Published in final edited form as:

Drug Test Anal. 2018 January ; 10(1): 137–147. doi:10.1002/dta.2262.

Kinetic and metabolic profiles of synthetic cannabinoids NNEI and MN-18

Richard C. Kevin^{a,b,*}, Timothy W. Lefever^c, Rodney W. Snyder^c, Purvi R. Patel^c, Thomas F. Gamage^c, Timothy R. Fennell^c, Jenny L. Wiley^c, Iain S. McGregor^{a,b}, and Brian F. Thomas^c

^aSchool of Psychology, The University of Sydney, NSW, 2006, Australia

^bThe Lambert Initiative for Cannabinoid Therapeutics, The University of Sydney, NSW, 2050, Australia

^cRTI International, 3040 Cornwallis Road, Research Triangle Park, NC, 27709, USA

Abstract

In 2014 and 2015, synthetic cannabinoid receptor agonists NNEI (*N*-1-naphthalenyl-1-pentyl-1*H*-indole-3-carboxamide) and MN-18 (*N*-1-naphthalenyl-1-pentyl-1*H*-indazole-3-carboxamide) were detected in recreationally used and abused products in multiple countries, and were implicated in episodes of poisoning and toxicity. Despite this, the pharmacokinetic profiles of NNEI and MN-18 have not been characterized. In the present study NNEI and MN-18 were incubated in rat and human liver microsomes and hepatocytes, to estimate kinetic parameters and to identify potential metabolic pathways, respectively. These parameters and pathways were then examined *in vivo*, via analysis of blood and urine samples from catheterized male rats following intraperitoneal (3 mg/kg) administration of NNEI and MN-18. Both NNEI and MN-18 were rapidly cleared by rat and human liver microsomes, and underwent a range of oxidative transformations during incubation with rat and human hepatocytes. Several unique metabolites were identified for the forensic identification of NNEI and MN-18 intake. Interestingly, NNEI underwent a greater number of biotransformations (20 NNEI metabolites *versus* 10 MN-18 metabolites), yet parent MN-18 was eliminated at a faster rate than NNEI *in vivo*. Additionally, *in vivo* elimination was more rapid than *in vitro* estimates. These data highlight that even closely related synthetic cannabinoids can possess markedly distinct pharmacokinetic profiles, which can vary substantially between *in vitro* and *in vivo* models.

Keywords

Synthetic cannabinoid; Pharmacokinetics; Metabolism; NNEI; MN-18

*Corresponding author: Richard C. Kevin, Brain and Mind Centre (M02F), The University of Sydney, NSW 2050, Australia. richard.kevin@sydney.edu.au.

Conflict of Interest The authors have no conflict of interest to declare.

Introduction

Novel synthetic cannabinoid receptor agonists continue to be detected in products used for recreational purposes.^[1] Synthetic cannabinoid receptor agonists are comprised of several increasingly complex chemical classes, but are generally thought to exert their physiological and psychoactive effects via cannabinoid 1 (CB₁) receptors.^[2–3] Although blanket bans on novel psychoactive substances or novel “cannabimimetics” exist in some countries, new synthetic cannabinoids continue to be developed, possibly in an attempt to evade legislation.^[1, 4] These new compounds often possess greater potency at CB₁ receptors than their predecessors.^[5–6] Recent years have seen the proliferation of compounds that possess a carboxamide moiety, which usually acts as a linker between an indole or indazole core and a lipophilic substituent. Prominent examples include CUMYL-PICA, 5F-CUMYL-PICA, AB-PINACA, and AB-FUBINCA, among others. Several compounds of this type have been implicated in poisonings and toxicity worldwide.^[5, 7]

NNEI (*N*-1-naphthalenyl-1-pentyl-1*H*-indole-3-carboxamide) and its indazole analogue MN-18 (*N*-1-naphthalenyl-1-pentyl-1*H*-indazole-3-carboxamide) are two carboxamide synthetic cannabinoids that have been recently detected in recreational products. NNEI and MN-18 are carboxamide analogues of JWH-018 (1-pentyl-3-(1-naphthoyl)indole) and THJ-018 (1-pentyl-3-(1-naphthoyl)indazole), respectively, where a carboxamide group is substituted for a ketone linker (Figure 1). NNEI was first described in scientific literature in 2011,^[8] and was detected in recreational products shortly thereafter in Finland in 2012 and later in Japan in 2014,^[9–10] where it was subsequently implicated in a fatal poisoning.^[11] Similarly, MN-18 was first detected in Sweden in 2014 and in online products sold in Japan in 2014 and 2015,^[10, 12–13] although to our knowledge, no fatal cases involving MN-18 have been reported.

To our knowledge, the pharmacokinetic properties of NNEI and MN-18 have not been characterized. Most synthetic cannabinoids undergo extensive metabolic biotransformations, so a detailed understanding of metabolic pathways is important for identification of these compounds in biological matrices.^[14–16] Moreover, similar synthetic cannabinoids may also produce identical metabolites,^[17–18] which necessitates careful choice of targets for forensic screens. Some synthetic cannabinoid metabolites can also retain cannabinoid receptor efficacy and could be relevant to the overall pharmacological profile following drug administration.^[19–21]

This study aimed to characterize the pharmacokinetics and metabolism of NNEI and MN-18, using both *in vitro* and *in vivo* models. NNEI and MN-18 were first incubated with rat and human liver microsomes and hepatocytes to predict kinetic parameters and to identify metabolic pathways, respectively. These assays were followed by *in vivo* kinetic and metabolic assessment and confirmation in rat blood and urine. These data were used to propose metabolic pathways for NNEI and MN-18 in rats and humans, to compare the metabolic profiles of these closely related compounds, and to suggest useful analytical targets for future forensic analysis.

Materials and methods

Chemicals and reagents

NNEI and MN-18 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Rat and human liver microsomes and hepatocytes were obtained from XenoTech (Kansas City, KS, USA). Acetonitrile and formic acid were purchased from Fisher Scientific (Raleigh, NC, USA). Polysorbate 80 was purchased from Fisher (Pittsburgh, PA, USA), and saline from Patterson Vet Supply (Devens, MA, USA). All chemicals and solvents were at least ACS or high-performance liquid chromatography grade, respectively.

