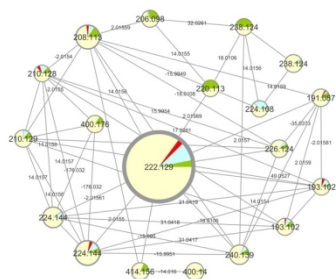


CLEAN COPY - ACCEPTED MANUSCRIPT



Metabolism elucidation of 2-Fluoro-Deschloroketamine (2F-DCK) using molecular networking in three complementary models (*in vitro*, *in cadaver*)

Thomas Gicquel, Romain Pelletier, Camille Richeval, Alexandr Gish, Florian Hakim, Vadim Mesli, Delphine Allorge, Isabelle Morel, Jean-michel Gaulier

Molecular network approach (1) allows a 2F-DCK metabolic profile proposal with evaluation using authentic biological *post mortem* samples, and (2) suggests nor-2F-DCK and dihydro-nor-FDCK metabolites as robust, specific and sensitive biomarkers of 2F-DCK use.

Graphical Abstract

318x90mm (150 x 150 DPI)

1
2
3
4
5
6
7
8
9

**Metabolite elucidation of 2-Fluoro-Deschloroketamine (2F-DCK) using
molecular networking across three complementary *in vitro* and *in vivo* models.**

10
11
12
13
14
15
16
17
18
19
20
21
22

Thomas Gicquel^{1,2*}, Romain Pelletier¹, Camille Richeval^{3,4}, Alexandr Gish³, Florian Hakim^{3,4}, Pierre-Jean Ferron², Vadim Mesli⁵, Delphine Allorge^{3,4}, Isabelle Morel^{1,2}, Jean-michel Gaulier^{3,4}

23
24
25

1 - CHU Rennes, Laboratoire de toxicologie biologique et médicolégale, F-Rennes 35033, France

26
27
28

2 - Univ Rennes, INSERM, INRAE, CHU Rennes, Institut NuMeCan (Nutrition, Métabolismes et Cancer), F-35033 Rennes, France.

29
30

3 - CHU Lille, Unité Fonctionnelle de Toxicologie, F-59000 Lille, France

31
32
33

4 - Univ. Lille, ULR 4483 – IMPECS – IMPact de l'Environnement Chimique sur la Santé humaine, F-59000 Lille, France

34
35
36

5 - CHU Lille, Service de Médecine Légale, F-59000 Lille, France

37
38

* Correspondence to:

39
40
41
42
43

Dr. Thomas Gicquel (ORCID: 0000-0002-2354-1884 and Publons: Q-1880-2019) Laboratoire de toxicologie biologique et medico-légale, CHU Pontchaillou, 2 Rue Henri Le Guilloux, 35000 Rennes.

44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

thomas.gicquel@chu-rennes.fr

Abstract

This work first aims to investigate metabolites of 2-Fluoro-Deschloroketamine (2F-DCK), a new arylcyclohexylamine derivatives (a group of dissociative ketamine-based substances) using two *in vitro* experimental approaches, and to compare obtained results by means of molecular networking. Metabolites of 2F-DCK were investigated using both human liver microsomes (HLMs) and hepatic (HepaRG) cell line incubates using molecular networking approach: 2F-DCK pure substance was incubated with HLMs for up to 1h at two concentrations (100 and 500 μM) and with HepaRG cells for two time periods (8h and 24h) at one concentration (20 μM). *In vitro* obtained results were subsequently applied to a 2F-DCK-related fatality case. *In vitro*-produced metabolites were investigated using high-resolution accurate mass spectrometry using Orbitrap mass analyzer technology. Thirteen metabolites were *in vitro* produced and several metabolic pathways can be postulated. Seven additional metabolites were found in *post-mortem* samples (bile and urine) of the case, comprising three phase II metabolites, which appear to be minor *in vivo* metabolites. HLMs and HepaRG cell models appear to be complementary and obtained data allowed the identification of several specific 2F-DCK metabolites in biological samples. In practical terms, observed metabolic ratios suggested that nor-2F-DCK (208.1137 m/z) and a hydrogenated metabolite (224.1443 m/z) could be proposed as reliable metabolites to be recorded in HRMS libraries in order to improve detection of 2F-DCK use.

