Cannabinoids and zebrafish

Muhammad Tayyab Akhtar

Muhammad Tayyab Akhtar

Cannabinoids and zebrafish

ISBN: 978-94-6203-345-0

Printed by: Wöhrmann Print Service

Cover art and designed by

M khurshid and MT Akhtar

Cannabinoids and zebrafish

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 22 mei 2013
klokke 10:00 uur

door

Muhammad Tayyab Akhtar

geboren te Rahim Yar Khan (Pakistan)

in 1984

Promotiecommissie

Promotor:	Prof. Dr. R. Verpoorte
Co-promotores:	Dr. F. van der Kooy Dr. Y.H. Choi
Overige leden:	Prof. Dr. S. Gibbons (The School of Pharmacy, London)
	Dr. F. Hollmann (TU Delft)
	Prof. Dr. M.K. Richardson
	Prof. Dr. P.G.L. Klinkhamer
	Prof. Dr. C.J. ten Cate

To My Father and Family!

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Muhammad Tayyab Akhtar

Natural Products Laboratory, Institute of Biology, Leiden University,
The Netherlands.

Cannabis is an annual, dioecious plant, more rarely monoecious, native to central Asia. Cannabis most likely originates from Neolithic China and its preparations were known to the ancient Assyrians, Scythians, Chinese, Indians, and Persians. However the complete history of domestication is not known but the first known evidence of the use of cannabis as a medicine was found in China 500 years ago (Hanuš and Mechoulam 2005). Preparations of cannabis have been used by man for their euphoric effects for over 4000 years (Uhl 2006). More than 700 varieties of Cannabis have been reported, however, the species and chemotaxonomic classification of this plant is quite controversial. Schultes et al. (1974) described three putative varieties of cannabis, *Cannabis sativa* L. (tall branched plants for fiber, seed and psychotropic use), *Cannabis indica* Lam. (short, broad leaflets plants utilized to produce hashish), *Cannabis ruderalis* Jan. (short, unbranched road side plants). More recently, de Meijer developed a classification system of cannabis based on the cannabinoid contents (de Meijer et al. 2003). For forensic and legislative purposes, the cannabis types are classified into the drug types and the fiber types (Hazekamp and Fischedick 2012). This classification is based on the contents of the psychoactive cannabinoid Tetrahydrocannabinol (THC). The ratio (THC + CBN)/CBD is determined for identifying the phenotypes of cannabis plants (Fetterman et al. 1971). If the ratio found is greater than 1, the cannabis variety is classified as drug type; if it is less than 1, it is a fiber type. More simply, cannabis strains cultivated for fiber and/or seed purposes (with low content of THC) are referred as fiber type, while the strains cultivated for medicinal or recreational use (with high content of THC) are recognized as drug type (Hillig and Mahlberg 2004, Hossein and Isaac 2007).

The basic material of all the cannabis products is the plant *Cannabis sativa* L. (Hazekamp 2007). Therefore, in literature more often, the variety *C. sativa* L. refers to cannabis. Likewise, in this thesis *C. sativa* will be used to denote

cannabis and vice versa. *Cannabis sativa* encompass both drug and nondrug varieties. Nondrug varieties are called hemp and drug varieties are referred to as marijuana (Hossein and Isaac 2007). The enormous number of products derived from *C. sativa* have greatly increased the attention for the chemistry and pharmacology of the plant. The demand for hemp made products is increasing in the global market, including fibers hemp leaves, hemp seed derivatives, oil, flour, beverages (beer, lemonade and liqueur) and cosmetic products (Lachenmeier et al. 2004). Hemp is becoming the centre of attention for sustainable economic development in USA (Tun 2005). The medicinal applications of marijuana are also intensively investigated. A large number of clinical reports favor the use of marijuana as an effective remedy against e.g. central thalamic pain, dystonia, familial Mediterranean fever, multiple sclerosis, chronic pain, depression, anxiety, migraine and sleeping difficulties (Ware et al. 2005). *Cannabis sativa* is a rich source of a variety of compounds; more than 500 chemical compounds have been identified in this plant, including cannabinoids, terpenoids and flavonoids. The synergism between these compounds might play a role in the therapeutic potential of cannabis (Stott and Guy 2004).

The term "cannabinoids" represents a group of C1, C3 and C5 side chains terpenophenolic compounds only found until now in *C. sativa* (Cannabaceae). The highest concentration of cannabinoids is present in resinous form in the buds and flowering tops of the female plants (Mechoulam and Goani 1967). After the purification and structure elucidation of Δ^9-Tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam 1964), Cannabidiol (CBD), Cannabiniol (CBN), and Δ^8-Tetrahydrocannabinol (Δ^8-THC) (El-Sohly 2006) were extensively studied and found to possess a variety of potentially useful pharmacological activities in addition to the psychotropic effects for which cannabis is well known (Monique and Stephen 1976). Cannabinoids, the active compounds in *C.*

sativa have many distinct therapeutic properties (Sirikantaramas et al. 2007). The analgesic, anti-emetic, anti-oxidative, neuroprotective and anti-inflammatory properties could be helpful in the treatment of many neurological disorders (Carter and Weydt 2002). Physiological actions as anticonvulsant, antidepressant, hypotensive, bronchodilation and lowering of intraocular pressure have led to a number of investigations on the possible development of useful medicines from the naturally occurring and synthetic cannabinoids. The Δ^9-THC is the psychoactive component of the cannabis plant (Scotter et al. 2010), while the major non psychoactive constituents include CBD, CBN, cannabigerol (CBG) and cannabichromene (CBC) (Gaoni and Mechoulam 1966). In the plant these compounds occur in their respective acidic form. i.e. having a carboxylic acid group. The acidic cannabinoids, which are non–psychotropic, undergo decarboxylation upon heat treatment (e.g. smoking) to yield the physchotropic cannabinoids.

The Δ^9-THC is known to be responsible for the main psychotropic effects of the cannabis made preparations (Ashton 2001). It was first identified in 1964 by Mechoulam and Gaoni (Gaoni and Mechoulam 1964). Sometimes, Δ^9-THC is used as a marker in evaluating the drug intensity of various cannabis based preparations (hashish, marijuana, hash oil) (Gambaro et al. 2002). The Δ^9-THC acts on the central nervous system (CNS) and caused euphoria, relaxation, tachycardia and alteration in blood pressure; hallucinations also appear at high doses (Pellegrini et al. 2005). It has been used in a number of disease states (e.g. pain, anxiety, asthma, glaucoma, and hypertension) (Abbott et al. 1977). Δ^9-THC mediates many of these effects by acting on the cannabinoid receptor type 1 (CB_1) (Tseng and Craft 2004, Varvel et al. 2005). The synthetic Δ^9-THC is being prescribed with the brand name “Marinol”. In 1986, the U.S food and drug administration (FDA) approved Marinol for the treatment of nausea and vomiting caused by cancer therapy. Marinol has also been used as an antiemetic

drug to treat loss of appetite and weight loss in patients infected with HIV (the virus causes AIDS). A U.K based company (GW Pharmaceuticals) sells the drug named Sativex, which contains the two best known cannabinoids, Δ^9-THC and CBD. Sativex has been approved in Canada, New Zealand and eight European countries. This drug is effective against muscle spasms associated with multiple sclerosis (MS), cancer and neuropathic pain.

After 5 decades of research, a lot is known about the chemistry and pharmacology of Δ^9-THC. Surely, Δ^9-THC has been approved by FDA and is being marketed to the pharmacies of U.S.A and Canada. But still the poor water solubility of Δ^9-THC is an issue which needs to be addressed. Δ^9-THC is a lipophilic compound and only slightly soluble in water. The human body contains high lipid contents, which are not only the body fats but also present in brain and cell membranes. Lipid-soluble drugs leave the blood rapidly and tend to accumulate in the fatty tissues (Hollister 1998). Likewise, Δ^9-THC binds strongly to plasma protein and other fatty tissues, which prolongs its release from the body (Paton 1975). The intravenously administered Δ^9-THC to a human can persists longer than three days in the plasma and its metabolites can be detected in the urine or feces even for 8 days (Kanter and Hollister 1977, Lemberger et al. 1970). Although, there is no report of continuing effects of the drug, the long persistence of Δ^9-THC in the body and the slow clearance of its metabolites might cause side effects. So, there is a need to structurally transform the compound to increase its polarity and its rapid release from the body.

Biotransformations are the chemical reactions carried out by cells, organs and enzymes. These reactions are used to structurally modify the compounds by exploring the unique properties of biocatalysts (Giri et al. 2001). Δ^9-THC was transformed into a number of new derivatives by using mammalian enzyme systems, leading to a better understanding of their pharmacodynamics and

pharmacokinetics. Mammalian transformations were useful to reveal the various metabolic pathways of Δ^9-THC in different mammalian species, but for the industrial scale production of the potential compounds by means of mammalian cell culture or tissue culture is too expensive to develop cannabinoid based drugs. Microorganisms may perform similar biotransformations as mammalians and could be suitable source for large scale production of new cannabinoids. The biotransformation potential of microorganisms and their enzymes for the production of new modified compounds is well acknowledged. The attractive characteristics of microorganisms make them favorable for biotransformation studies because of reduction in the number of animals used, ease of setup and manipulation (can easily be scaled up), and maintenance of stock cultures is simple and cost effective. Plants are able to produce a number of different chemicals which cannot easily be produced by synthetic means. Plant cell cultures are used to produce a variety of secondary metabolites including phenolics, steroids, alkaloids, coumarins and terpenoids. Plant enzymes act as unique biocatalysts and can successfully transform exogenous substrate to novel substances. Microorganisms and plant cell cultures have successfully produced new cannabinoids, which are structurally similar to the compounds obtained from mammalian transformations (This thesis). The only need is to scale up the production of these metabolites for further bioassays, clinical studies and potential commercial uses.

The zebrafish embryo is emerging as a prominent model of developmental biology, for disease studies and for drug discovery. A number of unique characteristics of the zebrafish embryo makes it an attractive model: small size, ease of maintenance, rapid development, large number of offspring, small amount of compound required for testing, and its optical transparency (Dahm and Geisler 2006). The embryos hatch approximately 2-3 dpf (Days post fertilization) and organogenesis of major organs is completed at 5 dpf

(Rubinstein 2003). The transparency of embryos and young larvae provide insight in the organ formation and their functions in developmental processes (Schwerte and Fritsche 2003). This optical transparency and access to all the developmental stages give an opportunity to study developing pathologies at different stages (Lieschke and Currie 2007). Genome sequencing has revealed a great deal of homology between the zebrafish and other vertebrates (including humans) (Schwerte and Fritsche 2003). A number of sophisticated mutagenesis and screening approaches have been developed in zebrafish embryos and made it a model of choice for the study of a wide variety of human diseases. Different protocols and automated imaging systems have been established for the behavioral analyses of zebrafish larvae. These systems produce a large set of data and facilitate high-throughput genetic, pharmacological and environmental screening (Creton 2009). A range of simple sensorimotor responses appear in the early developmental stages of the zebrafish larvae. These features represent the brain development and are useful for behavioral investigations (Souza and Tropepe 2011).

The zebrafish larvae are not only useful for high throughput analyses or acute toxicity but also helpful to understand the mechanisms of toxicity and possible adverse and long term effects of hazardous chemicals. The effect of added chemical entities finally leads to an alteration in gene transcription and protein expression, which ultimately affects the metabolic profile of the organism. Metabolites are the final product of gene expression and metabolomic profiling leads to the understanding of possible important events taking place in a cell, tissue or organism (Hayashi et al. 2011). A qualitative and quantitative measurement of all the metabolites present in a system at a particular time is called Metabolomics. Metabolomics, together with transcriptomics and proteomics, reflects the condition of the system and may show e.g. the effect of a medicine or toxin on the organism (Scholz et al. 2008). Metabolic

fingerprinting has been successfully introduced in zebrafish larvae to predict different embryonic stages (Hayashi et al. 2011, Hayashi et al. 2009). A multi-analytical approach (including 1H NMR, GC/MS and LC/MS) was also used to study the biochemical profile of livers of male and female zebrafish (Ong et al. 2009).

The CB1 (Lam et al. 2006) and CB2 (Rodriguez-Martin et al. 2007) receptors have been identified in zebrafish. A high level of sequence conservation of the CB_1 receptor has been shown for zebrafish and mammals (Lam et al. 2006). Involvement of the CB_1 receptor is also reported in the hatching process of zebrafish embryo (Migliarini and Carnevali 2009). The cannabinoid receptors in zebrafish embryos can help in studies 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 (Rodriguez-Martin et al. 2007).

Aim of the thesis

The aim of the present study was:

- To investigate the potential of bacterial strains and plant cell cultures to produce polar derivatives of Δ^9-THC.

- To examine the possibilities of using the zebrafish embryo to screen for cannabis receptor affecting compounds.

For the biotransformation, a library of microorganisms able to survive on hydrocarbons was available for screening. Methods for the analysis of cannabinoids and their metabolites and for their isolation and structure elucidation by spectroscopic methods were required for this study.

For the zebrafish screening the developmental, behavioral and metabolic effects of known cannabinoids in zebrafish embryos by using whole mount staining, visual motor response test needed to be explored. Furthermore a novel systems biology approach, using metabolomics was developed to study the effect of the cannabinoids.

References

Abbott BJ, Fukuda DS, Archer RA. 1977. Microbiological transformation of cannabinoids. Experientia 33: 718-720.

Ashton CH. 2001. Pharmacology and effects of cannabis: a brief review. Br J Psychiatry 178: 101-106.

Carter GT, Weydt P. 2002. *Cannabis*: Old medicine with new promise for neurological disorders. Curr Opin Investig Drugs 3: 437-440.

Creton R. 2009. Automated analysis of behavior in zebrafish larvae. Behav Brain Res 203: 127-136.

Dahm R, Geisler R. 2006. Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY) 8: 329-345.

de Meijer EP, Bagatta M, Carboni A, Crucitti P, Moliterni VM, Ranalli P, Mandolino G. 2003. The inheritance of chemical phenotype in *Cannabis sativa L.* Genetics 163: 335-346.

El-Sohly M, ed. 2006. Marijuana and the Cannabinoids New york: Humana Pr Inc.

Fetterman PS, Keith ES, Waller CW, Guerrero O, Doorenbos NJ, Quimby MW. 1971. Mississippi-grown *cannabis sativa* L.: Preliminary observation on chemical definition of phenotype and variations in tetrahydrocannabinol content versus age, sex, and plant part. J Pharm Sci 60: 1246-1249.

Gambaro V, Dell'Acqua L, Fare F, Froldi R, Saligari E, Tassoni G. 2002. Determination of primary active constituents in *Cannabis* preparations by high-resolution gas chromatography/flame ionization detection and high-performance liquid chromatography/UV detection. Anal Chim Acta 468: 245-254.

Gaoni Y, Mechoulam R. 1964. Isolation strucure and partial synthesis of an active constituent of hashish. J Amer Chem Soc 86: 1646.

Gaoni Y, Mechoulam R. 1966. Cannabichromene, a new active principle in hashish. Chem Commun 20-21.

Giri A, Dhingra V, Giri C, Singh A, Ward OP, Narasu ML. 2001. Biotransformations using plant cells, organ cultures and enzyme systems: current trends and future prospects. Biotech Adv 19: 175-199.

Hanuš L, Mechoulam R. 2005. Cannabinoid chemistry: an overview Cannabinoids as Therapeutics. Mechoulam R, Eds, Birkhäuser, Basel p. 23-46.

Hayashi S, Yoshida M, Fujiwara T, Maegawa S, Fukusaki E. 2011. Single-embryo metabolomics and systematic prediction of developmental stage in zebrafish. Z Naturforsch C 66: 191-198.

Hayashi S, Akiyama S, Tamaru Y, Takeda Y, Fujiwara T, Inoue K, Kobayashi A, Maegawa S, Fukusaki E. 2009. A novel application of metabolomics in vertebrate development. Biochem Biophys Res Commun 386: 268-272.

Hazekamp A. 2007. Cannabis: extracting the medicine. Leiden university , Leiden.

Hazekamp A, Fischedick JT. 2012. *Cannabis* - from cultivar to chemovar. Drug Test Anal 4: 660-667.

Hillig KW, Mahlberg PG. 2004. A chemotaxonomic analysis of cannabinoid variation in *Cannabis* (Cannabaceae). Am J Bot 91: 966-975.

Hollister LE. 1998. Health aspects of cannabis: revisited. Int J Neuropsychopharmacol 1: 71-80.

Hossein H, Isaac K. 2007. Hypercholesterolemic Effect of Drug-Type *Cannabis sativa* L. Seed (Marijuana Seed) in Guinea Pig. Pak j nutr 6: 59-62.

Kanter SL, Hollister LE. 1977. Marihuana metabolites in urine of man. VII. Excretion patterns of acidic metabolites detected by sequential thin layer chromatography. Res Commun Chem Pathol Pharmacol 17: 421-431.

Lachenmeier DW, Kroener L, Musshoff F, Madea B. 2004. Determination of cannabinoids in hemp food products by use of headspace solid-phase microextraction and gas chromatography-mass spectrometry. Anal Bioanal Chem 378: 183-189.

Lemberger L, Silberstein SD, Axelrod J, Kopin IJ. 1970. Marihuana: studies on the disposition and metabolism of Delta-9-tetrahydrocannabinol in man. Science 170: 1320-1322.

Lieschke GJ, Currie PD. 2007. Animal models of human disease: zebrafish swim into view. Nat Rev Genet 8: 353-367.

Mechoulam R, Goani Y. 1967. Recent advances in the chemistry of hashish. Fortschr Chem Org Naturst 25: 175-213

Migliarini B, Carnevali O. 2009. A novel role for the endocannabinoid system during zebrafish development. Mol Cell Endocrinol 299: 172-177.

Monique CB, Stephen S, Eds. 1976. Pharmacology of Marihuana: Philadelphia, PA, U.S.A. : Lippincott Williams & Wilkins.

Ong ES, Chor CF, Zou L, Ong CN. 2009. A multi-analytical approach for metabolomic profiling of zebrafish (*Danio rerio*) livers. Mol Biosyst 5: 288-298.

Paton WD. 1975. Pharmacology of marijuana. Annu Rev Pharmacol 15: 191-220.

Pellegrini M, Marchei E, Pacifici R, Pichini S. 2005. A rapid and simple procedure for the determination of cannabinoids in hemp food products by gas chromatography-mass spectrometry. J Pharm Biomed 36: 939-946.

Rodriguez-Martin I, de Velasco EM, Rodriguez RE. 2007. Characterization of cannabinoid-binding sites in zebrafish brain. Neurosci Lett 413: 249-254.

Rubinstein AL. 2003. Zebrafish: from disease modeling to drug discovery. Curr Opin Drug Discov Devel 6: 218-223.

Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D. 2008. The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. Environ Sci Pollut Res Int 15: 394-404.

Schultes RE, Klein WM, Plowman T, Lockwood TB. 1974. Cannabis: An example of taxonomic neglect. Bot museum leaf 23: 337-367.

Schwerte T, Fritsche R. 2003. Understanding cardiovascular physiology in zebrafish and Xenopus larvae: the use of microtechniques. Comp Biochem Phys A 135: 131-145.

Scotter EL, Abood ME, Glass M. 2010. The endocannabinoid system as a target for the treatment of neurodegenerative disease. Br J Pharmacol 160: 480-498.

Sirikantaramas S, Taura F, Morimoto S, Shoyama Y. 2007. Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology. Curr Pharm Biotechnol 8: 237-243

Souza BR, Tropepe V. 2011. The role of dopaminergic signalling during larval zebrafish brain development: a tool for investigating the developmental basis of neuropsychiatric disorders. Rev Neurosci 22: 107-119.

Stott CG, Guy GW. 2004. Cannabinoids for the pharmaceutical industry. Euphytica 140: 83-93.

Tseng AH, Craft RM. 2004. CB(1) receptor mediation of cannabinoid behavioral effects in male and female rats. Psychopharmacology (Berl) 172: 25-30.

Tun L. 2005. Sustainable development: Building a case for hemp. Journal of textile and apparel, technology and management 4.

Uhl M. 2006. Determination of Cannabinoids in Human Hair. Analytical and Practical Aspects of Drug Testing in Hair, CRC Press P. 127-141.

Varvel SA, Bridgen DT, Tao Q, Thomas BF, Martin BR, Lichtman AH. 2005. Delta-9-tetrahydrocannbinol accounts for the antinociceptive, hypothermic, and cataleptic effects of marijuana in mice. J Pharmacol Exp Ther 314: 329-337.

Ware MA, Adams H, Guy GW. 2005. The medicinal use of cannabis in the UK: results of a nationwide survey. Int J Clin Pract 59: 291-295.

CHAPTER 2

Biotransformation of Cannabinoids

Muhammad Tayyab Akhtar, Frank van der Kooy, and Robert Verpoorte

Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

Abstract

Cannabinoids are terpenophenolic compounds consisting of an aromatic polyketide and derived from the geranyl diphosphate C_{10} terpenoid unit. They are the active constituents in *Cannabis sativa* and have been utilized in a number of cannabis-based medicines. Biotransformation of cannabinoids is an important field of xenobiochemistry and toxicology and the study of the metabolism of these compounds can lead to the discovery of new compounds, unknown metabolites with unique structures and new therapeutic entities. Different fungi, bacteria, plants and animal cells have been used for the regio- and stereoselective transformation of cannabinoids. All of the above mentioned organisms have distinct enzymes which catalyze the conversion of a specific cannabinoid at different positions and thus provide a variety of derivatives. All organisms are able to transform the alkyl side chain where as mammalians are unique in the formation of the carboxy derivatives. This review article assesses the current knowledge on the biotransformation of Δ^8-THC, CBN, CBD with particular focus on Δ^9-THC.

Introduction

Pharmacological properties of cannabis are well known and documented in literature (Carter and Weydt 2002; Russo 2007). The term "cannabinoids" represents a group of C_1, C_3 and C_5 side chains terpenophenolic compounds found until now only in *Cannabis sativa L* (Cannabaceae) (Mechoulam and Goani 1967). Cannabinoids, the active compounds in *C. sativa* (Sirikantaramas et al. 2007) have distinct therapeutic properties. After the purification and structure elucidation, the best known cannabinoids, Δ^9-Tetrahydrocannabinol (Δ^9-THC) (Gaoni and Mechoulam 1964), Cannabidiol (CBD), Cannabiniol (CBN), and Δ^8-Tetrahydrocannabinol (Δ^8-THC) (Fig. 1) were extensively studied and found to possess a variety of potentially useful pharmacological

activities in addition to the psychotropic effects for which cannabis is well known (Monique and Stephen 1976). Such physiological actions as anticonvulsant, anti-emetic, anti-oxidative, neuroprotective, anti-inflammatory, antidepressant, hypotensive, bronchodilation and lowering of intraocular pressure have led to a number of investigations on the possible development of useful medicines from the naturally occurring and synthetic cannabinoids. THC is the psychoactive component of the cannabis plant (Scotter et al. 2010), while the major non psychoactive constituents include CBD, CBN, cannabigerol (CBG) and cannabichromene (CBC) (Fig. 1) (Gaoni and Mechoulam 1966). In the plant these compounds occur in their respective acidic form. i.e. having a carboxylic acid group. The acidic cannabinoids, which are non psychotropic, undergo decarboxylation upon heat treatment (e.g. smoking) to yield the physcotropic cannabinoids. Δ^9-THC has been used to treat number of disease states (e.g. pain, anxiety, asthma, glaucoma, hypertension) but also possesses some undesirable pharmacological side effects (e.g. euphoria, tachycardia, etc.) (Abbott et al. 1977). To minimize these undesirable side effects new cannabinoids have been prepared by de novo synthesis (Pars and Howes 1977) and by chemical and microbiological conversions (Abbott et al. 1977; Binder and Meisenberg 1978; Christie et al. 1978; Robertson et al. 1978b) of both naturally occurring and synthetic cannabinoids. This review assesses the available information regarding the production of various compounds obtained from the biotransformation of cannabinoids by using different microorganisms, plant cells and mammalian cells. In this review the benzoypyran numbering system is used instead of the monoterpene numbering system (under the monoterpene system Δ^9-THC is known as Δ^1-THC) (Fig. 2).

Biotransformation

Biotransformation can be defined as the enzymatic conversion of natural and

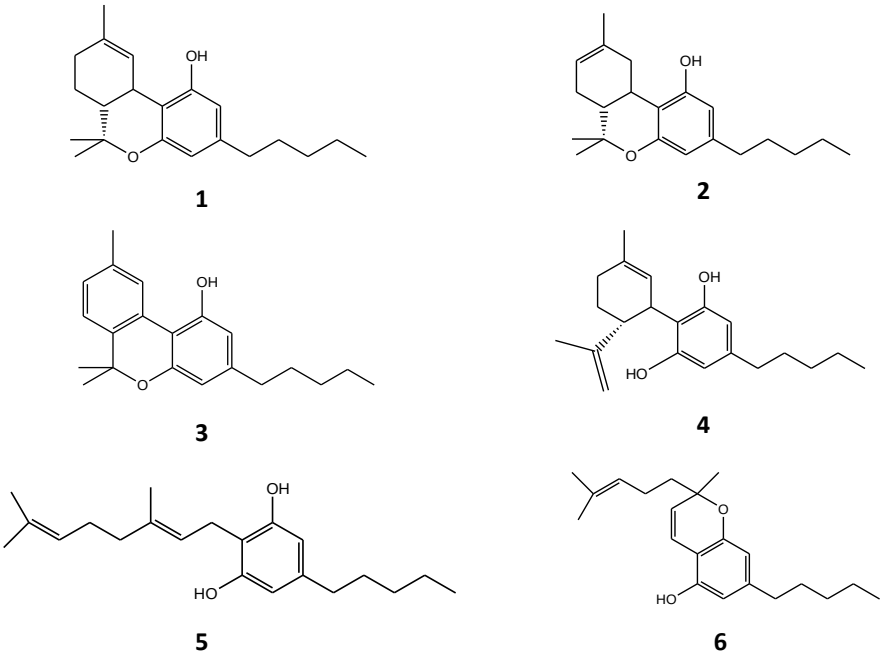


Figure. 1: Chemical structures of the major cannabinoids. **1**: Δ^9-THC, **2**: Δ^8-THC, **3**: CBD, **4**: CBN, **5**: CBG, **6**: CBC.

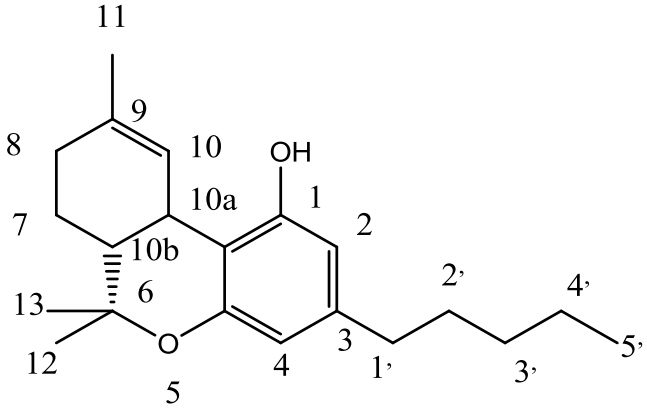


Figure 2: Δ^9-THC (Benzopyran numbering system)

synthetic compounds into substances having specifically modified structures (Venisetty and Ciddi 2003). Biotransformation is a vital process for survival, in that it transforms absorbed nutrients into substances required for normal bodily functions and to detoxify xenobiotics. These reactions convert non polar compounds into more polar compounds, making them water soluble and more easily excretable for the organism (Asha and Vidyavathi 2009). In this way biotransformation serves as an important defense mechanism in that, toxic xenobiotics and body wastes are converted into less harmful substances which can be easily excreted from the body. The pathways of xenobiotic metabolism can be divided into phase I and phase II reactions. Phase I includes oxidative, reductive and hydrolytic reactions. In these reactions, a polar group is introduced, so the molecule becomes more polar and thus improves excretion from the cell/or body. In phase II, polar endogenous molecules like glucoronic acid, sulfate, glycine, glutathione etc. are attached to form highly water soluble substances. These reactions are known as conjugation reactions (Smith and Rosazza 1983).

Active metabolites obtained from biotransformation

Plant, animal, microbial cells and purified enzymes are being used to carry out specific conversions of complex substances. This approach has great potential to generate novel products (Giri et al. 2001). Biotransformation reactions yield chemically stable metabolites which can be pharmacologically active (Baillie et al. 2002, Fura et al. 2004, Garattini 1985, Guengerich 2001, Kumar and Surapaneni 2001) and might thus significantly contribute to the therapeutic effect of a drug. Some of these have even been developed as drugs in their own right. Important examples are atorvastatin (Lennernas 2003; Williams and Feely 2002), simvastatin (Thomayant et al. 2003; Williams and Feely 2002),

fluoxetine (Cheer and Goa 2001), cetirizine (Golightly and Greos 2005) and fexofenadine (Golightly and Greos 2005, Meeves and Appajosyula 2003).

In a traditional prodrug-based approach to drug discovery, biotransformation reactions can play a key role to convert pharmacologically inactive compounds to active metabolites with strong therapeutic effects through phase I (oxidative or reductive) and phase II (conjugative) metabolism. Some of the important biotransformation reactions are aliphatic or aromatic carbon hydroxylation, epoxidation, heteroatom oxidation (N, S, and P), reduction, glucuronidation, sulfation and acetylation.

Microorganisms in biotransformation

The biotransformation potential of microorganisms and their enzymes for the production of a large library of novel metabolites is well documented (Nikolova and Ward 1993, Schulze and Wubbolts 1999, Ward and Singh 2000). The use of microbial cell cultures for transformation of natural products is favorable because they mimic mammalian biotransformation (Smith and Rosazza 1983). Microbial models offer a number of advantages over the use of animals in metabolism studies, such as a reduction in the number of animals used (particularly in the early phases of drug development), ease of setup and manipulation (microbial models can be scaled up easily for the preparation of large quantities of metabolites) and cost effectiveness. In addition, methods for genetic modification of microbes are well established (Rathbone and Bruce 2002). Microbial strains with specific properties for certain chemical conversions may be selected or even engineered for stereoselective and regiospecific conversions (Abourashed et al. 1999, Demain 2000, Rathbone and Bruce 2002, Smith and Rosazza 1983). The maintenance of stock cultures of microorganisms is also easier and cheaper than the maintenance of mammalian cell and tissue cultures or laboratory animals. Microorganisms can also be

utilized to perform reactions which are difficult to perform chemically (Venisetty and Ciddi 2003).

Plants and plant cells in biotransformation

Plants are able to produce a number of diverse biochemicals including drugs, flavors, pigments and agrochemicals which cannot easily be achieved by synthetic means. Plant cell cultures have great ability to produce specific secondary metabolites like a wide variety of chemical compounds including aromatics, steroids, alkaloids, coumarins and terpenoids. Production of secondary metabolites and the biotransformation of precursor compounds by plant tissue cultures may lead to both the synthesis of valuable substances and elucidation of their biosynthetic pathways (Berlin et al. 1989, Loh et al. 1983). Production and accumulation of plant specific secondary metabolites are not always possible in plant cell cultures. However such cultures may retain an ability to transform exogenous substrates into products of interest. Plant enzymes act as suitable biocatalysts to perform complex biochemical reactions (Giri et al. 2001). The chemical compounds, which can undergo biotransformation by plant enzymes, are diverse in nature (Franssen and Walton 1999) and are not necessarily natural intermediates in plant metabolism but can also be of synthetic origin (Pras et al. 1995). Plant cell cultures and enzymes have thus the potential to transform substances, such as industrial byproducts, into novel, valuable products. Plant biotransformation systems can be used alone or in combination with organic synthesis to produce novel chemicals.

Biotransformation of cannabinoids

Biotransformation of cannabinoids by mammalian cells

Extensive *in vivo* and *in vitro* studies have been carried out on the biotransformation of cannabinoids by mammalian cell cultures.

Tetrahydrocannabinol (THC) and other cannabinoids are shown to be good substrates for cytochrome P450 (CYP450) enzymes because of their hydrophobic properties (Yamamoto et al. 1995). Mammalian CYP450s metabolise Δ^9-THC into a number of new compounds. Many oxidative metabolites of tetrahydrocannabinols (THCs) such as 11-hydroxy-THC, 11-oxo-Δ^8-THC, 7-oxo-Δ^8-THC, 8β,9β-epoxyhexahydrocannabinol (EHHC), 9α,10α-EHHC and 3'-hydroxy-Δ^9-THC (Table 3) are pharmacologically more active than THC (Yamamoto et al. 2003).

Metabolism of THC in humans

The metabolism of Δ^9-THC and related cannabinoids in human has been studied by utilizing parenteral, oral, and smoking as a route of administration. The general pattern of metabolism is the same in all cases with as major metabolic reactions the formation of an 11-hydroxy derivative and as minor product the 8-hydroxy derivative (Wall and Perez-Reyes 1981). The monocarboxylated derivatives have been isolated from human urine (Table 3, compound 20 and 23), further side chain carboxylated and their hydroxylated derivatives occur and were also found in urine (Table 4, compound 27, 28, 29, 30, 31, 32, 34, 35, 36, 37, 39) (Halldin et al. 1982a, Halldin et al. 1982b). Among the acid derivatives, Δ^9-THC-11-oic acid (Table 3, compound 20) (Halldin et al. 1982b, Kanter and Hollister 1978), 4',5'-bisnor-Δ^9-THC-11,3'-dioic acid (Table 4, compound 35) (Halldin et al. 1982a) and Δ^9-THC-11-oic acid glucoronide (Fig. 3a) (Williams and Moffat 1980) are identified as the major metabolites in human urine. *In vitro* human liver metabolism yields 8α-hydroxy- (Table 1, compound 2) (Bornheim et al. 1992, Widman et al. 1979), 11-hydroxy-, 8β-hydroxy- (Table 1, compound 1, 3) (Bornheim et al. 1992, Halldin et al. 1982c, Wall et al. 1972, Widman et al. 1979), traces of 11-hydroxy-

epoxyhexahydrocannabinol (11-OH-EHHC) (Fig. 3b) (Halldin et al. 1982c) and 8-keto-Δ^9-THC (Fig. 3c) (Bornheim et al. 1992).

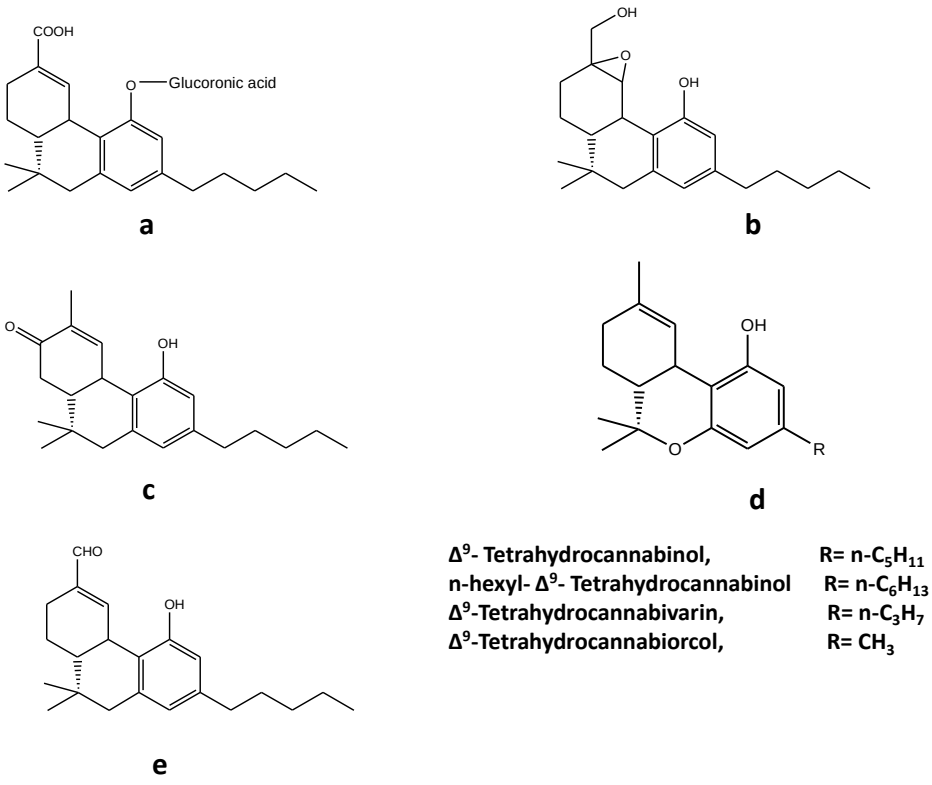


Figure 3: a; Δ^9-THC-11-oic acid glucoronide **b**; 11-hydroxy-EHHC **c**; 8-keto-Δ^9-THC **d**; Derivatives of THC **e**; 11-oxo- Δ^9- THC.

Metabolism of THC in rabbits

After *in vivo* administration of the Δ^9-THC to rabbits, a number of mono- and dihydroxy derivatives (Table 1 ,2) (Nordqvist et al. 1979a), monocarboxylic acid (Table 3, Compound 20) and side chain acids (Table 4, compound 26, 27, 29, 31, 32, 33, 34, 35, 38, 39) (Burstein et al. 1972, Nordqvist et al. 1979a) were found in the rabbit urine including the 4',5'-bisnor-Δ^9-THC-11,3'-dioic acid

(Table 4, compound 35) as a major compound (Nordqvist et al. 1974). Furthermore, three side-chain monocarboxylic acids hydroxylated in the allylic position in the isoprene moiety were also identified as O-glucuronide in rabbit urine (Nordqvist et al. 1979b). Rabbit *in vitro* liver microsomal metabolism of Δ^9-THC formed 11-hydroxy- (Table 1, compound 1) (Burstein and Kupfer 1971a, Nilsson et al. 1970; Wall et al. 1970) and 8α-hydroxy-Δ^9-THC (Table 1, compound 2) (Benzvi and Burstein 1975). Microsomal oxygenase was found to catalyze the oxidation of 11-hydroxy-Δ^8-THC to 11-oxo-Δ^8-THC (Watanabe et al. 1979).

Table 1. Monohydroxylated metabolites of Δ^9- THC. (1) man, (2) rhesus monkey**,** (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms.

		Species								References
No	Position of OH	1	2	3	4	5	6	7	8	
1	11-OH	+	+	+	+	+	+	+	+	(Burstein and Kupfer 1971a, Burstein and Kupfer 1971b, Matsunaga et al. 1995, Nilsson et al. 1970, Wall et al. 1970)
2	8α-OH	+	+	+	+	+	+	+	+	(BenZvi et al. 1974, Harvey and Paton 1976, Jones et al. 1974, Matsunaga et al. 1995, Wall et al. 1972, Widman et al. 1975a)
3	8β-OH	+	+	+	+	+	+	+	+	(Harvey et al. 1977, Matsunaga et al. 1995, Wall 1971, Wall et al. 1972, Widman et al. 1975a)
4	1'-OH	−	−	−	−	−	+	−	+	(Binder and Popp 1980, Harvey et al. 1978b, Harvey et al. 1980a)
5	2'-OH	+	−	−	−	−	+	−	+	(Binder and Popp 1980, Halldin et al. 1982c, Harvey et al. 1978b, Harvey et al. 1980a)
6	3'-OH	−	−	+	+	+	+	+	+	(Binder 1976, Christie et al. 1978, Harvey et al. 1977, Harvey et al. 1978b, Harvey et al. 1980a, Widman et al. 1975a)
7	4'-OH	−	−	+	−	−	+	+	+	(Binder 1976, Binder and Meisenberg 1978, Binder and Popp 1980, Harvey and Paton 1978, Harvey et al. 1980a, Robertson and Lyle 1975, Widman et al. 1975a)

Metabolism of THC in mouse

Harvey and Paton (1978) identified multiple substituted metabolites formed by *in vivo* metabolism of Δ^9-THC in the mouse liver among which, 11-; 2'-; and 3'-

Table 2. Di- and trihydroxy metabolites of Δ^9-THC. (1) man, (2) rhesus monkey, (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms.