In vitro incubations

Rat and human liver microsomes—*In vitro* incubations were performed as detailed previously.^[18] NNEI and MN-18 were incubated at 1 μ M with male human (pooled, 10 donors) or rat (pooled IGS Sprague-Dawley, 500 donors) liver microsomes at 37 °C in triplicate. An assay mixture containing microsomes (1 mg protein/mL final concentration), NADPH (1 mol/m³ final) and a buffer consisting of 50 mol/m³ potassium phosphate buffer, pH 7.4 with 3 mM MgCl₂ was prepared and preincubated at 37 °C for 5 min. 10 μ L of a 0.1 mol/m³ drug solution in acetonitrile was added to 990 μ L of assay mixture in a glass test tube in a 37 °C water bath. As a control, an additional microsome incubation was performed as specified above with no drug added. At 0, 5, 10, 30, and 60 min timepoints, 100 μ L samples were removed added to 100 μ L acetonitrile. Samples were centrifuged (11,000 *g* for 1 min) and stored at –80 °C until analysis.

Rat and human hepatocytes—Incubations of NNEI and MN-18 at 0.01 mol/m³ with male human (pooled, 10 donors) or rat hepatocytes (pooled, 3 donors) were performed in triplicate, with 1 mL cell suspension (rat: 0.86×10^6 cells/mL, 80 % viability; human: 0.80×10^6 cells/mL, 81.6% viability) in 24-well polystyrene cell culture plates in a 37 °C incubator with 5% CO₂ atmosphere. 10 μ L of a 1 mol/m³ drug solution in acetonitrile was added to 990 μ L of hepatocyte suspension. Concurrently, an incubation was conducted using 1 mL incubation cell blank containing live cells and solvent blank as a control; this incubation was treated exactly as the cell incubations. 100 μ L samples were removed and terminated with 100 μ L acetonitrile at 0, 10, 60 and 180 min timepoints. Samples were centrifuged (11,000 *g* for 1 min) and stored at –80 °C until analysis.

In vivo dosing and sampling in rats

Animals—Eight male Long Evans rats with jugular vein catheters were purchased from Charles River Laboratories (Raleigh, NC, USA), aged 49 days. Different groups of rats (*n* = 4 per group) were used to test NNEI or MN-18. Until testing, all animals were kept in a temperature controlled environment (20–22 °C) on a 12 h light-dark cycle (lights on at 6 a.m.), with ad libitum access to food and water. All *in vivo* work was carried out in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals,^[22] and were IACUC approved.

Procedures—After dose administration, animals were placed individually into glass metabolic cages for urine sample collection (Prism Research Glass, Raleigh, NC, USA).

Animals were injected intraperitoneally with 3 mg/mL NNEI or 3 mg/mL MN-18 dissolved in vehicle solution comprised of 7.8 % Polysorbate 80 N.F. (Fisher, Pittsburgh, PA, USA) and 92.2 % Saline USP (Patterson Vet Supply, Devens, MA, USA). The injection volume was 1 mL/kg of rat body weight. 200 μ L blood samples were drawn into chilled K₃EDTA collection tubes at 15 min pre-injection and 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, and 24 h post-injection. Blood samples were centrifuged at 2800 *g* for 10 min at 4 °C, and the plasma supernatant was decanted and stored at –80 °C until further analysis. Additionally, urine samples were collected at 8 and 24 h post-injection. At 24 h post-injection, rats were euthanized via CO₂ asphyxiation, and trunk blood was rapidly collected and stored as specified above.

Analyte extraction

All extractions of analytes (parent compounds and metabolites) from all matrices (microsomes, hepatocytes, plasma, and urine) were performed as previously described.^[18] Briefly, acetonitrile was added in a 3:1 ratio to the sample volume (microsomes/hepatocytes sample volume 50 μ L; plasma 25 μ L; urine 100 μ L) and centrifuged at 4000 *g* for 15 min at 4 °C. Supernatants were transferred to vials for immediate analysis via liquid chromatography-tandem mass spectroscopy (LC-MS/MS), as detailed below.

LC-MS/MS analyses

NNEI and MN-18 were quantified using LC-MS/MS following microsome incubation and in rat plasma. The LC-MS/MS system consisted of a Waters Acquity UPLC system, equipped with a Waters BEH C18 column (100 mm $\times$ 2.1 mm i.d., particle size 1.7 μ m) (Waters Corp., Milford, MA, USA), coupled to an API 5000 tandem-mass spectrometer (AB Sciex, Framingham, MA, USA). Gradient elution was used with mobile phase solutions 0.1 % formic acid in water (A) and acetonitrile (B), which began at 10 % B for 0.5 min and was then increased to 95 % B at 10 min, was held at 95 % B until 12.5 min and then returned to 10 % B for 2.5 min with a total run time of 15 min. The mass spectrometer was operated in positive electrospray ionization mode with multiple reaction monitoring. The quantified transitions were m/z 357.0 $\rightarrow$ 214.2 for NNEI and m/z 358.1 $\rightarrow$ 215.1 for MN-18. Secondary transitions m/z 357.0 $\rightarrow$ 144.1 and 358.1 $\rightarrow$ 145.1 were used to confirm NNEI and MN-18 identity, respectively, in addition to comparison with reference standards. The calibration curve for each analyte ranged from 1 – 500 ng/mL, and JWH-018 was used as an internal standard.

Metabolites were also identified using LC-MS/MS, using a Thermo LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). This was also coupled to a Waters Acquity UPLC system (Waters Corp.), and chromatographic conditions were identical to those specified above. The mass spectrometer was operated in positive electrospray ionization mode, with scan range m/z 100–910 and capillary temperature 250 °C. Higher-energy collisional dissociation (HCD) was implemented with a collision energy of 35 eV in order to increase fragmentation for metabolite identification. Full scan data was collected at a resolution of 30,000 FWHM at m/z 400, and MS/MS data was collected, at a for the six most abundant parent ions.