Keywords: New Psychoactive Substances, 2F-DCK, Metabolism, molecular networking, microsomes, HepaRG cell line

1. Introduction

New arylcyclohexylamine derivatives (NADs), a group of dissociative substances obtained by slight chemical modification on ketamine scaffold, are worldwide emerging (1,2). A ketamine analogue called “2-Fluoro-Deschloroketamine”, “2F-DCK” or “2FK”, was recently identified (2–7). A large retrospective study in New York City between 2003 and 2020 showed an increase in traffic fatalities related to ketamine and derivative use, including 2F-DCK (8). 2F-DCK emerged especially in Asian countries where a recent study showed the extent of ketamine and 2F-DCK consumption through their quantification in wastewater from large Chinese cities (9).

Based on its arylcyclohexylamine structure, 2F-DCK is a dissociative anaesthetic N-methyl-D-aspartate (NMDA) glutamatergic receptor antagonist. Nevertheless, there is currently not much information available about the pharmacology, toxicology, and metabolism of 2F-DCK. Furthermore, due to the high frequency of new products in the new psychoactive substances (NPS) area and, consequently, rapidly changing NPS landscape, the detection of NPS-related intoxication case represents a general analytical challenge for toxicologists (10). This issue is linked to several pitfalls including the fact that NPS, such as 2F-DCK, cannot be generally detected using routine laboratory immunoassay tests (*i.e.* screening tests for ecstasy/amphetamine, opiates, cocaine, and cannabis). Moreover, due to their extensive metabolism, the identification of target metabolites is often necessary in order to increase the chance of detecting NPS consumption and to enlarge the detection window in biological matrices from clinical and forensic cases (11–13).

To address this latter problem, NPS metabolism investigations using *in vitro* models, such as pooled human liver microsomes (HLMs) and HepaRG differentiated cell line, are now commonly used (14,15).

In addition, molecular networking (MN) approach offers valuable insights into xenobiotic metabolism by propagating structural information within the network and facilitating sample-to-sample comparison (16). Indeed, this original tool allows organization and visualization of tandem mass spectrometry (MS/MS) data in a graphical representation (17). In this way, MN displays visual representation of chemical similarity among analytes of an untargeted MS/MS analysis: each node represents an ion and its associated fragmentation spectrum, and the links between different nodes indicate similarities between MS² spectra. Applied in a multimatrix approach, MN provides a semi-quantitative visualization of molecule repartition in different

1
2
3 samples. MN has already shown its benefit in drug metabolism studies, *in vitro* and *in*
4 *vivo* (18–21), specifically in NPS intoxication cases (16, 22).
5
6
7

8 For the first time, we investigated 2F-DCK metabolism using two complementary *in vitro*
9 models, namely HLMs and HepaRG cell line, and compared obtained results using molecular
10 networking. *In vivo* 2F-DCK metabolism was then investigated by the analysis of authentic
11 *post-mortem* biological samples (peripheral blood, urine, bile, vitreous humor) from a recently
12 reported 2F-DCK-related fatal case (5).
13
14
15
16
17
18

