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Biotransformation of synthetic cannabinoids JWH-018, JWH-073 and AM2201 by *Cunninghamella elegans*

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ABSTRACT

Being marketed as "legal" smoking blends or mixtures, synthetic cannabinoids are abused widely owing to its cannabis-like effect. Due to the rapid introduction of new generation analogues of synthetic cannabinoids to escape from legislative/judicial control, the investigation of the metabolic pathways of these substances is of particular importance for drug control, abstinence and forensic toxicology purposes. In this study, the *in vitro* metabolism of JWH-018, JWH-073 and AM2201 by the fungus *Cunninghamella elagans* has been investigated with the purpose of validating its potential as a complementary model for investigating synthetic cannabinoid metabolism. JWH-018, JWH-073 and AM2201 were incubated for 72 h with *C. elegans*. Detection of metabolites was based on liquid chromatography–tandem mass spectrometry and high resolution mass spectrometry analysis. *C. elegans* was found capable of producing the majority of the phase I metabolites observed in earlier *in vitro* and *in vivo* mammalian studies as a result of monohydroxylation, dihydroxylation, carboxylation, dehydrogenation, ketone formation, dihydrodiol formation, dihydrodiol formation with *N*-dealkylation and combinations thereof. *C. elegans* can thus be a useful and economic model for studying synthetic cannabinoid metabolism.

1. Introduction

Abusive consumption of synthetic cannabinoids that are agonists at cannabinoid CB_1 receptors has been commonly reported since 2008 [1]. Usually sold as herbal blends or research chemicals in powder, synthetic cannabinoids mimic psychoactive effects of cannabis. However, unlike cannabis some synthetic cannabinoids are reported to be full agonists and thus create more serious public health issues [2]. When a synthetic cannabinoid is scheduled due to increased prevalence and health concerns, new molecules with similar or even stronger psychoactive effects are synthesized by slight structural modifications to bypass the laws [3,4]. The lack of metabolism data of these new psychoactive molecules together with the lack of reference standards has made optimized detection in biological matrices, especially urine, difficult.

Due to rapid changes in product composition and continuous emergence of new compounds, identifying the unique fingerprint of drug metabolites is of vital importance for forensic-toxicological, clinical–toxicological and doping analysis. Several *in vivo* and *in vitro* models are being used to identify metabolites of synthetic cannabinoids. *In vivo* studies involving the researcher himself administering drug have been reported [5]. Despite the reliability of data obtained from such experiments, the adverse effects of these drugs are unknown and due to both health and ethical reasons it is difficult to perform *in vivo* human excretion studies to investigate the metabolism.

Other *in vivo* animal systems such as mouse, rat, and primates can be used as alternatives to human administration in metabolism studies. Unfortunately these models suffer from a number of limitations such as ethical constraints, cost of experimental models, time that must be spent on breeding animals, etc. [6]. Moreover species differences in the excretory pathway may make the extrapolation from experimental animals to humans difficult [7]. Recently *in vivo* chimeric mouse models based upon the transplantation of primary human hepatocytes in uPA-SCID

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mice that suffer from a transgene-induced liver disease have been evaluated. This model proved to be an efficient alternative for human administration studies for the investigation of steroid metabolism and was recently used to reveal both phase I and phase II metabolism of synthetic cannabinoids, JWH-200 and JWH-122 [8–10]. Although the chimeric mouse is a promising model with respect to the array of metabolic pathways, the cost and complexity involved in the development of such a model with a high level of hepatocyte repopulation, low amount of urine that can be collected and the concentrated mouse urine matrix are some inherent limitations [10,11].

In vitro platforms like perfused liver, hepatocytes or human liver microsomes are other valuable models for the elucidation of drug metabolism [6]. In particular, primary human hepatocytes give the closest metabolic profile of a drug to that of *in vivo* human and are hence considered as the 'gold standard' for predicting *in vivo* metabolic pathways of drugs [12]. Metabolic defects, restricted accessibility to suitable liver samples, unsuitability for quantitative estimations, inability of the cells to proliferate, quick degradation of P450 enzyme activities during culture and the requirement for specific culturing conditions are limitations of these *in vitro* models [10,13].

The concept of using microorganisms, and in particular *Cunninghamella elegans*, as models of mammalian metabolism has been well documented [14–16]. It has been proved that *C. elegans* has CYP509A1 enzymes that are synonymous to that involved in xenobiotic detoxification in mammals [17] and can metabolize a wide variety of xenobiotics in a regio- and stereoselective manner similar to mammalian enzyme systems [14,15]. A recent review on *C. elegans* reports that the fungus shows similarities with mammalian metabolism for a wide variety of drugs [15]. It is highly efficient in its production of metabolites, especially from antidepressants, antibiotics, steroids, alkaloids and related drugs [15]. The cultures of the fungus can be grown by transferring to new agar plates without complexity, adding to advantages of the model [18].