Data analysis

Kinetic parameters were calculated as previously reported.^[17–18] Microsomal intrinsic clearance ($CL_{int, micr}$) and half-life were calculated from plots of chromatographic peak areas against time. Intrinsic clearance (CL_{int}) was calculated by scaling $CL_{int, micr}$ to whole liver dimensions for rats and humans.^[23–24] Hepatic clearances (CL_H) and extraction ratios (ER) were estimated based on the corresponding rat and human CL_{int} values and estimates of liver blood flow rate (rat 13.8 mL/min; human 1400 mL/min).^[25] CL_H was normalized to body weight, assuming mean body weight of 0.25 kg for rats and 70 kg for humans.^[23] Plasma concentrations of NNEI and MN-18 were calculated against a standard curve generated from corresponding calibrator samples using a reference standard. Plasma kinetic parameters were then computed from plasma concentrations of each compound via a noncompartmental analysis performed with Phoenix WinNonlin® version 6.3 (Certara, Princeton, NJ, USA).

Metabolites were identified in hepatocyte incubations, rat plasma, and rat urine using Compound Discoverer 2.0 software (Thermo Fisher Scientific) and manual inspection and interpretation of mass spectra.

Results

Liver microsome clearance

Clearance of NNEI and MN-18 occurred rapidly in rat and human liver microsome incubations (Table 1), with human microsome half-lives of 2.3 and 1.7 min, respectively. MN-18 was cleared more rapidly than NNEI in both rat and human microsomal incubations, and kinetic parameters were broadly similar between rat and human microsome incubations.

Plasma kinetics

The *in vivo* plasma kinetics of NNEI and MN-18 in rat plasma differed, as shown in Table 1 and in Figure 2. MN-18 was eliminated in a first order fashion and could not be detected in plasma after 24 h. Plasma concentrations of NNEI were approximately constant over the first 4 h, after which NNEI underwent slow and incomplete elimination over the remaining 20 h of the assay. Accordingly, MN-18 had a shorter-half life and higher apparent clearance (CL/F) than NNEI. Similar to CUMYL-PICA and 5F-CUMYL-PICA,^[18] *in vivo* clearance was less rapid than values predicted by *in vitro* microsomal incubations.

NNEI metabolism

Phase I metabolites—NNEI underwent several phase I metabolic transformations, which were primarily oxidative in nature (Table 2; Figures 3–5). The parent compound was identified by comparison to a reference standard, and eluted with a retention time (RT) of 8.87 min and a $[M+H]^+$ molecular ion at m/z 357.1932. One primary product ion was produced at m/z 214.1220, corresponding to cleavage of the carboxamide C–N bond, and a minor product ion was present at m/z 188.1449, following subsequent neutral loss of CO. A minor product ion at m/z 144.0444 was also detected, corresponding to the formation of an indole oxonium ion, which has been observed in several studies involving indole synthetic cannabinoids.^[18, 26–28]

Six monohydroxylated metabolites were observed with molecular ion m/z 373.1897. Four of these (N11, N14, N16, and N17) produced a product ion at m/z 230.1149, 15.99 Da greater than NNEI product ion 214.1220, indicating a hydroxylation on the indole or *N*-pentyl portions of the molecule. N19 and N20 eluted later and lacked this product ion, and instead retained product ion m/z 214.1220, indicating hydroxylation on the naphthyl moiety. N11 produced product ion m/z 144.0444, suggesting a hydroxylation on the *N*-pentyl chain rather than the indole group, probably on the ω or ω -1 carbon. N14, N16, and N17 instead produced an ion at m/z 160.0376 (15.99 Da greater than m/z 144.0444), pointing to an indole hydroxylation. Interestingly, N16 was not detected in human hepatocyte incubations, while N17 was exclusively detected in human hepatocyte incubations.

Three dihydroxylated metabolites were detected with a $[M+H]^+$ molecular ion at m/z 389.1837 (N3, N8, and N18). N8, which was only observed in rat hepatocyte incubations and plasma, produced a product ion at m/z 230.1149, indicating one hydroxylation on the indole or *N*-pentyl moieties, and the other on the naphthyl group. Product ion m/z 144.0444 further localized the first hydroxylation to the *N*-pentyl chain. N18 instead produced a product ion at m/z 214.1220, suggesting that both hydroxylations occurred on the naphthyl moiety, consistent with N18's late retention time compared to the other dihydroxylated metabolites. N3 produced a very small chromatographic peak, and mass spectral data was insufficient to assign a specific hydroxylation location beyond exclusion of the naphthyl group via a product ion at m/z 246.1079.

A single carbonylated metabolite (N13) was observed with molecular ion $[M+H]^+$ m/z 371.1739. This produced an ion at m/z 228.1023, 13.98 Da greater (i.e. +O -2H) than NNEI fragment 214.1220, and 2.01 Da less (i.e. -2H) than monohydroxylated metabolite (N11, N14, N16, and N17) fragment m/z 230.1149. Product ions at m/z 144.0444 and 85.0646 localized this transformation to the *N*-pentyl chain. Similar carbonylation on the *N*-pentyl has been reported for several synthetic cannabinoids.^[17, 26]

N13 was further oxidized to four additional metabolites with $[M+H]^+$ molecular ion m/z 387.1691 (N6, N9, N12 and N15). N6 had a product ion at m/z 244.096, restricting the hydroxylation to the indole or *N*-pentyl groups, and a product ion at m/z 101.0711 suggested that the hydroxylation occurred on the *N*-pentyl chain. In combination with previous reports concerning synthetic cannabinoids with an *N*-pentyl group,^[17, 26, 29] this strongly suggests the formation of a carboxylic acid metabolite. N9 had a product ion at m/z 160.0376, 15.99 Da greater than indole oxonium ion m/z 144.0444, indicating indole hydroxylation. N12 and N15 retained product ions at m/z 228.1023 and 85.0646, suggesting hydroxylation on the naphthyl group.