		Species								References
No	Position of OH Groups	1	2	3	4	5	6	7	8	
9	8α , 11-di-OH	+	+	+	+	+	+	–	–	(Benzvi Z. and Burstein 1975, Halldin et al. 1982c, Harvey and Paton 1976, Harvey et al. 1978b, Harvey et al. 1980a, Jones et al. 1974, Wall and Brine 1976, Wall et al. 1972)
10	8β, 11-di-OH	+	+	+	+	–	+	+	–	(Halldin et al. 1982c, Harvey et al. 1977, Harvey et al. 1978b, Harvey et al. 1980a, Wall and Brine 1976)
11	1', 11-di-OH	–	+	–	–	–	+	–	–	(Harvey et al. 1980a, Wall and Brine 1976)
12	2', 11-di-OH	+	+	+	–	–	+	–	–	(Halldin et al. 1982c, Harvey et al. 1977, Harvey et al. 1978b, Harvey et al. 1980a, Wall and Brine 1976)
13	3', 11-di-OH	–	+	+	+	–	+	–	+	(Christie et al. 1978, Halldin et al. 1982c, Harvey et al. 1977, Harvey et al. 1978b, Wall and Brine 1976)
14	4', 11-di-OH	+	+	+	–	–	+	–	+	(Binder 1976, Halldin et al. 1982c, Harvey and Paton 1978, Harvey et al. 1980a, Wall and Brine 1976)
15	4', 8α-di-OH	–	–	–	–	–	–	–	+	(Binder 1976)
16	1'-4', 8β-di-OH	–	–	–	–	–	+	–	–	(Harvey et al. 1980a)
17	1', 2'-4'-di-OH	–	–	–	–	–	+	–	–	(Harvey et al. 1980a)
18	2'-4',8α,11-tri- OH	–	–	+	–	–	+	–	–	(Harvey and Paton 1978, Harvey et al. 1977, Harvey et al. 1978b, Harvey et al. 1980a)
19	2'-4', 8β,11-tri- OH	–	–	–	–	–	+	–	–	(Harvey et al. 1980a)

the monohydroxy derivatives (Table 1, compound 1, 5, 6), 8β, 11-; 2', 11-; and 3', 11-dihydroxy derivatives (Table 2, compound 10, 12, 13), 2', 8α, 11- and 3', 8α, 11- the most abundant trihydroxy derivatives (Table 2, compound 18). Harvey and Paton (1978) also found the formation of 2',11-, and 3',11-dihydroxy-8-oxo-Δ^9-THC (Table 6, compound 48) in mouse liver and 4'-hydroxylation (Table 1, compound 7) was suggested as a major metabolic route for the Δ^9-THC transformation (Harvey et al. 1977). Mono- and dihydroxy-acids of Δ^9-THC have also been found in mouse liver. Δ^9-THC-11-oic acid

(Table 3, compound 20) was identified from the mouse liver together with its 8α- and 2'- monohydroxy derivative (Table 5, compound 41 and 43), 2',8α- and 3',8α-dihydroxy derivatives (Table 5, compound 45) (Harvey and Paton 1976; Harvey et al. 1978b). *In vitro* mouse liver microsomes transformed Δ^9-THC into 11-hydroxy- (Jones et al. 1974) and 8α-hydroxy-Δ^9-THC (BenZvi et al. 1974) (Table 1, compound 1,2). Later on, transformation of 8α-; 8β- and 11-hydroxy-Δ^9-THC was studied (Burstein and Shoupe 1981), metabolism of 8α-hydroxy-Δ^9-THC was parallel to that of Δ^9-THC but the presence of 8β-hydroxy group was found to suppress the hydroxylation and oxidation at C-11 and to increase the β-oxidation of the side-chain (Harvey et al. 1980b).

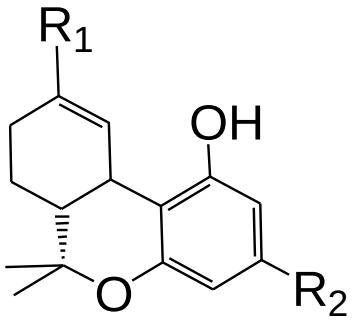
The *in vivo* metabolism of Δ^8-THC produced three alcohols, five diols, six triols, five monohydroxy acids, six dihydroxy acids, two substituted ketones, an epoxide, three dihydroxy metabolites and a glucuronide conjugate (Harvey and Paton 1980). Δ^{11}-THC was transformed into 26 metabolites produced by epoxidation among which 9α,11- and 9β,11-dihydroxyhexahydrocannabinols were the key metabolites (Harvey et al. 1980c). 11-oxo-Δ^8-tetrahydrocannabinol (11-oxo- Δ^8-THC) was identified from the mouse brain as a new *in vivo* metabolite of Δ^8-THC (Watanabe et al. 1980).

Metabolism of THC in monkey, guinea pig and dog

Purified Cytochrome P450 isozyme of monkey metabolized Δ^9-THC to 11-; 8α-; 8β- and 3'- hydroxy-Δ^9-THC (Table 1, compound 1, 2, 3, 6) (Matsunaga et al. 1995). Moreover, Δ^9-THC was also metabolized to some dihydroxy derivatives, such as 8α,11-; 8β,11-; 1',11-; 2',11-; 3',11- and 4',11-Δ^9-THC (Table 2, compound 9, 10, 11, 12, 13, 14) (Wall and Brine 1976).

Metabolism and autoradiography distribution showed the biotransformation of Δ^8-THC to the more potent and pharmacologically active 11-hydroxylated

Table 3. Mono-carboxylic acid metabolites of Δ^9-THC. (1) man, (2) rhesus monkey, (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms.



No	R^1	R^2	Species								References
			1	2	3	4	5	6	7	8	
20	COOH	C_5H_{11}	+	+	+	+	+	+	+	-	(BenZvi and Burstein 1974, Halldin et al. 1982c, Harvey and Paton 1976, Harvey et al. 1978b, Harvey et al. 1980a, Kanter and Hollister 1978, Nordqvist et al. 1979a)
21	CH_3	COOH	-	-	-	-	-	+	-	-	(Harvey et al. 1980a)
22	CH_3	CH_2COOH	-	-	-	-	-	+	-	-	(Harvey et al. 1978b, Harvey et al. 1980a)
23	CH_3	C_2H_2COOH	+	+	+	-	-	+	-	+	(Halldin et al. 1982b, Harvey et al. 1978b, Harvey et al. 1980a, Nordqvist et al. 1979a, Robertson et al. 1978a)
24	CH_3	C_3H_6COOH	-	-	-	-	-	+	-	-	(Harvey et al. 1978b, Harvey et al. 1980a)
25	CH_3	C_4H_8COOH	-	-	-	-	-	-	-	+	(Robertson et al. 1978a)

metabolite in the monkey *Callithrix jacchus* (Just et al. 1975). The rhesus monkey metabolized Δ^8-THC to various monohydroxy and dihydroxy metabolites. 11-hydroxy-Δ^8-THC was the most abundant metabolite. Furthermore, all the side-chain hydroxy metabolites except 5'-hydroxy- Δ^8-THC were identified. The total 4'-hydroxy- derivative was present in about one-third and 3'-hydroxy- in about one-sixth of the amount of 11-hydroxy-Δ^8-THC. Only minor amounts of 1'- and 2'-hydroxy- Δ^8-THC were isolated (Halldin et al. 1979, Widman et al. 1979).

Several mono-, di- and trihydroxy derivatives were formed by *in vivo* liver metabolism of Δ^9-THC by guinea pigs. The 11-; 8α-; 8β-; 1'-; 2'- and 3'- were the monohydroxy derivatives (Table 1, compound 1,2 ,3 4, 5, 6), 8α,11-; 8β,11-; 2,11-; and 3,11-dihydroxy derivatives (Table 2, compound 9, 10, 12, 13), 2,8α,11- and 3,8α,11- were major trihydroxy derivatives (Table 2, compound 18) (Harvey et al. 1978b). Guinea pigs also produced large amounts of the monocarboyxlic acids (Table 3, compound 20, 21, 22, 23, 24), substituted side-chain carboxylic acid (Table 4, compound 32), mono- and dihydroxy-derivatives of Δ^9-THC-11-oic acid (Table 5, compound 41, 42, 43, 44). The major metabolic pathways involved were allylic and aliphatic hydroxylations, oxidation of alcohols to ketones and acids, β oxidative degradation of the pentyl side chain and conjugation with glucuronic acid (Harvey et al. 1978a, Harvey et al. 1980a).

Twenty-nine metabolites were reported in a study of Δ^8-THC metabolism by guinea pigs. The 1'-hydroxy- and 4'-oxo-Δ^8-THC-11-oic acid were new metabolites with other metabolites having β-oxidation at the side chain (Harvey and Paton 1981). Hepatic microsomes of guinea pig oxidized Δ^8-THC to 7α-OH- and 7β-OH-Δ^8-THC which were further converted into 7-oxo-Δ^8-THC but with different mechanisms in both cases (Narimatsu et al. 1988).

Isolated perfused dog lung metabolized Δ^9-THC into 3'-hydroxy- and 4'-hydroxy- derivatives (Table 1, compound 6, 7) as the major metabolites, whereas small amounts of 8α- and 8β-hydroxy-Δ^9-THC (Table 1, compound 2, 3) were also produced which are predominant metabolites identified from *in vitro* dog liver microsomal metabolism of Δ^9-THC (Widman et al. 1975a).

Table 4. Substituted side-chain carboxylic acid metabolites of Δ^9-THC (1) man, (2) rhesus monkey, (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms.

			Species								References
No	Side-chain	Substituents	1	2	3	4	5	6	7	8	
26	COOH	11-OH, 8α-OH, 8β-OH, 8β, 11-di-OH	–	–	–	–	+	–	–	–	(Nordqvist et al. 1979a)
27	COOH	9-COOH	+	–	–	–	+	–	–	–	(Halldin et al. 1982a, Nordqvist et al. 1979a)
28	CH_2COOH	11-OH	+	–	–	–	–	–	–	–	(Halldin et al. 1982b)
29	CH_2COOH	8β-OH	+	–	–	–	+	–	–	–	(Halldin et al. 1982b, Nordqvist et al. 1979a, Nordqvist et al. 1979b)
30	CH_2COOH	9-COOH	+	–	–	–	–	–	–	–	(Halldin et al. 1982a)
31	C_2H_4COOH	11-OH	+	–	–	–	+	–	–	–	(Halldin et al. 1982b, Nordqvist et al. 1979b)
32	C_2H_4COOH	8β-OH	+	–	–	–	+	+	–	–	(Halldin et al. 1982b, Harvey et al. 1980a, Nordqvist et al. 1979a, Nordqvist et al. 1979b)
33	C_2H_4COOH	8β, 11-di- OH	–	–	–	–	+	–	–	–	(Nordqvist et al. 1979a)
34	C_2H_4COOH	9-COOH	+	–	–	–	+	–	–	–	(Halldin et al. 1982a, Nordqvist et al. 1979a, Nordqvist et al. 1974)
35	C_2H_4COOH	11-COOH	+	–	–	–	+	–	–	–	(Halldin et al. 1982a, Nordqvist et al. 1974)
36	C_3H_6COOH	8β-OH	+	–	–	–	–	–	–	–	(Halldin et al. 1982b)
37	C_3H_6COOH	9-COOH	+	–	–	–	–	–	–	–	(Halldin et al. 1982a)
38	C_4H_8COOH	9-COOH	–	–	–	–	+	–	–	–	(Nordqvist et al. 1979a, Nordqvist et al. 1979b)
39	CH=CH-COOH	9-COOH	+	–	–	–	+	–	–	–	(Halldin et al. 1982a, Nordqvist M. et al. 1979a)
40	CH_2-CH=CH-COOH	9-COOH	+	–	–	–	–	–	–	–	(Halldin et al. 1982b)

Metabolism of THC in mice and rat

Several derivatives have been synthesized by administering Δ^8- and Δ^9-THC to

Table 5. Hydroxy and dihydroxy derivatives of Δ^9-tetrahydrocannabinol-11-oic acid. (1) man, (2) rhesus monkey, (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms

		Species								References
No	Position of OH	1	2	3	4	5	6	7	8	
41	8α-OH	–	–	+	+	–	+	–	–	(Harvey and Paton 1976, Harvey et al. 1978b, Harvey et al. 1980a)
42	1'-OH	+	–	–	–	+	+	–	–	(Burstein et al. 1972, Halldin et al. 1982b, Harvey et al. 1980a)
43	2'-OH	–	–	+	+	+	+	–	–	(Burstein et al. 1972, Halldin et al. 1982b, Harvey and Paton 1976, Harvey et al. 1978b, Harvey et al. 1980a, Nordqvist et al. 1979b)
44	3', 4'-di-OH	–	–	+	+	+	+	–	–	(Halldin et al. 1982b, Harvey and Paton 1976, Harvey et al. 1978b, Harvey et al. 1980a, Nordqvist et al. 1979b)
45	2',8α-, 3',8α-, 4',8α-di-OH	–	–	+	+	–	–	–	–	(Harvey and Paton 1976, Harvey and Paton 1978, Harvey et al. 1978b)

Table 6. Mono- and di-hydroxy derivatives of 8-oxo-Δ^9-tetrahydrocannabinol. (1) man, (2)rhesus monkey, (3) mouse, (4) rat, (5) rabbit, (6) guinea pig, (7) dog, (8) microorganisms.

		Species								References
No	Position of OH	1	2	3	4	5	6	7	8	
46	7-, 2'-, 3'-OH	–	+	–	–	–	+	–	–	(Harvey et al. 1977, Harvey et al. 1978b, Harvey et al. 1980a)
47	4'-OH	–	–	–	–	–	+	–	+	(Binder 1976, Harvey et al. 1980a)
48	2', 11-, 3', 11-, 4', 11-di-OH.	–	–	+	–	–	–	–	–	(Harvey and Paton 1978, Harvey et al. 1977, Harvey et al. 1978b)

male mice (Charles River CD-1) to predict metabolic pathways and to find new metabolites. Brown and Harvey (1988a) reported the metabolism of Δ^9-tetrahydrocannabiorcol (Δ^9-THCO) (Fig. 3d) and Δ^8-Tetrahydrocannabiorcol (Δ^8-THCO) into Δ^9-THC-11- and Δ^8-THCO-11-oic acid respectively. Sixteen metabolites were identified from n-hexyl-Δ^9-THC (Fig. 3d) and eleven from n-hexyl-Δ^8-THC (Brown and Harvey 1988b). Seven metabolites were formed from each Δ^9-tetrahydrocannabivarin (Δ^9-THCV) (Fig. 3d) and Δ^8-

tetrahydrocannabivarin (Δ^8-THCV). The major biotransformation pathway led to the production of 11-hydroxy-tetrahydrocannabivarin and their further oxidation to carboxylic acid metabolites, other metabolites were mainly the hydroxy derivatives of these compounds (Brown and Harvey 1988c). Metabolism of 2'-, 3'- and 4'-hydroxy-Δ^9-THC were also studied in mice. Hydroxylation at the allylic 11-position followed by oxidation to the carboxylic acid was found to be an important metabolic pathway (Harvey 1990). Little oxidative degradation of the pentyl side chain was found for 2'-hydroxy-Δ^9-THC (Table 1, compound 5) but abundant metabolism occurred by oxidative cleavage of the pentyl side chain from 3' and 4'-hydroxy-Δ^9-THC (Table 1, compound 6, 7) (Harvey 1989).

Harvey and Paton (1979) described the conversion of deuterium-labeled Δ^8-, Δ^{11}- and Δ^9-THC into a number of metabolites containing 1-3 additional groups at positions 2'; 3'; 4'; 8α; 8β and 11 with the only difference that Δ^{11}-THC did not undergo allylic hydroxylation at position 11. *In vivo* liver metabolites of Δ^9-THC were extracted from rats. The 11-; 8α-; 8β- and 3'- (Table 1, compound 1, 2, 3 and 6) were the monohydroxy-, 8α,11-; 8β,11- and 3',11- (Table 2, compound 9, 10, 13) were the major dihydroxy- derivatives (Harvey et al. 1978b). Rat liver microsomes transformed Δ^9-THC into 11-oxo- (Fig. 3e) and further reduction of this derivative produced 11-hydroxy-Δ^9-THC (Table 1, compound 1) (BenZvi and Burstein 1974, Burstein and Kupfer 1971a). Rat lever homogenates led to the formation of 11-hydroxy- (Table 1, compound 1) (Burstein and Kupfer 1971b) and 8α,11-dihydroxy-Δ^9-THC (Table 2, compound 9) (Burstein and Kupfer 1971a, Wall 1971, Wall et al. 1970).

Rat liver incubation of Δ^8-THC produced 11-hydroxy-; 7α,11- and 7β,11-dihydroxy-Δ^8-THC (Wall 1971). Purified cytochrome P450 isozymes from rat hepatic microsomes are reported to be able to catalyze the oxidation of 11-oxo-

Δ^8-THC to Δ^8-THC-11-oic acid (Watanabe et al. 1991). Sex related differences were observed in the oxidative metabolism of Δ^9-THC between male and female rats. Liver microsomes of male rats biotransformed Δ^9-THC into various metabolites unlike female rats in which it was mainly oxidized selectively to 11-hydroxy-Δ^9-THC (Table 1, compound 1) (Burstein and Kupfer 1971a, Narimatsu et al. 1991).

Biotransformation of THC in mammalian cells shows that cytochrome P450 enzymes play an important role in the hydroxylation of THC. THC undergoes allylic hydroxylation at C-11 to form 11-hydroxy-THC which is the most common metabolite in almost every species studied. Allylic hydroxylation is also common at position 8 to give 8α- and 8β-hydroxy-THC. Other than allylic hydroxylations there are a number of monohydroxy and dihydroxy metabolites formed, being hydroxylated at all the positions of the alkane side chain of THC. There is however considerable species variation in the position of substitution. In addition to monohydroxy and dihydroxy, some polyhydroxylated metabolites have also been found in mice with hydroxyl substitution at position 2' and 3'. In monkeys and dogs, hydroxyl substitution was more common on the positions 3' and 4' while the monkey also formed minor amounts of metabolites having hydroxylation on position 1' and 2'. The major metabolic pathway of THC (Fig. 4) after monohydroxy metabolism is further oxidation to either a carbonyl compound or, in the case of the primary alcohols to a carboxylic acid, followed by glucuronidation of the acid. Following this biotransformation pathway there are a number of THC acids which are formed in man, rats, rabbits and guinea pigs where the acids link with glucuronide and become the major urinary excretion product.

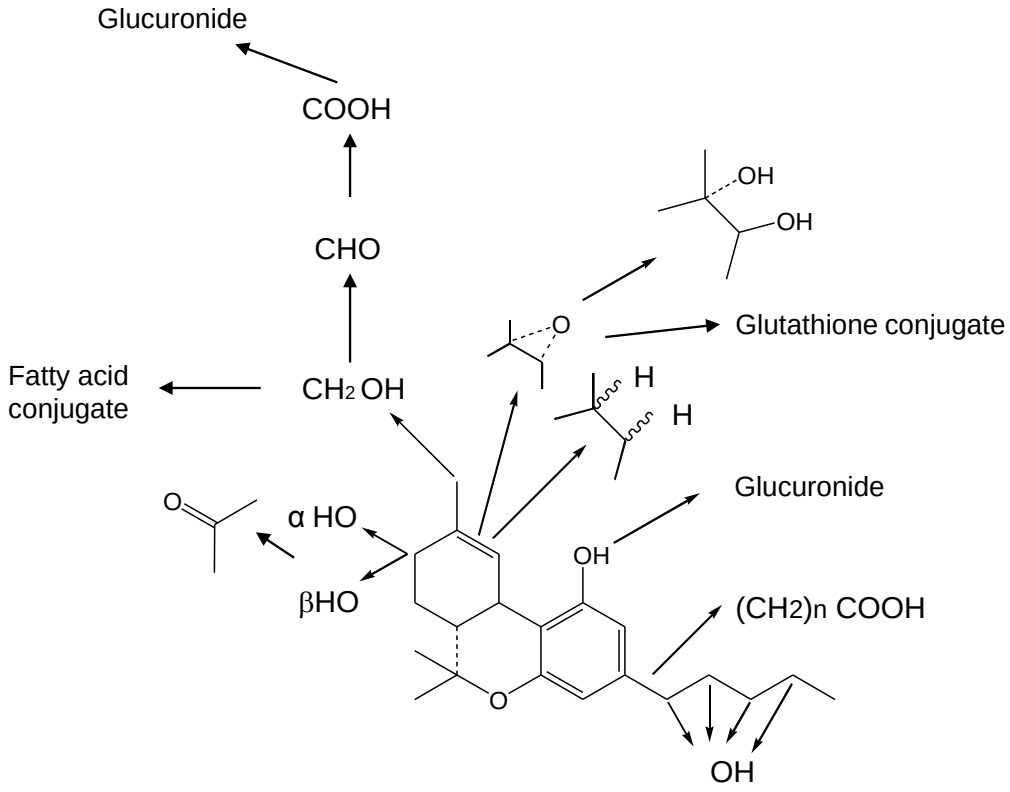


Figure 4: Metabolic pathway of Δ^9-THC

Biotransformation of Cannabidiol

Biotransformation of cannabidiol (CBD) has extensively been studied in mice and rats. Fourteen metabolites were isolated from the liver of mice after the administration of CBD. The most significant biotransformation step was glucoronide conjugation and to a lesser extent CBD-11-oic-acid was formed (Martin et al. 1977). Eight monohydroxy (Martin et al. 1976b) and ten dihydroxy metabolites were found in the *in vitro* transformation of CBD in rats

(Martin et al. 1976a), in both cases mainly 11-hydroxylation was formed with further hydroxylation at C-4 of the side chain. Over fifty metabolites of CBD were identified with considerable species variation in dog, rat and human urine. Thirty three metabolites were identified from the urine of dystonic patients treated with CBD. The major metabolic pathway was the hydroxylation and oxidation at C-11 and further hydroxylation in the pentyl and propyl groups produced 1'-; 2'-; 3'; 4'- and 10-hydroxy derivatives of CBD-11-oic acid. Acids were formed by β-oxidation and further biotransformations from the pentyl side-chain resulted in the formation of the oxidized metabolite CBD-11-oic acid (Harvey and Mechoulam 1990). In dog unusual glucoside conjugates were found of the metabolites 4'-; 5'-hydroxy- and 8-oxo-CBD. Other metabolites in all three species were mainly acids. Side-chain hydroxylated derivatives of CBD-11-oic acid were abundant in human urine (Harvey et al. 1991). CBD followed almost the same pattern of biotransformation as observed in Tetrahydrocannabinol, with several variations in pathways caused by different species. The monohydroxylation of the side chain and also at position 11 and 8 found in CBD is quite similar to THC. Dihydroxy compounds are also reported but unlike THC no trihydroxy metabolites are found in any species treated with CBD. A large number of metabolites are formed by β-oxidation of the side chain and Glucoronidation of CBD.

Biotransformation of Cannabinol

Cannabinol was metabolized by rat liver into side chain hydroxylated compounds: the 2'-; 3'-; 4'- and 5'-hydroxy were isolated as minor metabolites and 11-hydroxy-CBN was formed as the major metabolite (Widman et al. 1975b). Cannabinol-11-oic acid was found as a major compound from rat feces (Yisak et al. 1977). The administration of ^{14}C-labeled cannabinol to rats resulted in mono- and dihydroxy metabolites. The monohydroxy 11- and 4'-

hydroxy- (Lindgren et al. 1977, Yisak et al. 1977) and the dihydroxy- 1',11-; 4',11-; 2',11- and 3',11-dihydroxycannabinol were the most abundant compounds (Fonseka and Widman 1977, Yisak et al. 1977).

In rabbits, 4'-hydroxy-CBN was found as a major compound together with smaller amounts of 5'- and 3'-hydroxy-CBN (Widman et al. 1975b). In mice, the 11-position and the side chain of cannabinol was hydroxylated followed by further oxidation to acidic metabolites (Burstein and Varanelli 1975). Biotransformation of CBN also follows the same pattern like other cannabinoids but unlike other cannabinoids allylic hydroxylation of the terpene ring is absent in the metabolism of CBN. The 11-hydroxylation is the major metabolic route and further oxidation of this monohydroxy compound produces a common acidic metabolite, CBN-11-oic acid.

Biotransformation of cannabinoids by fungi and bacteria

Robertson et al. (1978a, 1978b) screened more than 100 species of fungi and bacteria to investigate microbial transformations of the four common cannabinoids, namely CBD, CBN, Δ^8-THC and Δ^9-THC. *Syncephalastrum racemosum* and *Mycobacterium rhodochrous* partially degraded the n-pentyl side chain of all these four compounds. Carboxylic acid and alcohol side chain derivatives were found to be the major metabolites. Among side chain derivatives, the 4'-hydroxy- metabolites were the most abundant compounds in the transformation of CBD, CBN, Δ^8-THC and Δ^9-THC by *Syncephalastrum racemosum* (Robertson and Lyle 1975).

Δ^8-Tetrahydrocannabinol was hydroxylated at the ring system and at the side chain by fermentation with *Pellicularia filamentosa, Streptomyces lavendulae* or *P. filamentosa* and yielded the compounds 7β,3'- and 7β,4'-dihydroxy-Δ^8-THC. *Streptomyces lavendulae* leads to the formation of 7α–hydroxy-, 7α,2'-;

7α,3'-; 7α,4'-dihydroxy- Δ^8-THC and 4'-hydroxy-7-oxo-Δ8-THC (Vidic et al. 1976).

Binder and Meisenberg (1978) indentified 51 fungal and bacterial strains which actively transformed Δ^9-THC into 11-; 8α-; 8β-; 3'- and 4'-hydroxy- (Table 1, compound 1, 2, 3, 6) and 4',11-dihydroxy-Δ^9-THC (Table 2, compound 14). *Cunninghamella blakesleeana* also produced 4'-hydroxy-8-oxo-Δ^9-THC (Table 6, compound 47) (Binder 1976).

Binder and Popp (1980) further studied the metabolic transformations of Δ^9-THC by using cultures of *Fusarium nivale, Gibberella fujikuroi* (Ascomycetes) and *Thamnidium elegans* (Phycomycetes). A number of metabolites were isolated from these species after which they were partly purified and their structures elucidated by combined gas chromatography/mass spectrometry. *Thamnidium elegans* formed 11-; 8α-; 8β-; and 1'-hydroxy- (Table 1, compound 1, 2, 3, 4) and 2',8β-dihydroxy-Δ^9-THC (Table 2, compound 16). *Fusarium nivale* and *Gibberella fujikuroi* both converted Δ^9-THC to the metabolites 2'-, 3'- and 4'-hydroxy-Δ^9-THC (Table 1, compound 5, 6, 7). These results show that there are two different enzyme systems capable of hydroxylating the substrate. System 1, which is common to the "*Fusarium*" type and microorganisms are restricted in its hydroxylating capacity to the side chain C-atoms 2', 3' and 4' of cannabinoids. In addition to this 'aliphatic hydroxylase' *Thamnidium elegans* posseses an 'allylic hydroxylase' capable of hydroxylating Δ^9-THC in positions 1', 8 and 11.

Microorganisms show similarities with mammalian hydroxylation of cannabinoids. Cannabinoids undergo allylic hydroxylation at C-11 to form 11-hydroxy- derivatives. Allylic hydroxylation is also seen at position 8 to give 8α- and 8β-hydroxy derivatives but unlike mammals, hydroxylation has also been found at position 7 (only found in guinea pig in mammals) and further oxidation

to form a carbonyl compound. In microorganisms, side chain degradation is found as one of the major metabolic pathways of cannabinoids. Side chain hydroxyl and carboxyl substitution is also common; hydroxylation is more likely on C-atom 2', 3' and 4' with their corresponding dihydroxy metabolites. Trihydroxy derivatives of naturally occurring cannabinoids are not found in bacteria or fungi. Side chain acid derivatives are present but carboxylation of the monoterpene moiety and glucoronidation is not reported.

Plant biotransformation of cannabinoids

Cannabielsoin (CBE) and its diastereoisomers have been isolated from the suspension cultures of *C. sativa*, *Saccharum officinarum* and tissue culture of *C. sativa*, after the administration of cannabidiol (Braemer and Paris 1987, Tanaka et al. 1997). Δ^9-THC was converted into cannabicoumaronon by a cell suspension culture of *C. sativa* (Braemer and Paris 1987). Tissue culture of *Pinelli ternatata* transformed Δ^8-THC and CBN into their glucopyranoside derivatives Δ^8-THC-2'-O-β-D-Glucopyranoside (Tanaka et al. 1997) and CBN-2'-O-β-D-Glucopyranoside, respectively (Tanaka et al. 1993).

Discussion and conclusions

Despite having useful psychomimetic and pharmacological activities, research into cannabis has been mainly focused on its use as a recreational drug and the subsequent legal aspects controlling the possession of this material. Some of this research has been conducted on the metabolism of cannabinoids in mammalian systems, leading to a better understanding of their pharmacodynamics and pharmacokinetics. A large library of THC isomers and their derivatives have been developed by using mammalian cytochrome P450 systems, which is a successful approach to finding metabolic pathways but little is known about the activity and interaction of these metabolites with the CB1 and CB2 receptors..

Perhaps the industrial scale production of potential compounds by means of mammalian cells or tissue is an expensive tool and thus an obstacle to develop cannabinoid based drugs. There are a number of other sources which can be used to enlarge the library of active metabolites obtained from the transformation of cannabinoids. Cannabinoids have been successfully transformed by fungal and bacterial strains, while there are only a few reports on the biotransformation with plant cell cultures. Microorganisms mimic some of the mammalian biotransformation pathways and their enzyme systems have great potential to produce improved or novel compounds in large quantities keeping production at a low cost.

Bacterial cytochrome P450 (monooxygenases) enzymes have the ability to hydroxylate exogenous and endogenous compounds at different positions. In the case of cannabinoid transformations, hydroxylation is likely to produce more polar cannabinoids which might be more suitable for medicinal use than parent compounds, which generally tend to accumulate in fat tissues. The significant role of the alkane side chain has been reported in the pharmacological action of THC (Billy et al. 1995) and further oxidation may change the chemistry of the side chain and lead to enhanced or modified activity of the compound. By taking into account these findings, we used alkane degrading bacterial strains for the transformation of Δ^9-THC and found eight major metabolites produced in mg scale. All these transformations were limited to the side chain and included two carboxylic acid derivatives formed by oxidation and β-oxidation of the terminal hydroxyl group of 5$^{'}$-hydroxy-Δ^9-THC (Rashidi et al. 2009). Numbers of new lead candidates are possible to obtain by using the enzymatic systems of bacterial, yeast and plant cells in cannabinoid transformation in combination with advanced chromatographic and analytical techniques.

There are more than 72 known cannabinoids but few reports on the metabolism or transformation of any other natural cannabinoid, except Δ^9-THC, CBN and CBD. Low availability of most of the cannabinoids limits the study of their pharmacological properties. There is a demand for non-psychoactive cannabinoids and their metabolites produced after biotransformation, as this might be a source of new compounds with interesting pharmacological profiles. Further studies with different microorganisms and a diverse range of cannabinoids is thus of great interest. New cannabinoid derivatives devoid of the psycho activity with only the desired profile of pharmacological activity are of great interest to the scientific community.

References

Abbott BJ, Fukuda DS, Archer RA. 1977. Microbiological transformation of cannabinoids. Experientia 33: 718-720.

Abourashed EA, Clark AM, Hufford CD. 1999. Microbial models of mammalian metabolism of xenobiotics: An updated review. Curr Med Chem 6: 359-374.

Asha S, Vidyavathi M. 2009. *Cunninghamella* - A microbial model for drug metabolism studies - A review. Biotech Adv 27: 16-29.

Baillie TA, Cayen MN, Fouda H, Gerson RJ, Green JD, Grossman SJ, Klunk LJ, LeBlanc B, Perkins DG, Shipley LA. 2002. Drug metabolites in safety testing. Toxicol Appl Pharmacol 182: 188-196.

BenZvi Z, Burstein S. 1974. 7-Oxo-delta1-tetrahydrocannabinol: a novel metabolite of Δ^1-tetrahydrocannabinol. Res Commun Chem Pathol Pharmacol. 8: 223-229.

Benzvi Z, Burstein S. 1975. Transformation of Δ^1-tetrahydrocannabinol (THC) by rabbit liver-microsomes. Biochem Pharmacol 24: 1130-1131.

BenZvi Z, Burstein S, Zikopoulos J. 1974. Metabolism of Δ^1-tetrahydrocannabinol by mouse hepatic microsomes: Identification of 6alpha-hydroxytetrahydrocannabinol. J. Pharm. Sci 63: 1173-1174.

Berlin J, Martin B, Nowak J, Witte L, Wray V, Strack D. 1989. Effect of permeabilization on the biotransformation of phenylalanine by immobilized tobacco cell cultures. J Biosci 44: 249-254.

Billy RM, David RC, William RP, Rita LB, Raj KR. 1995. Pharmacological evaluation of dimethylheptyl analogs of Δ^9-THC: reassessment of the putative three-point cannabinoid-receptor interaction. Drug Alcohol Depen 37: 231-240.

Binder M. 1976. Microbial transformation of (-)-D 1- 3 , 4 - trans - tetrahydrocannabinol by *Cunninghamella blakesleeana Lender*. Helv Chima Acta 59: 1674.

Binder M, Meisenberg G. 1978. Microbial transformation of cannabinoids 2. A screening of different microorganisms. Eur J Appl Microbiol Biotechnol 5: 37-50.

Binder M, Popp A. 1980. Microbial transformation of cannabinoids. Part 3: Major metabolites of (3R,4R) -Δ^1-tetrahydrocannabinol. Helv Chima Acta 63: 2515-2518.

Bornheim LM, Lasker JM, Raucy JL. 1992. Human hepatic-microsomal metabolism of Δ^1-tetrahyrocannabinol. Drug Metab Dispos 20: 241-246.

Braemer R, Paris M. 1987. Biotransformation of cannabinoids by a cell suspension culture of *Cannabis sativa* L. Plant Cell Rep 6: 150-152.

Brown NK, Harvey DJ. 1988a. *In vivo* metabolism of the methyl homologues of Delta-8-tetrahydrocannabinol, Delta-9-tetrahydrocannabinol and abn-delta-8-tetrahydrocannabinol in the mouse. Biomed Environ Mass Spectrom 15: 389-398.

Brown NK, Harvey DJ. 1988b. Metabolism of n-hexyl-homologues of Δ^8-tetrahydrocannabinol and Δ^9-tetrahydrocannabinol in the mouse. Eur J Drug Metab Pharmacokinet 13: 165-176.

Brown NK, Harvey DJ. 1988c. *In vivo* metabolism of the n-propyl homologs of Δ^8- and Δ^9-tetrahydrocannabinol in the mouse. Biomed Environ Mass Spectrom 15: 403-410.

Burstein S, Kupfer D. 1971a. Hydroxylation of trans-Δ^1-tetrahydrocannabinol by hepatic microsomal oxygenase. Ann N Y Acad Sci 191.

Burstein S, Shoupe TS. 1981. Metabolic pathways for the transformation of Δ^1-tetrahydrocannabinol in mouse hepatic microsomes. Drug Metab Dispos 9: 94-96.

Burstein S, Rosenfeld J, Wittstruck T. 1972. Isolation and characterization of two major urinary metabolites of Δ^1-Tetrahydrocannabinol. Science 176: 422-423.

Burstein SH, Kupfer D. 1971b. Hydroxylation of trans- 1 -tetrahydrocannabinol by a hepatic microsomal monooxygenase. Chem Biol Interact 3: 316.

Burstein SH, Varanelli C. 1975. Transformations of cannabinol in the mouse. Res Commun Chem Pathol Pharmacol 11: 343-354.

Carter GT, Weydt P. 2002. Cannabis: Old medicine with new promise for neurological disorders. Curr Opin Investig Drugs 3: 437-440.

Cheer SM, Goa KL. 2001. Fluoxetine - A review of its therapeutic potential in the treatment of depression associated with physical illness. Drugs 61: 81-110.

Christie RM, Rickards RW, Watson WP. 1978. Microbial transformation of cannabinoids. 1. Metabolism of (-)-Δ^9-6a, 10a, 10a-trans-tetra-hydrocannabinol by *Chaetomium-globosum.* Aust J Chem 31: 1799-1807.

Demain AL. 2000. Small bugs, big business: The economic power of the microbe. Biotech Adv 18: 499-514.

Fonseka K, Widman M. 1977. Dihydroxylated metabolites of cannabinol formed by rat liver i*n vitro*. J Pharm Pharmacol 29: 12-14.

Franssen MCR, Walton MJ. 1999. Biotransformations. Pages 277-325 in Walton M, Brown D, eds. Chemicals from Plants: Perspectives on Plant Secondary Products. New York, Plant Physiol, Academic Press

Fura A, Shu YZ, Zhu M, Hanson RL, Roongta V, Humphreys WG. 2004. Discovering Drugs through Biological Transformation: Role of Pharmacologically Active Metabolites in Drug Discovery. J Med Chem 47: 4339-4351.

Gaoni Y, Mechoulam R. 1964. Isolation, Structure, and Partial Synthesis of an Active Constituent of Hashish. J Am Chem Soc 86: 1646-1647.

Gaoni Y, Mechoulam R. 1966. Cannabichromene, a new active principle in hashish. Chem. Commun: 20.

Garattini S. 1985. Active drug metabolites. An overview of their relevance in clinical pharmacokinetics. Clin Pharmacokinet 10: 216-227.

Giri A, Dhingra V, Giri C, Singh A, Ward OP, Narasu ML. 2001. Biotransformations using plant cells, organ cultures and enzyme systems: current trends and future prospects. Biotech Adv 19: 175-199.

Golightly LK, Greos LS. 2005. Second-generation antihistamines - Actions and efficacy in the management of allergic disorders. Drugs 65: 341-384.

Guengerich FP. 2001. Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. Chem Res Toxicol 14: 611-650.

Halldin MM, Widman M, Martin B. 1979. The importance of side-chain hydroxylated metabolites of Δ^6-tetrahydrocannabinol in rhesus monkey. Acta Pharm Suec 16: 34-40.

Halldin MM, Andersson LK, Widman M, Hollister LE. 1982a. Further urinary metabolites of Δ^1-tetrahydrocannabinol in man. Arznei-Forschung 32: 1135-1138.

Halldin MM, Carlsson S, Kanter SL, Widman M, Agurell S. 1982b. urinary metabolites of Δ^1-tetrahydrocannabinol in man. Arznei-Forschung 32: 764.

Halldin MM, Widman M, Vonbahr C, Lindgren JE, Martin BR. 1982c. Identification of invitro metabolites of Δ^1-tetrahydrocannabinol formed by human livers. Drug Metab Dispos 10: 297-301.

Harvey DJ. 1989. Further studies on the oxidative cleavage of the pentyl side-chain of cannabinoids: identification of new biotransformation pathways in the metabolism of 3'-hydroxy-delta-9-tetrahydrocannabinol by the mouse. Xenobiotica 19: 1437-1447.

Harvey DJ. 1990. Oxidative cleavage of the pentyl side-chain of cannabinoids. Identification of new biotransformation pathways in the metabolism of 4'-hydroxy-Δ^9-tetrahydrocannabinol in the mouse. Drug Metab Dispos 18: 350-355.

Harvey DJ, Paton WD. 1976. Characterization of three monohydroxyacid and two dihydroxyacid metabolites of Δ^1-tetrahydrocannabinol in mouse liver. Res Commun Chem Pathol Pharmacol. 13: 585-599.

Harvey DJ, Paton WD. 1978. Identification of six 4"- hydroxy - metabolites of Δ^1-tetrahydrocannabinol in mouse liver. Res Commun Chem Pathol Pharmacol 21: 435.