The aim of the study was to elucidate the metabolite profile of (1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone (JWH-018), (1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone (JWH-073) and [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone (AM2201), three amino alkyl indoles with well-defined metabolic profiles, using the *C. elegans* model and to compare with previously reported *in vivo* and *in vitro* data to examine the potential of this model [19–23].

2. Materials and methods

2.1. Chemicals

JWH-018 and JWH-073 were synthesized in-house following previously reported methods and characterized by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy with no evidence of cross contamination [24,25]. AM2201 (purity 99.4%) was obtained from the National Measurement Institute (North Ryde, NSW, Australia). Reference standards of JWH-018 metabolites namely JWH-018 *N*-(4-hydroxypentyl), JWH-018 *N*-(5-hydroxypentyl) and JWH-018 *N*-pentanoic acid and JWH-073 metabolites namely JWH-073 *N*-(3-hydroxybutyl), JWH-073 *N*-(4-hydroxybutyl) and JWH-073 *N*-butanoic acid were obtained from PM separations (Capalaba, QLD, Australia). Reagent grade dichloromethane and LC grade acetonitrile and methanol were obtained from Chemsupply (Gilman, SA, Australia). Potato dextrose agar, glucose, peptone, and yeast extract were purchased from Oxoid Australia (Adelaide, SA, Australia).

2.2. Microbial culture and biotransformation conditions

Cultures of *C. elegans* ATCC 10028b (Cryosite Ltd., South Granville, NSW, Australia) were propagated on potato dextrose agar plates [26] at 27 °C for 5 days. The mycelia from five plates were then transferred to 20 mL of sterile physiological saline solution and homogenized for 5 min. Approximately 3 mL aliquots of the homogenate were used to inoculate 250 mL Erlenmeyer flasks containing 100 mL of growth media prepared according to the methods in [18]. The cultures were incubated for 48 h at 26 °C on an Infors HT Multitron rotary shaker (*In vitro* Technologies, Noble Park North, VIC, Australia) operating at 180 rpm. After 48 h, 10 mg of JWH-018, JWH-073 or AM2201 dissolved in 0.5 mL of methanol was added to the culture and incubated for further 72 h [18]. Control experiments consisted of cultures without cannabinoids and flasks containing only media and cannabinoid [27,28].

2.3. Extraction, isolation, and identification of metabolites

After 72 h of incubation, the contents of each flask, including the controls, were filtered through Buchner funnel into a separating funnel and extracted with three aliquots of dichloromethane (3× 50 mL). The combined organic extracts were evaporated to dryness under vacuum at 40 °C using a Buchi rotary evaporator (*In vitro* Technologies, Noble Park North, VIC, Australia) and placed under high vacuum to remove traces of moisture. The residue was dissolved in acetonitrile to prepare 1 mg/mL stock solution and was filtered through 0.22 μM syringe filter before analysis. Cannabinoid parent drugs and metabolites were chromatographically separated using an Agilent Zorbax Eclipse XDB-C18 analytical column (150 mm × 4.6 mm, 5 μm). Mobile phases consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The gradient used consisted of 30% B (0–2 min), linear gradient from 30% B to 50% B (2–5 min), 50% B to 90% B (5–30 min, hold for 5 min) and 90% B to 30% B (35–40 min) run at 0.4 mL/min. MS data were acquired on an Agilent 6490 Triple Quadrupole mass spectrometer with an electrospray ionization source (ESI) source (positive ion mode), interfaced with an Agilent 1290 LC system. Samples prepared were injected in 2 μL volume to obtain full scan and product ion scan spectra. Product ion scan experiments were conducted on precursor ions that were presumed to be metabolites based on the comparison of full scan spectra of the samples and controls. A fragmentor voltage of 380 V with discrete collision energy of 10, 20, 30 and 40 eV (for product ion scan) was applied. The scanning mass range was set at *m*/*z* 100–1000 (scan time = 500 ms). The sheath gas temperature and flow were set to 250 °C and 11 L/min, respectively. The capillary and nozzle voltages were 3000 V and 1500 V, respectively.

High resolution quadrupole time-of-flight mass spectrometry (HRQToFMS) experiments were carried out on an Agilent 6510 Accurate Mass QToF Mass Spectrometer, equipped with ESI source operated in positive ion mode, in order to determine accurate masses of the metabolites. The following operation parameters were used: injection volume 2 μL (full scan) and 10 μL (product ion scan); capillary voltage 3500 V; nebulizer pressure 40 psi (275,790 Pa); drying gas 10.0 L/min; gas temperature 350 °C; fragmentor voltage 160 V; collision energy 10, 20 and 40 eV; skimmer voltage 60 V. HRQToFMS accurate mass spectra were recorded across the range from *m*/*z* 100 to *m*/*z* 1000. The mass axis was calibrated using the mixture provided by the manufacturer over the *m*/*z* 50–3200 range. A second orthogonal sprayer with a reference solution was used as a continuous calibration using the following reference masses: *m*/*z* 121.0509 and *m*/*z* 922.0098. The chromatographic conditions and column used were same as described above. The controls were subjected to the same analysis.