome at any concentration tested. Likewise, both the aqueous buffers also resulted in entirely different embryos metabolome. Taken together, these results show that a critical evaluation of carrier solvents and aqueous medium is important to avoid false negative results. Particularly in case of zebrafish embryos metabolomic studies, DMSO has a relatively more pronounced effect than ethanol and used as a carrier solvent, DMSO concentration should be lower than 0.01%. Morevover, ^{1}H NMR based metabolomics can successfully be applied for the identification of markers of stress- or toxicity-induced metabolic shifts **(Chapter 7)**.

In conclusion, considering the aims of the thesis it has been shown:

that bacterial cell culture and also plant cell culteres are a promising tool for the large scale production of highly polar derivatives of Δ^{9}-THC. Further evaluation of these derivatives with respect to their binding affinity for CB receptors could be helpful in understanding the pharmacological properties of these more polar metabolites. .

- To develop novel tools to measure cannabinoids in-vivo assays studying the behavioral effects on zebrafish embryos/larvae, using the visual motor response test proved to be useful. A similar physiological response of zebrafish embryos to cannabinoids was found as reported for rodents. The zebrafish embryo can be applied early in the drug discovery pipeline and early assessment of drug safety for novel cannabinoid agonists and antagonists.

Future Prospects

A number of studies on the metabolic pathways of THC in several mammalian species have led to the discovery of a large collection of THC metabolites. Microorganisms and plant cell cultures have proved to be efficient sources for the large scale production of polar derivatives of THC. However, only a small fraction of these THC derivatives have been evaluated pharmacologically and the medicinal potential of a large proportion of these compounds still remains to be fully explored. The derivatives with differential binding affinities to CB receptors or relatively weaker binding affinities are particularly attractive in this regard as these might possess interesting pharmacological promise including the opportunity to develop drugs that are more specific and have less side effects.

Although zebrafish embryos have shown a rodent-like physiological response to CB_1 agonists and antagonists, still much work remains to be done to fully assess their role as an alternative model system to study different aspects of cannabinoid pharmacology. Particularly the question of the different functions of the two known cannabinoid receptors in humans translates to zebrafish will be of interest to study in more detail. Amongst the issues that require particular attention in this regard, are the amino acid sequence homology of zebrafish, human and rodent CB receptors, distribution and expression levels of CB receptors at different stages of zebrafish development and study of other receptors that might interact with cannabinoids such as the G protein-coupled receptor 55 (GPR_{55}) in rodents (Pertwee 2007). Future research along these lines would lead to a more comprehensive understanding the effects of natural, synthetic and endogenous cannabinoids in zebrafish. Moreover, combining molecular techniques like in-situ hybridization (ISH), quantitative PCR (qPCR) and whole mount staining of embryos would not only further establish the

usefulness of zebrafish as a model system but would also further improve our understanding of cannabinoid associated effects on animal physiology.

In conclusion, future explorations of zebrafish for pharmacokinetic studies of a plethora of psychoactive and non-psychoactive cannabinoids holds great promise for the field. This promise will certainly be further boosted by combining the classical genetic methods, state of the art molecular techniques and cutting edge metabolomic technologies such as NMR and LC/GC-MS with the high throughput that zebrafish embryos offer.

Reference

Pertwee RG. 2007. GPR55: a new member of the cannabinoid receptor clan? Br J Pharmacol 152: 984-986.