19 2. Material and Methods

20 *Material*

21 β -OH-ethyltheophyllin (internal standard), methyl-clonazepam (internal standard),
22 ammonium formate, formic acid, sulfosalicylic acid dihydrate, alamethicin (*Trichoderma*
23 *viride*), uridine diphosphate glucuronic acid (UDPGA), glucose-6-phosphate dehydrogenase
24 (G6PD), glucose-6-phosphate (G6P), and 5-sulfosalicylic acid, were all purchased from
25 Sigma-Aldrich (Saint-Quentin-Fallavier, France). Hydrocortisone hemisuccinate was
26 purchased from Serb (Paris, France). Dimethyl sulfoxide (DMSO) was obtained from Sigma-
27 Aldrich (Saint Louis, MO USA). Tetra-sodium salt of a reduced form of nicotinic acid
28 adenine dinucleotide phosphate (NADPH) was purchased from Roche (Mannheim, Germany).
29 Insulin was obtained from Sigma-Aldrich (Saint Louis, MO, USA). William's E medium
30 (Gibco, ThermoFischer Scientific, San Jose, CA) supplemented with 10% fetal bovine serum
31 (Lonza, Verviers, Belgium), penicillin-streptomycin, and L-glutamine were purchased from
32 Life Technologies (Eugene, OR, USA). William's E medium, penicillin-streptomycin, L-
33 glutamine and trypsin were purchased from ThermoFisher Scientific (Saint Aubin, France).
34 2F-DCK powder was a seized sample of purity greater than 95% (the remaining 5%
35 consisting in inorganic compounds) and in which no significant organic impurity was detected
36 using both liquid chromatography with high-resolution mass spectrometry detection (LC-
37 HRMS) and nuclear magnetic resonance (NMR) methods (5). LC-MS grade water, methanol,
38 and acetonitrile were purchased from Biosolve (Dieuze, France) and all other chemicals such
39 as zinc sulfate were of analytical grade and obtained from common commercial sources. Fetal
40 Bovine Serum (FBS) was purchased from Eurobio (Courtaboeuf, France) and from Hyclone
41 GE Healthcare Life Sciences (Logan, UT USA).
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57

58 *HLM incubates*

1
2
3
4 HLM incubates were prepared as already reported at concentrations usually used (13,14).
5
6 Once thawed, HLMs (2 mg protein/mL for a final volume of 100 μ L) were pre-activated by
7
8 alamethicin on ice in an intermediate volume of 50 μ L in 0.1 M Tris-HCl-MgCl₂(10 mM
9
10 MgCl₂ and 100 mM Tris-HCl solution) at pH 7.4. This mixture was added to a ~~50~~-dried
11
12 residue of two different concentrations of a 2F-DCK solution (100 and 500 μ M). Fifty μ L of a
13
14 cofactor mixture (5 mM UDPGA, 1.3 mM NADPH, 3.3 mM G6P and 0.5 U/mL G6PD) in
15
16 0.1 M Tris-HCl was then added. The enzymatic reaction was performed at 37 °C for 60 min
17
18 and stopped by the addition of 100 μ L of methanol. Samples were freezed at -20°C until
19
20 analysis.

21 ***HepaRG cell culture and treatment.***

22
23 The HepaRG cell line was obtained from a liver tumor of a female patient suffering from
24
25 hepatocarcinoma and was cultured and treated as described previously (21). Briefly, HepaRG
26
27 cells were seeded at a density of 10⁵ cells/well in 96-well plates and cultured during two
28
29 weeks in culture medium (William's E medium supplemented with 10% FBS, 50 U/mL
30
31 penicillin, 50 μ g/mL streptomycin, 5 μ g/mL insulin, 2 mM glutamine, 50 μ M sodium
32
33 hydrocortisone hemisuccinate and 2% DMSO. Cells were then cultured during two additional
34
35 weeks in the same type of medium supplemented with 2% DMSO to induce cell
36
37 differentiation into cholangiocyte- and hepatocyte-like cells expressing liver-specific
38
39 functions. The detection of 2F-DCK and its metabolites was performed using this coculture
40
41 model. Differentiated HepaRG cells were incubated with 100 μ L of 2F-DCK for 8 or 24 hours
42
43 at 20 μ M, a non-cytotoxic concentration. Cell culture supernatants were removed and freezed
44
45 at -20°C until analysis.