NNEI also underwent dealkylation, losing the *N*-pentyl chain to form metabolite N7, with $[M+H]^+$ molecular ion at m/z 287.1160, and primary product ions at m/z 144.0444 and at 144.0810. This is consistent with cleavage of the C-N bond of the carboxamide group and formation of two ions with the same nominal mass but different mass defect (indole oxonium ion $C_9H_6NO^+$ and 1-naphthylammonium ion $C_{10}H_{10}N^+$). N7 was hydroxylated to metabolites N1 and N5, which both possessed a $[M+H]^+$ molecular ion at m/z 303.1115, 15.99 Da greater than N7. N1 retained the product ion at m/z 144.0810, but also produced a

product ion at m/z 160.0376, indicating hydroxylation of the indole group. N5 only produced a product ion at m/z 144.0444, indicating hydroxylation on the naphthyl group, consistent with its later retention time compared to N1. However, N1 and N5 were not detected in human hepatocyte incubations.

No other phase I metabolites or metabolites eluting after the parent compound were detected. We considered that metabolic amide hydrolysis could liberate 1-naphthylamine, or its oxidized derivatives, but these compounds were not observed in any *in vitro* incubation or *in vivo* sample. Peak areas for all detected NNEI metabolites at each hepatocyte incubation timepoint can be found in Table S1, Supporting Information.

Phase II metabolites—NNEI underwent few phase II transformations. Following monohydroxylation, three glucuronides with $[M+H]^+$ molecular ion m/z 549.2221 were observed; N2, N4, and N10. N2 was only found in human hepatocyte incubations, and exhibited product ions at m/z 373.1922, as a result of deglucuronidation to the monohydroxylated precursor, and at m/z 230.1149 and 160.0376, indicating a glucuronide of an indole hydroxylation (i.e. N14, N16, or N17). N4 also produced a product ion at m/z 230.1149, but there was no clear ion at either m/z 160 or 144 that might be used to distinguish between indole and *N*-pentyl hydroxylations. N10 instead produced an ion at m/z 214.1220, suggesting a glucuronide of naphthyl hydroxylations N19 or N20. No other phase II transformations were observed.

MN-18 metabolism

Phase I metabolites—Only 10 metabolites were identified for MN-18, compared to 20 metabolites for NNEI (Table 3; Figures 3, 4 and 6). The RT of MN-18 was 10.13 (confirmed with a reference standard), and the $[M+H]^+$ molecular ion was m/z 358.1901, 0.99 Da greater than NNEI, as expected. Major product ions were observed at m/z 233.1305 and 215.1199, corresponding to fragmentation along the carboxamide linking group, and at m/z 145.0399, corresponding to formation of an indazole oxonium ion.

Four monohydroxylated metabolites (M5, M7, M8, and M10) were observed with a $[M+H]^+$ molecular ion at m/z 374.1848. M7 and M8 produced ions at m/z 249.1238 and 231.1153, 15.99 Da greater than MN-18 fragments 233.1305 and 215.1199, respectively, indicating hydroxylation on the indazole or *N*-pentyl moieties. A product ion at m/z 145.0399 (and absence of an ion at m/z 161) suggested that hydroxylation occurred on the *N*-pentyl chain (likely on the ω and ω -1 carbons). M5 and M10 instead retained NNEI product ions m/z 233.1305, 215.1199, and 145.0399 suggesting a hydroxylation on the naphthyl group.

A dihydroxylated metabolite (M4) was observed with m/z 390.1846 and product ions at m/z 249.1238 and 231.1153, suggesting that one hydroxylation occurred on the naphthyl group, while m/z 145.0399 localised the other hydroxylation to the *N*-pentyl chain. However, this metabolite was only detected in rat hepatocyte incubations.

Like NNEI, MN-18 was carbonylated, forming metabolite M9, which produced a molecular ion of 372.1703, 13.98 Da greater (i.e. +O –2H) than parent MN-18 at m/z 358.1901. This transformation was localized to the *N*-pentyl chain via product ions at m/z 229.0948,

145.0399, and 85.0646, using reasoning similar to that used for identification of NNEI metabolite N13. This metabolite was further oxidized (or alternatively, M5 or M10 was carbonylated), forming metabolite M2 with a molecular ion at m/z 388.1645 and product ions identical to M9, suggesting hydroxylation on the naphthyl group. Metabolite M6 also possessed a molecular ion at m/z 388.1645, but also product ions at m/z 245.0906 and m/z 217.0982, localizing the hydroxylation to the indole or *N*-pentyl moieties. A product ion at m/z 163.0508 further localized this transformation to the *N*-pentyl chain, which likely indicates a carboxylation. Surprisingly, this metabolite was only observed in human hepatocyte incubations. No other phase I metabolites or metabolites eluting after the parent compound were detected. Peak areas for all detected MN-18 metabolites at each hepatocyte incubation timepoint can be found in Table S2, Supporting Information.

Phase II metabolites—Like NNEI, MN-18 underwent few phase II transformations. Two metabolites (M1 and M3) with a $[M+H]^+$ molecular ion at m/z 550.2156 were observed – 176 Da greater than monohydroxylated metabolites, suggesting glucuronidation. M1 produced a product ion at m/z 374.1857, corresponding to deglucuronidation to the monohydroxylated precursor, and additional product ions at m/z 249.1238, 231.1100, and 145.0399, suggesting this glucuronidation occurred after hydroxylation of the *N*-pentyl chain (i.e. M7 or M8). However, this metabolite was only found following incubation with human hepatocytes. M3 also produced a product ion at m/z 374.1857, but also at m/z 233.1305 and m/z 215.1199, indicating that M3 is a glucuronide of metabolites with a monohydroxylation on the naphthyl moiety (i.e. M5 or M10).