Harvey DJ, Paton WD. 1979. The metabolism of deuterium - labeled analogs of Δ^1-, Δ^6-, and Δ^7-tetrahydrocannabinol and the use of deuterium labeling. Recent Dev. Mass Spectrom. Biochem Med 2: 127-147.

Harvey DJ, Paton WD. 1980. Identification of *in vivo* liver metabolites of Δ^6-tetrahydrocannabinol produced by the mouse. Drug Metab Dispos 8: 178-186.

Harvey DJ, Paton WD. 1981. *In vivo* metabolism of Δ^6-tetrahydrocannabinol by the guinea pig : identification of two new hydroxyacid and ketoacid metabolites. Res Commun in Substances Abuse 2: 193-201.

Harvey DJ, Mechoulam R. 1990. Metabolites of cannabidiol identified in human urine. Xenobiotica 20: 303 - 320.

Harvey DJ, Martin BR, Paton WD. 1977. Identification of di- and tri-substituted hydroxy and ketone metabolites of Δ^1-tetrahydrocannabinol in mouse liver. J Pharm Pharmacol 29: 482-486.

Harvey DJ, Martin BR, Paton WD. 1978a. Identification and measurement of cannabinoids and their *in vivo* metabolites in liver by gas chromatography-mass spectrometry. Adv Biosci 22-23: 45-62.

Harvey DJ, Martin BR, Paton WD. 1978b. Comparative *in vivo* metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC), cannabidiol (CBD) and cannabinol (CBN) by several species. Recent Dev Mass Spectrom. Biochem. Med 1: 161-184.

Harvey DJ, Martin BR, Paton WD. 1980a. Identification of *in vivo* liver metabolites of Δ^1-tetrahydrocannabinol , cannabidiol and cannabinol produced by the guinea pig. J Pharm Pharmacol 32: 262.

Harvey DJ, Leuschner JTA, Paton WD. 1980b. Influence of 6β-hydroxylation on the metabolism of Δ^1-tetrahydrocannabinol: switching of the major site of biotransformation from C-7 to the side-chain. Res Commun Substance 1: 159-167.

Harvey DJ, Samara E, Mechoulam R. 1991. Urinary metabolites of cannabidiol in dog, rat and man and their identification by gas chromatography-mass spectrometry. J Chromatogr 562: 299-322.

Harvey DJ, Gill EW, Slater M, Paton WD. 1980c. Identification of the *in vivo* liver metabolites of (--)-Δ^7- tetrahydrocannabinol produced by the mouse. Drug Metab Dispos 8: 439-445.

Jones G, Widman M, Agurell S, Lindgren JE. 1974. Monohydroxylated metabolites of Δ^1-tetrahydrocannabinol in mouse brain. Comparison with *vitro* liver metabolites. Acta Pharm Suec 11: 283-294.

Just WW, Erdmann G, Thel S, Werner G, Wiechmann M. 1975. Metabolism and autoradiographic distribution of Δ^8- and Δ^9-tetrahydrocannabinol in some organs of monkey *Callithrix jaccus*. N-S Arch Pharmacol 287: 219-225.

Kanter SL, Hollister LE. 1978. Marihuana metabolism in urine of man. 9. Identification of Δ^9-tetrahydrocannabinol-11-oic acid by thin-layer chromatography. J Chromatogr 151: 225-227.

Kumar GN, Surapaneni S. 2001. Role of drug metabolism in drug discovery and development. Med Res Rev 21: 397-411.

Lennernas H. 2003. Clinical pharmacokinetics of atorvastatin. Clin Pharmacokinet 42: 1141-1160.

Lindgren JE, Agurell S, Yisak Wb, Widman M. 1977. Neutral *in vivo* metabolites of cannabinol isolated from rat faeces. J Pharm Pharmacol 29: 387-390.

Loh WHT, Hartsel SC, Robertson LW. 1983. Tissue culture of *cannabis sativa* L. and *in vitro* biotransformation of phenolics. Z Pflanzenphysiol 111: 355-400.

Martin B, Agurell S, Nordqvist M, Lindgren JE. 1976a. Dioxygenated metabolites of cannabidiol formed by rat liver. J Pharm Pharmacol 28: 603-608.

Martin B, Nordqvist M, Agurell S, Lindgren JE, Keander L, Binder M. 1976b. Identification of monohydroxylated metabolites of cannabidiol formed by rat liver. J Pharm Pharmacol 24: 275-279.

Martin BR, Harvey DJ, Paton WD. 1977. Biotransformation of cannabidiol in mice. Identification of new acid metabolites. Drug Metab Dispos 5: 259-267.

Matsunaga T, Iwawaki Y, Watanabe K, Yamamoto I, Kageyama T, Yoshimura H. 1995. Metabolism of delta-9-tetrahydrocannabinol by cytochrome P450 isozymes purified from hepatic microsomes of monkeys. Life Sci 56: 2089-2095.

Mechoulam R, Goani Y. 1967. Recent advances in the chemistry of hashish. Fortschr Chem Org Naturst 25: 175-213.

Meeves SG, Appajosyula S. 2003. Efficacy and safety profile of fexofenadine HCL: A unique therapeutic option in H1-receptor antagonist treatment. J Allergy Clin Immun 112: 69-77.

Monique CB, Stephen S, Eds. 1976. Pharmacology of Marihuana: Philadelphia, PA, U.S.A. Lippincott Williams & Wilkins.

Narimatsu S, Watanabe K, Yamamoto I, Yoshimura H. 1991. Sex difference in the oxidative metabolism of Δ^9-tetrahydrocannabinol in the rat. Biochem Pharmacol 41: 1187-1194.

Narimatsu S, Matsubara K, Shimonishi T, Watanabe K, Yamamoto I, Yoshimura H. 1988. Enzymatic oxidation of 7-hydroxylated Δ^8-tetrahydrocannabinol to 7- oxo-Δ^8-tetrahydrocannabinol by hepatic microsomes of the guinea pig. Drug Metab Dispos 16: 156-161.

Nikolova P, Ward OP. 1993. Whole cell biocatalysis in nonconventional media. J Ind Microbiol 12: 76-86.

Nilsson IM, Agurell S, Nilsson JL, Ohlsson A, Sandberg F, Wahlqvist M. 1970. Δ^1-tetrahydrocannabinol: structure of a major metabolite. Science 168: 1228-1229.

Nordqvist M, Lindgren JE, Agurell S. 1979a. Acidic metabolites of Δ^1-tetrahydrocannabinol isolated from rabbit urine. J Pharm Pharmacol 31: 231.

Nordqvist M, Agurell S, Binder M, Nilsson I. 1974. Structure of an acidic metabolite of Δ^1-tetrahydrocannabinol isolated from rabbit urine. J Pharm Pharmacol 26: 471.

Nordqvist M, Agurell S, Rydberg M, Falk L, Ryman T. 1979b. More acidic metabolites of Δ^1-tetrahydrocannabinol isolated from rabbit urine. J Pharm Pharmacol 31: 238-243.

Pars HG, Howes JF. 1977. Potential therapeutic agents derived from the cannabinoid nucleus. Adv Drug Res 11: 97-189.

Pras N, Woerdenbag HJ, Van Uden W. 1995. The power of plant enzymes in bioconversions. Agric Biotechnol News info p. 231–243..

Rashidi H, Akhtar MT, Kooy FVD, Verpoorte R, Duetz WA. 2009. Hydroxylation and further oxidation of Δ^9-tetrahydrocannabinol by alkane-degrading bacteria. Appl Environ Microbiol 75: 7135–7141.

Rathbone DA, Bruce NC. 2002. Microbial transformation of alkaloids. Curr Opin Microbiol 5: 274.

Robertson LW, Lyle MA. 1975. Biotransformation of cannabinoids by *Syncephalastrum racemosum*. Biol Mass Spectrom 2: 266-271.

Robertson LW, Huff SR, Ghosh A, Malhotra R. 1978a. Acidic biotransromation products of cannabinoids produced by *Mycobacterium rohodchrous.* Lloydia 41: 659.

Robertson LW, Koh SW, Huff SR, Malhotra RK, Ghosh A. 1978b. Microbiological oxidation of pentyl side-chain of cannabinoids. Experientia 34: 1020-1022.

Russo EB. 2007. History of cannabis and its preparations in saga, science, and sobriquet. . Chem Biodivers 4: 1614-1648. .

Schulze B, Wubbolts MG. 1999. Biocatalysis for industrial production of fine chemicals. Curr Opin Biotechnol 10: 609-615.

Scotter EL, Abood ME, Glass M. 2010. The endocannabinoid system as a target for the treatment of neurodegenerative disease. Br J Pharmacol 160: 480-498.

Sirikantaramas S, Taura F, Morimoto S, Shoyama Y. 2007. Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology. Curr Pharm Biotechnol 8: 237-243

Smith RV, Rosazza JP. 1983. Microbial Models of Mammalian Metabolism. J Nat Prod 46: 79-91.

Tanaka H, Morimoto S, Shoyama Y. 1993. Cannabis, 21. Biotransformation of Cannabinol to Its Glycosides by *In vitro* Plant Tissue. J Nat Prod 56: 2068-2072.

Tanaka H, Takahashi RN, Morimoto S, Shoyama Y. 1997. A new Cannabinoid, Δ^6-Tetrahydrocannabinol 2'-*O-beta*-D-glucopyranoside, Biotransformed by Plant Tissue. J Nat Prod 60: 168-170.

Thomayant P, Bennett M, Nathan Y. 2003. The human hepatic metabolism of simvastatin hydroxy acid is mediated primarily by CYP3A, and not CYP2D6. Br J Clin Pharmacol 56: 120-124.

Venisetty RK, Ciddi V. 2003. Application of Microbial Biotransformation for the New Drug Discovery Using Natural Drugs as Substrates. Curr Pharm Biotechnol 4: 153-167.

Vidic HJ, Hoyer GA, Kieslich K, Rosenberg D. 1976. Microbiological transformations of nonsteroidal structures, IX. Microbiological hydroxylation of Δ^8-tetrahydrocannabinol. Chem Ber 109: 3606-3614

Wall ME. 1971. The *in vitro* metabolism of tetrahydrocannabinol (THC). Ann N Y Ascad Sci 191: 23.

Wall ME, Brine DR. 1976. Identification of cannabinoids and metabolites in biological materials by combined gas-liquid chro- matography-mass spectrometry. Nahas GG, Eds. Marihuana : chemistry, biochemistry, and cellular effects. New York : Springer-Verlag p. 51.

Wall ME, Perez-Reyes M. 1981. The metabolism of Δ^9-tetrahydrocannabinol and related cannabinoids in man. J Clin Pharmacol 21: 178-189.

Wall ME, Brine DR, Pitt CG, Perez-Reyes M. 1972. Identification of Δ^9-tetrahydrocannabinol and metabolites in man. J Am Chem Soc 94: 8579-8581.

Wall ME, Brine DR, Brine GA, Pitt CG, Freudenthal RI, Christensen HD. 1970. Isolation, structure, and biological activity of several metabolites of Δ^9-tetrahydrocannabinol. J Am Chem Soc 92: 3466-3468.

Ward OP, Singh A. 2000. Enzymatic asymmetric synthesis by decarboxylases. Curr Opin Biotechnol 11: 520-526.

Watanabe K, Yamamoto I, Oguri K, Yoshimura H. 1979. Microsomal oxygenase catalyzed oxidation of 11-hydroxy -Δ^8-tetrahydrocannabinol to 11-oxo-Δ^8-tetrahydrocannabinol. Biochem Biophys Res Commun 88: 178-182.

Watanabe K, Yamamoto I, Oguri K, Yoshimura H. 1980. Identification and determination of 11 - oxo -Δ^8-tetrahydrocannabinol as an intermediate metabolite of Δ^8-tetrahydrocannabinol in the mouse brain and liver. J Pharmacobiodyn 3: 686-691.

Watanabe K, Matsunaga T, Narimatsu S, Yamamoto I, Imaoka S, Funae Y, Yoshimura H. 1991. Catalytic activity of cytochrome P450 isozymes purified from rat liver in converting 11-oxo-Δ^8-tetrahydrocannabinol to Δ^8-tetrahydrocannabinol-11-oic acid. Biochem Pharmacol 42: 1255-1259.

Widman M, Halldin MM, Martin B. 1979. *In vitro* metabolism of tetrahydrocannabinol by rhesus monkey liver and human liver. Adv Biosci 22-23: 101-103.

Widman M, Nordqvist M, Dollery CT, Briant RH. 1975a. Metabolism of Δ^1-tetrahydrocannabinol by the isolated perfused dog lung. Comparison with *in vitro* liver metabolism. J Pharm Pharmacol 27: 842.

Widman M, Dahmen J, Leander K, Petersson K. 1975b. *In vitro* metabolism of cannabinol in rat and rabbit liver. Syntheses of 2"-, 3"- and 5"-hydroxycannabinol. Acta Pharm Suec 12: 385-392.

Williams D, Feely J. 2002. Pharmacokinetic-Pharmacodynamic Drug Interactions with HMG-CoA Reductase Inhibitors. Clin Pharmacokinet 41: 343-370.

Williams PL, Moffat AC. 1980. Identification in human urine of Δ^9-tetrahydrocannabinol- 11-oic acid glucuronide: a tetrahydrocannabinol metabolite. J Pharm Pharmacol 32: 445-448.

Yamamoto I, Watanabe K, Narimatsu S, Yoshimura H. 1995. Recent advances in the metabolism of cannabinoids. Int J Biochem Cell Biol 27: 741-746.

Yamamoto I, Watanabe K, Matsunaga T, Kimura T, Funahashi T, Yoshimura H. 2003. Pharmacology and toxicology of major constituents of marijuana-on the metabolic activation of cannabinoids and its mechanism. Toxicology 22: 577-589.

Yisak WA, Widman M, Lindgren JE, Agurell S. 1977. Neutral *in vivo* metabolites of cannabinol isolated from rat faeces. J Pharm Pharmacol 29: 487-490.

Braemer R, Paris M. 1987. Biotransformation of cannabinoids by a cell suspension culture of *Cannabis sativa* L. Plant Cell Rep 6: 150-152.

CHAPTER 3

Hydroxylation and further oxidation of Δ^9-tetrahydrocannabinol by alkane-degrading bacteria

Hassan Rashidi[‡], Muhammad Tayyab Akhtar[†], Frank van der Kooy[†], Robert Verpoorte[†], Wouter A. Duetz[‡]

[†] Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

[‡] Enzyscreen BV, Biopartner Centre, Wassenaarseweg 72, 2333 AL, Leiden, The Netherlands.

Abstract

The microbial biotransformation of Δ^9–tetrahydrocannabinol was investigated using a collection of 206 alkane-degrading strains. 15% of these strains, mainly Gram-positive strains from the genera *Rhodococcus, Mycobacterium, Gordonia*, and *Dietzia* yielded more polar derivatives. Eight derivatives were produced on a mg scale, isolated, purified, and their chemical structures were elucidated with the use of LC-MS, ^{1}H-NMR, and 2-D NMR (^{1}H-^{1}H COSY and HMBC). All eight biotransformation products possessed modified alkyl chains, with hydroxy, carboxy and ester functionalities. In a number of strains, β-oxidation of the initially formed C5 carboxylic acid led to the formation of a carboxylic acid lacking two methylene groups.

Introduction

Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the decarboxylated product of the corresponding Δ^9-THC-acid, the major cannabinoid present in the cannabis plant (*Cannabis sativa* L., Cannabaceae). This compound is officially registered as a drug for the stimulation of appetite and antiemesis in patients under chemotherapy and HIV therapy regimes. Other biological activities ascribed to this compound include lowering of intraocular pressure in glaucoma, as analgesic for muscle relaxation, immunosuppression, sedation, bronchodilation, and neuroprotection (Grotenhermen 2003).

Δ^9-Tetrahydrocannabinol and many of its derivatives are highly lipophilic and poorly water-soluble with a logPo/w > 4.5 for Δ^9-THC (Thomas et al. 1990). Calculations of the n-octanol/water partition coefficient (Ko/w) of Δ^9-THC at neutral pH, vary between 6000 using the shake-flask method (Mechoulam 1982), and 9.44×106 by reverse-phase HPLC estimation (Thomas et al. 1990). The poor water solubility and high lipophilicity of cannabinoids cause their absorption across the lipid bi-layer membranes and fast elimination from blood

circulation. In terms of the 'Lipinsky rule of 5' (Lipinski 2000), the high lipophilicity of cannabinoids is hindering the further development of these compounds into large scale pharmaceutical products.

To generate more water-soluble analogues, one can either apply *de novo* chemical synthesis, or modify naturally occurring cannabinoids, e.g. by introducing hydroxy, carbonyl or carboxy groups. Chemical hydroxylation of compounds such as cannabinoids is difficult (Δ^9-THC is converted into Δ^8-THC under mild conditions), microbial biotransformation of cannabinoids is potentially a more fruitful option to achieve this goal.

So far, studies on biotransformation of Δ^9-THC were mainly focused on fungi, which lead to the formation of a number of mono- and dihydroxylated derivatives. Previous reports on the biotransformation of cannabinoids by various microorganisms are summarized in Table 1.

The aim of the present study was to test bacterial strains capable of transforming Δ^9-THC to new products (with potentially better pharmaceutical characteristics) at a higher yield and specificity than previously found for fungal strains. For this purpose, we have chosen to use a collection of alkane-degrading strains, since it was shown in previous studies (Duetz et al. 2001, Smits et al. 2002, Beilen et al. 2005) that alkane oxygenases often display a broad substrate range. Production of novel cannabinoid derivatives that might have interesting pharmacological activities was another objective of this project.

Materials and methods

Bacterial strains and growth conditions: A total of 206 alkane-degrading strains (mainly isolated from soil and surface water, see (Beilen et al. 2005) were screened. An optimized Evans mineral medium according to (Duetz et al. 2000) with four times more trace elements (E4T) supplemented with a carbon

Table 1. Previous biotransformation experiments conducted using various microorganims to transform cannabinoids

Cannabinoid(s)	Microorganism(s) used	No. of transformed Products	Reference
Δ^9-THC	Cunninghamella blakesleeana	6	(Binder 1976)
Δ^8-THC	*Pellicularia filamentosa*	4	(Vidic et al. 1976)
Δ^8-THC	*Streptomyces lavendulae*	4	(Vidic et al. 1976)
$\Delta^{6a, 10a}$-THC	400 cultures (Soil microorganisms)	various	(Abbott et al. 1977)
Nabilone	400 cultures (Soil microorganisms)	various	(Abbott et al. 1977)
$\Delta^{6a, 10a}$-THC	358 cultures containing bacteria, actinomycetes, and molds	3	(Fukuda et el. 1977)
Δ^9-THC, Δ^8-THC, CBD, CBN	*Syncephalastrum racemosum*, *Mycobacterium rhodochrous*	various	(Robertson et al. 1978)
Δ^9-THC	*Chaetomium globosum*	3	(Christie et al. 1978)
Δ^9-THC	51 fungal strains	8	(Binder and Miesenber 1978)
Nabilone	Microbes	various	(Archer et al. 1979)
Δ^9-THC	*Fusarium nivale*, *Gibberella fujikuroi* and *Thamnidium elegans*	8	(Binder and pop 1980)

sources was used throughout this study. All cultures were grown aerobically at 30 ^{o}C. To grow bacteria on agar-based media, pre-warmed Evans mineral medium (E4T) was mixed with a molten 4 % agar no.2 solution (1:1 v/v) and dispensed in either Petri dishes or microtiter plates. For growth on n-alkanes, Petri dishes containing E4T medium were incubated at 30 ^{o}C with n-alkanes

supplied through the vapor phase by placing an open Erlenmeyer flask with the mixture of alkanes in a sealed container holding the Petri dishes.

Miniaturized screening: The alkane degrading bacteria were screened using a microtiter based technology platform as described before (Duetz et al. 2000, Beilen et al. 2005). Briefly, multiple strains stored in one 96-well microtiter plate at -80°C were sampled simultaneously without thawing the bulk cultures by using a spring-loaded 96-pin replicator (Kühner, Basel, Switzerland) as previously described (Duetz et al. 2000) and were transferred to a regular sterile polystyrene microtiter plate (type 3072; Costar, Cambridge, Mass.). Each well (working volume, 350 µl) contained 180 µl of a solidified mineral medium agar (2% [wt/vol]) without a carbon source. The inoculated microtiter plate was placed in a desiccator together with a beaker of water and a 50-ml beaker containing 10 ml of a 1:1:1:1:1 (vol/vol/vol/vol/vol) mixture of hexane, octane, decane, dodecane, and hexadecane. The lid on top was kept 2 mm from the wells in order to allow for a sufficient supply of gaseous alkanes to the wells. After 7 days of growth at the ambient temperature (30°C), the cell mass that developed on the agar surface was harvested as follows. First, 110 µl of potassium phosphate buffer (50 mM, pH 7.0) was added to each well. Repeated lateral movement of the spring-loaded replicator in the wells resulted in suspension of a large part of the cell mass. The suspensions were subsequently transferred by using a 12-channel multipipette and wide-orifice tips to a microtiter plate with 0.5-ml conical wells (Maxi-plaque; Polylabo, Geneva, Switzerland). The microtiter plate was centrifuged for 15 min at 4,000 rpm in an Eppendorf type 5403 centrifuge. After disposal of the supernatant, the cells were resuspended in 100 µl of a buffer (50 mM potassium phosphate buffer [pH 7.0]) by repeated filling and emptying of wide-orifice pipette tips by using a 12-channel multipipette. Subsequently, 2 µL substrate (20 g/L Δ^9-THC in methanol) which was purified as previously described (Hazekamp et al. 2004),

was added to each well and the wells were closed by using a sandwich cover consisting of a pierced layer of soft silicone combined with a rigid polypropylene plate. The microtiter plate was incubated for 6h at 30 ^{o}C with orbital shaking at 300 rpm and an amplitude of 50 mm. To extract the metabolites, 50 µL of chloroform was added to each well after incubation. The microtiter plate's contents were transferred into 1.5 ml tubes and then centrifuged for 10 min at 12,000 ×g. The chloroform layer was transferred to another 1.5 ml tube and air-dried. The residue was re-dissolved in MeOH and used for analysis by HPLC and LC-MS.

Large scale Biotransformation: Three selected alkane degrading bacteria (ENZHR1, ENZHR3 and ENZHR5) were cultured in 500 ml flasks containing 100 ml E4T medium aerobically at 30 ^{o}C. A mixture of n-alkanes was supplied through the vapor phase using a small plastic container attached to the flask's cap, as described above. The flasks were incubated for 18-24h at 30 ^{o}C with orbital shaking at 300 rpm and an amplitude of 50 mm. Then 2 ml of substrate (20 g/L Δ^{9}-THC in methanol) was added to each flask and incubated for a further 24 hrs. Metabolites were produced on a 1-10 mg scale and were extracted as described above. In order to increase the extraction efficiency of acid derivatives, hydrochloric acid (HCl) was added to aqueous phase before extraction.

Purification of metabolites: All transformed metabolites were purified with a semi-preparative Agilent 1200 HPLC. Various ratios of aqueous methanol were employed to purify the different products. The column employed was a RP18 Phenomenex (250X10mm, 5µm). The yields of all metabolites were in the 1-10 mg range.

HPLC-APCI-MS Analysis: Analysis was performed by using a high-performance liquid chromatography (Agilent 1100 series, Hannover, Germany)

equipped with a diode array detector, and a mass detector in series. Solvent 1 (methanol containing 0.1% formic acid) and 2 (water containing 0.1% formic acid) were used at a 70:30 (v/v) ratio for 7 min isocratically. This was followed by a gradient to 100% solvent 1 over a period of 6 min. After 1 min the mobile phase was returned to the starting conditions and left to re-equilibrate for a further 3 min. (total run time of 17 min). A Macherey-Nagel (Duren, Germany) Nucleosil C_{18} column (70 mm length, 3µm internal diameter, 5µm particle size) equipped with an 8 mm-long pre-column of the same material was used to separate the components. The settings of the mass spectrometer (MS) were as follows: APCI mode; positive ionization; fragmentor voltage 100 and 240 V; gas temperature, 350 ^{o}C; vaporizer temperature, 400 ^{o}C; drying gas (N_2) flow rate, 4 liters min^{-1}; nebulizer pressure, 45 psig (lb/in); capillary voltage, 4000 V; corona current, 4.0 µA.

Determination of the maximal solubility of metabolites: 20 µL of 10 mM of each sample in methanol was dried in eppendorf tubes. 20 µL of 50 mM phosphate buffer pH 7.0 were added to each eppendorf, vortexed for 5 minutes and were left over night. After centrifugation for 10 minutes in 2000 g in room temperature, 5 µL of supernatant was diluted 20 times in methanol and was analysed by HPLC under the same condition as described above.

NMR: The purified compounds after evaporation of MeOH were re-dissolved in CD_3OD and ^{1}H-NMR (400 MHz) spectra were recorded by a Bruker model AV-400 NMR spectrometer with reference to residual solvent as standard.

Results

Screening. To identify appropriate bacterial strains for the bioconversion of cannabinoids to more polar derivatives, we screened 206 (largely unidentified) alkane-degrading strains grown with a mixture of alkanes as their sole source of carbon and energy. Strains selected for further study were identified by partial

16S RNA sequencing. Formation of more polar compounds was observed for 76 strains, of which 44 strains converted more than 50% of substrate after 6 hours incubation at a cell density of approximately 5 g dry wt / L. In seventy of the strains capable of converting Δ^9-THC into more polar derivatives, only compounds [**3]** and/or [**4]**, (Table 2) were formed in significant amounts. In only 6 of the strains other compounds were formed. In total eight different metabolites were identified (based on relative retention time, UV spectra, and mass spectra). The proposed structures (based on MS and NMR data; see further below) are given in (Table 2).

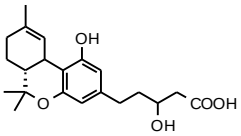
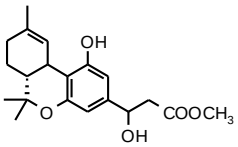
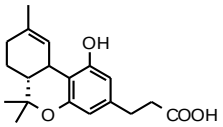
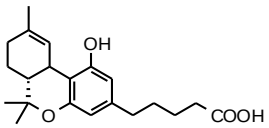
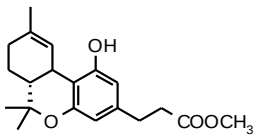
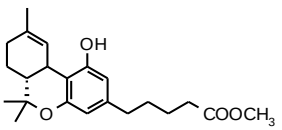
Metabolite **[1]** was the most polar derivative identified during the screening procedure and it was produced only by *Gordonia* sp ENZHR5 in relatively small quantities. Metabolite [**2]** was the second most polar derivative and was produced by only 4 of the screened strains, in a very low yield. The molecular mass of 347 [M+H], and the absence of any significant change in the UV spectra in comparison to Δ^9-THC suggested modification of the alkyl moiety. Metabolites [**3]** and [**4]** were the most abundant and were produced by all 76 strains, though in varying ratios. This ratio was observed to be not only strain-dependent, but was also influenced by the concentration of substrate, cell density and incubation time. In general, higher cell densities and longer incubation times resulted in increasing concentrations of compound [**3]**, suggesting compound [**3]** was a product of through-conversion of [**4]** to [**3]**. Metabolite [**5]** was produced by three strains, all of them also capable of production of metabolite [**2]**. A steady decrease in the concentration of metabolite [**5]** and an increase of metabolite [**2]** with a prolonged incubation time suggests that metabolite [**5]** is a precursor of metabolite [**2]**. Metabolite [**6]** was produced only by microorganisms capable of production of metabolites [**2]** and [**5]** in a time-dependent manner and a very low yield. Metabolites [**7]** and [**8]** were formed by only 1 of the screened strains.

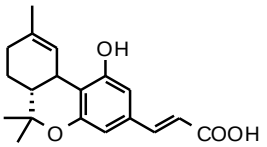
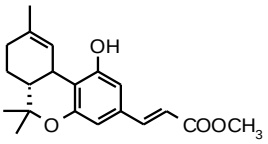
After preliminary analysis of the results, we focused on three strains that biotransformed Δ^9-THC at relatively high yields, and which covered all produced derivatives based on retention time of peaks and the molecular mass as determined by the LC-MS analysis. These strains were a *Dietzia* sp ENZHR1 yielding one major metabolite, a *Mycobacterium* sp ENZHR3 and a *Gordonia* sp ENZHR5, both producing a mixture of derivatives.

Structure elucidation. The chemical structures of metabolites [**1-8]** were further elucidated (Table 2) using ^{1}H-NMR, ^{1}H-^{1}H COSY and HMBC. The ^{1}H-NMR spectrum of all eight metabolites were in accordance with Δ^9-THC with regard to the signals due to two angular methyl groups [3H each, *s* at δ 1.41, C6 β methyl and δ 1.09, C6 α methyl], and three aromatic and olefinic protons (1H, *q*, *J*= 1.6 Hz, at δ 6.31, H-10; 1H, *d*, *J*=1.6 Hz at δ 6.14, H-4; 1H, *d*, *J*= 1.6 Hz at δ 6.06, H-2) (Choi et al. 2004). The changes were mostly limited to the signals representing protons in the alkyl side chain. The ^{1}H-NMR chemical shifts of the main signals of the metabolites and Δ^9-THC are shown in Table 3. On the basis of these proton NMR data, MS spectra and UV spectra, we propose compounds [**1-8]** to have the chemical structures as mentioned below, with the following argumentation:

3'-hydroxy-Δ^9-THC-5'-oic acid [1]: ^{1}H NMR (CD_3OD, 400MHz). The signals representing H-5' (tertiary methyl group at δ 0.87) disappeared and H-4' (2H, *m*, at δ 1.29) shifted up-field to δ 2.32 ppm. The signal representing H-3' (2H, *m*, at δ 1.29) could not be detected; instead a new single proton signal at δ 3.92 ppm was observed. The aromatic protons of H-2 and H-4 showed a slightly downfield shift from δ 6.06 and δ 6.14 to δ 6.09 and δ 6.16, respectively. The signal of H-2' (2H, *m* at δ 1.55) shifted to δ 1.70 (2H, *m*) and the signals representing the two H-1' (2H, *td* at δ 2.42 in (Δ^9-THC) divided into two signals with a slightly downfield shift to δ 2.45 and δ 2.57 with an intensity of one

Table 2: The proposed structures for the Δ^9-THC derivatives formed in the present study with their respective differences in physicochemical properties

Compound No	Chemical Name	rRT	UV_{max}	MW [b]	Proposed structure
	Δ^9-THC	1.000	210,280	314	NA
1	3'-Hydroxy- Δ^9-THC-5'-oic acid	1.000	210,280	314	
2	1'–hydroxy- Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester	0.225	210,280	346	
3	Δ^9-THC-4',5'-bis, nor-3'-oic acid	0.241	210,280	316	
4	Δ^9-THC-5'-oic acid	0.350	210,280	344	
5	Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester	0.375	210,280	330	
6	Δ^9-THC-5'-oic acid-methyl ester	0.540	210,280	358	

7	1',2'-dehydro- Δ ^{9}-THC-4',5'-bis, nor-3'-oic acid	0.350	232,300	314	
8	1',2'-dehydro- Δ ^{9}-THC-4',5'-bis, nor-3'-oic acid methyl ester	0.658	232,300	328	

proton each. APCI-MS *m/z* 361 $[M]^+$ (100), 343 (54), 325 (11). Based on the information provided above and ^{1}H-^{1}H cosy data (data not provided), we propose the structure as given in Table 2 for this compound.

1'-hydroxy-Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [2]. ^{1}H NMR (CD_3OD, 400MHz). The signals representing H-5' (tertiary methyl group at δ 0.87), H-4' (2H, *m*, at δ 1.29) and H-3' (2H, *m*, at δ 1.29) was not observed. The aromatic protons of H-2 and H-4 showed a slightly downfield shift from δ 6.06 and δ 6.14 to δ 6.25 and δ 6.35 respectively. The signal of H-2' (2H, *m* at δ 1.55) shifted to δ 2.67 (1H, dd) and δ 2.61 (1H, dd); the signal representing H-1' (2H, *td* at δ 2.42 in Δ^9-THC) showed a strong downfield shift to δ 4.80 with an intensity of one proton. Presence of a new methyl ester signal at δ 3.66 was confirmed by HMBC which shows a long range coupling of a new signal representing a methyl ester group with carbonyl group around 173 ppm. APCI-MS *m/z* 347 $[M]^+$ (10), 329 (100). Based on this information we propose the structure as in Table 2 for this compound.

Δ^9-THC-5'-oic acid [4]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum is in accordance with Δ^9-THC as explained above but the tertiary methyl group at δ 0.87 representing H-5' was not observed and signals representing H-3' and

4' (2H, *m*, at δ 1.29, H-3'; 2H, *m*, at δ 1.29, H-4') have shifted downfield to δ 1.59 and δ 2.29 respectively. A slightly downfield shift of the signal representing proton H-2' (2H, *m*) from δ 1.55 to δ 1.60 was observed but there were no significant change in the signal of H-1' (2H, *m*, at δ 2.42). APCI-MS *m/z* 345 $[M]^+$ (100), 327 (11). Based on the collected data, we propose that compound [**4]** is Δ^9-THC 5'-oic acid (Table 2).

Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [5]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum lacked the signals representing H-5' (tertiary methyl group at δ 0.87) H-3' (2H, *m*, at δ 1.29) and H-4' (2H, *m*, at δ 1.29). A significant downfield shift in the signal representing proton H-2' (2H, *t*) from δ 1.55 to δ 2.58 and slight downfield shift of H-1' (2H, *td*) from δ 2.42 to δ 2.74 and appearance of a new signal (3H, *s*) at δ 3.68 a typical signal of methyl ester group, were the main changes in the ^{1}H-NMR in comparison to Δ^9-THC. Long-range coupling of this new signal with a carbonyl group at 173 ppm in the HMBC spectra confirmed the presence of methyl ester substitution. APCI-MS *m/z* 331 $[M]^+$ (100), 299 (70). Based on the information provided above, we propose the structure as given in Table 2 for compound [**5]**.

Δ^9-THC-5'-oic acid-methyl ester [6]. ^{1}H NMR (CD_3OD, 400MHz). The ^{1}H-NMR spectrum is in accordance with Δ^9-THC as explained above and identical to compound [**4]** with only the presence of one additional signal (3H, *s*, at δ 3.63 typical for a O-methyl ester (confirmed by HMBC). APCI-MS *m/z* 359 $[M]^+$ (100), 327 (22). Based on the collected data, we propose that compound [**6]** is Δ^9-THC-5'-oic acid-methyl ester (Table 2).

1',2'-dehydro-Δ^9-THC-4',5'-bis, nor-3'-oic acid [7]. ^{1}H NMR (CD_3OD, 400MHz). Most of the ^{1}H-NMR spectrum is in accordance with Δ^9-THC as described above but the tertiary methyl group at δ 0.87 representing H-5' and signals representing H-3' and 4' (2H, *m*, at δ 1.29, H-3'; 2H, *m*, at δ 1.29, H-4';

Table 3. ^{1}H NMR chemical shifts (ppm) of the altered signals of new metabolites as compared to Δ^{9}-THC**.**

	Chemical shifts (ppm) for compound								
position	**Δ^{9}-THC**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
2	*6.06, d*	6.09, *d*	6.25, *d*	6.11, *d*	6.06, *d*	6.11, *d*	6.06, *d*	6.47, *d*	6.47, *d*
	(1.6)	(~1)	(1.5)	(1.6)	(1.2)	(~1)	(~1)	(~1)	(~1)
4	6.14, *d*	6.16, *d*	6.35, *d*	6.19, *d*	6.14, *d*	6.19, *d*	6.14, *d*	6.55, *d*	6.55, *d*
	(1.6)	(~1)	(1.5)	(1.6)	(1.2)	(~1)	(~1)	(~1)	(~1)
1'	2.42, *td*	2.45, *m*	4.8, *dd*	2.7, *td*	2.42, *td*	2.74, *td*	2.42, *td*	6.32, *d*	6.33, *d*
	(7.3, 1.6)		(8.8, 2.4)	(7.6, ~1)	(7.2, ~1)	(6.0, ~1)	(7.2, ~1)	(16)	(16)
1' (new signal)		2.57, *m*							
2'	1.55, *q*	1.7, *m*	2.67, *dd*	2.4, *td*	1.6, *m*	2.58, *td*	1.6, *m*	7.42, *d*	7.46, *d*
	(7.8)		(15.2, 8.8)	(7.6, ~1)		(6.0, ~1)		(16)	(16)
2' (new signal)			2.61, dd						
			(15.2, 2.4)						
3'	1.29, *m*	3.92, *bs*			1.59, *m*		1.59, *m*		
4'	1.29, *m*	2.32, *m*			2.29, *m*		2.32, *td*		
							(7.2, ~1)		
5'	0.9, *t* (7.0)								
New signal			3.66, *s*			3.68, *s*	3.63, *s*		3.76, *s*

respectively) disappeared. A significant downfield shift observed in the signals representing the aromatic protons of H-2 (1H, *d*) and H-4 (1H, *d*) from δ 6.06 and δ 6.14 to δ 6.47 and δ 6.55 respectively. Signals representing protons H-2' (2H, *t*, at δ 1.55) and H-1' (2H, *td*, at δ 2.42) changed to 1H doublets and shifted to 7.42 and 6.32 ppm, respectively. Significant changes in UV absorption of the compound suggested a further conjugation of a chromophore with the aromatic ring. A coupling constant of H-1 and H-2 (*J*=16 Hz) indicated a *trans* formation of the vicinal protons of a double bond between C-1' and C-2'. Fig. 1 shows the proposed structure for compound [**7]** with a *trans* configuration of the protons at C-1' and C-2'. APCI-MS *m/z* 315 $[M]^+$ (100), 297 (15).

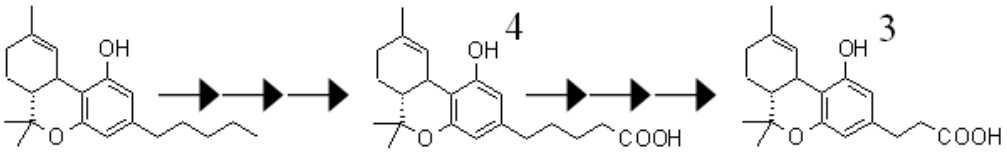


Figure 1: Biotransformation of Δ^9-THC by *Dietzia* sp ENZHR1

1',2'-dehydro-Δ^9-THC-4',5'-bis, nor-3'-oic acid-methyl ester [8]. ^{1}H NMR (CD_3OD, 400MHz). Most of the ^{1}H-NMR spectrum is in accordance with Δ^9-THC as described above and identical to compound **7** with only the presence of one additional signal (3H, *s*, at δ 3.68 typical for a O-methyl ester (confirmed by HMBC). APCI-MS *m/z* 329 $[M]^+$ (100), 297 (10). Table 2 shows the proposed structure for compound [**8]** with a *trans* configuration of protons at C-1' and C-2'. As shown in Fig. 1, production of new metabolites in *Dietzia* sp ENZHR1 was limited to [**3]** and [**4]** while the production of compound [**3]** yielded less than 5% of the total conversion based on their respective HPLC chromatogram areas. In *Mycobacterium* sp ENZHR3, compound [**3]** is one of the major

products (35-70% of total conversion based on the incubation condition) while compound [**5]** is the second most abundant metabolite. Compounds **[2], [6]** and **[8]** were found to be minor products. Formation of [**4]** is totally dependent on incubation conditions. Production of [**5]**, [**6]** and [**8]** in *Mycobacterium* ENZHR3 increased in a time-dependent manner accompanied by a decreasing amount of [**3]** and [**4]** (Fig. 2). *Gordonia* sp ENZHR5 also showed a multi-roduct pattern of production, although [**1]**, [**3]** and [**7]** were the most abundant metabolites formed (Fig. 3).