46 ***Biological samples***

47
48 Peripheral blood, bile, vitreous humor, and urine were *post-mortem* samples collected in a
49
50 fatal self-intoxication case related to 2F-DCK (5). This fatality involved two other NPS (3-
51
52 MeO-PCE and 5-MeO-DMT) as well as recent use of classical illicit drugs (THC, cocaine,
53
54 and amphetamine). 2F-DCK concentrations determined by LC-HRMS were 1780, 6100,
55
56 12000, and 1500 μ g/L in the victim's peripheral blood, urine, bile, and vitreous humor,
57
58 respectively (5).

59 ***Extraction step***

60
61 Biological, HLM incubates, and HepaRG supernatant samples were extracted as reported
62
63 previously (19,21). Briefly, 200 μ L of each sample were supplemented with ~~500~~ of
64
65 methanol containing internal standards (methyl-clonazepam at 1.25 mg/L and β -OH-
66
67 ethyltheophyllin at 16 mg/L) and then extracted with 300 μ L of 0.1 M zinc sulfate solution.

1
2
3 After supernatant evaporation, the residue was dissolved in 200 μL of LC–MS grade water
4 and transferred into chromatographic vials for LC–HRMS analysis.

6 ***LC–HRMS/MS analysis***

7
8 Liquid chromatography–mass spectrometry (LC–MS) analyses were carried out using
9 Orbitrap Q Exactive mass spectrometer coupled to an UltiMate 3000 pump (Thermo
10 Scientific, San Jose, CA). A heated electrospray ionization source (HESI-II) was used for the
11 ionization of the target compounds. Data acquisition, calibration, and instrument control were
12 performed using Xcalibur 2.1 (Thermo Scientific) software. Samples were maintained at 15
13 $^{\circ}\text{C}$ in the autosampler and quality controls were injected before each analysis. A non-targeted
14 screening LC–HRMS/MS method used for MN building was performed as follows : the
15 mobile phases were composed of ammonium formiate at 2 mM and formic acid 0.1% in water
16 (phase A) and ammonium formiate at 2 mM and formic acid 0.1% in methanol and
17 acetonitrile (50/50) (phase B). LC was performed on a Accucore Phenyl Hexyl (100 \times 2.1
18 mm, 2.6 μm) (Thermo Scientific) using the following gradient elution: initial conditions of
19 99:1 (A:B) maintained for 1 min, increasing to 1:99 (A:B) for 9 min, followed by a 1.5 min
20 plateau with 1:99 (A:B) and return to initial conditions 99:1 (A:B) for equilibration. This
21 corresponded to a total chromatographic run of 15 min. The flow rate was 500 $\mu\text{L}/\text{min}$, the
22 column temperature was maintained at 40 $^{\circ}\text{C}$, and the injection volume was 10 μL . For mass
23 spectrometry, the instrument operated in an ESI positive mode and the range for acquisition
24 was 150–450 m/z. Ion precursor selection was performed in the data dependent mode of
25 operation where the most intense ion from the previous scan was selected for fragmentation.
26 Full scan (MS1) data were acquired for each ionization mode at a resolution of 35,000
27 FWHM, with an AGC target of 1e6 and a maximum injection time of 120 ms. Source
28 parameters were as follows: source voltage + 3.0 kV, sheath gas flow 60 units, auxiliary gas
29 flow 10 units, capillary temperature 320 $^{\circ}\text{C}$, S-Lens RF level 60 units. MS/MS (MS2) data
30 were acquired at a resolution of 17,500 FWHM with an AGC target of 1e5, maximum
31 injection time was 50 ms, a TopN of 5, an isolation window of 2.0 m/z. The normalized
32 collision energy (NCE) was stepped at 17.5, 35, and 52.5, and the dynamic exclusion time set
33 at 3 s.