Discussion

This study examined the clearance and metabolism of synthetic cannabinoids NNEI and MN-18 in rat and human liver microsomes and hepatocytes, and in whole animals following intraperitoneal administration of each compound in adult male rats. Both compounds underwent a series of oxidative transformations, but NNEI was more extensively metabolized than MN-18. Despite this, the *in vivo* half-life of NNEI was greater than that of MN-18, which was eliminated rapidly. Most metabolites found in rat hepatocyte incubations were also detected in rat plasma, although only a small number of metabolites were detected in urine. Additionally, *in vitro* kinetic calculations overestimated actual *in vivo* elimination rates.

Comparison of metabolic processes for indole and indazole synthetic cannabinoids

Metabolism of NNEI and MN-18 proceeded primarily via phase I oxidations followed by phase II glucuronidation. However, a point of interest was the relatively small number of metabolic transformations for MN-18 compared to NNEI. 20 metabolites were detected for NNEI compared to only 10 for MN-18. MN-18 did not undergo oxidations on the indazole ring system, and did not undergo dealkylation of the *N*-pentyl chain. Some other indazole synthetic cannabinoids partially reflect this pattern; AB-PINACA and ADB-FUBINACA do not appear to be hydroxylated on the indazole moiety.^[15] However, AB-FUBINACA is apparently susceptible to indazole hydroxylations,^[14] as are AMB and 5F-AMB.^[17] Perhaps unsurprisingly, this suggests that the overall metabolic profile of a given synthetic

cannabinoid is underpinned by complex interactions between the structural subunits that comprise each compound, such as the lipophilic substituent (e.g. a naphthyl group) or “tail” (e.g. an *N*-pentyl chain or *N*-(4-fluorobenzyl) group). At present, these interactions are difficult to predict, which underscores the need for further *in vitro* and *in vivo* studies of the metabolic profiles of novel synthetic cannabinoids. In this case, further kinetic evaluation of indole and indazole pairings could prove valuable in determining whether the differences seen between indole NNEI and indazole MN-18 is a general trend.

In rat and human liver microsomes and in rat plasma, MN-18 was consistently cleared more quickly than NNEI (Table 1). This was reflected by urinary metabolite concentrations; semi-quantitatively, MN-18 metabolites were more abundant at 8 h post-injection compared to 24 h post-injection, whereas the reverse was true for NNEI (Figure 4). At present we can only speculate as to why this is the case; presumably MN-18 undergoes rapid transformation to one or more metabolite whereas NNEI is more stable, despite being eventually metabolized into a wider array of compounds. Rat urinary chromatographic data suggests that *in vivo*, MN-18 was primarily metabolized to the naphthyl-monohydroxylated metabolite M5, before further glucuronidation to metabolite M3 (Figure 4). Further investigation with metabolite reference standards could confirm predominant metabolic pathways for each compound.

***In vitro* versus *in vivo* kinetics**

Estimation of the pharmacokinetic parameters of novel synthetic cannabinoids using liver microsomes and hepatocytes is common,^[15–17, 26, 30–32] but such reports are not always accompanied by *in vivo* data. In the present study, it was noteworthy that *in vivo* elimination of NNEI and MN-18 from rat blood was slower than that predicted by *in vitro* incubations in rat microsomes. We have reported similar findings concerning the kinetics of synthetic cannabinoids CUMYL-PICA and 5F-CUMYL-PICA.^[18] Synthetic cannabinoids are usually lipophilic, and it has been demonstrated that AB-CHMINACA and 5F-AMB are sequestered into adipose tissue.^[33] Similar sequestration of NNEI and MN-18 into rat adipose tissue could account for delayed elimination in our *in vivo* model. Alternatively, blood protein binding of NNEI and MN-18 could contribute to this delay. Our kinetic calculations did not account for this factor because blood protein binding is not characterized for these compounds. Regardless of the underlying mechanism, these data emphasize that some caution is necessary when attempting to translate *in vitro* synthetic cannabinoid kinetic data to *in vivo* models.

Useful metabolites for the identification of NNEI and MN-18 intake

Close structural relationships between many synthetic cannabinoids necessitates careful selection of analytical targets for forensic screens. Importantly, convergence of metabolic pathways for NNEI and MN-18 was not observed, because the indole or indazole core remained intact in all detected metabolites. Therefore, any of the metabolites presented here could be used to distinguish between NNEI and MN-18 intake. However, it should be noted that the 5-fluoropentyl analogues of NNEI and MN-18 could produce identical metabolites to some of those detected in the present study. For example oxidative defluorination of 5F-NNEI (1-(5-fluoropentyl)-*N*-(naphthalen-1-yl)-1*H*-indole-3-carboxamide) could lead to formation of an identical carboxylic acid metabolite (metabolite N6). This metabolic

convergence occurs for other fluorinated and *des*-fluorinated pairings including JWH-018 and AM-2201, PB-22 and 5F-PB-22, AMB and 5F-AMB, and CUMYL-PICA and 5F-CUMYL-PICA.^[16–18, 27] Therefore, metabolites that arise from hydroxylations on the naphthyl moiety of NNEI or MN-18 may be the most useful for unique identification of NNEI and MN-18 exposure - particularly N19, N10, M3, and M10, which were found in both blood and urine (Tables 2 and 3, Figure 4). These metabolites were observed at levels clearly above detection thresholds, can be readily distinguished from other hydroxylations by product ions at *m/z* 214 and 215 for NNEI and MN-18, respectively, and should not form during the metabolism of 5-fluoropentyl analogues.