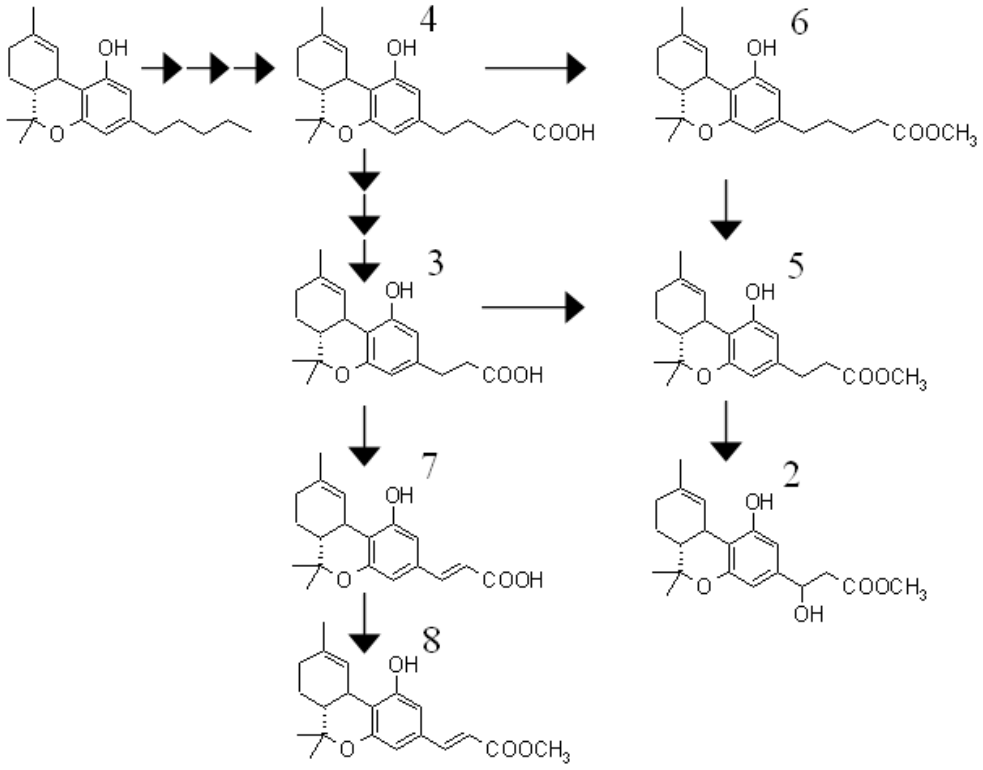


Figure 2: Production of new derivatives from Δ^9-THC by *Mycobacterium* sp ENZHR3

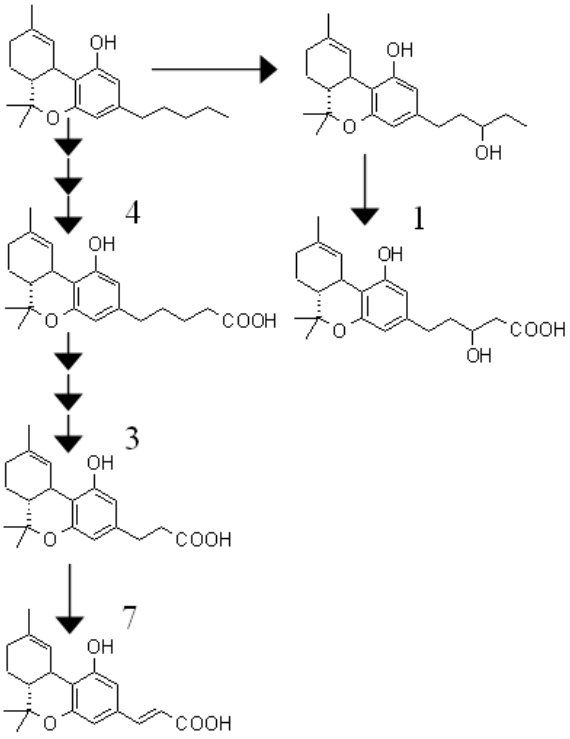


Figure 3: Metabolites by *Gordonia* sp ENZHR5 during biotransformation of Δ^9-THC

Solubility studies. The physicochemical properties of the derivatives were found to be significantly different from those of the parent compounds. For [3] and [4], the water-solubility at pH 7 are up to 8.5 and 4.5 mM respectively which is a dramatical increase in comparison to the solubility of Δ^9-THC and CBD (50 and 135 µM, respectively). Solubility of the methyl ester derivatives is also assumed to be improved based on their HPLC retention time in comparison to the parent compounds. The double bond between C-1' and C-2' has a negative effect on solubility; for example [7] has a 1.4 fold lower solubility compared to the saturated metabolite (6.2 mM for the dehydro metabolite).

Discussion

In this study, we established that a significant part (approx 37%) of a randomly-isolated collection of bacterial alkane degraders is capable of hydroxylation and further modification of the pentane moiety of tetrahydrocannabinol. A total of eight compounds were produced on a 1-10 mg scale. Their structures were elucidated using MS and UV spectrometry, ^{1}H-NMR and 2-D NMR (^{1}H-^{1}H COSY and HMBC).

The finding that Gram-positive alkane degraders from the CMN group (including Rhodococci and Mycobacteria) were overrepresented in the group of THC-converters possibly reflects their relatively good cell wall permeability for lipophilic compounds as a result of the absence of a LPS layer. The best producers were from the genera of Dietzia, Mycobacterium and Gordonia. A similar overrepresentation of Gram-positives was observed in similar screenings for the bioconversion of D- and L-limonene (van Beilen et al. 2005). A possibly broader substrate range of the (mainly P450) alkane monooxygenases in these strains may also have played a role in this finding.

The most frequently found metabolites were [**3]** and [**4].** Probably compound **[4]** was formed as a result of the action of an alkane monooxygenase, in combination with two aspecific dehydrogenases (possibly, but not necessarily alkanol dehydrogenases). The assumed initial product (Δ^9-THC-5'-OH) was not detected in significant amounts, probably reflecting the relatively high specific activities of the dehydrogenases responsible for the further oxidation of the alcohol. Compound **[3]** is also a carboxylic acid, but its alkane chain is two methylene groups shorter. In the light of the gradual conversion of compound **[4]** to compound **[3]** (as was observed for a number of strains) it seems reasonable to assume that compound **[3]** is formed as a result of β-oxidation of compound **[4]**.

Other metabolites were produced by only a few screened strains and normally in low yields, except compounds [**1]** and [**7]** that were produced in high yields by *Gordonia* sp ENZHR5. Further work is needed to assess which enzymes are responsible for these conversions.

Formation of Δ^9-THC derivatives with a C-3 side chain has been observed during an *in vivo* study by feeding mice with Δ^9-THC-5'-OH, Δ^9-THC-5'-oic acid and Δ^8-THC-5'-oic acid in another study (Harvey and Leuschner 1985).

The possible (pharmaceutical) application of the isolated derivatives is presently the subject of further study. The improved water solubility (8.5 and 4.5 mM for compounds [**3]** and [**4]**) would make them better pharmaceuticals in terms of the Lipinski rules. This positive effect might however be offset by changes in their affinities for the relevant receptors. Therefore, the isolated compounds are presently being tested with respect to their binding affinities for the CB1 and CB2 receptors.

References

Abbott BJ, Fukuda DS, Archer RA. 1977. Microbiological transformation of cannabinoids. Experientia 33: 718-720.

Archer RA, Fukuda DS, Kossoy AD, Abbott BJ. 1979. Microbiological transformations of nabilone, a synthetic cannabinoid. Appl Environ Microbiol 37: 965-971.

Binder M. 1976. Microbial transformation of (-)-D 1- 3 , 4 - trans - tetrahydrocannabinol by Cunninghamella blakesleeana Lender. Helv Chima Acta 59: 1674.

Binder M, Meisenberg G. 1978. Microbial transformation of cannabinoids 2. A screening of different microorganisms. Eur J Appl Microbiol Biotechnol 5: 37-50.

Binder M, Popp A. 1980. Microbial transformation of cannabinoids. Part 3: Major metabolites of (3R,4R) -Δ^1-tetrahydrocannabinol. Helv Chima Acta 63: 2515-2518.

Choi YH, Hazekamp A, Peltenburg-Looman AM, Frederich M, Erkelens C, Lefeber AW, Verpoorte R. 2004. NMR assignments of the major cannabinoids and cannabiflavonoids isolated from flowers of Cannabis sativa. Phytochem Anal 15: 345-354.

Christie RM, Rickards RW, Watson WP. 1978. Microbial transformation of cannabinoids. 1. Metabolism of (-)-Δ^9-6a, 10a, 10a-trans-tetra-hydrocannabinol by Chaetomium-globosum. Aust J Chem 31: 1799-1807.

Duetz WA, Fjallman AHM, Ren S, Jourdat C, Witholt B. 2001. Biotransformation of D-Limonene to (+) trans-Carveol by Toluene-Grown Rhodococcus opacus PWD4 Cells. Appl Environ Microbiol 67: 2829-2832.

Duetz WA, Ruedi L, Hermann R, O'Connor K, Buchs J, Witholt B. 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Appl Environ Microbiol 66: 2641-2646.

Fukuda D, Archer RA, Abbott BJ. 1977. Microbiological transformations of {Delta}6a,10a-tetrahydrocannabinol. Appl Environ Microbiol 33: 1134-1140.

Grotenhermen F. 2003. Pharmacokinetics and pharmacodynamics of cannabinoids. Clin Pharmacokinet 42: 327-360.

Harvey DJ, Leuschner JT. 1985. Studies on the beta-oxidative metabolism of Δ^1- and Δ^6- tetrahydrocannabinol in the mouse. The *in vivo* biotransformation of metabolites oxidized in the side chain. Drug Metab Dispos 13: 215-219.

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, van Zweden R, Verpoorte R. 2004. Preparative Isolation of Cannabinoids from *Cannabis sativa* by Centrifugal Partition Chromatography. J Liq Chromatogr Relat Technol 27: 2421 - 2439.

Lipinski CA. 2000. Drug-like properties and the causes of poor solubility and poor permeability. J Pharmacol Toxicol Methods 44: 235-249.

Robertson LW, Koh SW, Huff SR, Malhotra RK, Ghosh A. 1978. Microbiological oxidation of pentyl side-chain of cannabinoids. Experientia 34: 1020-1022.

Smits TH, Balada SB, Witholt B, van Beilen JB. 2002. Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. J Bacteriol 184: 1733-1742.

Thomas BF, Compton DR, Martin BR. 1990. Characterization of the lipophilicity of natural and synthetic analogs of delta 9-tetrahydrocannabinol and its relationship to pharmacological potency. J Pharmacol Exp Ther 255: 624-630.

van Beilen JB, Holtackers R, Luscher D, Bauer U, Witholt B, Duetz WA. 2005. Biocatalytic production of perillyl alcohol from limonene by using a novel Mycobacterium sp. cytochrome P450 alkane hydroxylase expressed in Pseudomonas putida. Appl Environ Microbiol 71: 1737-1744.

Vidic HJ, Hoyer GA, Kieslich K, Rosenberg D. 1976. Microbiological transformations of nonsteroidal structures, IX. Microbiological hydroxylation of Δ^8-tetrahydrocannabinol. Chem Ber 109: 3606-3614.

CHAPTER 4

Hydroxylation and glycosylation of Δ^9-THC by *Catharanthus roseus* cell suspension culture analyzed by HPLC-PDA and mass spectrometry.

Muhammad Tayyab Akhtar, Natali Rianika Mustafa, Robert Verpoorte, and Young Hae Choi.

Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

Abstract

The aim of this study was to test the capability of *Catharanthus roseus* suspension cultured cells for converting Δ^9-THC into more polar derivatives. The transformed metabolites were analysed and isolated by HPLC. Structures of some new derivatives were proposed on the basis of molecular ion peaks, fragmentation pattern and spectroscopic data obtained from LC-MS. Δ^9-THC was rapidly absorbed by *Catharanthus roseus* cultured cells and upon biotransformation new glycosylated and hydroxylated derivatives were isolated by preparative HPLC and identified using LC-MS molecular ion peaks, fragmentation patterns and spectroscopic data. In addition, cannabinol was detected as degradation product, including its glycosylated derivative. In the compound uptake efficiency, Δ^9-THC was rapidly absorbed by *Catharanthus roseus* cell suspension cultures line CRPP. Based on these results, it is concluded that *Catharanthus* cultured cells have great potential to transform Δ^9-THC into more polar derivatives and can be used for the large scale production of new cannabinoids, which can be a source of new compounds with interesting pharmacological profiles.

Introduction

Besides microbial and fungal cultures, plant cell cultures have also been utilized for biotransformation. Even though plant cell cultures most often have less capacities and resources to produce specific secondary metabolites compared to intact plants, they often retain the ability to perform all biocatalytic steps and are well able to efficiently transform exogenously applied substrates into products of interest. Moreover, it is often noted that plant cell cultures can take up and transform xenobiotic compounds according to metabolic pathways of endogenous metabolites with structural similarities, which may well lead to novel and valuable products with improved properties regarding stability, bio-

activity and solubility (Ishihara et al. 2003). Some attempts have also been made to test the plants and plant cells for the transformation of cannabinoids into new chemical entities. For example, some suspension cultures of *Cannabis sativa* (*C sativa*) and *Saccharum officinarum* were used to convert cannabidiol to cannabeilsoin (Braemer et al. 1987, Hartsel et al. 1983) and tetrahydrocannabinol to cannabicoumaron (Braemer and Paris 1987).

Catharanthus roseus cell enzyme system has been successfully used for the development of new compounds by the hydroxylation and glycosylation of the parent compound into its derivatives with enhanced water solubility. Furuya et al. (1992) reported the epoxidation of tabersonine into lochnericine and further methoxylation into lochnerinine by using a suspension culture of *C. roseus*. *Catharanthus roseus* cell suspension culture spiked with vanillin was able to convert the compound into glucovanillin (Sommer et al. 1997, Yuana et al. 2002), vanillic acid and glucovanillic acid (Yuana et al. 2002). Feeding/elicitation of a *C. roseus* cell suspension culture with salicylic acid resulted in hydroxylation of this compound into 2.5-dihydroxybenzoic acid (gentisic acid) followed by a glycosylation of the hydroxyl group at C-5 (Shimoda et al. 2002, Mustafa et al. 2009). *Catharanthus roseus* was also used to transform triptolide (Ning et al. 2004) and cinobufagin (Ye et al. 2003) into the new products 12β, 13α-dihydroxytriptonide and 3-epi-desacetylcinobufotalin respectively. Deoxyartemisinine was obtained from a suspension culture of *C. roseus* after feeding the antimalarial compound artimisinine (Patel et al. 2010). The aim of the current study is to investigate the potential of *C. roseus* cultured cells for the transformation of Δ^9-THC for producing more polar compounds, while maintaining pharmacological activity. In addition, we studied the effect of the compound on the biomass accumulation of the cell cultures and the effect of growth conditions on the biotransformation

of THC. LC-MS with APCI was employed for analytical evaluation and characterisation of Δ^9-THC derivatives.

Material and Method

Isolation of Δ^9-THC

Δ^9-THC was purified according to Hazekamp et al. (2004).

Plant cell cultures:

Catharanthus roseus cell suspension culture line CRPP was grown in Gamborg's B5 medium (Gamborg et al. 1968) supplemented with 2% glucose and 1.86 mg/L naphthalene-3-acetic acid (NAA). The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL of the medium and cultivated at 24 (± 1)°C under continuous light (500-1500 lux) at 100 rpm. Subculturing the cells for maintenance was performed every 3 weeks by diluting the cultures 1:1 with fresh medium. For the experiment, 30 ml of the 3-week old cultures were pipetted into 50 ml fresh medium in a 250 mL-Erlenmeyer flask.

Feeding experiments:

For the feeding experiment 18 cultures were inoculated as described above and three medium flasks were maintained at similar conditions; treatments were performed in triplicate for all conditions at day five after subculturing, which corresponded to mid-exponential phase. The cell cultures received either Δ^9-THC (8 mg; dissolved in 0.8ml of ethanol at 24 and 48h), ethanol as solvent control (0.8ml) for 24 and 48h or remained untreated as non-fed control for 24 and 48h. Subsequently cells and medium were harvested at 24 and 48 hours after treatment. The medium flasks were spiked with Δ^9-THC at the same time and collected after 48 hours to determine effect of medium, pH and light on Δ^9-THC Cells were harvested by vacuum filtration using a Bűchner funnel and Whatman filter paper (d=90 mm; Ref No: 10311809, Whatman GmbH, Dassel,

Germany) ; after_initial filtration the medium was collected and cells were washed with 100 ml of Millipore water. The cells were transferred into 50 ml falcon tubes, frozen in liquid nitrogen and stored at -20 °C before further analysis.

Extraction

Collected cells were lyophilized and weighed. Fifty mg of cells (DW) were weighed into a 1,5 mL microtube and extracted with 1 ml methanol by vortexing (1 min) followed by ultrasonication for 15 minutes (at room temperature). The supernatants were collected by centrifugation (20 min at 13,000 rpm), and extraction of the remaining cell biomass was repeated twice, each time using 1 ml methanol. The pooled supernatant was evaporated to dryness and dissolved in 1 ml of ethanol p.a for LC-MS analysis. Media were extracted three times with an equal volume of EtOAc (50 ml). The media extracts were dried with a rotary evaporator, weighed, and dissolved in 1 ml ethanol p.a.; 10 ul was injected for HPLC analysis.

Hydrolysis of extracts

Cell extracts were hydrolysed in order to release Δ^9-THC from its glucoside forms and for confirmation of the glucosylated products. For this purpose, 1 mg cells (DW) from the Δ^9-THC -fed cultures were weighed into 1,5 ml microtubes and extracted with 0.5 ml methanol combined with 0.5 ml of 6 N HCl. The mixture was placed in a water bath at 80°C for 1 h (Meuwly and Metraux 1993). The hydrolysed samples were extracted with 1 ml of chloroform (three times), the pooled chloroform fractions were dried under a nitrogen flow, re-dissolved in ethanol and 10 µl injected to the LC-MS system.

HPLC Conditions

All quantitative analysis were carried out on an Agilent 1200 HPLC system equipped

with an autosampler, injector and a photo diode array (PDA) detector. A Phenomenex C18 (150 x 4.6 mm, 5µm particle size) column was used for chromatographic analysis. The mobile phase consisted of methanol and water, acidified with 25 mM of formic acid. The column was run with a gradient of methanol (65 to 100%) over 25 minutes. The column was further run for 3 min with 100% methanol followed by re-equilibration of the column under initial conditions for 4 min. Flow rate was 1.0 ml/min and the UV detection was set at 228 nm.

Isolation of some target compounds were performed using a preparative Phenomenex C18 (250 x 4.6 mm, 5 µm particle size) column using an isocratic elution system with MetOH-water (85-15) with a run-time of 25 minutes, a flow-rate of 2.0 ml/min and detection at 228 nm.

LC-MS Analyses

LC-MS analyses for product identification were performed by using an Agilent 1100 HPLC single-quadrupole mass-spectrometer. Instrument operation and data acquisition were controlled through Agilent LC/MSD Chemstation A.10.02 software (Santa Clara,CL, United states). The HPLC system and column conditions were the same as mentioned above. The settings of the mass spectrometer (MS) were as follows: APCI mode; Positive and negative ionization; fragmenter voltage, 150 and 250 V; gas temperature, 350C; vaporizer temperature, 350 $^{\circ}$C; drying gas (N_2) flow rate, 5 liters min^{-1}; nebulizer gas pressure, 35 psig (lb/in^2); capillary voltage, 4000 V; corona current, 4.0 µA. Ions were detected in the range of 100-1000 u.

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs. To analyze the impact of EtOH and

Δ^9-THC on total dry cell weight, we used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance.

Results and discussion

Quantitative analysis of Δ^9-THC in the media and the cells was performed in order to study the efficiency of uptake of this compound by the *C. roseus* cells line CRPP. The presence of a small proportion of the compound/substrate in the cells and only traces in the media (Figure 1A) shows the quick uptake of compound by the *C. roseus* cells. Further, the transformed products were released back into the media. The effect of growth conditions (temperature, light, humidity etc.) on the stability of Δ^9-THC was studied by incubating the media spiked with Δ^9-THC for 48 h (as negative control). The identification and quantification of cannabinol (CBN) from these flasks showed that 28 to 30 % of the Δ^9-THC was degraded to CBN only because of the growth conditions.

The effect of Δ^9-THC on the growth of *Catharanthus* cell line was studied by measuring the fresh weight and dry weight of the cells fed with Δ^9-THC for 24 and 48 h, and compared with those of the control cells which were treated with ethanol only (as control solvent) or not treated at all (control blank). The dry weight of the cells treated with Δ^9-THC was significantly reduced as compared to the controls, which could be due to toxicity of Δ^9-THC to the cells. A longer treatment with Δ^9-THC (48h) further reduced the dry weight of the cells slightly (Figure 1B).

Transformation of product

The potential of *Catharanthus roseus* cell suspension culture as tool for drug transformation is well documented (Bourgogne et al. 1989). For example, the

transformation of cinobufagin was performed using cell suspension culture of *C. roseus* and its four glycosylated derivatives were identified (Ye et al. 2002).

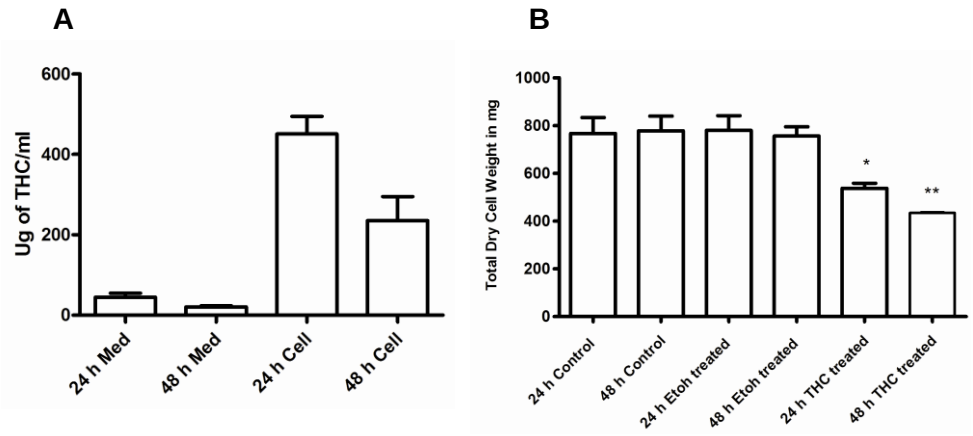


Figure 1: A, Quantification of Δ^9-THC (ug/ml) in media and cells after 24 and 48 hours of incubation. **B**, Total dry cell weight of CRPP incubated in 11-Glu media (Control), treated with EtOH and Δ^9-THC for 24 and 48 hours. ***** depict differences between 24h control and cells treated with EtOH and Δ^9-THC for 24 and 48 hours. Statistical icons: *= p< 0.05, **= p< 0.01.

Previously, cannabinoids were transformed by using different plant cell cultures; however, there is no report on the use of *C. roseus* as a transformation tool for cannabinoids. The Δ^9-Tetrahydrocannabinol (**1)** was transformed into four different derivatives named as **2, 3, 4** and **5** (Figure 2). Structures are proposed on the basis of molecular mass, fragmentation pattern, and spectroscopic data of the transformed compounds obtained from LC-MS.

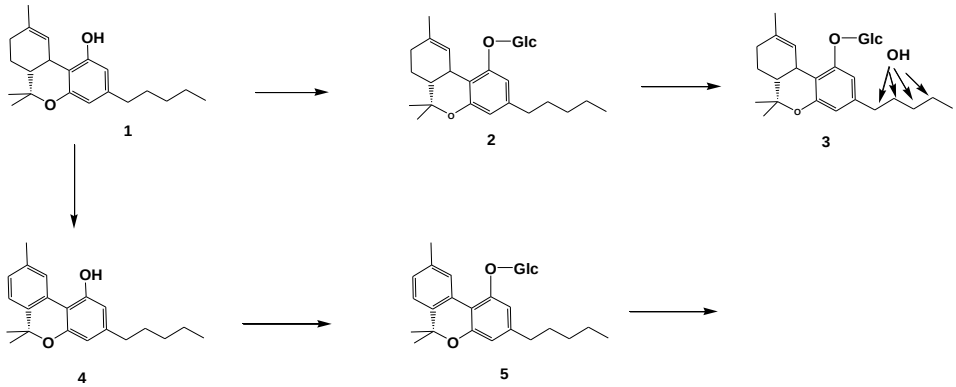


Figure 2: Chemical structures of products 1, 2, 3, 4 and 5.

The compound **1** was transformed to its glycosylated and further hydroxylated derivative **2** and **3** respectively. The positive parent ion peak at 477 $[M+H]^+$ (Figure 3A) is indicating the addition of one glucose molecule to compound **1** and its fragmentation to base peak at 315 $[M+H]^{+,}$ suggested that **2** is composed of **1** and one molecule of glucose. The compound **3** eluted before **2** with a molecular ion peak at 493 $[M+H]^+$ (Fig. 3B) proposing a further addition of an hydroxyl group to compound **2**. This was also confirmed by the fragmentation of this derivative in which **3** was fragmented to m/z 331 and 313. The positive peak at m/z 331 indicates the removal of a glucose molecule resulting in a molecular weight which is a sum of compound **1** and a hydroxyl group. After the hydrolysis of the EtOAc extract (medium extract), molecular peaks of m/z 331 and m/z 315 were found. These peaks are evidence that after loss of glucose molecule, either **1** only or its hydroxyl derivative was present in the extract. The hydroxyl group is probably attached to the 5 carbon side chain or non-phenolic ring of the product **3**. Literature shows that the chromophore of the cannabinoids corresponds to its substituted phenolic ring, whereas alkyl-side chain and cyclization of non-phenolic part has no influence on the absorbance (Hazekamp et al. 2005). In the current study, spectroscopic data show the same

UV-spectrum (Figure 4, 5) but different retention times for the products **1**, **2** and **3**. The elution sequence was **3**, **2** and **1**.

An earlier study had revealed that **1** is quite an unstable compound at high temperature, light and acidic medium (Tanaka et al. 1993). This was confirmed by chromatographic and spectroscopic analyses which showed that after an exposure of 48 h to light and a temperature of 35 °C in the presence of 11-Glu media, 28-30 % of **1** was degraded into 4. The product 4 was quantified by making the calibration curve of the reference compound and obtained the r^2-value equal to 0.998. The positive molecular ion peak at m/z 473 $[M+H]^+$ (Fig. 3D) shows that 4 was further converted to product 5 with an addition of a

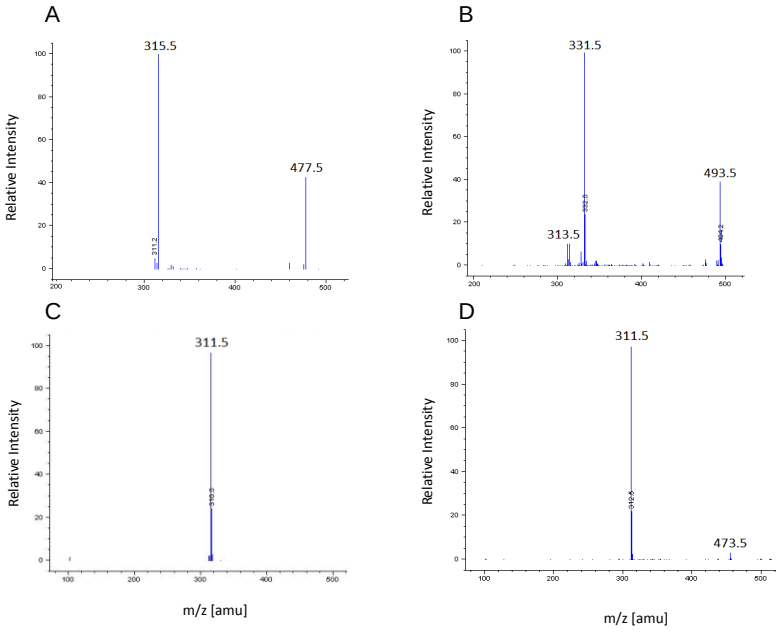


Figure 3: LC-MS spectra of the isolated products. A) Product 2; B): Product 3; C): Product 4; D): Product 5.

glucose molecule. The MS fragmentation of **5** also confirms that m/z 473 $[M+H]^+$ was fragmented to a base peak at m/z 311 $[M+H]^+$, which is the molecular weight of compound **4**. This assumption was also verified by the

acidic hydrolyses of 5 which released a glucose molecule and a molecular ion peak at m/z 311 was found. The UV spectrum of compound **4** (Fig. 4C) and **5** (Fig. 4D) is similar to that of CBN reference compound (Fig. 5B). Previously, the tissue cultures of tubers of *Pinelli ternate* were reported to be able to transform Δ^8-tetrahydrocannabinol and cannabinol to their glycosylated derivatives (Tanaka et al. 1993, 1997). Our data is in good agreement with these previous reports and shows that the CRPP cell line has not only transformed **1** but interestingly, its enzymatic system can also efficiently convert **4** to its glycosylated derivative. Although, **1** was degraded to **4** because of growth but interestingly, its enzymatic system can also efficiently convert **4** to its glycosylated derivative. Although, **1** was degraded to **4** because of growth

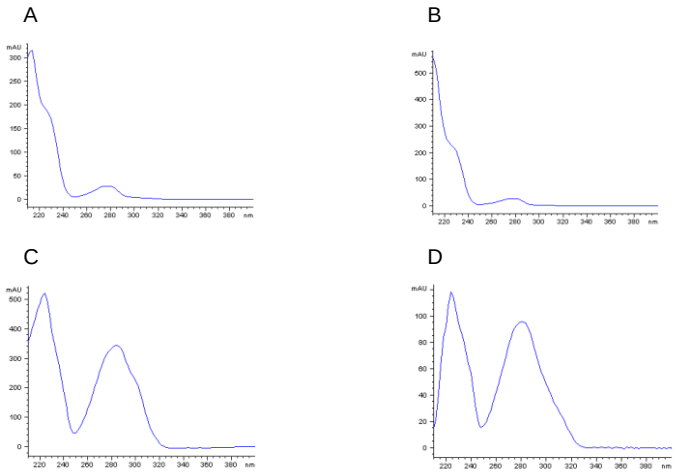


Figure 4: UV-spectra of the isolated products in the range of 190-400 nm. A) Product 2; B): Product 3; C): Product 4; D): Product 5.

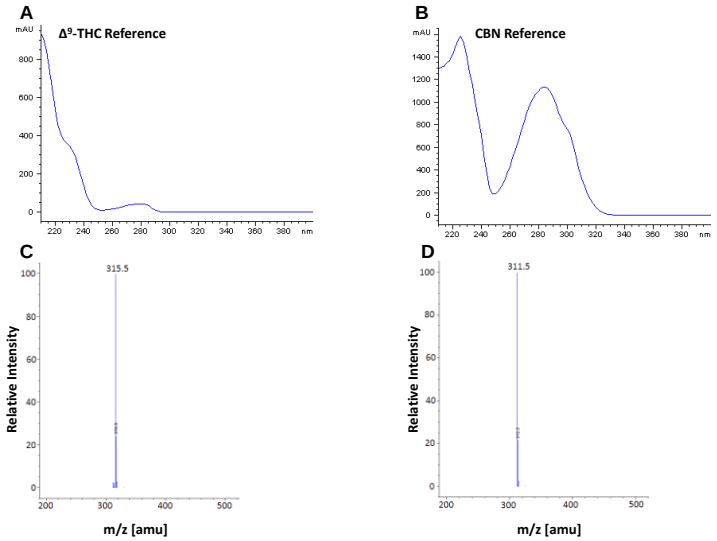


Figure 5: LC-MS and UV spectra of Δ^9-THC and CBN reference compounds. A): Δ^9-THC UV spectra; B): CBN UV spectra; C): Δ^9-THC MS spectra; D): CBN MS spectra.

conditions but its further glycosylation was done in the presence of CRPP cultured cells. Compound **3** was the major compound indentified in the EtOAc extract, whereas 4 and 5 were minor compounds. The low quantity of **4** and **5** might be due to the fast uptake of **1** leaving only very little amounts to be degraded into **4**. *Pinelli ternata* tissue segments also successfully transformed cannabidiol and cannabidiolic acid into their glycosylated derivatives. Cannabidiol was transformed into cannabidiol- *O-β*-D-glucopyranoside and cannabidiol- *O-β*-D-diglucopyranoside. In both derivatives, glucose molecules were conjugated with the phenolic hydroxyl group (Tanaka et al. 1996). These studies suggest that the phenolic hydroxyl group is the most favorable site for glucose linkage in the transformation of cannabinoids.

Conclusion

The rapid uptake and conversion of Δ^9-THC into glycosylated and further hydroxyl derivatives shows the conversion capabilities of *Catharanthus roseus* cell suspension culture line CRPP. A growing body of literature including data presented in this study establishes that the glycosylation of cannabinoids is a common metabolic pathway in plant tissue or cell cultures. Glycosylated cannabinoids are phase ii reaction products in mammals and could easily be produced on larger scale by using cell suspension cultures of *C. roseus*. However, we incubated cells for two days after adding the substrate. Larger quantity of substrate and longer incubation time in dark conditions could minimize the substrate degradation and might be helpful to obtain a higher quantity of new derivatives for NMR studies.

References

Abbott BJ, Fukuda DS, Archer RA. 1977. Microbiological transformation of cannabinoids. Experientia 33: 718-720.

Binder M, Meisenberg G. 1978. Microbial transformation of cannabinoids 2. A screening of different microorganisms. Eur J Appl Microbiol Biotechnol 5: 37-50.

Bourgogne V, Labidalle S, Galons H, Miocque M, Foulquier M, Jacquin-Dubreuil A, Cosson L. 1989. Biotransformation of a synthetic compound, 1,5-diphenylsulphinyl-3-methyl-3-nitropentane, by cell suspensions of *Catharanthus roseus*. Phytochemistry 28: 2345-2347.

Braemer R, Paris M. 1987. Biotransformation of cannabinoids by a cell suspension culture of *Cannabis sativa* L. Plant Cell Rep 6: 150-152.

Breithaupt D, Kraut S. 2006. Simultaneous determination of the vitamins A, E, their esters and coenzyme Q_{10} in multivitamin dietary supplements using an RP-C30 phase. Eur Food Res Technol 222: 643-9.

Carter GT, Weydt P. 2002. Cannabis: Old medicine with new promise for neurological disorders. Curr Opin Investig Drugs 3: 437-440.

Chen G, Pramanik BN, Liu YH, Mirza UA. 2007. Applications of LC/MS in structure identifications of small molecules and proteins in drug discovery. J Mass Spectrom 42: 279-87.

Fitzgerald RL, Rivera JD, Herold DA. 1999. Broad spectrum drug identification directly from urine, using liquid chromatography-tandem mass spectrometry. Clin Chem 45: 1224-34.

Fukuda D, Archer RA, Abbott BJ. 1977. Microbiological transformations of {Delta}6a,10a-tetrahydrocannabinol. Appl Environ Microbiol 33: 1134-1140.

Furuya T, Sakamoto K, Iida K, Asada Y, Yoshikawa T, Sakai S, Aimi N. 1992. Biotransformation of tabersonine in cell suspension cultures of *Catharanthus roseus*. Phytochemistry 31: 3065-8.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50: 151-158.

Gaoni Y, Mechoulam R. 1966. Cannabichromene, a new active principle in hashish. Chem Commun 1: 20.

Hartsel SC, Loh WHT, Robertson LW. 1983. Biotransformation of cannabidiol to cannabielsoin by suspension-cultures of *cannabis-sativa* and *Saccharum-officinarum*. Planta Med 48: 17-19.

Hazekamp A, Peltenburg A, Verpoorte R, Giroud C. 2005. Chromatographic and Spectroscopic Data of Cannabinoids from *Cannabis sativa* L. J Liq Chromatogr Relat Technol 28: 2361-2382.

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, van Zweden R, Verpoorte R. 2004. Preparative Isolation of Cannabinoids from *Cannabis sativa* by Centrifugal Partition Chromatography. J Liq Chromatogr Relat Technol 27: 2421-2439.

Meuwly P, Metraux JP. 1993. Ortho-anisic acid as internal standard for the simultaneous quantitation of salicylic acid and its putative biosynthetic precursors in cucumber leaves. Anal Biochem 214: 500-5.

Michael B, Astrid P. 1980. Microbial transformation of cannabinoids. Part 3: Major metabolites of (3R,4R) -Δ^1-tetrahydrocannabinol. Helv Chima Acta 63: 2515-2518.

Mustafa NR, Kim HK, Choi YH, Verpoorte R. 2009. Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. Biotechnol Lett 31: 1967-1974.

Ning LL, Han J, Zhang XY, Guo HZ, Bi KS, Guo DA. 2004. Biotransformation of triptolide and triptonide by cell suspension cultures of *Catharanthus roseus*. J Asian Nat Prod Res 6: 93-7.

Patel S, Gaur R, Verma P, Bhakuni RS, Mathur A. 2010. Biotransformation of artemisinin using cell suspension cultures of *Catharanthus roseus* (L.) G.Don and Lavandula officinalis L. Biotechnol Lett 32: 1167-71.

Pitt JJ. 2009. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev 30: 19-34.

Rashidi H, Akhtar MT, Kooy FDR, Verpoorte R, Duetz WA. 2009. Hydroxylation and further oxidation of Δ^9-tetrahydrocannabinol by alkane-degrading bacteria. Appl Environ Microbiol 75: 7135–7141.

Shimoda K, Yamane S-y, Hirakawa H, Ohta S, Hirata T. 2002. Biotransformation of phenolic compounds by the cultured cells of *Catharanthus roseus*. J Mol Catal B: Enzym 16: 275-281.

Sirikantaramas S, Taura F, Morimoto S, Shoyama Y. 2007. Recent Advances in *Cannabis sativa* Research: Biosynthetic Studies and Its Potential in Biotechnology. Curr. Pharm. Biotechnol 8: 237-243.

Sommer J, Schröder C, Stöckigt J. 1997. *In vivo* formation of vanillin glucoside. Plant Cell Tiss Org Cult 50: 119-123.

Tanaka H, Morimoto S, Shoyama Y. 1993. Cannabis, 21. Biotransformation of Cannabinol to Its Glycosides by In Vitro Plant Tissue. J Nat Prod 56: 2068-2072.

Tanaka H, Takahashi R, Morimoto S, Shoyama Y. 1996. Cannabis 25, biotransformation of cannabidiol and cannabidiolic acid by *Pinellia ternata* tissue segments. Plant Cell Rep 15: 819-823.

Tanaka H, Takahashi R, Morimoto S, Shoyama Y. 1997. A new Cannabinoid, Δ^6-Tetrahydrocannabinol 2'-*O-beta*-D-glucopyranoside, Biotransformed by Plant Tissue. J Nat Prod 60: 168-170.

Xiao JF, Zhou B, Ressom HW. 2012. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. Trends Analyt Chem 32: 1-14.

Ye M, Dai J, Guo H, Cui Y, Guo D. 2002. Glucosylation of cinobufagin by cultured suspension cells of *Catharanthus roseus*. Tetrahedron Lett 43: 8535-8538.

Ye M, Ning L, Zhan J, Guo H, Guo D. 2003. Biotransformation of cinobufagin by cell suspension cultures of *Catharanthus roseus* and *Platycodon grandiflorum*. J Mol Catal B-Enzym 22: 89-95.

Yuana, Dignum MJW, Verpoorte R. 2002. Glucosylation of exogenous vanillin by plant cell cultures. Plant Cell Tiss Org Cult 69: 177-182.

Ishihara K, Hamada H, Hirata T, Nakajima N. 2003. Biotransformation using plant cultured cells. J Mol Catal B-Enzym 23: 145-70.