34 ***Molecular networking generation***

35 Spectral data allowed us to generate MN using semi-quantitative bioinformatic approach.
36 Data processing, visualization, and network analysis, using open source softwares, have been
37 described in details elsewhere (16). Briefly, raw data were converted to an open MS format
38 (.mzXML) with ProteoWizard's MSConvert module (23). The mzXML files were

preprocessed (deconvolution, deisotoping, alignment, gap-filling, filtering) with MZmine 2 software (24). The single .mgf output file was then loaded on the Global Natural Products Social networking (GNPS) web-based platform in order to generate the multi-matrix molecular network (17). Parameters were used at m/z 0.02 for the mass tolerance of precursor and fragment ions used for MS/MS spectral library searching, and m/z 0.02 for the mass tolerance of fragment ions used for MN. Links between nodes were created when the cosine score was greater than 0.7, and the minimum number of common fragment ions shared by two MS/MS spectra was 6. Full data processed through the GNPS platform are accessible through the following links:

Figure 1 : HLM :

<https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=fe6e296f47bb451eadeba745bf7456f2>

Figure 2 : HepaRG :

<https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=594d314272a3475289317b4683bc7aca>

Figure 3 : Biological sample :

<https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=d9878185cfc642308b46d413c56fb689>

The molecular network was visualized using Cytoscape 3.8.0 software (25). The nodes were annotated by spectral matching using SIRIUS 4.0 software (26) and information propagation (identified node can serve as a starting point to identify another node in the same cluster) as already reported (21).

3. Results

2F-DCK metabolism in an acellular model (HLM)

A molecular network was built in order to visualize *in vitro* 2F-DCK metabolism in HLMs (Figure 1A). A cluster containing 9 nodes including a node for 2F-DCK (222.1294 m/z) was observed (*the MS2 spectra of 2F-DCK including the structure of the main fragments is provided as supplementary material*). This node is the central one on the figure. A specific color was assigned to each condition: 2F-DCK was present in the standard solution at 1 mg/L (dark blue), HLM incubation at 100 μ M (light blue) and 500 μ M (turquoise) (Figure 1B). In the node, respective parts display observed relative analytical signals. Eight other nodes were observed in this cluster and correspond to putative but consistent metabolites produced by HLMs. None of these compounds were found in the standard solution (dark blue). Mass shifts were labelled on the edge links between nodes and permit to identify visually biotransformation reaction (reported in Table 1) by information propagation between the 2F-DCK structurally-closed molecules. Table 1 summarized 2F-DCK and related metabolites

1
2
3 produced by HLMs or HepaRG cells, together with those observed (*in vivo*) in the victim's
4 peripheral blood, urine, bile, and vitreous humour.

5
6 Among identified metabolites, nor-2F-DCK (208.1137 m/z) was the main metabolite and
7 corresponds to a dealkylated metabolite of 2F-DCK, a loss of methyl corresponding to a mass
8 shift of 14.016 as labelled in the edge between these two nodes. Nodes linked to 2F-DCK and
9 labelled (238.1243 m/z) with a mass shift of +15.995 correspond to an oxidation reaction.
10 Hydrogenation of this ion leads to a compound labelled 240.139 m/z , such as hydrogenation
11 of nor-2F-DCK (208.1137 m/z) leads to compounds labelled 210.1294 m/z with a mass
12 shift of 2.0155 m/z . At the opposite, dehydrogenation of 2F-DCK (222.1287 m/z)
13 produces compounds labelled 224.1443 m/z . In this *in vitro* model, 2F-DCK metabolism
14 seems to be more intense at 500 μM considering the appearance of a metabolite (210.1286 m/z)
15 which was not produced at 100 μM , larger peak surface for most of the other molecules
16 (238.1243 m/z ; 224.1443 m/z ; 240.1393 m/z , and 208.1130 m/z), and a smaller surface for
17 the parent ion, 2F-DCK. Our data showed also three couples of compounds with the same
18 exact mass but with different retention times (4.5 and 5.0 min for 224.1443 m/z , 3.2 and 6
19 min for 238.1243 m/z , 4.1 and 4.5 min for 210.1286 m/z , respectively), corresponding to
20 isomers with different fragment ions (Table 1). The nodes labelled 208.1130 and
21 224.1443 m/z (RT=4.5 min) are bigger than the other metabolites suggesting interesting
22 biomarkers. These visual results give a good understanding of the 2F-DCK metabolism
23 pathway and allow to identify the structures of metabolites.
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38