Conclusions

This study characterized, for the first time, the metabolism of NNEI and MN-18 in rat and human hepatocytes and *in vivo* in male rats. Comparison of the metabolic profile of NNEI to its indazole analogue MN-18 revealed marked differences in pharmacokinetic parameters and metabolic pathways. While both NNEI and MN-18 were rapidly cleared by rat and human liver microsomes, NNEI underwent a greater number of biotransformations (hepatocyte incubations generated 20 NNEI metabolites *versus* 10 MN-18 metabolites), but parent MN-18 was eliminated at a faster rate than NNEI *in vivo*. Moreover, *in vitro* kinetic calculations overestimated *in vivo* elimination rates. Further pharmacokinetic evaluation of similar indole/indazole pairings could prove informative. With respect to analytical screening, metabolites formed via oxidation of the naphthyl moiety of NNEI and MN-18 are proposed as useful analytes for unique identification of NNEI and MN-18 intake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RCK was supported by an Australian Postgraduate Award. ISM was supported by a National Health and Medical Research Council fellowship. This work was supported by research grant funding to BFT from the National Institute on Drug Abuse (1R01DA-040460), to JLW from the National Institutes of Health (DA-03672), internal research and development funds provided by the Research Triangle Institute, NC, USA, and by the Lambert Initiative for Cannabinoid Therapeutics, NSW, Australia.

References

1. EMCDDA. [Accessed May 5, 2017] European Drug Report. 2016. <http://www.emcdda.europa.eu/system/files/publications/2637/TDAT16001ENN.pdf>
2. Wiley JL, Marusich JA, Lefever TW, Grabenauer M, Moore KN, Thomas BF. Cannabinoids in disguise: ⁹-tetrahydrocannabinol-like effects of tetramethylcyclopropyl ketone indoles. *Neuropharmacology*. 2013; 75:145–154. [PubMed: 23916483]
3. Banister SD, Moir M, Stuart J, et al. The pharmacology of indole and indazole synthetic cannabinoid designer drugs AB-FUBINACA, ADB-FUBINACA, AB-PINACA, ADB-PINACA, 5F-AB-PINACA, 5F-ADB-PINACA, ADBICA and 5F-ADBICA. *ACS Chem. Neurosci*. 2015; 6(9):1546–1559. [PubMed: 26134475]
4. EMCDDA. [Accessed May 5, 2017] European Drug Report. 2015. http://www.emcdda.europa.eu/attachements.cfm/att_239505_EN_TDAT15001ENN.pdf

5. Adams AJ, Banister SD, Irizarry L, Trecki J, Schwartz M, Gerona R. “Zombie” outbreak caused by the synthetic cannabinoid AMB-FUBINACA in New York. *N. Engl J Med.* 2017; 376(3):235–242. [PubMed: 27973993]
6. Banister SD, Longworth M, Kevin RC, et al. Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. *ACS Chem. Neurosci.* 2016; 7(9):1241–1254. [PubMed: 27421060]
7. Schwartz MD, Trecki J, Edison LA, Steck AR, Arnold JK, Gerona RR. A common source outbreak of severe delirium associated with exposure to the novel synthetic cannabinoid ADB-PINACA. *J Emerg Med.* 2015; 48(5):573–580. [PubMed: 25726258]
8. Blaazer AR, Lange JHM, van der Neut MAW, et al. Novel indole and azaindole (pyrrolopyridine) cannabinoid (CB) receptor agonists: Design, synthesis, structure-activity relationships, physicochemical properties and biological activity. *Eur J Med Chem.* 2011; 46(10):5086–5098. [PubMed: 21885167]
9. EMCDDA. [Accessed May 5, 2017] New drugs in Europe. 2012. http://www.emcdda.europa.eu/system/files/publications/734/EMCDDA-Europol_2012_Annual_Report_final_439477.pdf
10. Uchiyama N, Matsuda S, Kawamura M, et al. Characterization of four new designer drugs, 5-chloro-NNEI, NNEI indazole analog, α -PHPP and α -POP, with 11 newly distributed designer drugs in illegal products. *Forensic Sci Int.* 2014; 243:1–13. [PubMed: 24769262]
11. Sasaki C, Saito T, Shinozuka T, et al. A case of death caused by abuse of a synthetic cannabinoid N-1-naphthalenyl-1-pentyl-1H-indole-3-carboxamide. *Forensic Toxicol.* 2015; 33(1):165–169.
12. Uchiyama N, Shimokawa Y, Kikura-Hanajiri R, Demizu Y, Goda Y, Hakamatsuka T. A synthetic cannabinoid FDU-NNEI, two 2H-indazole isomers of synthetic cannabinoids AB-CHMINACA and NNEI indazole analog (MN-18), a phenethylamine derivative N-OH-EDMA, and a cathinone derivative dimethoxy- α -PHP, newly identified in illegal products. *Forensic Toxicol.* 2015; 33(2): 244–259. [PubMed: 26257833]
13. EMCDDA. [Accessed May 5, 2017] Annual Report on the Implementation of the Council Decision 2005/387/JHA. <http://www.emcdda.europa.eu/system/files/publications/1018/TDAN15001ENN.pdf>
14. Vikingsson S, Green H, Brinkhagen L, Mukhtar S, Josefsson M. Identification of AB-FUBINACA metabolites in authentic urine samples suitable as urinary markers of drug intake using liquid chromatography quadrupole tandem time of flight mass spectrometry. *Drug Test Anal.* 2015; 8(9): 950–956. [PubMed: 26560240]
15. Takayama T, Suzuki M, Todoroki K, et al. UPLC/ESI-MS/MS-based determination of metabolism of several new illicit drugs, ADB-FUBINACA, AB-FUBINACA, AB-PINACA, QUPIC, 5F-QUPIC and α -PVT, by human liver microsome. *Biomed. Chromatogr.* 2014; 28(6):831–838. [PubMed: 24861751]
16. Wohlfarth A, Gandhi AS, Pang S, Zhu M, Scheidweiler KB, Huestis MA. Metabolism of synthetic cannabinoids PB-22 and its 5-fluoro analog, 5F-PB-22, by human hepatocyte incubation and high-resolution mass spectrometry. *Anal Bioanal Chem.* 2014; 406(6):1763–1780. [PubMed: 24518903]
17. Andersson M, Diao X, Wohlfarth A, Scheidweiler KB, Huestis MA. Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. *Rapid Commun Mass Spectrom.* 2016; 30(8):1067–1078. [PubMed: 27003044]
18. Kevin RC, Lefever TW, Snyder RW, et al. In vitro and in vivo pharmacokinetics and metabolism of synthetic cannabinoids CUMYL-PICA and 5F-CUMYL-PICA. *Forensic Toxicol.* 2017; 35(2): 333–347. [PubMed: 28824730]
19. Seely KA, Brents LK, Radomska-Pandya A, et al. A major glucuronidated metabolite of JWH-018 is a neutral antagonist at CB1 receptors. *Chem Res Toxicol.* 2012; 25(4):825–827. [PubMed: 22404317]
20. Brents LK, Gallus-Zawada A, Radomska-Pandya A, et al. Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity. *Biochem Pharmacol.* 2012; 83(7): 952–961. [PubMed: 22266354]