CHAPTER 5

Developmental effects of cannabinoids on zebrafish larvae

Muhammad Tayyab Akhtar(¶, #), Shaukat Ali(*,#), Hassan Rashidi(¶), Frank van der Kooy(¶), Robert Verpoorte (¶) and Michael K. Richardson(*)

¶ Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

*Institute of Biology, Leiden University Leiden, Sylvius Laboratory, Sylviusweg 72, 2333 BE, Leiden, the Netherlands.

Abstract

Here, we examine the effects of the cannabinoids Δ^9-THC, (R)-(+)-[2,3-Dihydro-5-methyl- 3-(4-morpholinylmethyl)pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]- 1-napthalenylmethanone (WIN 55,212-2) and 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP 55,940), and the cannabinoid antagonist (AM251). Exposures were either acute (1-12 h exposure at 108 hour post fertilization [hpf]); or chronic (96 h exposure starting at 24 hpf). Geometric range-finding was used to determine the experimental concentrations. LC_{50} was determined based on mortality at 5 days post fertilisation (dpf). At day 5, behavioural analysis (visual motor response test) was carried out in which movement of individual larvae was analysed using automated video-tracking. With acute exposure, embryos showed a biphasic response to the dark challenge with all three cannabinoids tested. This response consisted of stimulation of locomotor activity at low concentrations, suppression at high doses. With chronic exposure, embryos habituated to the effects of all three cannabinoids when assayed with the dark challenge phase. Furthermore, the excitation was ameliorated when the antagonist AM251 was co-administered with the cannabinoid. When AM251 was administered on its own (chronically or acutely), the locomotor activity was suppressed at high concentrations. We examined the embryos for a range of malformations after chronic exposure to cannabinoid. Only Δ^9-THC was associated with a significant increase in malformations at 5d (yolk sac and pericardial oedema, bent tail). We conclude that cannabinoids have behavioural effects in zebrafish that are comparable to some of those reported in the literature for mammals. In particular, the acute exposure response resembles behavioural effects reported for adult rodents. Our data are consistent with these behavioural effects being mediated, at least in part, by the CB_1 receptor.

Introduction

Zebrafish embryos have great promise for use in high-throughput screening of new drug candidates (Bull and Levin 2000, Lieschke and Currie 2007, Ali et al. 2011, Ali et al. 2011a, Ali et al. 2011b, Ali et al. 2011c). The zebrafish model is not an alternative to rodent models in drug screening, but is complementary to them (Ali et al. 2011). It could be helpful in studies demanding rapid, high-throughput and low-cost assays, such as in the early (pre-regulatory) stages of drug testing (Teraoka et al. 2003, Redfern et al. 2008) and also for behavioral testing (Best et al. 2008, Champagne et al. 2010, Rihel et al. 2010, Ali et al. 2011a). Many basic cellular and molecular pathways regulated by different compounds, and by stress stimuli, are similar between the zebrafish and mammals (Voelker et al. 2007, Schaaf et al. 2008).

Purification and structural elucidation of Δ^9-THC (Gaoni and Mechoulam 1964) has led to the discovery of many pharmacological properties of cannabinoids. Δ^9-THC and its derivatives are being studied for their psychotropic properties and other pharmacological activities, including their possible actions as anticonvulsants, antidepressants, hypotensives, bronchodilators, analgesics and the ability to lower intraocular pressure (Holdcroft et al. 2006). Cannabinoids have also been examined for suitability in the symptomatic treatment of multiple sclerosis (Zajicek et al. 2005, Baker et al. 2007). Unfortunately, cannabinoids may have serious, undesirable effects such as dependency, a possible causative association with psychotic illness, and cognitive impairment including deleterious effects on memory (Niyuhire et al. 2007, Hoffman et al. 2007, Morgan et al. 2009, Cooper and Haney 2009, Justinova et al. 2009).

Rodent models have been used to explore the teratological, toxicological and behavioural effects of cannabinoids and their receptor agonists (Sulcova et al.

1998, Norwood et al. 2003, Drews et al. 2005, Wiley et al. 2007). Cannabinoid receptor type 1 (CB_1) (Lolait et al. 1990), and Cannabinoid receptor type 2 (CB_2) (Munro et al. 1993) are G-protein-coupled receptors (Pertwee 2008). Extensive work has been done to understand their role (Rodriguez-Martin et al. 2007, Braida et al. 2007, Migliarini and Carnevali 2009). Several previous studies showed that the behavioral effect of Δ^9-THC is mediated by the central CB_1 receptor in rats (Tseng and Craft 2004). CB_1 (Lam et al. 2006) and CB_2 (Rodriguez-Martin et al. 2007) receptors have also been reported in zebrafish. CB_1 receptor antagonist (rimonabant) has been reported to attenuate the salvinorin A inducing stimulation (swimming activity) of adult zebrafish (Braida et al. 2007). The zebrafish, CB1 receptor appeared in the preoptic area at 24 hour post fertilization (hpf) (Lam et al. 2006).

The cannabinoids used in this study (Δ^9-THC, WIN 55,212-2 and CP 55,940) are CB_1 and CB_2 agonists (Schatz et al. 1997, Pertwee 2008). A pronounced chronic and acute behavioral effect of cannabinoids has been observed in pubertal rats, which postulate that an immature brain could be more vulnerable to the externally exposed cannabinoid than an adult organism (Schneider et al. 2008). The aim of this study is to determine the teratology, toxicology and behavioural effects of Δ^9-THC, CP 55,940 and WIN 55,212-2 in zebrafish embryos.

Material and methods

Ethics statement

All animal experimental procedures were conducted in accordance with local and international regulations. The local regulation is the *Wet op de dierproeven* (Article 9) of Dutch Law (National) and the same law administered by the Bureau of Animal Experiment Licensing, Leiden University (Local). This local regulation serves as the implementation of *Guidelines on the protection of*

experimental animals by the Council of Europe, Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 days after fertilization). Because embryos used here were no more than 5 days old, no license is required by Council of Europe (1986), Directive 86/609/EEC or the Leiden University ethics committee.

Animals

Male and female adult zebrafish (*Danio rerio*) of AB wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, the Netherlands) who obtain stock from Europet Bernina International BV (Gemert-Bakel, the Netherlands). The AB strain is a wild type strain (see www.zfin.org) and shows high genetic diversity, increasing the likelihood that we will detect idiosyncratic responses to the toxins. Fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm; W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08.00). Water and air were temperature controlled (25±0.5 $^{\circ}$C and 23 $^{\circ}$C, respectively). The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., Burlingame, USA) and twice a week with frozen food (artemias; Dutch Select Food, Aquadistri BV, the Netherlands).

Defined embryo buffer

To produce a defined and standardized control and vehicle for these experiments, we used 10% Hank's balanced salt solution (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO) at a concentration 0.98 g/L in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma Cat S5761), and adjusted to pH 7.46. A similar medium has been used previously in other studies (Ali et al. 2011, Ali et al. 2011a, Ali et al. 2011b, Wielhouwer et al. 2011).

Embryo preparation

Embryo preparation was done according to Ali et al. 2011. Briefly, all incubations of embryos were carried out in an incubator with orbital shaking (50 rpm) under a light cycle of 14 h light: 10 h dark (lights on at 8.00 in the morning). The embryos were gently transferred at 24 hours post fertilization (hpf) using a sterile plastic Pasteur pipette into 96-well microtitre plates (Costar 3599, Corning Inc., NY). A single embryo was plated per well, so that embryos subsequently dying would not affect others; and also to allow individual embryos to be tracked for the whole duration of the experiment, including for behavioral recording.

Cannabinoid treatment

A significant proportion of zebrafish eggs cultured under laboratory conditions are either unfertilised or die within a few hours (Ali et al. 2011). For this reason, we began administration of cannabinoids at 24 hpf. Purification of Δ^9-tetrahydrocannabinol was done by using centrifugal partition chromatography (Hazekamp et al. 2004). The final concentration of dimethlysulphoxide (DMSO) in the water was 0.01%. All pipetting was done manually, with an 8-channel pipetter.

Preliminary range-finding

To determine a suitable range of concentrations for testing, we performed range-finding. The concentrations were in a geometric series in which each was 50% greater than the next lowest value (United States Environmental Protection Agency ,1996, Ali et al. 2011). We used 0.0, 12.5 25.0 50.0 and 100 mg/L of cannabinoids. A static replacement regime was used. Thus, there was no refreshment of buffer after the addition of compound. Each well contained 250 µL of either test compound or control (buffer only) or vehicle (0.01% DMSO in

buffer). We used 32 embryos for each concentration and 32 embryos each cannabinoid as control, and 32 embryos for each cannabinoid to control for the vehicle. The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates.

Mortality scoring

Mortality rate was recorded at 48, 72, 96 and 120 hpf in both range-finding and test concentration experiments, by examination under a dissecting stereomicroscope. Embryos were scored according to (Ali et al. 2011).

Refined geometric series and LC_{50} determination

After the range-finding experiments, a series of concentrations lying in the range between 0% and 100% mortality were selected for LC_{50} determination. The range for Δ^9-THC and WIN 55,212-2 was 0.3-9.6 mg/L and for CP 55,940 was 2.25-18.0 mg/L. Each geometric series of concentrations for each compound was repeated three times (in total 36 embryos per concentration and 36 embryos for vehicle for each compound). The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates in each independent experiment. The LC_{50} in mg/L was then converted into LC_{50} mmol/L. The LC_{50} (expressed in mg/L of buffer) was determined based on cumulative mortality at 120 hpf using Regression Probit analysis with SPSS Statistics v.17.0 (SPSS Inc., Chicago, USA).

Antagonist treatment

A geometric series of concentrations (0.5-8 mg/L) of AM251 was used to select effective concentrations for further testing. These selected concentrations were used together with one fixed concentration of each cannabinoid. The concentrations of cannabinoids selected were those on which hyper-locomotor activity was obtained.

Chronic and acute exposure

The exposure of cannabinoids for 96 h (24- 120 hpf) is defined as a chronic while for 1-12 h as acute exposure regime.

Behavioural analysis

The visual motor response test was performed at 5 days post fertilization (dpf) according to Ali et al. (2011a, 2011b) on all living larvae of both range finding experiments and geometric series. The test was performed in the presence of original solutions added at 24 h. Thus, there was no replacement or refreshment of buffer before test. The temperature used for testing was 28±0.5 °C. The visual motor response test has been previously characterized and typically consists of brief (less than 10 min) frequently alternating periods of light and dark. A key feature of this test is the robust but transient behavioral activity that occurs in response to sudden transitions from light to dark (Burgess and Granato 2007, Emran et al. 2008, Macphail et al. 2009, Rihel et al. 2010). Because such behavioral response has been shown to be highly sensitive to neuroactive chemical compounds, the visual motor response test has become a validated tool to assess the impact of a wider range of chemical agents on neuronal and physiological integrity of the developing zebrafish (Burgess and Granato 2007, Emran et al., 2008, Macphail et al. 2009, Rihel et al. 2010). Here we used a modified version of this test consisting of a single transition from light to dark. The activity of each larva was automatically recorded and analyzed in the ZebraBox recording apparatus equipped with VideoTrack software (both from Viewpoint S.A., Lyon, France). The white light intensity of the ZebraBox was 500 lux. The experimental recording consists of two steps. First, larvae were acclimated to the behavioral setup with lights ON for 2 min. This period was necessary and sufficient to ensure low and stable behavioral activity. Once basal levels of locomotor activity were stabilized following the acclimatizing period,

basal swimming activity was recorded during 4 min with lights ON. This period is referred to as 'basal context'. Immediately following the basal activity recording, the lights were suddenly turned off for 4 min. Behavioral activity in the dark was also automatically recorded during this period. This period is referred to as the 'dark challenge context'. We chose four-minute session to prevent habituation, and also to favor more robust behavioral changes. Because of the robustness of the behavioral changes induced by varying illumination, this task can be used to reveal more readily than any other tasks, defective brain function, aberrant nervous system development and/or locomotor and visual defects caused by toxic compounds (Ali et al. 2011a).

Morphological assessment of embryo phenotypes in the survivor population

Morphological assessment was done according to Ali et al. (2011a). All embryos remained in their original multi-well plates, so that every individual could be tracked throughout the entire experimental and analysis procedure. The phenotypes were scored according to the criteria listed in Table 1.

Table 1. Phenotype analysis.

Larval phenotype	Criteria
1. Normal	Absence of any of the phenotypes listed below:
2. Heart	Presence of pericardial oedema
3. Yolk	Presence of yolk sac oedema
4. Pigmentation	Dispersion of melanocytes (pigment cells)
5. Tail	Tail bent
6. Body axis	Body/primary axis bent/curved
7. Meckel's cartilage	Meckel's cartilage grossly hypoplastic, missing or unfused in midline. These effects may be unilateral or bilateral.
8. Branchial arches	One or more cartilages of the branchial skeleton hypoplastic or missing.

Description of the seven categories used to score larval phenotype at 5 dpf

Statistical analysis

Statistical analyses were performed using GraphPad Prism for Windows (version 5.03) and also used to plot graphs. To analyze the impact of compounds on embryo locomotion in the visual motor response test, we used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance. LC_{50} was determined using Regression Probit analysis (Chi-Squares test, Pearson Goodness-of-fit test and 95% confidence interval) with SPSS Statistics for windows version. 17.0 (SPSS Inc., Chicago, USA).

Results

LC_{50} of cannabinoids

The LC_{50} was determined for chronic exposure of zebrafish embryos to cannabinoids (96 h of exposure beginning at age 24 hpf). The following LC_{50} values were obtained at 5 dpf: Δ^9-THC, 3.37 mg/L (0.01 mmol/L); WIN 55,212-2, 1.8 mg/L (0.003 mmol/L); and CP 55,940, 16.92 mg/L (0.049 mmol/L).

Functional impairment at sub-lethal concentrations

We analysed the degree of behavioural change in zebrafish embryos exposed to cannabinoids. We used a behavioral test, the *visual motor response test*, which relies on the integrity of the central and peripheral nervous systems, including the visual system, and on normal locomotor and skeletal system development. The effects of three cannabinoids are illustrated in Figure 1and Figure 2.

We analysed the effects on total distance moved in the basal, challenge and recovery phases for both chronic and acute exposure regimes (Table 2, Table 3). The effects on this locomotory parameter fell into the categories of monotonic

stimulation; monotonic suppression; biphasic response (stimulation at lower and suppression at higher concentrations); or no significant effect.

Table 2. Concentration-dependent functional impairment by three cannabinoids (chronic exposure)

	Tdm[a] in basal phase (light on)			**Tdm in challenge phase (light off)**			**Tdm in recovery phase (light on)**		
	=	↓	↑	=	↓	↑	=	↓	↑
Compound	**Con[b] (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**
Δ^9-THC	0.3, 0.6, 1.2, 2.4	-	-	0.3, 0.6, 2.4	-	1.2	0.3, 0.6, 1.2, 2.4	-	-
WIN55,212-2	0.3, 0.6, 1.2	-	-	0.3, 0.6, 1.2	-	-	0.3, 0.6, 1.2	-	-
CP55, 940	2.25, 4.5, 9	-	-	2.25, 4.5, 9	-		2.25, 4.5, 9	-	-

Key: '=' equal to control; '↓' significantly lower than control; '↑' significantly higher than control; 'a' Total distance moved; 'b' Concentration.

Effect of chronic (96 h) exposure to cannabinoids on locomotor activity

We focus here on the dark challenge phase in order to be able to make comparisons with studies on mammals. The term 'chronic exposure' is here arbitrarily applied to 96 h of treatment since this covers the major stages of organogenesis (Kimmel et al. 1995). Compared to controls, embryos exposed chronically to all Δ^9-THC concentrations showed habituation (with increasing concentration. Only with a concentration of 1.2 mg/L was there any significant stimulation in the challenge phase with Δ^9-THC (Fig. 1A).

Table 3. Concentration-dependent functional impairment by three cannabinoids (acute exposure)

		Tdm in basal phase (light on)			**Tdm in challenge phase (light off)**			**Tdm in recovery phase (light on)**		
		=	↓	↑	=	↓	↑	=	↓	↑
Compound	**Exposure duration (hour)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**	**con (mg/L)**
Δ^9-THC	1	0.6, 1.2	-	2.4, 3.4	0.6, 3.4	-	1.2, 2.4	0.6	-	1.2, 2.4 3.4
	4	0.6, 1.2, 3.4	-	2.4	0.6, 1.2, 2.4	3.4	-	0.6, 1.2, 3.4	-	2.4
	12	0.6, 1.2, 2.4	3.4	-	-	2.4, 3.4	0.6, 1.2	0.6, 1.2, 2.4	3.4	-
WIN 55,212-2	1	-	0.6, 1.2, 1.8	-	0.6	-	1.2, 1.8	0.6, 1.2, 1.8	-	-
	4	0.6, 1.8	-	1.2	0.6, 1.8	-	1.2	0.6, 1.2, 1.8	-	-
	12	0.6, 1.2, 1.8	-	-	0.6, 1.8	-	1.2	0.6, 1.2, 1.8	-	-
CP55, 940	1	2.25, 4.5, 9	-	18	-	-	2.25, 4.5, 9, 18	2.25, 4.5, 9	-	18
	4	2.25, 4.5, 9, 18	-	-	-	-	2.25, 4.5, 9, 18	2.25, 9, 18	-	4.5
	12	18	-	2.25, 4.5, 9	-	-	2.25, 4.5, 9,	2.25, 9, 18	-	4.5

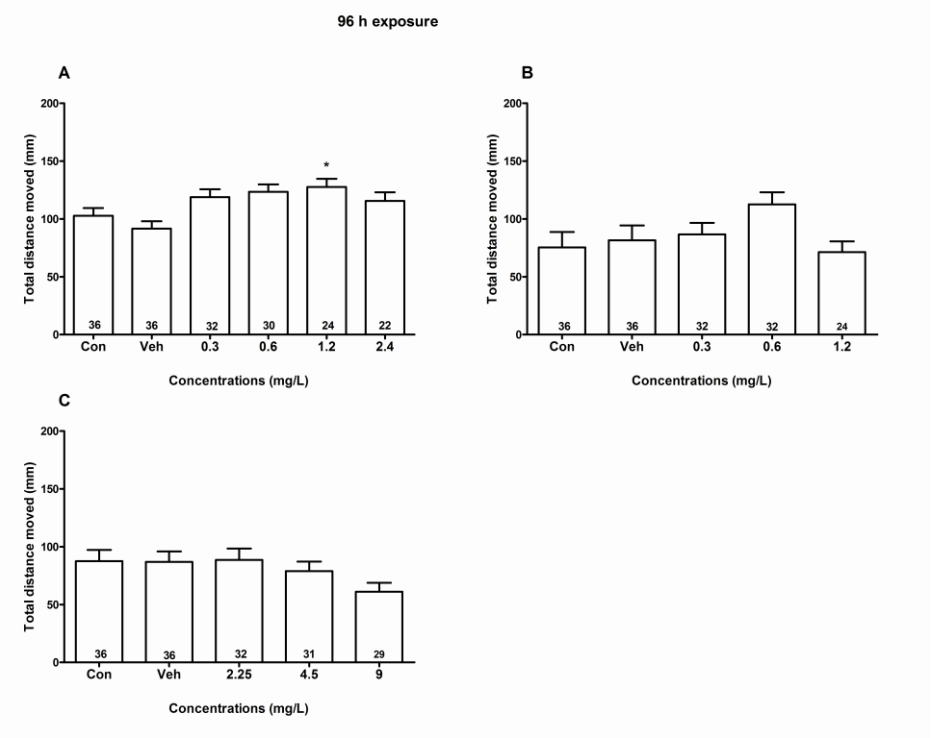


Figure 1. Behavior analysis of live zebrafish embryos treated with Δ^9-THC, WIN 55,212-2 and CP 55,940 for 96 h. **A**, locomotor activity induced by Δ^9-THC exposure; **B**, by WIN 55,212-2; **C**, by CP 55,940. ***** depict differences between controls and different used concentrations. In figures **A-C**, it can be seen that there is habituation that occurs with 96 h exposure. The number inside the base of the bars = *N* embryos .Statistical icons: *= $p < 0.05$.

Effect of acute exposure to cannabinoids on locomotor activity

Here, acute exposure is arbitrarily applied to a 1-12 h exposure starting at 108 hpf. For behavioral analysis, embryos were exposed at 4.5 dpf for 12 h, and at 5 dpf for 1-4 h (in order to provide a common endpoint of 5 d). With Δ^9-THC, there was an effect of both concentration and duration of exposure on locomotor activity. With ≥1 h exposure time, locomotor activity was stimulated at low concentrations (Fig. 2A); no effect was found with high concentrations, even after 4 h of treatment (Fig. 2B). A biphasic response (stimulation at low

concentrations and suppression at high concentrations) was found with 12 h exposure (Fig. 2C). By contrast, low concentrations continued to cause hyper-activity at 12 h exposure (Fig. 2A-C). Concerning the other cannabinoids, the action of WIN 55,212-2 on locomotor activity closely resembles that of Δ^9-THC and a biphasic response was found after 12 h of exposure (Fig. 2D-F). At low concentrations, CP 55,940 (Fig. 2G-I) and WIN 55,212-2 both gave a similar behaviour pattern as Δ^9-THC (hyper-activity from 1 h - 12 h of exposure).

Effect of exposure to the cannabinoid receptor antagonist AM251 on locomotor activity

We exposed zebrafish embryos to AM251 for 1-12 h (acute exposure) or 96 h chronic exposure. Concentration-dependent suppression of locomotor activity was found in both cases (Fig. 3A-D).

Embryos were co-exposed acutely to a cannabinoid plus antagonist (AM251) for 1-12 h. The antagonist caused a dose-dependent amelioration of the locomotor activity induced by the cannabinoid alone (Fig. 4A-I).

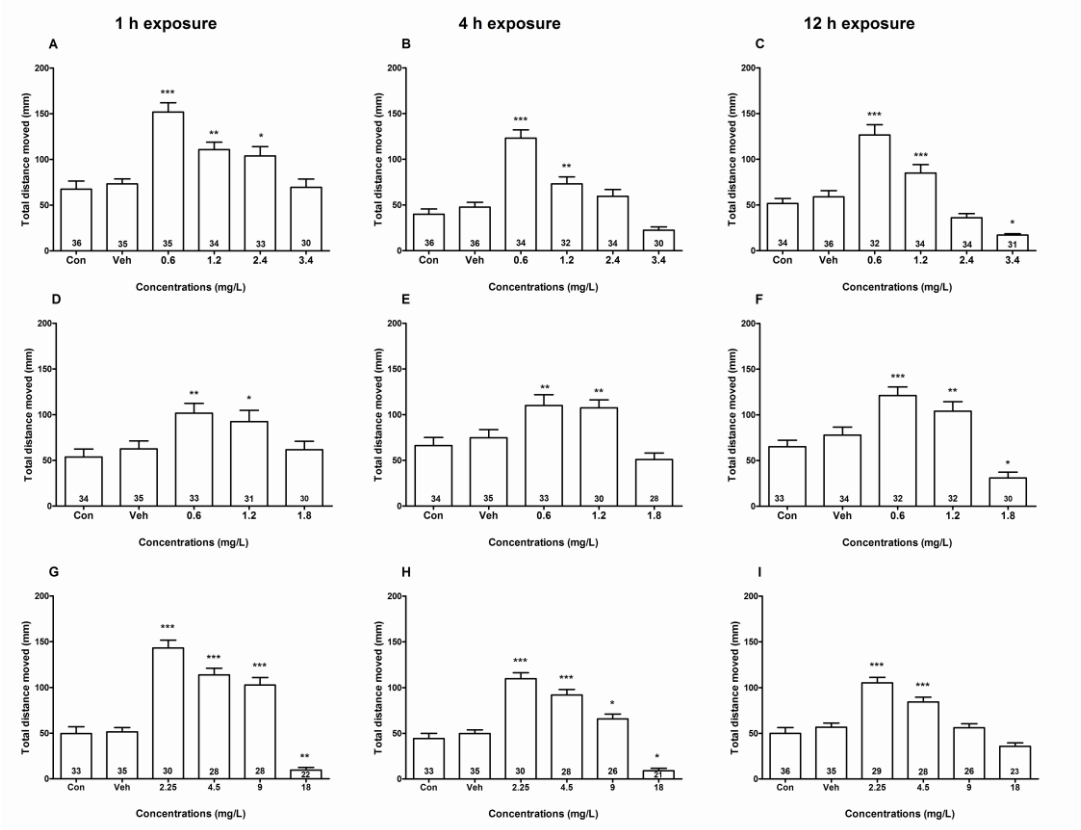


Figure 2. Behavior analysis of live zebrafish embryos treated with Δ^9-THC, WIN 55,212-2 and CP 55,940 for selected time points**.** The graphs represent locomotor activity at the following time points: **A**, **D** and **G** 1 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively; **B**, **E** and **H** 4 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively; **C**, **F** and **I** 12 h exposure with Δ^9-THC, WIN 55,212-2 and CP 55,940 respectively. ***** depict differences between controls and different used concentrations. In figures C, F and G, it can be seen that there is a biphasic response in acute regimes. The number inside the base of the bars = *N*. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

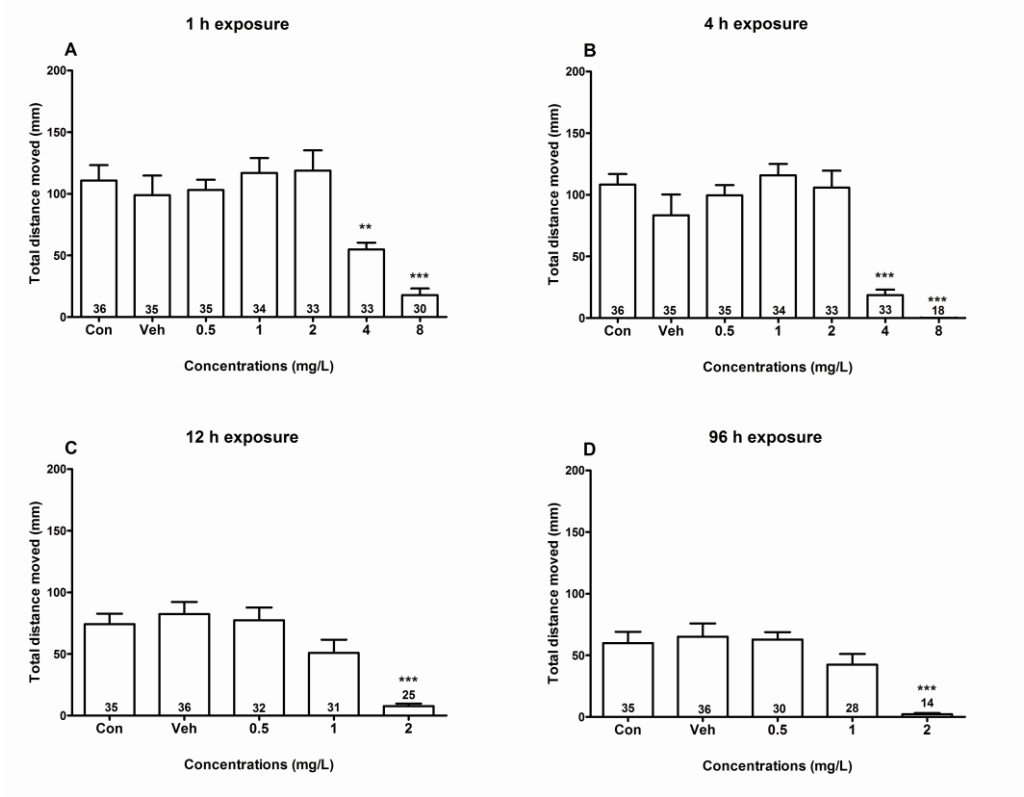


Figure 3. Behavior analysis of live zebrafish embryos treated with AM251 for selected timepoints. The graphs represent locomotor activity at the following time points: **A**, 1 h exposure **B**, 4 h; **C**, 12 h; **D**, 96 h. ***** depict differences between controls and different used concentrations. The number inside the base of the bars = *N*. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

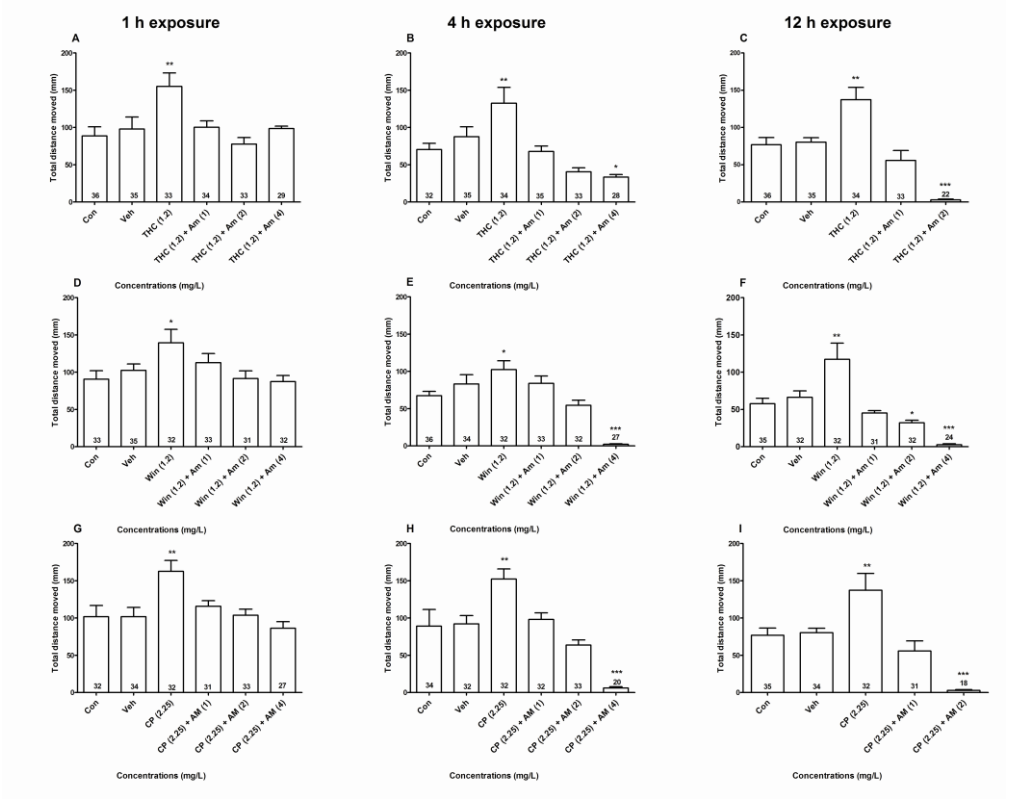


Figure 4. Behavior analysis of live zebrafish embryos co-administrated of AM251 with Δ^9-THC, WIN 55,212-2 and CP 55,940 for selected timepoints. The graphs represent locomotor activity at the following time points: A, D and G 1 h exposure to AM251 with Δ^9-THC, with WIN 55,212-2 and with CP 55,940 respectively; B, E and H 4 h exposure to AM251 with Δ^9-THC, with WIN 55,212-2 and with CP 55,940 respectively; C, F and I 12 h exposure to AM251 with Δ^9-HC, with WIN 55,212-2 and with CP 55,940 respectively. ***** depict differences between controls and different used concentrations. The number inside the base of the bars = *N*. Statistical icons: *= p< 0.05, **= p< 0.01 and ***= p< 0.001.

Morphological assessment of embryos

The results of morphological analysis of embryos are shown in Figure 5 and Table 4. With 0.3 to 2.4 mg/L Δ^9-THC treatment, the frequency of pericardial and yolk sac oedemas, and bent body, were significantly higher than in control (buffer only) and vehicle (DMSO and buffer only) experiments. With CP 55,940 and WIN 55,212-2 no significant increase in the frequency of any malformation was seen (the apparent increase in yolk sac oedema with CP 55,940 exposure was not statistically significant in view of the number of cases).

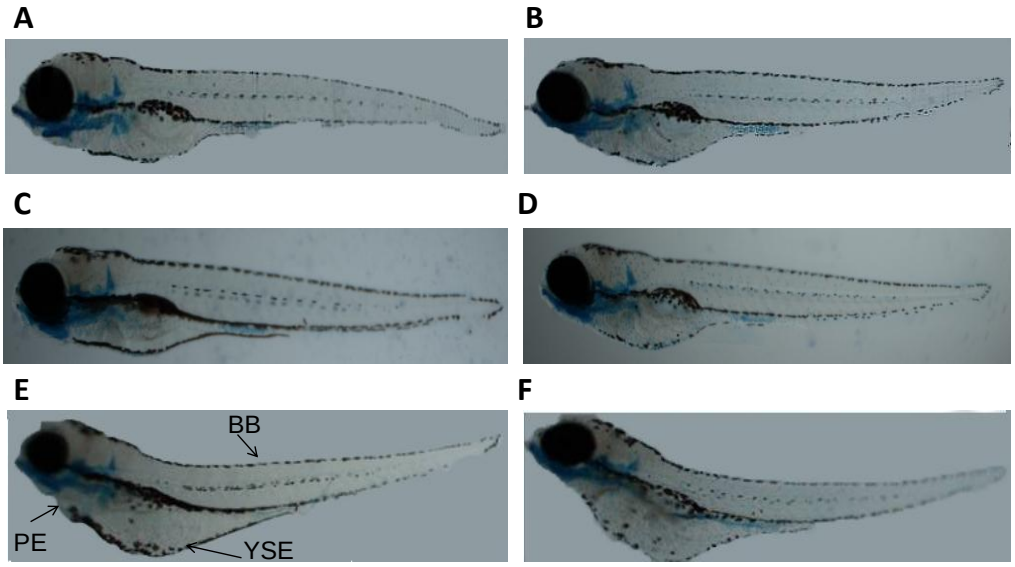


Figure 5. Morphological assessment of zebrafish embryos at 5 dpf treated with cannabinoids. Zebrafish embryos stained with alcian blue reveals the ventral view. The aim of this figure is to show the effects on body axis of embryos after 96 h exposure to cannabinoids. A, control; B, vehicle; C, 1.2 mg/L Win 55,212-2; D, 9 mg/L CP 55,940; E, 0.6 mg/L Δ^9-THC; F, 2.4 mg/L Δ^9-THC. PE, pericardial edema; YSE, yolk sac edema; BB, bent body.

Table 4. Statistical analysis of incidence of malformations in zebrafish embryos at different concentrations of Δ^9-THC.

	Significance level			
Categories	0.3 (mg/L)	0.6 (mg/L)	1.2 (mg/L)	2.4 (mg/L)
Pericardial Oedema	-	**	*	*
Yolk sac oedema	***	***	***	***
Bent tail	-	-	-	-
Body/primary axis (bent/curved)	***	***	*	*
Meckel's cartilage(hypoplasia)	-	-	-	-
Branchial arches (hypoplasia)	-	-	-	-

Key:* Statistical icons: (-), not significant; (*), $p< 0.05$; (**), $p< 0.01$; and (***), $p< 0.001$. Note that there were no significant increases in malformations after exposure to WIN 55,212 and CP 55,940. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

Discussion

We have studied the effects of cannabinoids on the survival, locomotor activity and morphological development of zebrafish embryos. Our readouts were mortality recording, the visual motor response test and morphological analysis. Both acute and chronic exposure regimes, and the effects of the cannabinoid receptor antagonist (AM251), were examined.

In acute regimes, Δ^9-THC showed a biphasic response with increasing hyperactivity succeeded by suppression of activity as the dose increased. These findings are consistent with studies in rodents which reported a stimulation of locomotor activity by Δ^9-THC at low concentrations, and suppression at higher

concentrations (Grisham and Ferraro 1972, Ferraro and Gluck 1974, Taylor and Fennessy 1977). A recent study (Freedland et al. 2002) suggested that high concentrations of Δ^9-THC in adult rats decreased cerebral metabolism. According to this study, this metabolic change was associated with the biphasic motor behaviour of Δ^9-THC. In zebrafish embryos, CB1 receptors are expressed in the preoptic area by 24 hpf (Lam et al. 2006). We therefore chose this time to begin chronic administration of cannabinoids. For acute exposure, we began to expose embryos of 4.5 dpf so that all embryos, regardless of treatment, were analysed at the same endpoint (day 5).

WIN 55,212-2 also caused a biphasic response in acute regimes. This is consistent with findings (Drews et al. 2005) in the open field test, where rats treated with low concentrations of WIN 55,212-2 covered more distance than controls, and those treated with high concentrations covered less distance. CP 55,940 also caused a biphasic response in acute exposure regimes. Biphasic locomotor activity has been reported in rats exposed to CP 55,940 (McGregor et al. 1996). Furthermore, a pre-treatment of CP 55,940 caused hyperactivity in rats subsequently exposed to morphine (Norwood et al. 2003).

In chronic regimes, all three cannabinoids showed habituation. It is interesting to notice that the habituation is probably not accompanied by general sedation of the embryos because their locomotor activity in the corresponding basal phase is normal (Table 2, Table 3). Several studies in different species have shown that chronic exposure of cannabinoids is accompanied by the development of tolerance to many of the acute effects. These effects include memory disorder, hypothermia and analgesia (reviewed by (Howlett et al. 2004). In rodents, the development of tolerance to motor-behavioural effects of chronic cannabinoids exposure has been studied. For example, chronic exposure to Δ9-THC (Abood et al. 1993, Rodriguez et al. 1994, Howlett et al. 2004),

WIN 55,212-2 (Martini et al. 2010) and CP 55,940 (Costa et al. 1996, Rubino et al. 1997) all caused tolerance to the effects of those cannabinoids on suppressing locomotor activity. This phenomenon was associated with down regulation of CB receptors after long-term exposure to cannabinoids (Abood et al. 1993, Rodriguez et al. 1994, Costa et al. 1996, Rubino et al. 1997, Howlett et al. 2004, Martini et al. 2010). Hence, the study of CB receptors expression level after a chronic exposure to cannabinoids can further extend our understanding of the phenomenon of tolerance in zebrafish embryos.

An increased incidence of curved body axis and bent tail were found in embryos exposed chronically to Δ^9-THC. It is necessary, therefore to consider the possibility that the changes in locomotion and behaviour were caused by these malformations, and not by an action of the cannabinoid on the nervous system. But it can be seen in Figure 1, embryos exposed chronically to Δ^9-THC have shown similar locomotor activity compared to control. Moreover, Win 55,212-2 and CP 55,940 have not shown any significant incidence of malformations at any concentration tested, yet do show changes in locomotor behaviour in acute regimes. This suggests that the locomotor effects of these cannabinoids is not a secondary one due to teratogenicitiy.

It has previously been reported in rodents that AM251 decreases the total distance travelled in open field test (Sink et al. 2010) and also blocks the locomotor excitation caused by CB_1 agonists (Kongkam et al. 2008). Our data suggest that AM251 attenuates the increased locomotor activity induced by CB_1 agonists. These results implicate the involvement of CB_1 receptors in the regulation of locomotor activity in zebrafish larvae and are in good agreement with previous rodent studies.

It has also been shown that AM251 attenuates the behavioural sensitization induced in rodents by amphetamine, nicotine and Δ^9-THC (Gatley et al. 1996,

Thiemann et al. 2008, Le et et al. 2008). These studies show that the blockade of CB1 receptor not only opposes the inducing effect of cannabinoids but can also alter the activity of other psychotropic compounds having binding sites other than CB receptors. Another study found that CP 55,940 has one, and WIN 55212-2 two different binding sites in the zebrafish brain (Rodriguez et al. 2007). So, It would be of great interest to explore the AM251 binding affinity in the zebrafish brain. It is also likely that CB receptor-knockout in zebrafish embryos will lead to a deeper understanding of the role of CB receptors in zebrafish physiology. Moreover, study of agonist and antagonist interactions could be helpful in understanding the zebrafish endogenous cannabinoid system.