39 **2F-DCK metabolism in a cellular model (HepaRG cells)**

40
41 In the HepaRG model, we obtained a MN (Figure 2A) exhibited a 2F-DCK-containing cluster
42 (Figure 2B). The 2F-DCK metabolism seems to be more intense when the incubation time is
43 longer (24h in dark pink *versus* 8h in light pink) considering the appearance of a metabolite
44 (238.124 m/z) which was not produced after 8h of incubation, a larger peak surface for most
45 of the metabolites, and a smaller peak surface for the parent ion (222.1294 m/z). As observed
46 with HLM metabolism, the nodes labelled 208.1137, 210.1286, 224.1443, and 238.1243 m/z
47 were present. Here, three nodes corresponding to two exact masses (191.0866 and 193.1024
48 m/z at RT= 4.4 and 5.1 min, respectively) and in agreement with the loss of an amino function
49 were observed in HepaRG cell supernatants but absent in the HLM model. Nor-2F-DCK
50 (208.1130 m/z) was the main metabolite produced in HepaRG cell model.
51
52
53
54
55
56
57
58
59
60

61 **2F-DCK metabolites in authentic biological samples**

1
2
3 In order to verify our *in vitro* results, we used *post-mortem* biological samples from a fatality
4 case involving 2F-DCK (5). Metabolites were mainly present in urine (yellow) and bile
5 (green), as shown by metabolic ratio visual observation (Figure 3). M09 and M12 were the
6 most intense metabolites and were observed in all biological matrices analyzed.
7
8
9

10
11 We have identified a total of eighteen metabolites in the biological samples that were
12 obviously formed either *via* hydrogenation, *N*-demethylation, hydroxylation, deamination,
13 dehydration, or glucuronidation, or a combination of these reactions, as reported in Figure
14 4. Metabolites were named from M01 (Hydroxy-2F-DCK at RT=3.2 min and 238.1243 *m/z*)
15 to M20 (Hydroxy-2F-DCK glucuronide at RT=6.1 min and 414.1558 *m/z*) according to
16 their retention time sorting.
17
18
19

20
21 Three compounds (M14, M18, and M20) were 2F-DCK glucuroconjugated metabolites
22 and identified in two (bile and urine) of all analysed biological samples at 400.1760,
23 400.1398, and 414.1558 *m/z*, respectively. Thirteen metabolites identified in HLM
24 incubates and/or in HepaRG supernatants were also detected in the studied biological
25 samples. Metabolic ratios between 2F-DCK and metabolites in biological samples, HLMs,
26 and HepaRG (at 8h and 24h) are reported in Table 1.
27
28
29
30
31
32
33

34 **4. Discussion**

35
36 2F-DCK metabolism remains currently poorly described. Davidsen *et al.* (2020) incubated
37 HLMs with 2F-DCK and identified three metabolites (ranked from highest to lowest intensity
38 observed): 208.1130 *m/z* (*N*-demethylation) ; 238.1238 *m/z* (cyclohexanone hydroxylation)
39 and 191.0866 *m/z* (deamination) (3). Tang *et al.* (2020) described a *N*-demethylation reaction
40 producing nor-2F-DCK followed by dehydrogenation (MH⁺ = 206) or hydroxylation (MH⁺ =
41 224) on cyclohexane (4). In the present work, we also identified those previously reported
42 metabolites and named them M09 (nor-2F-DCK at 208.1130 *m/z*), M01, M03, and M19 for
43 the three hydroxy-2F-DCK compounds (238.1238 *m/z*), M05 (206.0975 *m/z*) and M17
44 (224.1079 *m/z*). Recently, Mestria *et al.* (2021) reported two peaks corresponding to dihydro-
45 nor-2F-DCK (210.1294 *m/z*) and two others corresponding to dihydro-2F-DCK (224.1443 *m/z*)
46 in a *post-mortem* blood sample from a forensic case (7). These compounds have a similar
47 fragmentation pattern than that of metabolites we identified as M07, M13, M15, and M12.
48 This latter metabolite appears to be a major metabolite present in all matrices analysed (*in*
49 *vitro* and *in vivo*). We also identified 10 other newly described metabolites (Table 1).
50
51
52
53
54
55
56
57
58
59
60