21. Brents LK, Reichard EE, Zimmerman SM, Moran JH, Fantegrossi WE, Prather PL. Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLoS One*. 2011; 6(7):e21917. [PubMed: 21755008]
22. National Research Council. Guide for the Care and Use of Laboratory Animals. 8. Washington, D.C.: National Academies Press; 2011.
23. Davies B, Morris T. Physiological parameters in laboratory animals and humans. *Pharm Res*. 1993; 10(7):1093–1095. [PubMed: 8378254]
24. McNaney CA, Drexler DM, Hnatyshyn SY, et al. An automated liquid chromatography-mass spectrometry process to determine metabolic stability half-life and intrinsic clearance of drug candidates by substrate depletion. *Assay Drug Dev Technol*. 2008; 6(1):121–129. [PubMed: 18336089]
25. Baranczewski P, Stanczak A, Sundberg K, et al. Introduction to in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. *Pharmacol Rep*. 2006; 58(4):453–472. [PubMed: 16963792]
26. Diao X, Carlier J, Scheidweiler KB, Huestis MA. In vitro metabolism of new synthetic cannabinoid SDB-006 in human hepatocytes by high-resolution mass spectrometry. *Forensic Toxicol*. 2017; 35(2):252–262.
27. Sobolevsky T, Prasolov I, Rodchenkov G. Detection of urinary metabolites of AM-2201 and UR-144, two novel synthetic cannabinoids. *Drug Test Anal*. 2012; 4(10):745–753. [PubMed: 23042760]
28. Grigoryev A, Kavanagh P, Melnik A. The detection of the urinary metabolites of 1-[(5-fluoropentyl)-1H-indol-3-yl]-(2-iodophenyl)methanone (AM-694), a high affinity cannabinimimetic, by gas chromatography - mass spectrometry. *Drug Test Anal*. 2013; 5(2):110–115. [PubMed: 22522907]
29. Rajasekaran M, Brents LK, Franks LN, Moran JH, Prather PL. Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. *Toxicol Appl Pharmacol*. 2013; 269(2):100–108. [PubMed: 23537664]
30. Thomsen R, Nielsen LM, Holm NB, Rasmussen HB, Linnet K, Consortium TI. Synthetic cannabinimimetic agents metabolized by carboxylesterases. *Drug Test Anal*. 2014; 7(7):565–576. [PubMed: 25346527]
31. Wohlfarth A, Pang S, Zhu M, et al. First metabolic profile of XLR-11, a novel synthetic cannabinoid, obtained by using human hepatocytes and high-resolution mass spectrometry. *Clin Chem*. 2013; 59(11):1638–1648. [PubMed: 24014837]
32. Franz F, Angerer V, Brandt SD, et al. In vitro metabolism of the synthetic cannabinoid 3, 5-AB-CHMFUPPYCA and its 5, 3-regioisomer and investigation of their thermal stability. *Drug Test Anal*. 2017; 9(2):311–316. [PubMed: 26888282]
33. Hasegawa K, Wurita A, Minakata K, et al. Postmortem distribution of AB-CHMINACA, 5-fluoro-AMB, and diphenidine in body fluids and solid tissues in a fatal poisoning case: usefulness of adipose tissue for detection of the drugs in unchanged forms. *Forensic Toxicol*. 2015; 33(1):45–53.

Figure 1. Structures of **a** NNEI and **b** MN-18, compared to predecessor ketone analogues **c** JWH-018 and **d** THJ-018, and similar carboxamide synthetic cannabinoids **e** CUMYL-PICA and **f** CUMYL-PINACA.

Figure 2. Mean plasma concentrations of **a** NNEI and **b** MN-18 following a 3 mg/kg intraperitoneal injection in male rats ($n = 4$). Error bars are SEM

Figure 3.

Combined extracted ion chromatograms of **a** NNEI and **b** MN-18 metabolites after 3 h incubation with rat hepatocytes, or 3 h incubation with human hepatocytes (dashed trace, for metabolite peaks N2, N9, N17, M1, and M6), obtained by liquid chromatography – single-stage-mass spectrometry. Large peaks are truncated to maintain visibility of smaller peaks. *RT* retention time.

Figure 4. Combined extracted ion chromatograms of **a** NNEI and **b** MN-18 metabolites in rat urine 8 h post-injection (dashed trace) and 24 h (solid trace), obtained by liquid chromatography – single-stage-mass spectrometry of a single representative urine sample. *RT* retention time.

Figure 5.

Proposed NNEI metabolic pathways in rats and humans.

^R Metabolite was only observed in rat hepatocyte incubations, rat plasma, or rat urine^H Metabolite was only observed in human hepatocyte incubations[#] Product ion $m/z 144.08$ is distinct from indole oxonium product ion $m/z 144.04$. See text for further detail

Figure 6.

Proposed MN-18 metabolic pathways in rats and humans.