Conclusions

Our findings show that 96 h duration of exposure in zebrafish embryos starting at 24 hpf can be used to study the teratology of sub-lethal concentrations of cannabinoids. This regime also leads to habituation in behavioural response. In acute exposure, our findings are similar to the results found in rodents, with dose-dependent hyperactivity followed by suppression. The antagonist blocks the increased locomotor activity induced by cannabinoids. This suggests that some similarity in cannabinoid response pathways between zebrafish and mammals exists. Further validation, and study of receptor interactions, is needed before we can be sure that the zebrafish embryo can be a useful tool for the pre-clinical screening of natural, synthetic and endogenous cannabinoids.

References

Abood ME, Sauss C, Fan F, Tilton, CL, Martin BR (1993). Development of behavioral tolerance to delta-9-THC without alteration of cannabinoid receptor binding or mRNA levels in whole brain. Pharmacol Biochem Behav 46: 575-79.

Ali S, Champagne DL, Alia A, Richardson MK (2011a). Large-scale analysis of acute ethanol exposure in zebrafish development: a critical time window and resilience. PLoS ONE 6: e20037.

Ali S, Champagne DL, Richardson, MK, (2011b). Behavioral profiling of zebrafish embryos exposed to a panel of 60 water-soluble compounds. Behav Brain Res 228: 272-8.

Ali S, Champagne DL, Spaink HP, Richardson MK (2011c). Zebrafish embryos and larvae: A new generation of disease models and drug screens. Birth Defects Res C Embryo Today 93: 115-33.

Ali S, van Mil HG, Richardson MK (2011). Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. PLoS ONE 6: e21076.

Baker D, Jackson SJ, Pryce G (2007). Cannabinoid control of neuroinflammation related to multiple sclerosis. Br J Pharmacol 152: 649-54.

Best JD, Berghmans S, Hunt JJ, Clarke SC, Fleming A, Goldsmith P, Roach AG (2008). Non-associative learning in larval zebrafish. Neuropsychopharmacol 33: 1206-15.

Braida D, Limonta V, Pegorini S, Zani A, Guerini-Rocco C, Gori, E, Sala M (2007). Hallucinatory and rewarding effect of salvinorin A in zebrafish: kappa-opioid and CB1-cannabinoid receptor involvement. Psychopharmacology (Berl) 190: 441-8.

Bull J, Levin B, (2000). Perspectives: microbiology. Mice are not furry petri dishes. Science 287: 1409-10.

Burgess HA, Granato M (2007). Modulation of locomotor activity in larval zebrafish during light adaptation. J Exp Biol 210: 2526-39.

Champagne DL, Hoefnagels CC, de Kloet RE, Richardson MK (2010). Translating rodent behavioral repertoire to zebrafish (*Danio rerio*): relevance for stress research. Behav Brain Res 214: 332-42.

Cooper ZD, Haney M (2009). Actions of delta-9-tetrahydrocannabinol in cannabis: relation to use, abuse, dependence. Int Rev Psychiatr 21: 104-12.

Costa B, Parolaro D, Colleoni M (1996). Chronic cannabinoid, CP-55,940, administration alters biotransformation in the rat. Eur J Pharmacol 313: 17-24.

Dahm R, Geisler R (2006). Learning from small fry: the zebrafish as a genetic model organism for aquaculture fish species. Mar Biotechnol (NY) 8: 329-45.

Di FM, Morgan C, Dazzan P, Pariante C, Mondelli V, Marques TR, at al. (2009). High-potency cannabis and the risk of psychosis. Br J Psychiatry 195: 488-91.

Drews E, Schneider M, Koch M (2005). Effects of the cannabinoid receptor agonist WIN 55,212-2 on operant behavior and locomotor activity in rats. Pharmacol Biochem Behav 80: 145-50.

Emran F, Rihel J, Dowling JE (2008). A behavioral assay to measure responsiveness of zebrafish to changes in light intensities. J Vis Exp 20.

Ferrari F, Ottani A, Giuliani D (1999). Influence of the cannabinoid agonist HU 210 on cocaine- and CQP 201-403-induced behavioural effects in rat. Life Sci 65: 823-831.

Gamaleddin I, Wertheim C, Zhu AZ, Coen KM, Vemuri K, Makryannis A (2011). Cannabinoid receptor stimulation increases motivation for nicotine and nicotine seeking. Addict Biol 17: 47-61.

Gaoni Y, Mechoulam R (1964). Isolation strucure and partial synthesis of an active constituent of hashish. J Am Chem Soc 86: 1646.

Gatley SJ, Gifford AN, Volkow ND, Lan R, Makriyannis A (1996). 123I-labeled AM251: a radioiodinated ligand which binds in vivo to mouse brain cannabinoid CB1 receptors. Eur J Pharmacol 307: 331-8.

Guo S (2004). Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? Genes Brain Behav 3: 63-74.

Hazekamp A, Simons R, Peltenburg-Looman A, Sengers M, van Zweden R, Verpoorte R (2004). Preparative Isolation of Cannabinoids from *Cannabis sativa* by Centrifugal Partition Chromatography. J Liq Chromatogr Relat Technol 27: 2421-39.

Hoffman AF, Oz M, Yang R, Lichtman AH, Lupica CR (2007). Opposing actions of chronic Delta-9-tetrahydrocannabinol and cannabinoid antagonists on hippocampal long-term potentiation. Learn Mem 14: 63-74.

Holdcroft A, Maze M, Dore C, Tebbs S, Thompson S (2006). A multicenter dose-escalation study of the analgesic and adverse effects of an oral cannabis extract (Cannador) for postoperative pain management. Anesthesiology 104: 1040-6.

Howlett AC, Breivogel CS, Childers SR, Deadwyler SA, Hampson RE, Porrino LJ (2004). Cannabinoid physiology and pharmacology: 30 years of progress. Neuropharmacology 1: 345-58.

Huestis MA, Mazzoni I, Rabin O (2011). Cannabis in sport: anti-doping perspective. Sports Med 41: 949-66.

Justinova Z, Panlilio LV, Goldberg SR (2009). Drug addiction. Curr Top Behav Neurosci 1: 309-46.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995). Stages of embryonic development of the zebrafish. Dev Dyn 203: 253-310.

Kongkam P, Rerknimitr R, Punyathavorn S, Sitthi-Amorn C, Ponauthai Y, Prempracha N, et al. (2008). Propofol infusion versus intermittent meperidine and midazolam injection for conscious sedation in ERCP. J Gastrointestin Liver Dis 17: 291-7.

Lam CS, Rastegar S, Strahle U (2006). Distribution of cannabinoid receptor 1 in the CNS of zebrafish. Neuroscience 138: 83-95.

Le FB, Forget B, Aubin HJ, Goldberg SR (2008). Blocking cannabinoid CB1 receptors for the treatment of nicotine dependence: insights from pre-clinical and clinical studies. Addict Biol 13: 239-52.

Lieschke GJ, Currie PD (2007). Animal models of human disease: zebrafish swim into view. Nat Rev Genet 8: 353-67.

Macphail RC, Brooks J, Hunter DL, Padnos B, Irons TD, Padilla S (2009). Locomotion in larval zebrafish: Influence of time of day, lighting and ethanol. Neurotoxicology 30: 52-8.

Martini L, Thompson D, Kharazia V, Whistler JL (2010). Differential regulation of behavioral tolerance to WIN55,212-2 by GASP1. Neuropsychopharmacology 35: 1363-73.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561-4.

McGregor IS, Issakidis CN, Prior G (1996) Aversive effects of the synthetic cannabinoid CP 55,940 in rats. Pharmacol. Biochem Behav 53: 657-64.

Migliarini B, Carnevali O (2009). A novel role for the endocannabinoid system during zebrafish development. Mol Cell Endocrinol 299: 172-7.

Munro S, Thomas KL, Abu-Shaar M (1993). Molecular characterization of a peripheral receptor for cannabinoids. Nature 365: 61-5.

Niyuhire F, Varvel SA, Martin BR, Lichtman AH (2007). Exposure to marijuana smoke impairs memory retrieval in mice. J Pharmacol Exp Ther 322: 1067-75.

Norwood CS, Cornish JL, Mallet PE, McGregor, IS (2003). Pre-exposure to the cannabinoid receptor agonist CP 55940 enhances morphine behavioral sensitization and alters morphine self-administration in Lewis rats. Eur J Pharmacol 465: 105-14.

Pertwee RG (2008). The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: Delta-9-tetrahydrocannabinol, cannabidiol and Delta-9-tetrahydrocannabivarin. Br J Pharmacol 153: 199-215.

Postlethwait JH, Woods IG, Ngo-Hazelett P, Yan YL, Kelly PD, Chu F, et al. (2000). Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Research 10: 1890-1902.

Redfern WS, Waldron G, Winter MJ, Butler P, Holbrook M, Wallis R, Valentin JP (2008). Zebrafish assays as early safety pharmacology screens: paradigm shift or red herring? J Pharmacol Toxicol Methods 58: 110-7.

Rihel J, Prober DA, Arvanites A, Lam K, Zimmerman S, Jang S, et al. (2010) Zebrafish behavioral profiling links drugs to biological targets and rest/wake regulation. Science 327: 348-51.

Rodriguez de FF, Gorriti MA, Fernandez-Ruiz JJ, Palomo T, Ramos JA (1994). Downregulation of rat brain cannabinoid binding sites after chronic delta-9-tetrahydrocannabinol treatment. Pharmacol Biochem Behav 47: 33-40.

Rodriguez-Martin I, de Velasco EM, Rodriguez RE (2007). Characterization of cannabinoid-binding sites in zebrafish brain. Neurosci Lett 413: 249-54.

Rodriguez-Martin I, Herrero-Turrion MJ, Marron Fd, V Gonzalez-Sarmiento R, Rodriguez RE (2007). Characterization of two duplicate zebrafish Cb2-like cannabinoid receptors. Gene 389: 36-44.

Rubino T, Patrini G, Parenti M, Massi P, Parolaro D (1997). Chronic treatment with a synthetic cannabinoid CP-55,940 alters G-protein expression in the rat central nervous system. Brain Res Mol Brain Res 44: 191-7.

Rubinstein AL (2003). Zebrafish: from disease modeling to drug discovery. Curr Opin.Drug Discov Devel 6: 218-23.

Rubio P, Rodriguez de FF, Munoz RM, Ariznavarreta C, Martin-Calderon JL, Navarro M (1995). Long-term behavioral effects of perinatal exposure to delta-9-tetrahydrocannabinol in rats: possible role of pituitary-adrenal axis. Life Sci 56: 2169-76.

Sain NM, Liang A, Kane SA, Urban MO (2009). Antinociceptive effects of the non-selective cannabinoid receptor agonist CP 55,940 are absent in CB1(-/-) and not CB2(-/-) mice in models of acute and persistent pain. Neuropharmacology 57: 235-41.

Schaaf MJ, Champagne D, van L, I van W, Meijer AH, Meijer OC, Spaink HP, Richardson MK (2008). Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. Endocrinology 149: 1591-9.

Schatz AR, Lee M, Condie RB, Pulaski JT, Kaminski NE (1997). Cannabinoid receptors CB1 and CB2: a characterization of expression and adenylate cyclase modulation within the immune system. Toxicol Appl Pharmacol 142: 278-87.

Sink KS, Segovia KN, Sink J, Randall PA, Collins LE, Correa M, et al. (2010). Potential anxiogenic effects of cannabinoid CB1 receptor antagonists/inverse agonists in rats: comparisons between AM4113, AM251, and the benzodiazepine inverse agonist FG-7142. Eur Neuropsychopharmacol 20: 112-22.

Sulcova E, Mechoulam R, Fride E (1998). Biphasic effects of anandamide. Pharmacol Biochem Behav 59: 347-52.

Teraoka H, Dong W, Hiraga T (2003). Zebrafish as a novel experimental model for developmental toxicology. Congenit Anom (Kyoto) 43: 123-32.

Thiemann G, Di M, V Molleman A, Hasenohrl RU (2008). The CB(1) cannabinoid receptor antagonist AM251 attenuates amphetamine-induced behavioural sensitization while causing monoamine changes in nucleus accumbens and hippocampus. Pharmacol Biochem Behav 89: 384-91.

Tropepe V, Sive HL (2003). Can zebrafish be used as a model to study the neurodevelopmental causes of autism? Genes Brain Behav 2: 268-81.

Tseng AH, Craft RM (2004). CB(1) receptor mediation of cannabinoid behavioral effects in male and female rats. Psychopharmacology (Berl) 172: 25-30.

United States Environmental Protection Agency (1996). Ecological Effects Test Guidelines: OPPTS 850.1075: Fish Acute Toxicity Test, Freshwater and Marine. pp. 1-11.

van Hell HH, Bossong MG, Jager G, Kristo G, van Osch MJ, Zelaya F, et al. (2011). Evidence for involvement of the insula in the psychotropic effects of THC in humans: a double-blind, randomized pharmacological MRI study. Int J Neuropsychopharmacol 14: 1-12.

Veldman MB, Lin S (2008). Zebrafish as a developmental model organism for pediatric research. Pediatr Res 64: 470-6.

Voelker D, Vess C, Tillmann M, Nagel R, Otto GW, Geisler R, et al. (2007). Differential gene expression as a toxicant-sensitive endpoint in zebrafish embryos and larvae. Aquat Toxicol 81: 355-64.

Wielhouwer EM, Ali S, Al-Afandi A, Blom MT, Olde Riekerink MB, Poelma C, et al. (2011). Zebrafish embryo development in a microfluidic flow-through system. Lab Chip 11: 1815-24.

Wiley JL, O'connell MM, Tokarz ME, Wright MJ J (2007). Pharmacological effects of acute and repeated administration of Delta-9-tetrahydrocannabinol in adolescent and adult rats. J Pharmacol Exp Ther 320: 1097-1105.

Zajicek JP, Sanders HP, Wright DE, Vickery PJ, Ingram WM, Reilly SM, et al. (2005). Cannabinoids in multiple sclerosis (CAMS) study: safety and efficacy data for 12 months follow up. J Neurol Neurosurg Ps 76: 1664-9.

CHAPTER 6

Metabolic effects of cannabinoids in zebrafish (*Danio rerio*) embryo determined by ^{1}H NMR metabolomics.

Muhammad Tayyab Akhtar, Yahya Mushtaq, Michael K. Richardson, Robert Verpoorte and Young Hae Choi.

Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

Abstract

Development of efficient high throughput analysis methods is a crucial challenge to speed up the preliminary screening of potential psychostimulants. In this study, we used principal component analysis in combination with ^{1}HNMR to study the metabolic effects of cannabinoid receptor type 1 (CB_1) agonist Δ^9-THC and antagonist AM251in zebrafish embryos. The embryos were exposed at 24 hours post fertilization for 96 hours and analysed at 5dpf. A dose-dependent increase was found in the levels of choline, betaine, glycine, taurine, ATP and glucose upon exposure to Δ^9-THC. The levels of excitatory neurotransmitters glutamate and glutamine increased at lower doses of Δ^9-THC, whereas the higher, lethal dose resulted in abrupt decrease of glutamate levels. In contrast to Δ^9-THC, the antagonist AM251 caused a dose-dependent reduction in betaine, choline, taurine and also inhibited the accumulation of glutamate and glutamine. Interestingly, both compounds induce the production of the dopamine precursor's phenylalanine and tyrosine at higher doses. These findings suggest that the CB_1 receptor is involved in the regulation of metabolites, which are directly involved in neurotransmission in zebrafish embryos. Furthermore, our results show the importance of a ^{1}HNMR based metabolomics platform for the preliminary screening of psychostimulants and pharmaceutical drugs; and in providing a detailed overview of their general effects on the metabolome of a whole organism.

Introduction

Metabolomics is a comprehensive study of a collection of the metabolites present in a cell, a tissue, or an organism at a particular time. It is a very useful tool in research efforts aimed at understanding the metabolism and related physiological processes at a systems biology level (Weckwerth 2003). High-resolution proton nuclear magnetic resonance (H NMR) (1) is an ideal tool for

the metabolite profiling of biofluids, tissue extracts and intact tissues (Griffin 2004) as well as mass spectrometry. ^{1}HNMR spectroscopy has been used to investigate the biochemical composition of different tissues in an organism (Coen et al. 2003) and drug activity and toxicity assessment in blood serum, liver and testis of rodents (Ekman et al. 2006, Waters et al. 2006) as well as in body fluids such as serum, urine and feces (Le Gall et al. 2011, Mantle et al. 2011). In comparison to other analytical platforms such as mass spectrometry coupled with liquid or gas chromatography, the attractive features of NMR are: simple sample preparation; non-destructive sampling; short measurement time; ease of quantitation and high reproducibility (Kwon et al. 2011)

Many of the Δ^9-THC activities depend on the interaction with cannabinoid receptors (CB) (Lichtman and Martin 1996). Currently, two CB receptors have been identified, CB_1 and CB_2. CB_1 is expressed in brain and periphery, whereas CB_2 is known to be expressed in immune cells and referred as periphery CB_2 receptors (Onaivi et al. 2008). Since the discovery of CB receptors (Matsuda et al. 1990, Munro et al. 1993), a great deal of work has been done to find out their role in cannabinoids mediated activities (Alhamoruni et al. 2012, Fraga et al. 2011, Karmaus et al. 2012). Rodent models are used to study the behavioural and pharmacological effects of cannabinoids (Rubio et al. 1995, Wiley et al. 2007, Wise et al. 2011). Biphasic behavioral and physiological responses have been reported in rodents subjected to Δ^9-THC (Grisham and Ferraro 1972, Stiglick and Kalant 1982, Taylor and Fennessy 1977). These biphasic responses have also been reported in the level of brain monoamines and body temperature (Taylor and Fennessy 1977). Previously, an HPLC method was used to analyze the levels of excitatory and inhibitory amino acids in the brain tissue of rats treated with Δ^9-THC (Hikal et al. 1988). Here, we used NMR based metabolomics to study the effects of CB receptor type 1 (CB_1) agonist Δ^9-THC

and CB_1-antagonist AM251 on metabolic profile of 5 days old post fertilization (5 dpf) zebrafish embryos.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in accordance with national regulations, described in the *Wet op de dierproeven* (article 9) of Dutch Law administered by the Bureau of Animal Experiment Licensing. This national regulation serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 dpf).

Zebrafish handling

Male and female adult zebrafish (*Danio rerio)* of wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) which obtain stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). The fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm, W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08:00). Water and air were temperature controlled (25 ± 0.5 ^{o}C and 23 ^{o}C, respectively). All the zebrafish handling was in accordance with national regulations. The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., and Burlingame, CA, USA) and twice a week with frozen food (Dutch Select Food, Aquadistri BV, Leiden, The Netherlands).

Defined embryo buffer

Ten percentage (v/v) of Hank's balanced salt solution was used (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO, USA) at a concentration 0.98 g/L

in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma), and adjusted to pH 7.46. A similar medium has been used previously (Irons et al. 2010; Macphail et al. 2009).

Embryo care

Eggs were obtained by random pairwise mating of zebrafish. Three adult males and four females were placed together in small breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. The breeding tanks (L 26 cm; H 12.5 cm, W 20 cm) had mesh egg traps to prevent the eggs from being eaten. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml fresh embryo buffer. Eggs were washed four times to remove debris. Further unfertilized, unhealthy and dead embryos were screened under a dissecting microscope. At 3.5 hours post fertilization (hpf), embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28.5°C, in acclimatised room. All incubations of embryos were carried out in acclimatised room under a light cycle of 14 h light: 10 h dark.

Preliminary range-finding

To determine a suitable range of concentrations of testing compounds, we performed range-finding experiments. The concentrations were in a geometric series in which each was 50% greater than the next lowest value. We used 0.0, 12.5, 25.0, 50.0 and 100 mg/L of cannabinoids. A static replacement regime was used. Thus, there was no refreshment of buffer after the addition of compound. Each well contained 250 µL of either test compound or control (buffer only) or vehicle (0.01% DMSO in buffer). We used 32 embryos for each concentration and 32 embryos each cannabinoid as control, and 32 embryos for

each cannabinoid to control for the vehicle. The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates.

Refined geometric series and LC50 determination

After the range-finding experiments, a series of concentrations lying in the range between 0% and 100% mortality were selected for LC_{50} determination. The range for Δ^9-THC and AM251 was 0.3-9.6 mg/L and 0.5-8 mg/L, respectively. Each geometric series of concentrations for each compound was repeated three times (in total 36 embryos per concentration and 36 embryos for vehicle for each compound). The embryos for controls and treatment groups for each compound were plated in the same 96-well microtitre plates in each independent experiment. The LC_{50} (expressed in mg/L of buffer) was determined based on cumulative mortality at 120 hpf using Regression Probit analysis with SPSS Statistics v.17.0 (SPSS Inc., Chicago, USA).

Embryo treatment and collection

One hundred and twenty embryos per replicate were collected. After 24 hours the embryo were treated with 0.6, 1.2, 2.4 and 3.4 mg/L (LC_{50}) of Δ^9-THC; 0.6, 1.2, 2.4 and 4.8 mg/L (LC_{50}) of AM251. At 5dpf, embryos were collected in falcon tube. The collected embryos were immediately frozen in liquid nitrogen and stored at -80 $^{\circ}$C till further analysis.

Sample preparation and Extraction

A fairly simple sample preparation method already was used for plant metabolomics (Abdel-Farid et al. 2009, Ali Kashif et al. 2010, Jahangir et al. 2008, Kim et al. 2010a, Kim et al. 2010b). Embryos were freeze dried and transferred to a micro tubes (2 ml) to which 1ml of 50% CH_3OH-*d4* in D_2O (KH_2PO_4 buffer, pH 6.0) containing 0.01% TMSP (w/w) was added. The

mixture was vortexes for one min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for ten minutes. An aliquot (650 µL) was then transferred to a 5mm-NMR tube. NMR spectra were recorded at 25°C on a 500MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. CH_3OH-d 4 was used as the internal lock. each ^1H NMR spectrum consisted of 128 scans requiring with the parameters as used by our group (Kim et al. 2010b).

Data Pre Processing and Analysis

Spectral intensities of 1H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4–δ 10.0. The regions of δ 4.8–δ 4.9 and δ 3.30–δ 3.34 were excluded from the analysis because of the residual signal of the deuterated solvents. PCA was performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a Pareto scaling method. All of the spectra were referenced, base line, phase-corrected and visually inspected by superimposing and stacking the spectra (Fig. 1A) using MestReNova v.6.0.2 (Mestrelab research S.L., A Coruna, Spain).

Results

Principal component analysis

Principal component analysis (PCA) is a statistical tool applied to process a huge number of data sets by reducing the multidimensionality of the data without losing required information. This technique helps to assess the possible differences and similarities between the samples. In order to look into overview of metabolic changes firstly we applied PCA to binned ^1H NMR variables representing the key metabolites regulated differently in the Δ^9-THC treated and non-treated zebrafish embryos. The PCA revealed that four components were

enough to represent 84% of the variation in the data. The score plot shows that 40.6% of the separation is based on PC1 and 21.8% is based on PC2 scores (Fig 1B). Inspection of the loading plot reveals that the major source of variability among the samples is strongly correlated with the dose of Δ^9-THC (Fig 1C). The control (HBSS) and vehicle (HBSS with 0.01% DMSO) groups tend to cluster together along the positive side of PC1 (horizontal line), while the most affected group or best separated group from the control is the medium dose group (1.2mg/L). The high dose (2.4 and 3.4 mg/l) groups cluster together in between low and medium dose (Fig 1B).

PLS modeling with Orthogonal signal correction

The NMR data was subjected to Partial least square data analysis (PLS-DA). A 'Y' variable was included in the data representing different classes (Control, 0.01% DMSO treated, Low dose (0.6mg/L Δ^9-THC), medium dose (1.2mg/L Δ^9-THC), high dose (2.4mg/L Δ^9-THC) and LC50 (3.4mg/L Δ^9-THC), while each class was given a specific value. The objective of PLS-DA modeling is to relate two data matrices i.e X and Y to each other by a linear multivariate model (Wold et al. 2001). Each class was assigned a different value as a Y variable. After cross validation, the model showed three components, which were enough to describe 57% of the variation in data.

The PLS modeling was applied because less variation and discrepancy appeared in the score plot (Fig 1B), which might be because of un-correlated variables (metabolites), noise or systematic variations in NMR data effecting the separation in the score plot. To remove these uncorrelated data we developed a model with the use of a data filter called orthogonal signal correction (OSC). This filter had already been applied successfully in several studies (Gavaghan et al. 2002, Hauksson et al. 2001). The filter calculates a vector which is

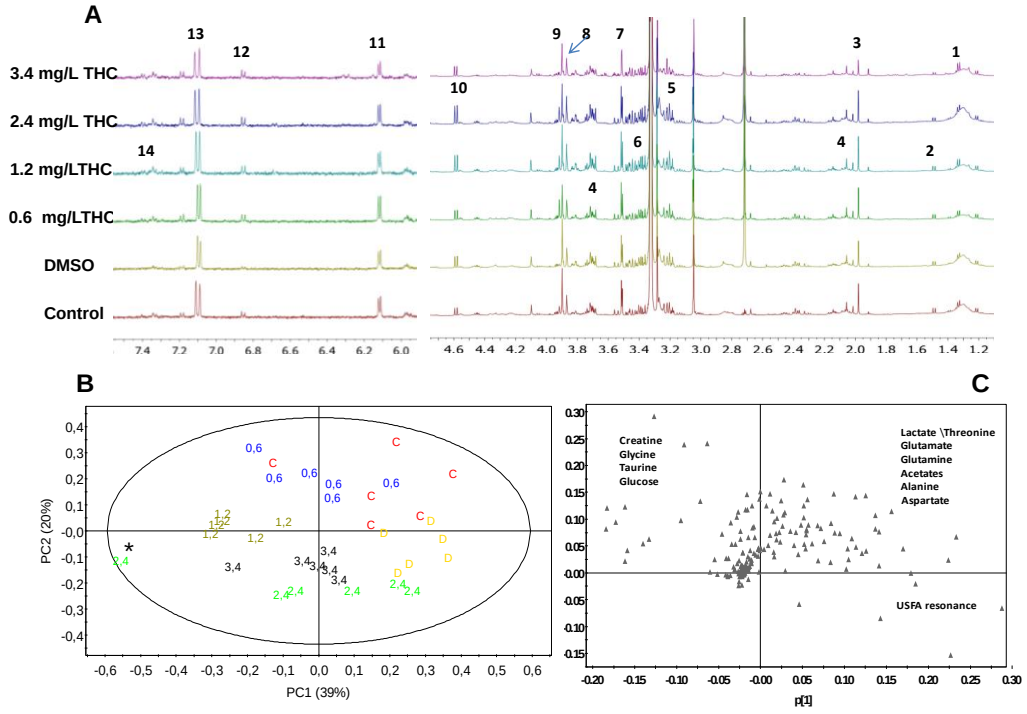


Figure 1. **A**; Comparison of ^{1}H NMR spectra of zebrafish embryos, showing the region of interest after treating the embryos with Control, 0.01% DMSO and 0.6, 1.2, 2.4 and 3.4 mg/L of Δ^{9}-THC. (1) Lactate (2) Alanine, (3) Acetate, (4) Glutamate (5) Choline, (6) Taurine, (7) Glycine, (8) Betaine, (9) Creatine, (10) Glucose, (11) ATP, (12) Tyrsoine, (13) Histidine, (14) Phenyalanine. **B**; Scatter plot from Principal component analysis. (C) Control, (D) 0.01% DMSO, (0.6) 0.6 mg/L Δ^{9}-THC , (1.2) 1.2 mg/L Δ^{9}-THC, (2.4) 2.4 mg/L Δ^{9}-THC, (3.4) 3.4 Δ^{9}-THC; * represent the outlier. **C;** Representative loading plot of PCA analysis.

orthogonal to Y and then by multiplying this vector to the loadings (represent the orientation of the model in the space) subtract it from X-data. This process removes the un-correlated data; the residual matrix left is the representative matrix of the variables responsible for the variation in the model. The filter was used before applying any model. After filtering, the data was subjected to partial least square analysis. The improvement in the model can be seen in Fig 2C where comparison of the cross validation of two components is shown. After

applying the filter three components of the model were able to describe 95% of the variation. The model was validated by using the permutation test with 100 permutations as shown in Fig 2C. Results indicate a good validation of the model as the intercept value of both R2Y and Q2Y are within the limits as described by Eriksson (2001). The scatter plot from this data shows the grouping of control, DMSO and low dose (0.6mg/L) treated groups on the negative side of PLS-DA1, while the moderate dose (1.2 mg/L) clustered on positive side of component 1 and negative side of component 2 (Fig 2A). The highest dose (3.4mg/L) group, on the other hand, belongs to the positive side of both component 1 and component 2.

S-Plot

The corresponding loading plot or S-plot shows the variables affecting the separation in the scatter plot (Fig 2B). The variables (^{1}H NMR-chemical shifts) related to certain key metabolites were identified and are shown in Chapter 7, Table 1. The identification of metabolites confirm that the levels of amino acids such as glutamate, glutamine, isoleucine, alanine, threonine, aspartate, taurine, phenylalanine, choline, creatine and glycine are affected in embryo populations treated with Δ^9-THC. The comparison of the S-plot with the Scatter plot reveals that the lower levels of alanine, threonine, aspartate, glutamate and glutamine are associated with the embryos treated with highest dose (3.4 mg/L) of Δ^9-THC. The levels of phenylalanine, taurine and choline were increased in the samples treated with higher doses of Δ^9-THC compared to the control group. Visual inspection of ^{1}H NMR spectra reveals that different doses of Δ^9-THC resulted in differential changes in the levels of metabolites in the embryo population (Fig 1A). The levels of glucose, glutamate, acetate and adenosine triphosphate (ATP) increased with the increasing dose of Δ^9-THC (0.6 to 2.4

mg/L of Δ^9-THC) and decreased at the highest dose of Δ^9-THC (3.4 mg/L). A dose dependent increase was observed in the level of choline, glycine and

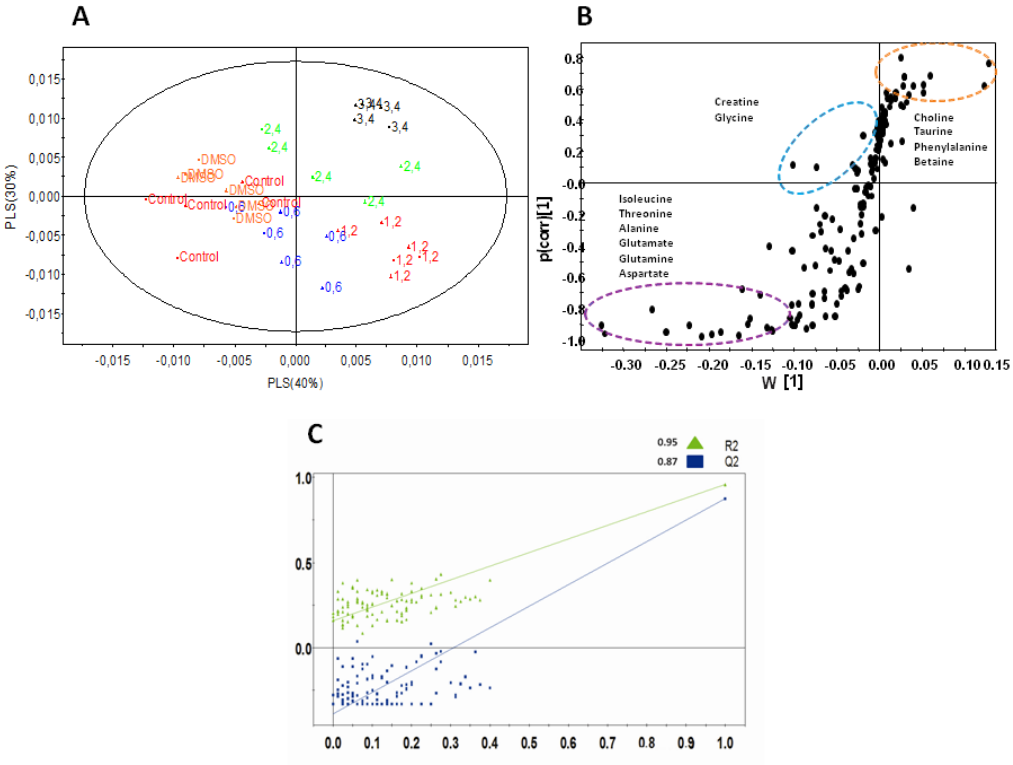


Figure 2. A; Scatter plot from Partial least square analysis with orthogonal signal correction filter. Figure shows the embryos treated with Control; 0.01% DMSO; 0.6, 1.2, 2.4 and 3.4 mg/L of Δ^9-THC. * represent the outlier. **B**; S-Plot shows the markers (variables) responsible for the separation. Upper right corner correspond to the high doses of Δ^9-THC while lower left corner correspond with the lower doses and control group of Δ^9-THC. **C;** Cross validation of two components. **C;** validation of PLS-DA model with permutation test (100 permutations).

betaine. Phenylalanine and tyrosine levels were increased at higher doses (2.4 and 3.4 mg/L).

In additional experiments, we exposed embryos to the CB_1 receptor antagonist AM251 (0.6, 1.2, 2.4 and 4.8 mg/L). The 24 hpf embryos were exposed for 96 h to the test compounds. The PCA score plot shows a clear separation of the groups treated with AM251 from the control group (Fig 3A). The treated groups were clustered to the negative side of PC1, whereas control samples had a positive PC1 score. This separation was based on 33% of PC1. The population exposed to higher (2.4 and 4.8 mg/L) and lower (0.6 and 1.2 mg/L) doses of AM251 were separated by PC2 (27%), having negative and positive PC2 scores, respectively (Fig 3A). The corresponding loading column plot shows the differentiating metabolites responsible for the separation between treated and control groups (Fig 3B). The ^{1}H NMR shows the signals of the discriminating metabolites identified (Fig 4).

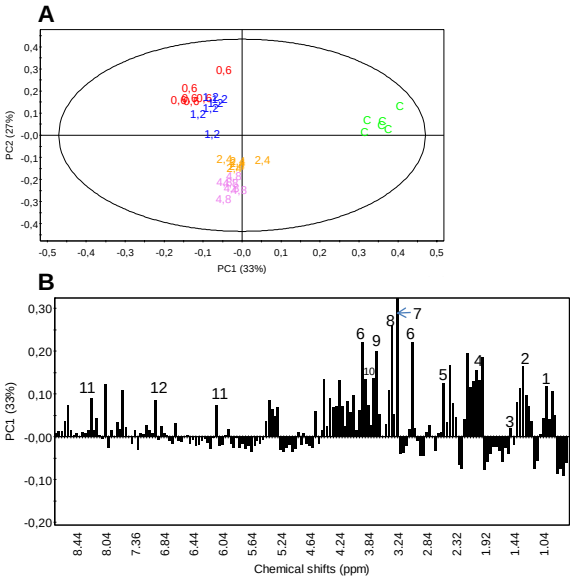


Figure 3. A, Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryos treated with AM 251, 0.6= 0.6 mg/L AM251; 1.2= 1.2 mg/L AM251; 2.4= 2.4 mg/L AM251; 4.8= 4.8 mg/L AM251 and C= Control (0.01% Dmso); **B**, Loading column plot of PC1. 1, leucine; 2, lactate;

3,alanine; 4, acetate; 5 glutamine; 6, creatine; 7, choline;
8, glycine; 9, glutamate; 10, betaine, 11; ATP; 12, histidine.

In the corresponding loading column plot, most of the signals are found at the PC1 positive side, corresponding with lower levels of the related metabolites in treated groups. From the ^{1}H NMR signals at positive side in column loading plot belongs to lactate, acetate, glutamate, glutamine, choline, glycine, betaine and ATP, which therefore all present at lower levels in the treated embryos. The ^{1}H NMR spectra (Fig 4) and loading column plot of PC2 (not shown) show increasing levels of phenylalanine and tyrosine in the embryo populations treated with higher doses (2.4 and 4.8 mg/L) of AM251. Lower doses (0.6 and 1.2 mg/L) do not show any significant rise in phenylalanine and tyrosine levels compared to the control group.

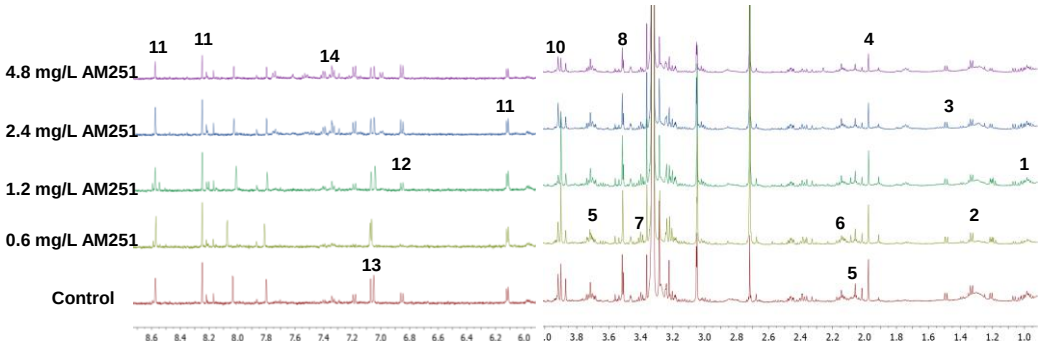


Figure 4. The ^{1}H NMR spectra of 5 days old zebrafish embryos raised in 0.01% DMSO (Control) and exposed to 0.6, 1.2, 2.4 and 4.8 mg/L of AM251. 1, leucine; 2, lactate; 3, alanine; 4, acetate; 5, glutamate; 6, glutamine; 7, taurine; 8, glycine; 9, betaine; 10, creatine; 11, ATP; 12, tyrosine; 13, histidine; 14, phenylalanine.

Discussion

There is an extensive dataset available on the stress and drug-driven metabolome of mammals (Diederich et al. 1997, Milakofsky et al. 1985). These data show the modulation of amino acid levels in the blood and tissues of

rodents and humans mediated by exogenous stimuli. Ethanol is reported to decrease the overall amino acid level in the plasma of rats (Hagman and Eriksson 1990, 1991). L-DOPA is known to significantly inhibit the production of glutamic acid and glutamine in the plasma, heart and brain stem of rats (Diederich et al. 1997). Aspartic acid is decreased in brain stem cells and plasma of rats subjected to L-DOPA and ethanol, respectively (Diederich et al. 1997, Milakofsky et al. 1989). Here, for first time, we report the effects of cannabinoids on the metabolome of zebrafish embryos exposed to Δ^9-THC and AM251.

Glutamate, choline and glycine are neurotransmitters involved in synaptic excitation or inhibition by acting on their respective receptors (Haga et al. 2012, Hollmann and Heinemann 1994, Liu J. et al. 2010, Lopez-Corcuera et al. 2001). Glutamate mediates synaptic excitation and inhibition by acting on N-methyl-D-aspartate (NMDA)-sensitive ionotropic glutamate receptors (Hollmann and Heinemann 1994, Liu J. et al. 2010). A number of Δ^9-THC regulated activities are mediated by cannabinoid receptors of the CB_1 class distributed throughout the central nervous system and are also present on the presynaptic terminals of glutamatergic synapses (Wiley et al. 2005).

In rodents, several studies have been focused on the effects of cannabinoids on the regulation of glutamate levels and subsequent glutamatergic neurotransmission (Antonelli et al. 2004, Castaldo et al. 2007, Ferraro et al. 2001). In one study, Δ^9-THC was shown to inhibit glutamatergic neurotransmission (Shen and Thayer 1999, Galanopoulos et al. 2011). In a study of Δ^9-THC effect on locomotor activity in rats, a higher level of glutamate was found in the rat brain tissues with increased motor activity at lower dose, while higher doses showed habituation (Galanopoulos et al. 2011).