1
2
3 Ketamine undergoes extensive hepatic *N*-demethylation to the predominant metabolite
4 norketamine, mainly through CYP2B6 and CYP3A4 pathways, in humans. Norketamine is
5 also further metabolized to hydroxynorketamine by CYP2D6 and CYP2A6 (27). We
6
7 hypothesized that the same enzymes are involved in the metabolism of 2F-DCK.
8
9

10 *N*-demethylation of 2F-DCK (nor-2F-DCK or M09) effectively appears to be the main
11 biotransformation reaction regarding our *in vitro* and *in vivo* results. Dehydro-nor-2F-DCK
12 (M05) and hydroxy-nor-2F-DCK (M17) are probably produced by the same metabolization
13 pathways as those of ketamine (28,29). Reduction of the ketone function was observed for 2F-
14 DCK although it has not been described for ketamine in the literature. The presence of
15 glucuronidated metabolites is consistent with the presence of phase I precursor identified by
16 information propagation (+176.032 *m/z*). For example, M14 and M18 are respective
17 glucuronides of M12 and M17.
18
19
20
21
22
23
24

25 Even though HepaRG cells are deemed capable of producing phase II metabolites (14,30), no
26 2F-DCK glucuronides were observed with this *in vitro* model in the present study.
27 Glucuronidation of 2F-DCK phase I metabolites have been already described (27). Here, we
28 identified three 2F-DCK-derived glucuronides in bile and urine (M14, M18, and M20) from a
29 forensic case.
30
31
32
33

34 The present study provides new data about the *in vitro* and *in vivo* metabolism of 2F-DCK.
35 The main 2F-DCK metabolites identified are identical between i) an acellular biological
36 model (HLM), ii) a cellular model (HepaRG), and iii) *post-mortem* biological samples from a
37 real case. The observed metabolic ratios suggest that nor-2F-DCK (M09) and dihydro-nor-2F-
38 DCK (M12) could be proposed as reliable metabolites to be recorded in MS/MS or
39 HRMS libraries in order to improve detection of 2F-DCK use by toxicologists.
40
41
42
43
44
45

46 **5. Conclusion**

47 Molecular networks building from high precision spectral data ($[M+H]^+$ and fragments)
48 obtained through *in vitro* (HLMs and differentiated HepaRG cells) and *in vivo* approaches
49 provide a solution for the identification of unknown metabolites of NPS, and offer a visual
50 representation that facilitates the interpretation and understanding of toxicological results in
51 the context of a death involving NPS. In the present study, this molecular network approach
52 (1) allowed to propose a 2F-DCK metabolic profile in agreement with that observed in
53 authentic biological samples, and (2) strongly suggests that nor-2F-DCK and dihydro-nor-2F-
54 DCK could be used as robust, specific, and sensitive biomarkers of 2F-DCK use.
55
56
57
58
59
60

Figures

Figure 1: Visualization of *in vitro* 2F-DCK metabolism in HLMs using molecular networking. HLMs were incubated with 2F-DCK (100 and 500 μM) during 1h. a) The multimatrix molecular network. Each concentration is depicted in a specific color: 100 μM (light blue) and 500 μM (turquoise) and standard solution (dark blue). b) Details of the specific 2F-DCK-containing cluster. Nodes are labelled with the exact protonated mass (m/z) and the links are labelled with the exact mass shift. Proposed metabolites of 2F-DCK structure are linked to the corresponding nodes (# exact position of the chemical function is not determined).