^R Metabolite was only observed in rat hepatocyte incubations, rat plasma, or rat urine^H Metabolite was only observed in human hepatocyte incubations

Table 1

Pharmacokinetic parameters of NNEI and MN-18 incubated in rat and human liver microsomes *in vitro* and in rat plasma *in vivo*

Pharmacokinetic parameter	NNEI	MN-18
<i>Rat liver microsomes</i>		
Half-life (min)	2.21	1.61
$CL_{int, \text{ micr}}$ (mL/min/mg)	0.31	0.43
CL_{int} (mL/min/kg body wt.)	564.4	774.8
CL_H (mL/min/kg body wt.)	50.28	51.53
ER	0.91	0.93
<i>Human liver microsomes</i>		
Half-life (min)	2.29	1.71
$CL_{int, \text{ micr}}$ (mL/min/mg)	0.30	0.41
CL_{int} (mL/min/kg body wt.)	350.2	469.0
CL_H (mL/min/kg body wt.)	18.92	19.18
ER	0.95	0.96
<i>Rat plasma</i>		
Half-life (h)	11.55	5.40
CL/F (mL/min/kg body wt.)	18.8	191.5
C_{max} (ng/mL)	296	213
T_{max} (h)	2.63	0.25
AUC 0–24 h (h.ng/mL)	4240	304
AUC 0– ∞ (h.ng/mL)	5964	314

AUC area under the curve, *CL/F* observed apparent clearance; *CL_H* estimated hepatic clearance; *CL_{int}* estimated intrinsic clearance; *CL_{int, micr}* intrinsic microsome clearance; *C_{max}* mean maximum observed concentration; *ER* extraction ratio; *T_{max}* mean time of *C_{max}* PK determinations in rat plasma represent the mean of 4 values.

Table 2

NNEI metabolites following incubation with rat and human hepatocytes, and in rat plasma and urine following a 3 mg/kg i.p. injection

Metabolite	RT (min)	Transformation	Molecular ion [M+H] ⁺ (m/z)	Mass error (ppm)	Major product ion(s) (m/z)	Rat hepatocyte	Human hepatocyte	Rat plasma	Rat urine
N1	5.30	Dealkylation (N-pentyl) + hydroxylation (indole)	303.1117	-3.6	160, 144.08, 144.04	✓			✓
N2	5.67	Hydroxylation (indole) + glucuronidation	549.2221	-1.8	373, 230, 160		✓		
N3	5.72	Dihydroxylation	389.1837	-5.7	246	✓	✓	✓	✓
N4	5.97	Hydroxylation (N-pentyl, indole) + glucuronidation	549.2220	-2.0	373, 230	✓	✓		✓
N5	6.14	Dealkylation (N-pentyl) + hydroxylation (naphthyl)	303.1115	-4.3	144	✓			
N6	6.33	Carboxylation (N-pentyl)	387.1691	-3.1	244, 210, 101, 85	✓	✓		
N7	6.36	Dealkylation (N-pentyl)	287.1260	-6.6	144	✓	✓		
N8	6.53	Dihydroxylation (N-pentyl, naphthyl)	389.1837	-5.7	230, 144	✓	✓	✓	
N9	6.61	Carboxylation + hydroxylation (indole)	387.1690	-3.4	244, 160		✓		
N10	6.64	Hydroxylation (naphthyl) + glucuronidation	549.2211	-3.6	373, 214	✓	✓	✓	✓
N11	6.71	Hydroxylation (N-pentyl)	373.1897	-3.8	230, 204, 144	✓	✓	✓	✓
N12	6.86	Carboxylation (N-pentyl) + hydroxylation (naphthyl)	387.1691	-3.1	228, 144, 85	✓			
N13	7.05	Carboxylation (N-pentyl)	371.1739	-4.0	228, 144, 85	✓	✓	✓	
N14	7.07	Hydroxylation (indole)	373.1897	-3.8	230	✓	✓	✓	
N15	7.17	Carboxylation (N-pentyl) + hydroxylation (naphthyl)	387.1691	-3.1	228, 144, 85	✓			
N16	7.35	Hydroxylation (indole)	373.1898	-3.5	230	✓			
N17	7.44	Hydroxylation (indole)	373.1897	-3.8	230		✓		
N18	7.54	Dihydroxylation (naphthyl)	389.1837		214	✓			
N19	7.92	Hydroxylation (naphthyl)	373.1898	-3.5	214	✓	✓	✓	✓
N20	8.72	Hydroxylation (naphthyl)	373.1897	-3.8	214	✓	✓		
NNEI	8.87	Parent compound	357.1932	-8.1	214, 188, 144	✓	✓	✓	

Ticks denote detection of compounds in a given matrix.

RT: retention time.

Table 3

MN-18 metabolites following incubation with rat and human hepatocytes, and in rat plasma and urine following a 3 mg/kg i.p. injection

Metabolite	RT (min)	Transformation	Molecular ion [M+H] ⁺ (m/z)	Mass error (ppm)	Major product ions (m/z)	Rat hepatocyte	Human hepatocyte	Rat plasma	Rat urine
M1	6.01	Hydroxylation (N-penty) + glucuronidation	550.2156	-5.1	374, 356, 249, 231, 213, 145		✓		
M2	6.40	Carbonylation (N-penty) + hydroxylation (naphthyl)	388.1645	-2.6	229, 163, 145, 85	✓	✓		
M3	6.89	Hydroxylation (naphthyl) + glucuronidation	550.2163	-3.8	374, 233, 215	✓	✓	✓	✓
M4	6.90	Dihydroxylation (naphthyl, N-penty)	390.1846	-8.7	233	✓			
M5	7.02	Hydroxylation (naphthyl)	374.1851	-3.2	233, 215, 145	✓	✓	✓	
M6	7.29	Carboxylation	388.1645	-2.8	245, 227, 217, 163		✓		
M7	7.46	Hydroxylation (N-penty)	374.1849	-3.7	249, 231, 213, 145	✓	✓	✓	
M8	7.84	Hydroxylation (N-penty)	374.1848	-4.0	249, 231, 213, 145	✓	✓	✓	
M9	7.91	Carbonylation (N-penty)	372.1703	-0.81	229, 163, 145, 85	✓	✓	✓	
M10	8.63	Hydroxylation (naphthyl)	374.1847	-4.3	233, 215, 145	✓	✓	✓	✓
MN-18	10.13	Parent compound	358.1901	-3.6	233, 215, 145	✓	✓	✓	

Ticks denote detection of compounds in a given matrix.

RT=retention time.