Previously, we determined the behavioral effects of Δ^9-THC on the locomotor activity of 5 day old zebrafish embryos (Thesis chapter 5). In a chronic exposure (96 hours) to Δ^9-THC and AM251, we found a significant locomotor stimulation of zebrafish embryos at the lower dose (1.2 mg/L) while a higher dose (2.4 mg/L) of Δ^9-THC caused habituation. Conversely, AM251 caused a significant suppression of locomotor activity. In the current study, Δ^9-THC induced a dose dependent increase in the levels of glutamate at doses below LC_{50} (1.2 mg/L and 2.4 mg/L) (Fig 1A). Exposure to 1.2 mg/L of Δ^9-THC results in locomotor hyperactivity (Thesis Chapter 5). In contrast to Δ^9-THC, AM251 produced a dose-dependent decrease in the quantity of glutamate (Fig 4) associated with locomotor suppression (Thesis chapter 5).

Previous data from rodent studies, together with present findings and our previous behavioural study, indicate an important role of CB_1 receptor mediated glutamate regulation in the behavior of zebrafish embryos. The discovery of CB receptors and glutamate receptors and transporters (Gesemann et al. 2010, Rico et al. 2010) in zebrafish embryos, and the opposite effect of CB_1 agonist and antagonist in the current study, together suggest a strong interaction of CB_1 receptors and glutamatergic neurotransmission in zebrafish (Tomasini et al. 2002).

It has been suggested that an overall increase of glutamate levels induced in brain tissues by Δ^9-THC might reduce synaptic glutamate levels and consequently affect glutamatergic neurotransmission (Galanopoulos et al. 2011). We have found an overall increase of glutamate levels in whole embryos treated with Δ^9-THC. Therefore, in order to investigate this question further, a detailed study of glutamate levels in zebrafish brain tissue could provide an insight into Δ^9-THC and glutamate interaction in the zebrafish brain.

The effects identified here on choline levels are significant because choline is the precursor of the neurotransmitter acetylcholine (ACh) which mediates cholinergic neurotransmission. Choline is metabolized into betaine which takes part in the biosynthesis of glycine (Friesen et al. 2007). Glycine is a mediator of glycinergic neurotransmission. ACh and glycine receptors have been reported in zebrafish embryos (Liu and Westerfield 1992, Rigo and Legendre 2006). In rodents, activation of CB receptors modulates cholinergic, glycinergic and noradrenergic neurotransmission (Szabo and Schlicker 2005). Δ^9-THC is known to interact with ACh and glycine receptors (Xiong et al. 2011).

Higher levels of choline and ACh have been found in the five brain regions of mice exposed to Δ^9-THC (Tripathi et al. 1987). However, there are also contradictory reports on the Δ^9-THC modulated effects on ACh release in *in vivo* studies (Acquas et al. 2001, Gessa et al. 1998, Pisanu et al. 2006). Subsequently, these conflicting findings were attributed to different doses of Δ^9-THC used in the different studies (Solinas et al. 2007). Tzavara et al. (2003) reported a biphasic effect of CB_1 agonist on the modulation of Ach release and proposed that higher doses of CB_1 agonists act as an ACh depressant and lower doses act as a stimulator of ACh neurotransmission. Δ^9-THC was also reported to activate glycine receptors in rodents, but there are no studies describing the effects of cannabinoids on the regulation of glycine. The visual analyses of ^{1}H NMR spectra reveal a dose dependent increase of choline, glycine and betaine in Δ^9-THC treated embryos (Fig 1A). On the contrary, AM251 shows a dose dependent inhibition of choline and betaine, while a biphasic effect was found in case of glycine (Fig 4). Although increase in choline at the LC_{50} value of Δ^9-THC (3.4 mg/L) is contradictory to previous findings in rodents, the reciprocal effect of the antagonist suggests that CB_1 might also play some role in the regulation of choline and glycine levels in zebrafish embryos.

Another metabolite whose levels were found to vary significantly between treatments in our study was phenylalanine. This is an essential amino acid and a precursor of tyrosine. Tyrosine hydroxylase (TH) converts tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) a precursor of the neurotransmitter dopamine. Visual inspection of ^{1}H NMR spectra showed a significant increase of phenylalanine and tyrosine levels in the embryos exposed to higher doses of Δ^{9}-THC (2.4 and 3.4 mg/L) and AM251 (2.4 and 4.8 mg/L) (Fig 1A, Fig 4).

A number of attempts have been made to determine the functional interaction between cannabinoids and dopamine neurotransmission. The presence of CB_1 receptors in TH containing cells is evidence of some connection between cannabinoids and catecholamine synthesis (Bosier et al. 2007). Many studies have shown that Δ^{9}-THC elevates dopamine levels in the brain, and that this effect can be blocked by a CB_1 receptor antagonist (Le Foll and Goldberg 2005). Δ^{9}-THC was reported to increase the TH expression in a cultured fetal mesencephalic cell line; by contrast, the CB_1 receptor antagonist SR141716A had no such effect on TH expression. It did, however, attenuate the effect of Δ^{9}-THC on TH expression (Hernandez et al. 2000, Hernandez et al. 1997). The strong correlation found in the present study between the ^{1}H NMR signals of tyrosine and phenyalanine suggests a co-regulation of both compounds after exposure to Δ^{9}-THC and AM251.

An important question is the specificity of the metabolomic patterns induced by Δ^{9}-THC and AM251 in this study. That is, whether similar might be due to any compound exposure. This seems unlikely because we have recently examined the metabolic effect of solvents (DMSO and ethanol) on the 5 days old zebrafish embryos and found that they induce a entirely different metabolic profile compared to that found here for cannabinoids (Thesis chapter 7). The previous and current metabolic data suggest that the accumulation of the

dopamine precursor (tyrosine) in cannabinoid administered embryos is not a general effect of drugs or solvent toxicity rather it is a specific effect of cannabinoid exposure on tyrosine regulation.

Interestingly, both the CB_1 receptor agonist and antagonist used here increased the levels of the dopamine precursor phenylalanine; this suggests that tyrosine might be regulated in a CB_1 receptor-independent manner in the embryos. Lam et al. 2006 found that CB_1-receptors and TH-expressing cells were co-localized in the caudal zone of the hypothalamus of zebrafish embryos. Together, these findings strengthen the possibility of cannabinoid receptor involvement in the regulation of the dopaminergic system in zebrafish embryos.

Based on the current data, it is not possible to draw conclusions on the impact of cannabinoids on TH activity, or on the exact role of CB_1 receptors in the regulation of tyrosine synthesis or of the dopaminergic system. Therefore, further molecular and functional studies are required to confirm the effects of cannabinoids on TH regulation and subsequently dopaminergic neurotranmission in zebrafish.

In addition to neurotransmitters, several other metabolites were also differentially regulated by Δ^9-THC and AM251 compared to controls. For example, the higher level of ATP and glucose might indicate a higher energy demand of the hyperactive embryos exposed to Δ^9-THC. A dose dependent decrease of acetate and a biphasic effect on creatine levels was induced by AM251. There is no straightforward explanation of the modulation of these metabolites, but it is at least possible that they might play some role in cannabinoid induced effects in zebrafish embryo.

Our study shows that zebrafish larvae are a useful model for performing metabolomic profiling in a whole animal exposed to pharmacological agents. This approach may help in identifying the changes a drug could elicit in

different biosynthetic pathways. The result of our study shows that cannabinoids are involved in the regulation of metabolites which are directly involved in neurotransmission in zebrafish embryos. Further investigations are needed to characterize the interactions of Δ^9-THC with different neurotransmitter pathways in different regions of the zebrafish brain. Recently, 1H NMR was used to study the metabolic profile of adult zebrafish liver (Ong et al. 2009). Meatbolomic studies need to be complemented by the use of techniques such as in situ hybridization, immunochemistry and Q-PCR in order to gain a complete picture of gene expression, protein distribution and metabolite profiles. Questions that remain to be answered are the distribution of CB receptors in the adult zebrafish brain, the differential roles of cannabinoid receptor subtypes, and the function of the zebrafish endogenous cannabinoid system.

Reference

Abdel-Farid IB, Jahangir M, van den Hondel CAMJJ, Kim HK, Choi YH, Verpoorte R. 2009. Fungal infection-induced metabolites in Brassica rapa. Plant Science 176: 608-615.

Acquas E, Pisanu A, Marrocu P, Goldberg SR, Di Chiara G. 2001. Delta-9-tetrahydrocannabinol enhances cortical and hippocampal acetylcholine release in vivo: a microdialysis study. Eur J Pharmacol 419: 155-161.

Alhamoruni A, Wright KL, Larvin M, O'Sullivan SE. 2012. Cannabinoids mediate opposing effects on inflammation-induced intestinal permeability. Br J Pharmacol 165: 2598-2610.

Ali K, Maltese F, Fortes AM, Pais MS, Choi YH, Verpoorte R. 2010. Monitoring biochemical changes during grape berry development in Portuguese cultivars by NMR spectroscopy. Food Chem 124: 1760-1769.

Ali S, Champagne DL, Spaink HP, Richardson MK. 2011. Zebrafish embryos and larvae: a new generation of disease models and drug screens. Birth Defects Res C Embryo Today 93: 115-133.

Antonelli T, Tanganelli S, Tomasini MC, Finetti S, Trabace L, Steardo L, Sabino V, Carratu MR, Cuomo V, Ferraro L. 2004. Long-term effects on cortical glutamate release induced by prenatal exposure to the cannabinoid receptor agonist (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-

naphthalenylmethanone: an in vivo microdialysis study in the awake rat. Neuroscience 124: 367-375.

Bosier B, Tilleux S, Najimi M, Lambert DM, Hermans E. 2007. Agonist selective modulation of tyrosine hydroxylase expression by cannabinoid ligands in a murine neuroblastoma cell line. J Neurochem 102: 1996-2007.

Castaldo P, Magi S, Gaetani S, Cassano T, Ferraro L, Antonelli T, Amoroso S, Cuomo V. 2007. Prenatal exposure to the cannabinoid receptor agonist WIN 55,212-2 increases glutamate uptake through overexpression of GLT1 and EAAC1 glutamate transporter subtypes in rat frontal cerebral cortex. Neuropharmacology 53: 369-378.

Coen M, Lenz EM, Nicholson JK, Wilson ID, Pognan F, Lindon JC. 2003. An integrated metabonomic investigation of acetaminophen toxicity in the mouse using NMR spectroscopy. Chem Res Toxicol 16: 295-303.

Costa B. 2007. On the pharmacological properties of Delta9-tetrahydrocannabinol (THC). Chem Biodivers 4: 1664-1677.

Diederich C, Milakofsky L, Hare TA, Hofford JM, Dadmarz M, Vogel WH. 1997. Effects of L-DOPA/carbidopa administration on the levels of L-DOPA, other amino acids and related compounds in the plasma, brain and heart of the rat. Pharmacology 55: 109-116.

Ekman D, Keun H, Eads C, Furnish C, Murrell R, Rockett J, Dix D. 2006. Metabolomic evaluation of rat liver and testis to characterize the toxicity of triazole fungicides. Metabolomics 2: 63-73.

Eriksson.L. JE, Kettaneh-Wold.s. 2001. Multivariate and Megavariate Data Analysis Principles and Application. Umea, Sweden: Umetrics.

Ferraro L, Tomasini MC, Gessa GL, Bebe BW, Tanganelli S, Antonelli T. 2001. The cannabinoid receptor agonist WIN 55,212-2 regulates glutamate transmission in rat cerebral cortex: an in vivo and in vitro study. Cereb Cortex 11: 728-733.

Fraga D, Raborn ES, Ferreira GA, Cabral GA. 2011. Cannabinoids Inhibit Migration of Microglial-like Cells to the HIV Protein Tat. J Neuroimmune Pharmacol 6: 566-77.

Freedland CS, Whitlow CT, Miller MD, Porrino LJ. 2002. Dose-dependent effects of Delta9-tetrahydrocannabinol on rates of local cerebral glucose utilization in rat. Synapse 45: 134-142.

Friesen RW, Novak EM, Hasman D, Innis SM. 2007. Relationship of dimethylglycine, choline, and betaine with oxoproline in plasma of pregnant women and their newborn infants. J Nutr 137: 2641-2646.

Galanopoulos A, Polissidis A, Papadopoulou-Daifoti Z, Nomikos GG, Antoniou K. 2011. Delta(9)-THC and WIN55,212-2 affect brain tissue levels of excitatory amino acids in a phenotype-, compound-, dose-, and region-specific manner. Behav Brain Res 224: 65-72.

Gavaghan CL, Wilson ID, Nicholson JK. 2002. Physiological variation in metabolic phenotyping and functional genomic studies: use of orthogonal signal correction and PLS-DA. FEBS Letters 530: 191-196.

Gesemann M, Maurer CM, Neuhauss SC. 2010. Excitatory amino acid transporters in the zebrafish: Letter to "Expression and functional analysis of Na(+)-dependent glutamate transporters from zebrafish brain" from Rico et al. Brain Res Bull 83: 202-206.

Gessa GL, Casu MA, Carta G, Mascia MS. 1998. Cannabinoids decrease acetylcholine release in the medial-prefrontal cortex and hippocampus, reversal by SR 141716A. Eur J Pharmacol 355: 119-124.

Griffin JL. 2004. Metabolic profiles to define the genome: can we hear the phenotypes? Philos Trans R Soc Lond B Biol Sci 359: 857-871.

Grisham MG, Ferraro DP. 1972. Biphasic effects of 9-tetrahydrocannabinol on variable interval schedule performance in rats. Psychopharmacologia 27: 163-169.

Haga et al. 2012. Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. Nature 482: 547-551.

Hagman M, Eriksson T. 1990. Dose-dependent decrease in rat plasma amino acids after acute administration of ethanol. J Pharm Pharmacol 42: 869-870.

Hagman M, Eriksson T. 1991. Development of tolerance to the plasma amino acid-decreasing effect of ethanol in the rat. J Pharm Pharmacol 43: 625-629.

Hauksson JB, Edlund U, Trygg J. 2001. NMR processing techniques based on multivariate data analysis and orthogonal signal correction. 13C CP/MAS NMR spectroscopic characterization of softwood kraft pulp. Magn Reson Chem 39: 267-275.

Hernandez M, Berrendero F, Suarez I, Garcia-Gil L, Cebeira M, Mackie K, Ramos JA, Fernandez-Ruiz J. 2000. Cannabinoid CB(1) receptors colocalize with tyrosine hydroxylase in cultured fetal mesencephalic neurons and their activation increases the levels of this enzyme. Brain Res 857: 56-65.

Hernandez ML, Garcia-Gil L, Berrendero F, Ramos JA, Fernandez-Ruiz JJ. 1997. delta 9-Tetrahydrocannabinol increases activity of tyrosine hydroxylase in cultured fetal mesencephalic neurons. J Mol Neurosci 8: 83-91.

Hikal AH, Lipe GW, Slikker WJ, Scallet AC, Ali SF, Newport GD. 1988. Determination of amino acids in different regions of the rat brain. Application to the acute effects of tetrahydrocannabinol (THC) and trimethyltin (TMT). Life Sci 42: 2029-2035.

Hollmann M, Heinemann S. 1994. Cloned glutamate receptors. Annu Rev Neurosci 17: 31-108.

Ingham PW. 2009. The power of the zebrafish for disease analysis. Hum Mol Genet 18: 107-112.

Jahangir M, Kim HK, Choi YH, Verpoorte R. 2008. Metabolomic response of Brassica rapa submitted to pre-harvest bacterial contamination. Food Chem 107: 362-368.

Karmaus PW, Chen W, Kaplan BL, Kaminski NE. 2011. Delta(9)-Tetrahydrocannabinol Suppresses Cytotoxic T Lymphocyte Function Independent of CB(1) and CB (2), Disrupting Early Activation Events. J Neuroimmune Pharmacol.

Kim HK, Choi YH, Verpoorte R. 2010a. NMR-based metabolomic analysis of plants. Nat Protocols 5: 536-549.

Kim HK, Saifullah, Khan S, Wilson EG, Kricun SDP, Meissner A, Goraler S, Deelder AM, Choi YH, Verpoorte R. 2010b. Metabolic classification of South American Ilex species by NMR-based metabolomics. Phytochemistry 71: 773-784.

Lam CS, Rastegar S, Strähle U. 2006. Distribution of cannabinoid receptor 1 in the CNS of zebrafish Neuroscience 138: 83-95.

Le Foll B, Goldberg SR. 2005. Cannabinoid CB1 receptor antagonists as promising new medications for drug dependence. J Pharmacol Exp Ther 312: 875-883.

Le Gall G, Noor SO, Ridgway K, Scovell L, Jamieson C, Johnson IT, Colquhoun IJ, Kemsley EK, Narbad A. 2011. Metabolomics of fecal extracts detects altered metabolic activity of gut microbiota in ulcerative colitis and irritable bowel syndrome. J Proteome Res 10: 4208-4218.

Lichtman AH, Martin BR. 1996. Delta 9-tetrahydrocannabinol impairs spatial memory through a cannabinoid receptor mechanism. Psychopharmacology (Berl) 126: 125-131.

Liu DW, Westerfield M. 1992. Clustering of muscle acetylcholine receptors requires motoneurons in live embryos, but not in cell culture. J Neurosci 12: 1859-1866.

Liu J, Wu DC, Wang YT. 2010. Allosteric potentiation of glycine receptor chloride currents by glutamate. Nat Neurosci 13: 1225-1232.

Lopez-Corcuera B, Geerlings A, Aragon C. 2001. Glycine neurotransmitter transporters: an update. Mol Membr Biol 18: 13-20.

Mandrekar N, Thakur NL. 2009. Significance of the zebrafish model in the discovery of bioactive molecules from nature. Biotechnol Lett 31: 171-179.

Mantle PG, Nicholls AW, Shockcor JP. 2011. H NMR spectroscopy-based metabolomic assessment of uremic toxicity, with toxicological outcomes, in male rats following an acute, mid-life insult from ochratoxin a. Toxins (Basel) 3: 504-519.

Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. 1990. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature 346: 561-564.

Migliarini B, Carnevali O. 2009. A novel role for the endocannabinoid system during zebrafish development. Mol Cell Endocrinol 299: 172-177.

Milakofsky L, Miller JM, Vogel WH. 1989. Effect of ethanol on plasma amino acids and related compounds of stressed male rats. Pharmacol Biochem Behav 32: 1071-1074.

Milakofsky L, Hare TA, Miller JM, Vogel WH. 1985. Rat plasma levels of amino acids and related compounds during stress. Life Sci 36: 753-761.

Munro S, Thomas KL, Abu-Shaar M. 1993. Molecular characterization of a peripheral receptor for cannabinoids. Nature 365: 61-65.

Onaivi ES, et al. 2008. Brain neuronal CB2 cannabinoid receptors in drug abuse and depression: from mice to human subjects. PLoS One 3: e1640.

Ong ES, Chor CF, Zou L, Ong CN. 2009. A multi-analytical approach for metabolomic profiling of zebrafish (Danio rerio) livers. Mol Biosyst 5: 288-298.

Pamplona FA, Takahashi RN. 2012. Psychopharmacology of the endocannabinoids: far beyond anandamide. J Psychopharmacol 26: 7-22.

Pisanu A, Acquas E, Fenu S, Di Chiara G. 2006. Modulation of Delta(9)-THC-induced increase of cortical and hippocampal acetylcholine release by micro opioid and D(1) dopamine receptors. Neuropharmacology 50: 661-670.

Rico E, de Oliveira DL, Rosemberg DB, Mussulini BH, Bonan CD, Dias RD, Wofchuk S, Souza DO, Bogo MR. 2010. Expression and functional analysis of Na+-dependent glutamate transporters from zebrafish brain. Brain Research Bulletin 81: 517-523.

Rigo JM, Legendre P. 2006. Frequency-dependent modulation of glycine receptor activation recorded from the zebrafish larvae hindbrain. Neuroscience 140: 389-402.

Rodriguez-Martin I, Herrero-Turrion MJ, Marron Fdez de Velasco E, Gonzalez-Sarmiento R, Rodriguez RE. 2007. Characterization of two duplicate zebrafish Cb2-like cannabinoid receptors. Gene 389: 36-44.

Rubio P, Rodriguez de Fonseca F, Munoz RM, Ariznavarreta C, Martin-Calderdn JL, Navarro M. 1995. Long-term behavioral effects of perinatal exposure to delta 9-tetrahydrocannabinol in rats: possible role of pituitary-adrenal axis. Life Sci 56: 2169-2176.

Shen M, Thayer SA. 1999. Delta9-tetrahydrocannabinol acts as a partial agonist to modulate glutamatergic synaptic transmission between rat hippocampal neurons in culture. Mol Pharmacol 55: 8-13.

Solinas M, Scherma M, Tanda G, Wertheim CE, Fratta W, Goldberg SR. 2007. Nicotinic facilitation of delta9-tetrahydrocannabinol discrimination involves endogenous anandamide. J Pharmacol Exp Ther 321: 1127-1134.

Stiglick A, Kalant H. 1982. Residual effects of prolonged cannabis administration on exploration and DRL performance in rats. Psychopharmacology (Berl) 77: 124-128.

Szabo B, Schlicker E. 2005. Effects of cannabinoids on neurotransmission. Handb Exp Pharmacol: 327-365.

Taylor DA, Fennessy MR. 1977. Biphasic nature of the effects of delta9-tetrahydrocannabinol on body temperature and brain amines of the rat. Eur J Pharmacol 46: 93-99.

Tomasini MC, Ferraro L, Bebe BW, Tanganelli S, Cassano T, Cuomo V, Antonelli T. 2002. Delta(9)-tetrahydrocannabinol increases endogenous extracellular glutamate levels in primary cultures of rat cerebral cortex neurons: involvement of CB(1) receptors. J Neurosci Res 68: 449-453.

Tripathi HL, Vocci FJ, Brase DA, Dewey WL. 1987. Effects of cannabinoids on levels of acetylcholine and choline and on turnover rate of acetylcholine in various regions of the mouse brain. Alcohol Drug Res 7: 525-532.

Tzavara ET, Wade M, Nomikos GG. 2003. Biphasic effects of cannabinoids on acetylcholine release in the hippocampus: site and mechanism of action. J Neurosci 23: 9374-9384.

Waters NJ, Waterfield CJ, Farrant RD, Holmes E, Nicholson JK. 2006. Integrated metabonomic analysis of bromobenzene-induced hepatotoxicity: novel induction of 5-oxoprolinosis. J Proteome Res 5: 1448-1459.

Weckwerth W. 2003. Metabolomics in systems biology. Annu Rev Plant Biol 54: 669-689.

Wiley JL, M. OCM, Tokarz ME, Wright MJJ. 2007. Pharmacological effects of acute and repeated administration of Delta(9)-tetrahydrocannabinol in adolescent and adult rats. J Pharmacol Exp Ther 320: 1097-1105.

Wiley JL, Burston JJ, Leggett DC, Alekseeva OO, Razdan RK, Mahadevan A, Martin BR. 2005. CB1 cannabinoid receptor-mediated modulation of food intake in mice. Br J Pharmacol 145: 293-300.

Wise LE, Varvel SA, Selley DE, Wiebelhaus JM, Long KA, Middleton LS, Sim-Selley LJ, Lichtman AH. 2011. delta(9)-Tetrahydrocannabinol-dependent mice undergoing withdrawal display impaired spatial memory. Psychopharmacology (Berl) 4: 485-94.

Wold S, Sjosstrom M, Eriksson L. 2001. PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems 58: 109-130.

Xiong W, Cheng K, Cui T, Godlewski G, Rice KC, Xu Y, Zhang L. 2011. Cannabinoid potentiation of glycine receptors contributes to cannabis-induced analgesia. Nat Chem Biol 7: 296-303.

Yamaori S, Okamoto Y, Yamamoto I, Watanabe K. 2011. Cannabidiol, a Major Phytocannabinoid, as a Potent Atypical Inhibitor for Cytochrome P450 2D6. Drug Metab Dispos 39: 2049-56.

Kwon B, Kim S, Lee DK, Park YJ, Kim MD, Lee JS. 2011. 1H NMR spectroscopic identification of a glue sniffing biomarker. Forensic Sci Int 209: 120-125.

CHAPTER 7

Metabolic effects of carrier solvents and culture buffers in zebrafish embryos determined by ^{1}H NMR metabolomics.

Muhammad Tayyab Akhtar, Yahya Mushtaq, Michael K. Richardson, Robert Verpoorte, and Young Hae Choi.

Natural Products Laboratory, Institute of Biology, Leiden University, The Netherlands.

Abstract

Any bioactivity or toxicity test needs to dissolve the target compounds in an appropriate solvent. This is also the case in zebrafish embryo assays, an increasingly popular assay system in biomedical research. Water-soluble compounds can simply be dissolved in the aqueous medium that the zebrafish embryo grows in. This may be egg water (EW) or Hanks' balanced salt solution (HBSS). Non-polar compounds require in addition an organic solvent. It is important to know whether the solvent itself – including the type of buffer - has an effect on the organisms or assay. Lower concentrations of some organic solvents have been shown to produce a pronounced change in the metabolic profile of an organism. For these reasons, we evaluated the effect of two commonly-used organic carrier solvents in drug screening: Dimethyl sulfoxide (DMSO) and Ethanol; and two commonly used aqueous buffers. The effects of three concentrations (0.01, 0.1 and 1%) of these solvents were tested on the metabolic profile of 5 day old zebrafish embryos. The metabolic difference was determined by using the ^{1}H NMR based metabolomics. The zebrafish embryos were exposed at 24 hours post fertilization for 96 h, and then collected at 5 days post fertilization and analyzed by ^{1}H NMR. DMSO (0.1 and 1% but not 0.01%) exposure significantly decreased the level of adenosine triphosphate (ATP), betaine, alanine, histidine, lactate, acetate and creatine. By contrast, ethanol exposure did not alter the embryo metabolome at any concentration tested. We next examined the effect of HBSS and EW on the zebrafish embryo and found that the two media result in different embryo metabolomes. These results suggest that DMSO has relatively more pronounced effect on the embryo metabolome than ethanol, and used as a carrier solvent, DMSO concentration should be lower than 0.1%. These findings also show the significance of ^{1}H NMR based metabolomics which can detect even a slight change in embryo

metabolome caused by different media, and can successfully be used for the identification of markers of stress- or toxicity-induced metabolic shifts.

Introduction

Aquatic toxicity testing of potentially hazardous chemicals is an important issue in environmental hazard assessment . Water solubility is the major hurdle for the testing of hydrophobic compounds in aqueous test systems such as, for example, the zebrafish model. Hydrophobic compounds have to be homogeneously distributed in water (Herzel and Murty 1984). Therefore, carrier solvents are crucial for reliable studies of a wide range of compounds (Rufli et al. 1998) including lipophilic compounds, in aquatic bioassays (Chen et al. 2011). In fact, appropriate solvents for the delivery of xenobiotics is a major issue in *in vivo* toxicology studies (Nazir et al. 2003). Solvents may themselves affect the test organisms, leading to false positive or negative results (Rayburn et al. 1991). So, there is always need to analyze the effects of the solvents separately from that of the toxicant i.e. negative controls (Calleja and Persoone 1993).

For these and other reasons, it is necessary to first validate the use of carrier solvents before starting any toxicity study. This should result in a protocol that describes the type and maximum allowable concentrations (MACs) of solvents, which can be used in different studies without having effect on the experimental results. For this, United States environmental protection agency (US EPA) has set a maximum acceptable limit of 0.05% for solvents for acute toxicity tests and of 0.01% for chronic toxicity tests (Okumura et al. 2001).

Dimethyl sulfoxide (DMSO) and ethanol are commonly used solvents for hydrophobic compounds in toxicology studies. Both of these solvents have been used for developmental, reproductive and behavioral studies in zebrafish embryos, frog embryos, marine microalgae and *Drosophila melanogaster* (Chen

et al. 2011, Hallare A. et al. 2006, Nazir et al. 2003, Rayburn et al. 1991). These solvents have a great capability to solubilize a wide range of polar and nonpolar compounds. Therefore it is of importance to know the effects of these compounds on model organisms.

The zebrafish embryo assay has been used for the screening of a growing library of chemicals to understand their mechanisms and the indication of possible acute and long term adverse effects (Ali et al. 2011, Scholz et al. 2008). By comparison of the metabolome of the zebrafish embryo treated with certain test compounds, one may learn more about the kinds of metabolic 'signature'of certain drugs. For these studies, however, we need first of all to establish the metabolomic changes during normal development (in the absence of compound exposure) as well as the effect of the various carrier solvents (including the different aqueous buffers) used during compound exposure.

Previously, the zebrafish embryo model was used to study the behavioral effects of DMSO and ethanol(Chen et al. 2011). The effects of these solvents on the zebrafish embryo metabolome is not known. Here, we used nuclear magnetic resonance spectroscopy (NMR) based metabolomics to study the effects of DMSO and ethanol on the metabolic profile of zebrafish embryo.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in accordance with national regulations, which are described in the *Wet op de dierproeven* (article 9) of Dutch Law administered by the Bureau of Animal Experiment Licensing. This national regulation serves as the implementation of "Guidelines on the protection of experimental animals" by the Council of Europe (1986), Directive 86/609/EEC, which allows zebrafish embryos to be used up to the moment of free-living (approximately 5-7 dpf).

Zebrafish

Male and female adult zebrafish (*Danio rerio)* of wild type were purchased from Selecta Aquarium Speciaalzaak (Leiden, The Netherlands) which obtain stock from Europet Bernina International BV (Gemert-Bakel, The Netherlands). The fish were kept at a maximum density of 100 individuals in glass recirculation aquaria (L 80 cm; H 50 cm, W 46 cm) on a 14 h light: 10 h dark cycle (lights on at 08:00). Water and air were temperature controlled (25 ± 0.5 ^{o}C and 23 ^{o}C, respectively). All the zebrafish handling was in accordance with national regulations. The fish were fed twice daily with 'Sprirulina' brand flake food (O.S.L. Marine Lab., Inc., and Burlingame, CA, USA) and twice a week with frozen food (Dutch Select Food, Aquadistri BV, Leiden, The Netherlands).

Defined embryo buffer

Ten percentage (v/v) of Hank's balanced salt solution was used (made from cell-culture tested, powdered Hank's salts, without sodium bicarbonate, Cat. No H6136-10X1L, Sigma-Aldrich, St Louis, MO, USA) at a concentration 0.98 g/L in Milli-Q water (resistivity = 18.2 MΩ·cm), with the addition of sodium bicarbonate at 0.035 g/L (Cell culture tested, Sigma), and adjusted to pH 7.46. A similar medium has been used previously (Irons et al. 2010, Macphail et al. 2009).

Embryo care

Eggs were obtained by random pair wise mating of zebrafish. Three adult males and four females were placed together in small breeding tanks (Ehret GmbH, Emmendingen, Germany) the evening before eggs were required. The breeding tanks (L 26 cm; H 12.5 cm, W 20 cm) had mesh egg traps to prevent the eggs from being eaten. The eggs were harvested the following morning and transferred into 92 mm plastic Petri dishes (50 eggs per dish) containing 40 ml

fresh embryo buffer. Eggs were washed four times to remove debris. Further unfertilized, unhealthy and dead embryos were screened under a dissecting microscope. At 3.5 hours post fertilization (hpf), embryos were again screened and any further dead and unhealthy embryos were removed. Throughout all procedures, the embryos and the solutions were kept at 28.5°C, in acclimatised room. All incubations of embryos were carried out in acclimatised room under a light cycle of 14 h light: 10 h dark.

Embryo Treatment and Collection

One hundred and twenty embryos per replicate were collected. After 24 hours the embryo were treated with 0.01%, 0.1% and 1% of both DMSO and ethanol. Embryos were collected on 5 days post fertilization (dpf) that is four days of exposure in falcon tube. The collected embryos were immediately frozen in liquid nitrogen and stored at -80° C till further analysis.

Sample preparation and Extraction

A fairly simple sample preparation method already was used for plant metabolomics (Abdel-Farid et al. 2009, Ali Kashif et al. 2010, Jahangir et al. 2008, Kim et al. 2010a, Kim et al. 2010b, Kim et al. 2010c). Embryos were freeze dried and transferred to a micro tubes (2 ml) to which 1ml of 50% CH_3OH-*d4* in D2O (KH_2PO_4 buffer, pH 6.0) containing 0.01% TMSP (w/w) was added. The mixture was vortexed for one min, sonicated for 20 min, and centrifuged at 13,000 rpm at room temperature for ten minutes. An aliquot (650 µL) was then transferred to a 5mm-NMR tube.

NMR measurements

NMR spectra were recorded at 25°C on a 500MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 500.13 MHz. CH_3OH-d_4 was used as the internal lock. Each ^1H

NMR spectrum consisted of 128 scans requiring with the parameters as used by our group (Kim et al. 2010c)

Data Pre Processing and Analysis

Spectral intensities of 1H-NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to the region of δ 0.4–δ 10.0. The regions of δ 4.8–δ 4.9 and δ 3.30–δ 3.34 were excluded from the analysis because of the residual signal of the deuterated solvents. PCA was performed with the SIMCA-P software (v. 12.0, Umetrics, Umeå, Sweden) based on a Pareto scaling method. Statistical analyses were performed using GraphPad Prism (v. 5.03 for Windows, GraphPad Software, La Jolla CA, USA, www.graphpad.com). We used one-way analysis of variance and a Dunnett's Multiple comparison test with probability level of 5% as the minimal criterion of significance. All of the spectra were referenced, base line, phase-corrected and visually inspected by superimposing and stacking the spectra (Fig. 3) using MestReNova v.6.0.2 (Mestrelab research S.L., A Coruna, Spain).

Results

Effect of DMSO and Ethanol on embryos metabolome

In this study three concentrations (0.01, 0.1 and 1 %) of DMSO and Ethanol and two commonly used fish media were selected to analyze their metabolic effects on the 5 days old zebrafish embryos. The embryos were exposed to DMSO and Ethanol at 24 hpf and incubated for further 96 h in Hanks balanced salt solution (HBSS). The broad range identification ability of NMR makes it useful tool to identify macro metabolites. Mostly the metabolites identified are primary metabolites present in living organism like amino acids, energy related compounds, sugars and lipids. In the aliphatic region of NMR spectra amino acids like alanine, glycine, glutamine, glutamate, arginine, asparagines,

isoleucine, leucine and methionine; while organic acids like citric and lactic acid; bases like, creatine, choline and betaine; energy relating compounds, sugars like glucose and adenosine triphosphate, tyrosine and phenylalanine were identified. The identification of the metabolites was made by comparing the chemical shifts and splitting patterns with our in-house library of more than 700 common metabolites and comparison with available literature (Foxall et al. 1993, Govindaraju et al. 2000, Nicholson et al. 1995). The ^{1}H NMR spectra of the control zebrafish embryos (embryos raised only in HBSS) and those exposed to 1% of DMSO and Ethanol are shown in Fig. 1. It is evident that no

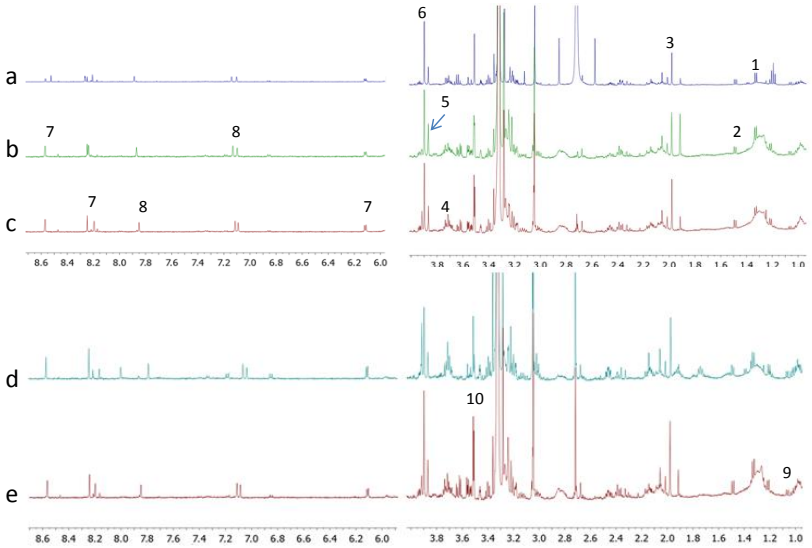


Figure 1. ^{1}H NMR spectra of 5 days old zebrafish embryo treated with (a) 1% of DMSO in HBSS, (b) 1% of ethanol in HBSS, (c) HBSS, (d) EW (e) HBSS,. 1, Lactate; 2, alanine; 3, acetate; 4, glutamate; 5, betaine; 6, creatine; 7, ATP; 8, Histidine; 9, valine; 10, glycine.

difference is present among the kind of metabolites, while significant differences exist among the level of metabolites. The signals of the discriminating metabolites are numbered (Fig 1). These signals were identified and are summarized in Table 1. Some of the major contributing metabolites

Table 1. Characteristic ^{1}H NMR chemical shifts and coupling contstants of indentified metabolites in 5 days old zebrafish embryo.

Metabolites	**Chemical shifts (δ) and Coupling constants (Hz)**
Acetate	δ 1.91 (s), δ 1.97 (s)
Alanine	δ 1.48 (d, J = 7.0 Hz)
ATP	δ 6.13 (d, J = 5.0 Hz), δ 8.26 (s), δ 8.56 (s)
Betaine	δ 3.29 (s), δ 3.87 (s)
Creatine	δ 3.06 (s), δ 3.91 (s)
Glucose	δ 4.60 (d, J = 7.94 Hz), δ 5.20 (d, 4.02 Hz)
Glutamate	δ 2.05 (m), δ 2.40 (m), δ 3.71 (m)
Glutamine	2.14,2.46,3.71
Glycine	δ 3.52 (s)
Histidine	δ 7.07 (s), δ 7.84 (s),
Lactate	δ 1.32(d, J= 7.0), δ 4.06 (m)
Valine	δ 1.01 (d, 7.01 Hz), δ 1.07 (d, J= 7.1 Hz)
3 methyl histidine	δ 7.05 (s)

were quantified and are shown in Fig. 2. The relative quantification was done by using the bucket data of ^{1}H NMR spectroscopy with the p value <0.01. It can be seen in the Fig. 2, compared to control group, the quantity of alanine, histidine, lactate, acetate, creatine and adenosine triphosphate (ATP) is greatly

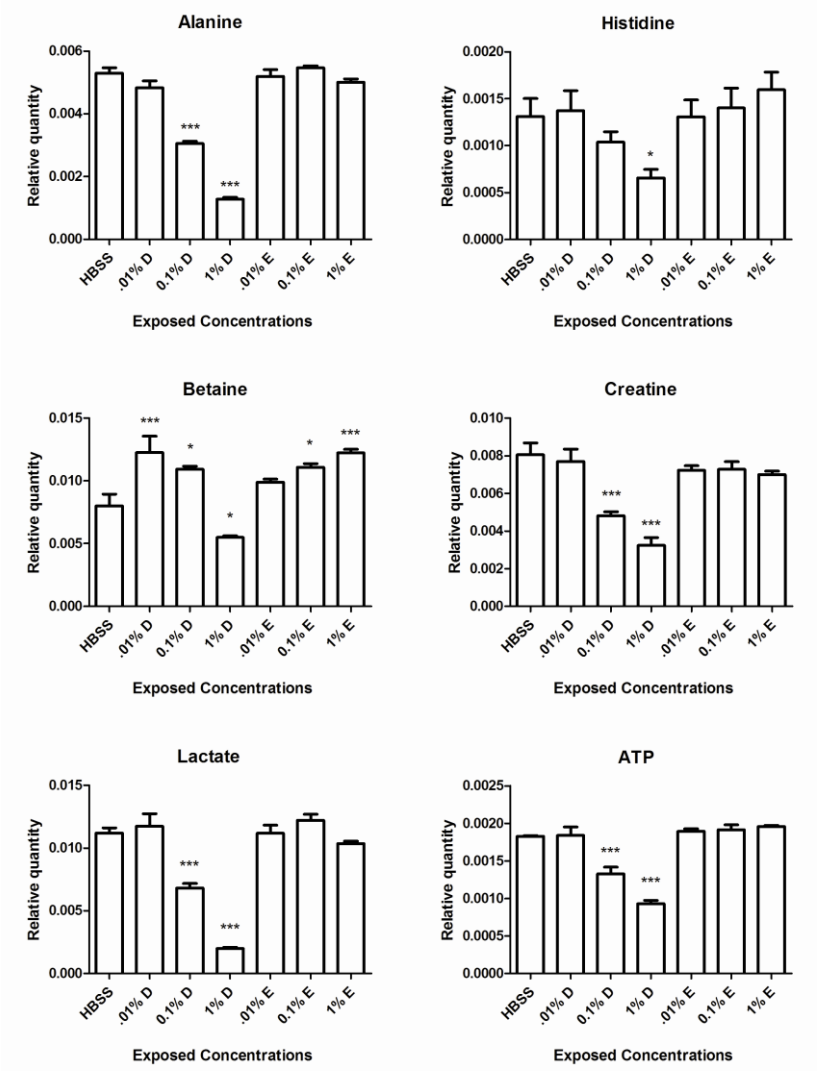


Figure 2. Relative quantification of the major differentiating metabolites based on mean peak area of the realted signals. "D" symbolizes the DMSO and "E" stands for Ethanol. ***** depict the differences between control (HBSS) and the different used concentrations of DMSO and Ethanol. Statistical icons: *= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$.

reduced after exposure to 0.1 and 1% of DMSO, However, 0.01% of DMSO does not show any effect on these metabolites but significantly increases the concentration of betaine. On the other hand, none of the ethanol concentrations affects any of the metabolite except betaine.

Multivariate data analyses (MvDA)

Principal component analysis (PCA) is a statistical technique used to analyze a large number of data by reducing the number of dimensions without losing important information. It is an unbiased and unsupervised method, which represents the data in a way that allows to observe similarities and differences in a score plot of PCA. The corresponding loading plot shows the chemical shifts of the discriminating metabolites responsible for the similarities and differences among the samples.

The PCA score plot shows the projections of Control (treated only with HBSS), DMSO and Ethanol treated groups (Fig. 3, A). A clear separation can be seen of the DMSO treated samples (0.1 and 1% DMSO) from control and Ethanol subjected groups. The major separation is characterized by PC1. The 0.1 and 1% DMSO samples were separated by PC2 and positioned in the positive and negative PC2 score, respectively. The 0.01% DMSO replicates were clustered more close to control having a positive PC1 score. The control and Ethanol groups were overlapping and placed on the positive side of PC1. The results show a clear metabolic change of the groups treated with higher concentrations of DMSO compared to control group. Whereas, there was hardly any distinction of metabolites observed between the control and the embryos exposed to Ethanol. The corresponding loading plot of the PCA was used to identify the ^{1}H chemical shifts of the subsequent metabolites responsible for the separation in score plot (not shown).

These results show that various solvents affect differently to zebrafish embryo metabolome. Dimethyl sulfoxide considerably changed the quantity of metabolites incubated in HBSS. To confirm these results, we also studied the effect of DMSO by growing the embryos in egg water containing 0.01, 0.1 and 1% of DMSO. The PCA score plot shows the separation of 0.1 and 1% of DMSO from 0.01% DMSO and control (embryo raised only in Egg water) group (Fig. 3B). The 95% of the separation was based on PC1 score. The 0.1

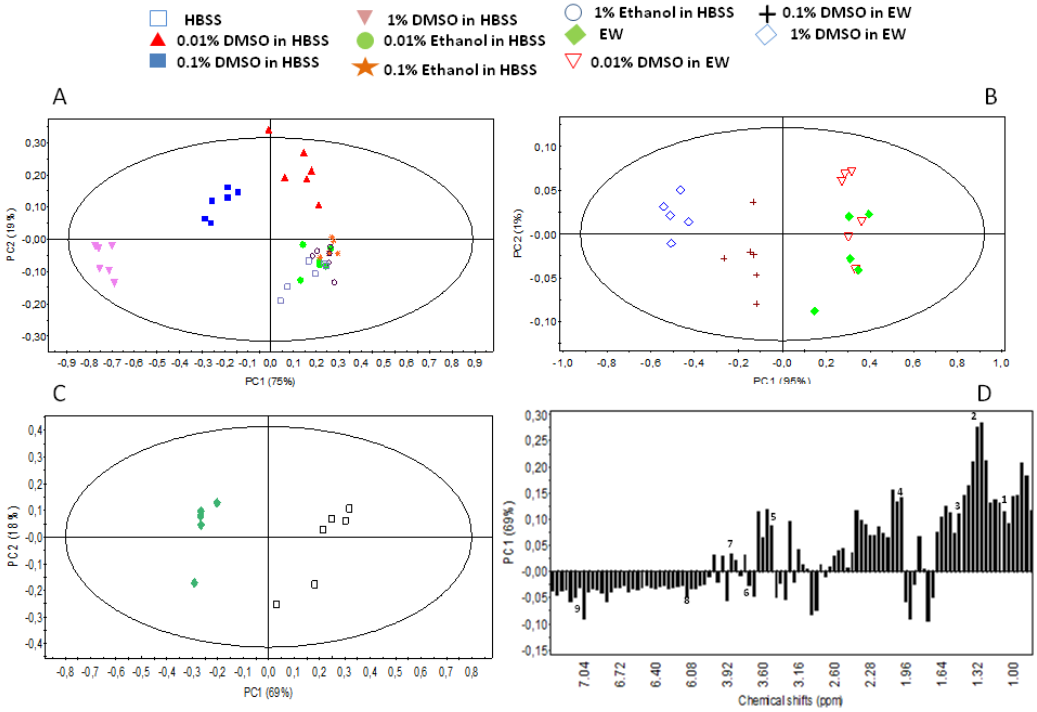


Figure 3. Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryo treated with A, .01, 0.1 and 1% of DMSO and Ethanol incubated in HBSS; B, .01, 0.1 and 1% of DMSO incubated in EW; C, HBSS and EW; D, Loading column plot of HBSS and EW. 1, valine; 2, lactate; 3, alanine; 4, acetate; 5 glycine; 6, glutamate; 7, ATP; 8, Histidine.

and 1% DMSO replicates were positioned on the negative, whereas 0.01% DMSO and control groups were overlapped on positive side of PC1. In the

corresponding loading column plot (not shown) all the signals were shifted to the positive side. The key metabolites responsible for the separation were quantified and are shown in Fig. 4. The 0.1 and 1% of DMSO showed a similar effect in EW and decreased the quantity of all those metabolites which were found suppressed in embryo populations exposed to 0.1 and 1% of DMSO and were raised in HBSS (Fig. 1a and Fig. 4). Whereas 0.01% of DMSO did not affect any of the metabolite compared to control group.

Effect of Aqueous media on embryos metabolome

We further analyzed the effect of commonly used fish media, Hanks balanced salt solution (HBSS) (Adams et al. 2005, Ali et al. 2011) and Egg water (EW) (Hentschel et al. 2005, Watanabe et al. 2010). An apparent separation can be observed by the PCA score plot for the PC1 score (Fig. 3C). The zebrafish embryo incubated in HBSS and Egg water is positioned on the positive and negative side of PC1, respectively. The differentiating metabolites were identified on the basis of chemical shifts obtained from the loading plot (Fig. 3D) and related signals are shown in the ^{1}H NMR spectra (Fig. 1d and 1e). The relative quantification (not shown) of these signals showed the reduction of valine, alanine, lactate, acetate, betaine and glycine levels in the samples raised in Egg water. Whereas glutamate, ATP and histidine were lower in the embryo incubated in HBSS. The comparison of HBSS and EW indicates that not only the choice of solvent but the selection of medium is also important for the reproducibility of the assay to study the effect of a drug on the zebrafish embryo metabolome.

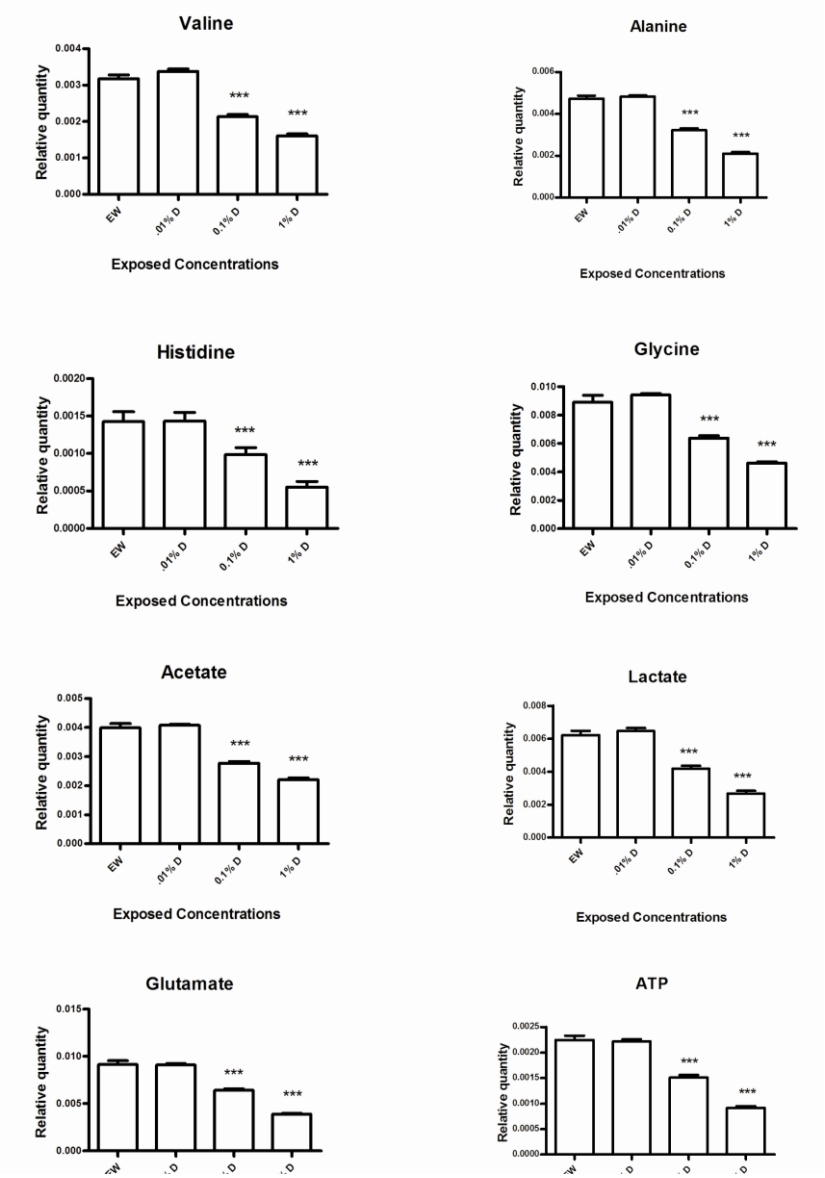


Figure 4. Relative quantification of the metabolites responsible for the separation in PCA score plot of DMSO treated samples (incubated in EW) and EW. The quantification based on mean peak area of the associated signals. ***** depict the differences between control (EW) and the different used concentrations of DMSO. Statistical icons: *= $p< 0.05$, **= $p< 0.01$ and ***= $p< 0.001$.

Discussion

In the present study, we examined the effects of 4 days of exposure to low concentrations of DMSO and ethanol on the metabolome of zebrafish embryo, as well as two aqueous buffers, EW and 10% HBSS. A significant difference was observed between the metabolic profiles of ^{1}H NMR analyses of embryo populations exposed to 0.1 and 1% DMSO and control (aqueous buffer only). DMSO was seen to have a large effect on the various metabolite levels and were well separated from ethanol and the aqueous buffer group in PCA by their PC1 and PC2 score. Particularly lower levels of amino and organic acids were observed in the treated groups. DMSO (0.1 and 1%) reduced the overall quantity of all the identified metabolites in a similar pattern. By contrast, ethanol did not alter the metabolite concentrations compared to control group. Finally, we noted that the choice of aqueous buffer (EW vs. HBSS) for raising zebrafish embryos had a significant effect on their metabolomic profiles.

Previously, NMR has been used to analyze the metabolic characterization of rat hepatocellular carcinoma formation, rat urine and serum, and human brain extracts for tumor biopsies (Maxwell et al. 1998, Wang et al. 2011, Wei et al. 2008). NMR based metabolomics and other targeted techniques (HPLC, LC-MS, GC-MS) have also been successfully applied for the metabolic fingerprinting of zebrafish embryo and liver of the adult fish (Ong et al. 2009, Papan and Chen 2009).

DMSO has previously been reported as a potential inducer of stress (Hallare et al. 2004). In a comparative study of embryotoxicity and proteotoxicity of carrier solvents to zebrafish embryos, DMSO at low concentrations (0.01, 0.05 and 0.1%) was shown to significantly increase the expression of the stress-related protein hsp 70 (heat shock protein). Ethanol and acetone showed such an activity at 1.5 and 0.1%, repectively (Hallare et al. 2006, Hallare et al. 2004).

As can be seen in Fig. 3 and 4 we find that DMSO exposure (0.1 and 1%) reduced the level of adenosine triphosphate (ATP). This is consistent with previous findings. Thus, it was reported that DMSO (0.1%) exposure, coupled with the inhibition of cytochrome P450 (CYPs) in zebrafish embryos, reduces the level of ATP in zebrafish ovarian follicles and fragments (David et al. 2012, Zampolla et al. 2009, Zampolla et al. 2011). CYP enzymes play an important role in the oxidative metabolism of endogenous and exogenous substances (Buhler and Wang-Buhler 1998). A further study found that in the kidneys of male wistar rats, DMSO reduced the level of ATP and slowed down the process of glycolysis by activating the FDPase (Fructose 1,6-diphaphatase) (Baxter and Lathe 1971).

Taken together, our results and previous findings could suggest that DMSO may cause a metabolic inhibition leading to greatly reduced levels of ATP, and in turn the suppression of primary metabolite levels in the zebrafish embryo. Further studies (including also, for example, proteomics and transcriptomics) are needed to confirm this hypothesis. What is perhaps surprising in our study is that ethanol exposure (0.01, 0.1 and 1.0%) had no significant effect on the zebrafish embryo metabolome, even though it is known to be a potent teratogen (Arenzana et al. 2006). These studies showed that even low concentrations of ethanol (0.05, 0.1 and 1%) can induce the hyper locomotor activity in zebrafish embryos (Echevarria et al. 2010). In a study of behavioral and teratological effects of ethanol on zebrafish larave, 0.01 and 0.1% of ethanol was found safe in not producing any developmental defect but still inducing hyper locomotion (Chen et al. 2011). By contrast, 1% ethanol not only altered the locomotor activity but also significantly increased the incidence of e.g pericardial edema, yolk sac edema, crooked body and cyclopia (Arenzana et al. 2006, Chen et al. 2011).

An interesting question is why, in our study, DMSO had such a pronounced metabolic effect, while previous studies showed it to have only mild phenotypic effects on embryos; while in contrast, ethanol had no effect on the metabolome in our study, even though it is known to have pronounced phenotypic effects on embryo development. The answer may lie in the fact that DMSO is known to produce pronounced effects on the liver enzymes and ATP production which might cause the large effect on embryo metabolism seen in our study. The use of DMSO should therefore be evaluated carefully, and the final concentration should be as low as possible; the concentration of 0.01% DMSO in either EW

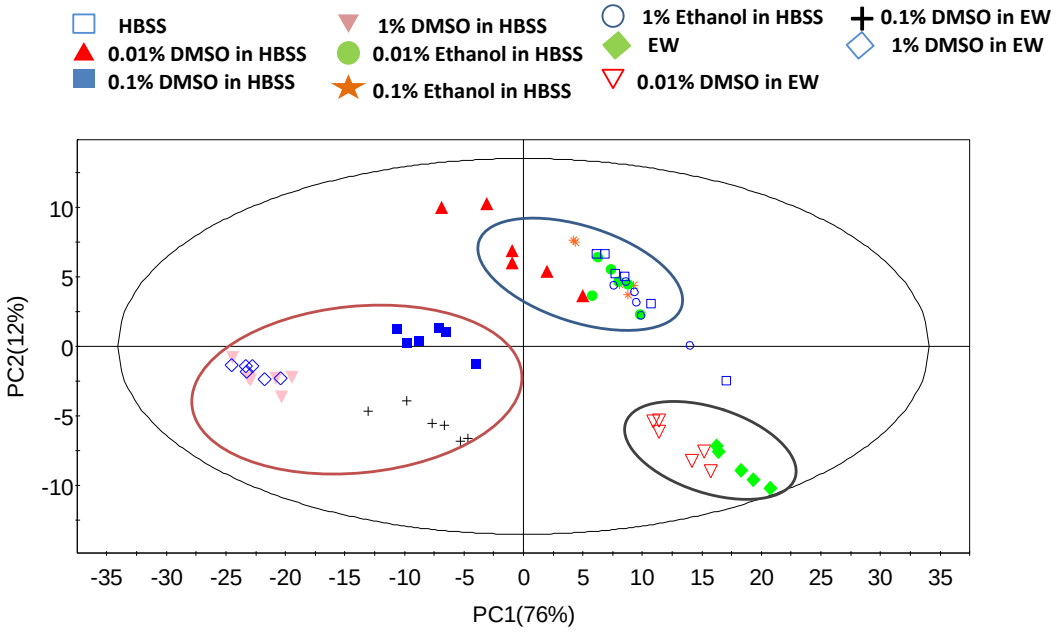


Figure 5: Score plot (PC1 vs PC2) of PCA based on whole range of ^{1}H NMR signals (δ 0.3- δ 10.0) of zebrafish embryo treated with Ethanol (0.01, 0.1 and 1%) raised in HBSS, DMSO (0.01, 0.1 and 1%) raised in HBSS and EW, raised only in HBSS and EW.

or HBSS had no significant effects on the zebrafish metabolome in this study.

Moreover, our findings suggest that the selection of the aqueous medium is also important because it may affect embryo metabolism. Given a fixed concentration of DMSO (0.01), the metabolic effect varies according to whether this concentration of DMSO was in HBSS or EW. It can be seen in the PCA analysis of all samples shown in Fig. 5 that, all three ethanol concentrations, 0.01% DMSO in HBSS and HBSS alone, were all clustered on the positive side of PC1. EW alone, and 0.01% of DMSO in EW replicates overlapped and had positive PC1 and PC2 scores. The points for DMSO (0.1%) in HBSS, and those for DMSO (0.1%) in EW, were separated by a small distance having negative PC1 score. Interestingly, the 1% DMSO samples (raised in either HBSS or EW) were found to cluster tightly together. Therefore, at this concentration of DMSO, the choice of aqueous medium no longer has an effect.

Conclusion

Our results demonstrate that NMR together with multivariate data analyses provides comprehensive information of amino and organic acids in the 5 dpf zebrafish embryo metabolome. NMR can successfully detect a even slight differences in metabolite levels induced by aqueous media or organic solvents, respectively. The study also shows that zebrafish embryos can be used for the preliminary screening of compounds by providing insight into the metabolic changes mediated by drugs or toxins. Finally, we recommend that for metabolomic studies of zebrafish embryos, the DMSO concentration should be lower than 0.1%.

References

Abdel-Farid IB, Jahangir M, van den Hondel CAMJJ, Kim HK, Choi YH, Verpoorte R. 2009. Fungal infection-induced metabolites in Brassica rapa. Plant Sci 176: 608-615.

Adams SL, Zhang T, Rawson DM. 2005. The effect of external medium composition on membrane water permeability of zebrafish (*Danio rerio*) embryos. Theriogenology 64: 1591-1602.

Ali K, Maltese F, Fortes AM, Pais MS, Choi YH, Verpoorte R. 2010. Monitoring biochemical changes during grape berry development in Portuguese cultivars by NMR spectroscopy. Food Chem 124: 1760-1769.

Ali S, van Mil HG, Richardson MK. 2011. Large-scale assessment of the zebrafish embryo as a possible predictive model in toxicity testing. PLoS One 6: e21076.

Arenzana FJ, Carvan MJr, Aijon J, Sanchez-Gonzalez R, Arevalo R, Porteros A. 2006. Teratogenic effects of ethanol exposure on zebrafish visual system development. Neurotoxicol Teratol 28: 342-348.

Baxter SJ, Lathe GH. 1971. Biochemical effects on kidney of exposure to high concentrations of dimethyl sulphoxide. Biochem Pharmacol 20: 1079-1091.

Buhler D, Wang-Buhler JL. 1998. Rainbow trout cytochrome P450s: purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 121: 107-137.

Busch W, Duis K, Fenske M, Maack G, Legler J, Padilla S, Strahle U, Witters H, Scholz S. 2011. The zebrafish embryo model in toxicology and teratology. Reprod Toxicol 31: 585-588.

Calleja MC, Persoone G. 1993. The influence of solvents on the acute toxicity of some lipophilic chemicals to aquatic invertebrates. Chemosphere 26: 2007-2022.

Chen TH, Wang YH, Wu YH. 2011. Developmental exposures to ethanol or dimethylsulfoxide at low concentrations alter locomotor activity in larval zebrafish: implications for behavioral toxicity bioassays. Aquat Toxicol 102: 162-166.

David RM, Jones HS, Panter GH, Winter MJ, Hutchinson TH, Kevin CJ. 2012. Interference with xenobiotic metabolic activity by the commonly used vehicle solvents dimethylsulfoxide and methanol in zebrafish (*Danio rerio*) larvae but not *Daphnia magna*. Chemosphere 8: 912-7.

Echevarria DJ, Hammack CM, Jouandot DJ, Toms CN. 2010. Does acute alcohol exposure modulate aggressive behaviors in the Zebrafish (*Danio rerio*), or is the bark worse than the bite? Int J Comp Psychol 23: 62.

Foxall PJD, Spraul M, Farrant RD, Lindon LC, Neild GH, Nicholson JK. 1993. 750 MHz 1H-NMR spectroscopy of human blood plasma. J Pharmaceut Biomed 11: 267-276.

Govindaraju V, Young K, Maudsley AA. 2000. Proton NMR chemical shifts and coupling constants for brain metabolites. NMR Biomed 13: 129-153.

Hallare A, Nagel K, Kohler HR, Triebskorn R. 2006. Comparative embryotoxicity and proteotoxicity of three carrier solvents to zebrafish (*Danio rerio*) embryos. Ecotoxicol Environ Saf 63: 378-388.

Hallare AV, Kohler HR, Triebskorn R. 2004. Developmental toxicity and stress protein responses in zebrafish embryos after exposure to diclofenac and its solvent, DMSO. Chemosphere 56: 659-666.

Hentschel DM, Park KM, Cilenti L, Zervos AS, Drummond I, Bonventre JV. 2005. Acute renal failure in zebrafish: a novel system to study a complex disease. Am J Physiol Renal Physiol 288: 923-929.

Herzel F, Murty AS. 1984. Do carrier solvents enhance the water solubility of hydrophobic compounds? Bull Environ Contam Toxicol 32: 53-58.

Jahangir M, Kim HK, Choi YH, Verpoorte R. 2008. Metabolomic response of Brassica rapa submitted to pre-harvest bacterial contamination. Food Chem 107: 362-368.

Kim HK, Choi YH, Verpoorte R. 2010a. NMR-based metabolomic analysis of plants. Nat. Protocols 5: 536-549.

Kim HK, Choi YH, Verpoorte R. 2010b. NMR-based metabolomic analysis of plants. Nat Protocols 5: 536-549.

Kim HK, Saifullah, Khan S, Wilson EG, Kricun SDP, Meissner A, Goraler S, Deelder AM, Choi YH, Verpoorte R. 2010c. Metabolic classification of South American Ilex species by NMR-based metabolomics. Phytochemistry 71: 773-784.

Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203: 253-310.

Maxwell RJ, et al. 1998. Pattern recognition analysis of 1H NMR spectra from perchloric acid extracts of human brain tumor biopsies. Magn Reson Med 39: 869-877.

Nazir A, Mukhopadhyay I, Saxena DK, Chowdhuri DK. 2003. Evaluation of the No Observed Adverse Effect Level of Solvent Dimethyl Sulfoxide in Drosophila melanogaster. Toxicol Mech Methods 13: 147-152.

Nicholson JK, Foxall PJD, Spraul M, Farrant RD, Lindon JC. 1995. 750 MHz 1H and 1H-13C NMR Spectroscopy of Human Blood Plasma. Anal Chem 67: 793-811.

Okumura Y, Koyama J, Takaku H, Satoh H. 2001. Influence of organic solvents on the growth of marine microalgae. Arch Environ Contam Toxicol 41: 123-128.

Ong ES, Chor CF, Zou L, Ong CN. 2009. A multi-analytical approach for metabolomic profiling of zebrafish (*Danio rerio*) livers. Mol Biosyst 5: 288-298.

Papan C, Chen L. 2009. Metabolic fingerprinting reveals developmental regulation of metabolites during early zebrafish embryogenesis. Omics 13: 397-405.

Parng C, Seng WL, Semino C, McGrath P. 2002. Zebrafish: a preclinical model for drug screening. Assay Drug Dev Technol 1: 41-48.

Postlethwait JH, Woods IG, Ngo-Hazelett P, Yan YL, Kelly PD, Chu F, Huang H, Hill-Force A, Talbot WS. 2000. Zebrafish comparative genomics and the origins of vertebrate chromosomes. Genome Res 10: 1890-1902.

Rayburn JR, DeYoung DJ, Bantle JA, Fort DJ, McNew R. 1991. Altered developmental toxicity caused by three carrier solvents. J Appl Toxicol 11: 253-260.

Rufli H, et al. 1998. Aquatic toxicity testing of sparingly soluble, volatile, and unstable substances and interpretation and use of data. Task Force of the European Centre for Ecotoxicology and Toxicology of Chemicals. Ecotoxicol Environ Saf 39: 72-77.

Scholz S, Fischer S, Gundel U, Kuster E, Luckenbach T, Voelker D. 2008. The zebrafish embryo model in environmental risk assessment--applications beyond acute toxicity testing. Environ Sci Pollut Res Int 15: 394-404.

Veldman MB, Lin S. 2008. Zebrafish as a developmental model organism for pediatric research. Pediatr Res 64: 470-476.

Wang J, Zhang S, Li Z, Yang J, Huang C, Liang R, Liu Z, Zhou R. 2011. (1)H-NMR-based metabolomics of tumor tissue for the metabolic characterization of rat hepatocellular carcinoma formation and metastasis. Tumour Biol 32: 223-231.

Watanabe K, et al. 2010. In vivo imaging of zebrafish retinal cells using fluorescent coumarin derivatives. BMC Neurosci 11: 116.

Wei L, Liao P, Wu H, Li X, Pei F, Li W, Wu Y. 2008. Toxicological effects of cinnabar in rats by NMR-based metabolic profiling of urine and serum. Toxicol Appl Pharmacol 227: 417-429.

Zampolla T, Spikings E, Zhang T, Rawson DM. 2009. Effect of methanol and Me2SO exposure on mitochondrial activity and distribution in stage III ovarian follicles of zebrafish (*Danio rerio*). Cryobiology 59: 188-194.

Zampolla T, Spikings E, Srirangarajah S, Rawson DM, Zhang T. 2011. Impact of cryoprotectants and cryopreservation on metabolic activity and cytoskeleton proteins of zebrafish (*Danio rerio*) ovarian fragments. Cryo Letters 32: 525-536.

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.

SAMENVATTING

Cannabinoïden vormen een groep van terpenophenolische verbindingen die enkel te vinden zijn in de Cannabis plant.(Cannabis sativa L). Δ9-tetrahydrocannabinol (Δ^9-THC) is de psychoactieve cannabinoïde. Het is pharmacologisch erkend voor de stimulatie van eetlust en werkt als antiemeticum bij patiënten met chemotherapie en HIV therapie en wordt verder gebruikt voor indicaties zoals pijn, MS, glaucoom en Tourette syndroom. Er is een groeiende belangstelling voor het ontwikkelen van nieuwe derivaten van Δ^9-THC met een hoge geneeskrachtige waarde. Echter, de hoge lipofiliteit van Δ^9-THC is een belemmering voor de verdere ontwikkeling van deze verbinding in een farmaceutisch product. Het menselijke lichaam heeft een hoog vetgehalte, niet alleen in het vetweefsel in het lichaam, maar ook in de hersenen en celmembranen. Δ^9-THC bindt sterk aan plasma eiwitten en aan vetweefsel, waardoor de uitscheiding uit het lichaam significant wordt verlengd. Het is dus noodzakelijk om de polariteit van de cannabinoïden te verhogen om een snelle afgifte uit het lichaam te verkrijgen. Dit proefschrift is gericht op het onderzoek van de mogelijke biotransformatie van cannabinoïden in meer polaire derivaten van Δ^9-THC door alkaan afbrekende bacteriën en *Catharanthus roseus* plantencellen.

In **hoofdstuk 2** hebben we een bibliotheek van Δ^9-THC metabolieten ontwikkeld door middel van bioconversies met celculturen van zoogdieren, schimmels, bacteriën en planten. Deze organismen hebben unieke enzymen die de omzetting van Δ^9-THC mogelijk katalyseren en die op verschillende plaatsen in het molecuul kunnen aangrijpen, en derhalve een bron zijn voor een verscheidenheid van derivaten. Deze verbindingen kunnen worden gebruikt om meer informatie over de farmacodynamiek en farmacokinetiek van Δ^9-THC te verkrijgen en aldus de weg vrijmaken voor de ontdekking van nieuwe verbindingen met verbeterde therapeutische eigenschappen.

In een poging om sterk polaire derivaten van Δ^9-THC te genereren hebben we 206 alkaan afbrekende bacteriestammen gescreend. De C5-alkylzijketen bleek de specifieke target site die alkaan afbrekende bacteriën gebruiken om Δ^9-THC om te zetten in meer polaire derivaten. Met name Gram-positieve stammen van de geslachten Rhodococcus, Mycobacterium, Gordonia en Dietzia bleken efficiënt in transformatie van Δ^9-THC. In totaal werden acht derivaten geproduceerd op milligram schaal. Al de eproduceerde transformatie producten hebben een gemodificeerde alkylketes met hydroxy, carboxy en ester functionaliteiten **(hoofdstuk 3).**

Naast micro-organismen, kunnen ook planten celcultures fungeren als biokatalysatoren om complexe biochemische reacties uit te voeren. Het enzymatische systeem van plantencellen kan vaak met succes exogeen toegepaste substraten omzetten naar nieuwe en waardevolle producten met verbeterde eigenschappen met betrekking tot de stabiliteit, bio-activiteit en oplosbaarheid. We onderzochten het potentieel van celsuspensiekweek van *Catharanthus roseus* voor de bioconversie van Δ^9-THC. Anders dan bacteriestammen, glycosyleren *Catharanthus roseus* cellen Δ^9-THC. Bovendien hydroxyleren ze cannabinoiden. In de celculture werd Δ^9-THC ook afgebroken tot cannabinol (CBN), dat vervolgens ook werd omgezet in zijn geglycosyleerde derivaten. Onze bevindingen suggereren dat micro-organismen en plantencelkweken verschillende cytochroom P450 enzymen bevatten en dat resulteert in verschillende catabole routes hebben voor Δ^9-THC **(Hoofdstuk 4).**

Het zebravis embryo bioassay model lijkt zeer geschikt voor high-throughput screening van nieuwe kandidaat-geneesmiddelen. Het maakt snelle, high-throughput en goedkope assays mogelijk, zoals voor het in vroege (pre-regulering) stadia testen van geneesmiddelen en voor gedrags testen. De gedragseffecten van Δ^9-THC worden gemedieerd door de centrale CB1

receptor. De ontdekking van CB receptoren in zebravis embryo's kan nuttig zijn om meer inzicht te krijgen in de farmacologie van cannabinoïden. Het kan ook nuttig zijn om een aantal onduidelijke aspecten van het cannabinoïden werkingsmechanisme, zoals het fenomeen van mogelijke tolerantie en afhankelijkheid veroorzaakt door cannabinoïd-gebaseerde geneesmiddelen te onderzoeken . We gebruikten kleuringen, visueel motorische respons test en 1H-NMR gebaseerde metabolomics om de fenotypische, gedrags- en metabole effecten van cannabinoïden in de zebravis embryo's te bestuderen.

Hoofdstuk 5 beschrijft de effecten van het natuurlijke cannabinoïd Δ^9-THC, en de synthetische cannabinoïden WIN 55,212-2 en CP 55940, en de cannabinoïd antagonist Am 251 op zebravis embryo motorische activiteit. We vonden een significante overeenkomst tussen fysiologische reacties van knaagdieren en zebravis embryo's op deze cannabinoïden. Het zebravis embryo lijkt daarom een betrouwbaar gedragsmodel voor het testen van deze psychoactieve verbindingen.

High-resolution proton nucleaire magnetische resonantie (1H-NMR) is een ideaal hulpmiddel voor het profileren van de metabolieten in biovloeistoffen, weefsel extracten en zelfs in intacte weefsels. Het is onder andere gebruikt om de biochemische samenstelling van verschillende weefsels te onderzoeken in een organisme en voor het bepalen van geneesmiddeltoxiciteit door analyse van bloedserum, lever en testis van knaagdieren. We hebben met succes 1H-NMR in combinatie met multivariate data-analyse toegepast voor de metaboliet profilering van zebravis embryos op 5 dagen na bevruchting (5dpf). Blootstelling van embryos aan Δ^9-THC en AM251 leidde tot een uitgesproken effect op de metabolieten die direct betrokken zijn bij neurotransmissie. De tegengestelde effecten van Δ^9-THC en AM251 laten zien dat de CB1-receptoren zijn betrokken bij hun werking in deze embryos. Onze resultaten

suggereren dat zebravisembryos kunnen worden gebruikt als modelorganisme om de metabole "footprint" van bepaalde geneesmiddelen **(hoofdstuk 6)** te bestuderen.

In de bioassays worden organische oplosmiddelen gebruikt om hydrofobe test stoffen op te lossen. Water oplosbare verbindingen kunnen eenvoudig worden opgelost in het waterige kweek medium van de verschillende experimentele cellen of organismen. Het is echter belangrijk te weten of het oplosmiddel zelf - inclusief het type buffer - een effect op de organismen, cellen of assay heeft. Daarom hebben we de metabole effecten gemeten van de meest gebruikte organische oplosmiddelen (dimethylsulfoxide, ethanol) en de twee gebruikte waterige buffers (HBSS en EW) waarin zebravis embryos worden gekweekt. Dimethylsulfoxide (DMSO) geeft een significant verlaagd niveau van veel primaire metabolieten. Ethanol heeft daarentegen geen invloed op het embryo's metaboloom bij de geteste concentraties. Ook beide waterige buffers leiden tot verschillende embryo metabolomen. Tezamen tonen deze resultaten dat een kritische evaluatie van carrier stoffen en waterige media belangrijk is om vals negatieve resultaten te voorkomen . Vooral bij zebravis embryo, heeft DMSO relatief een sterker effect op metabolietvorming dan ethanol heeft. Als drager oplosmiddel dienen DMSO concentraties lager te zijn dan 0,01%. 1H-NMR gebaseerde metabolomics kan met succes worden toegepast voor het in kaart bregen van metabolische veranderingen, bijvoorbeeld voor de identificatie van markers van stress-geïnduceerde toxiciteit **(hoofdstuk 7)**.

Concluderend, in relatie tot de doelstellingen van het proefschrift werd aangetoond dat:

- het gebruik van bacterieën en plantencelcultures is veelbelovend voor de grootschalige productie van polaire derivaten van Δ9-THC. Verdere evaluatie

van deze derivaten met betrekking tot hun bindingsaffiniteit voor CB-receptoren zou kunnen helpen bij het begrijpen van de farmacologische eigenschappen van deze polaire metabolieten.

- Bij het volgen van het gedrag van zebravis embryos / larven, met behulp van de visuele motorische respons test bleek dat embryo's een vergelijkbare fysiologische respons geven als knaagdieren na toedienen van cannabinoïden. Dit betekent mogelijk dat de zebravis embryos kunnen worden toegepast in het begin van de drug discovery pijplijn en bij de beoordeling van de veiligheid van geneesmiddelen, zoals bijvoorbeeld voor nieuwe cannabinoïde agonisten en antagonisten.

Toekomstperspectieven

Een aantal studies naar de catabole routes van THC in verschillende soorten zoogdieren hebben geleid tot de ontdekking van een groot aantal THC metabolieten. Micro-organismen en plantencel cultures bleken geschikt voor de grootschalige productie van polaire derivaten van THC. Echter, slechts een klein deel van deze THC-derivaten zijn farmacologisch geëvalueerd en de therapeutische mogelijkheden van een groot deel van deze verbindingen moeten nog worden onderzocht. Derivaten met verschillende bindingsaffiniteiten aan CB receptoren of met een relatief zwakke bindingsaffiniteit zijn interessant, met inbegrip van de mogelijkheid om geneesmiddelen te ontwikkelen die specifieker zijn en minder bijwerkingen hebben.

Hoewel zebravis embryos een knaagdier-achtige fysiologische respons hebben op CB1-agonisten en antagonisten, moet er nog veel werk worden gedaan om hun mogelijkheden als een alternatief model systeem in de cannabinoïde farmacologie volledig te kunnen beoordelen . Met name de vraag hoe de verschillende functies van de twee bekende cannabinoïdereceptoren bij de mens

zich vertalen naar het zebravis model is van belang. Onder de problemen die bijzondere aandacht in dit verband vereisen, zijn de aminozuursequentie homologie van zebravis, menselijke en knaagdieren CB receptoren; de verdeling en expressie van CB-receptoren in verschillende stadia van ontwikkeling van de zebravis; en de interactie van andere receptoren met de cannabinoïden G-eiwit-gekoppelde receptor 55 (GPR55) in knaagdieren (Pertwee 2007). Toekomstig onderzoek langs deze lijn zou kunnen leiden tot een meer omvattend begrip van de effecten van natuurlijke, synthetische en endogene cannabinoïden in de zebravis. Bovendien zou een combinatie van moleculaire technieken zoals in situ hybridisatie (ISH), kwantitatieve PCR (qPCR) en de kleuring van embryos niet alleen verder het nut van de zebravis als modelsysteem bewijzen, maar ook het begrip verbeteren van cannabinoïde geassocieerde effecten in de dierfysiologie.

Tot slot, het gebruik van de zebravis voor farmacokinetische studies naar nieuwe psychoactieve en niet-psychoactieve cannabinoïden houdt een grote belofte in voor nieuwe geneesmiddelen. Deze belofte zal zeker verder worden versterkt door het combineren van de klassieke genetische methoden, state-of-the-art moleculaire technieken en geavanceerde metabolomics technologieën gebaseerd op NMR of LC / GC-MS met de hoge doorvoer van analses die zebravis embryos mogelijk maken.

CURRICULUM VITAE

Muhammad Tayyab Akhtar was born on the 14th of April 1984 in Rahim Yar Khan, Pakistan. He got his early education in Rahim Yar Khan. In 2002, after passing his higher secondary school examination, he joined the faculty of Agriculture at University of Agriculture Faisalabad in Faisalabad, Pakistan. In June 2005, he received his BSc (Hons) in agriculture with the specialization in Plant Breeding and Genetics. From January 2006, he followed the master program of biotechnology and received his master's degree (M.Phil) from NIBGE Quaid-e-Azam University in Faisalabad, Pakistan. During his M.phil, he performed research at Industrial Biotechnology division (NIBGE), Faisalabad on the project "Effect of metal ions on the activity and thermal stability of α-amylases from *Aspergillus niger*". In 2007 he was awarded an overseas PhD Scholarship from the Higher Education Commission of Pakistan . Since March 2008 until October 2012, he worked on PhD research project "Zebrafish and cannabinoids" under the supervision of Prof. Dr. Rob Verpoorte at the Institute of Biology, Leiden University. The results of his PhD research are presented in this thesis.