Figure 2 : Visualization of *in vitro* 2F-DCK metabolism in HepaRG cells using molecular networking. Differentiated HepaRG cells were incubated with 2F-DCK (20 μM) during 8h (light pink) and 24h (pink). a) The multimatrix molecular network. b) Details of the specific 2F-DCK-containing cluster. Nodes are labelled with the exact protonated mass (m/z) and the links are labelled with the exact mass shift. Proposed metabolites of 2F-DCK structure are linked to the corresponding nodes (# exact position of the chemical function is not determined).

Figure 3: Visualization of *in vivo* 2F-DCK metabolism in an authentic case in the 2F-DCK-containing cluster. Nodes are labelled with the exact protonated mass (m/z) and the links are labelled with the exact mass shift. Four biological matrices are studied : blood (red), urine (yellow), vitreous humor (blue), and bile (green). Proposed metabolites of 2F-DCK structure are linked to the corresponding nodes (# exact position of the chemical function is not determined).

Figure 4: Proposed metabolites of 2F-DCK structure and postulated biotransformation combining both *in vitro* and *in vivo* studies (# exact position of the chemical function is not determined).

Tables

Table 1: Metabolites of 2F-DCK sorted by retention times (RT), the underlying name or biotransformation, formula, accurate mass of the precursor ions $[\text{M}+\text{H}]^+$, mass error (Δppm), main observed MS/MS product ions and metabolic ratio in the different matrices analysed [chromatographic peak area of the molecular ion $[\text{M}+\text{H}]^+$ of the compound of interest normalized to chromatographic peak area (100 %) of 2F-DCK]. Better biomarkers are in bold style.

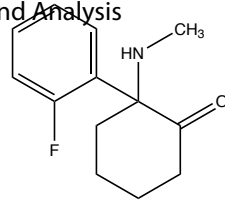
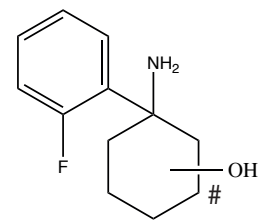
REFERENCES

1. European Monitoring Centre for Drugs and Drug Addiction. European Drug Report 2020: trends and developments. [Internet]. LU: Publications Office; 2020 [cited 2021 Jun 10]. Available from: <https://data.europa.eu/doi/10.2810/420678>
2. Li C, Lai CK, Tang MHY, Chan CCK, Chong YK, Mak TWL. Ketamine analogues multiplying in Hong Kong. *Hong Kong Med J*. 2019;25(2):169.
3. Davidsen AB, Mardal M, Holm NB, Andreasen AK, Johansen SS, Noble C, et al. Ketamine analogues: Comparative toxicokinetic in vitro-in vivo extrapolation and quantification of 2-fluorodeschloroketamine in forensic blood and hair samples. *J Pharm Biomed Anal*. 2020;180:113049.
4. Tang MHY, Li TC, Lai CK, Chong YK, Ching CK, Mak TWL. Emergence of new psychoactive substance 2-fluorodeschloroketamine: Toxicology and urinary analysis in a cluster of patients exposed to ketamine and multiple analogues. *Forensic Sci Int*. 2020;312:110327.
5. Gicquel T, Richeval C, Mesli V, Gish A, Hakim F, Pelletier R, et al. Fatal intoxication related to two new arylcyclohexylamine derivatives (2F-DCK and 3-MeO-PCE). *Forensic Sci Int*. 2021;324:110852.
6. Wallach J, Brandt SD. 1,2-Diarylethylamine- and Ketamine-Based New Psychoactive Substances. In: Maurer HH, Brandt SD, editors. *New Psychoactive Substances : Pharmacology, Clinical, Forensic and Analytical Toxicology* [Internet]. Cham: Springer International Publishing; 2018 [cited 2020 Nov 22]. p. 305–52. (Handbook of Experimental Pharmacology). Available from: https://doi.org/10.1007/164_2018_148
7. Mestria S, Odoardi S, Biosia G, Valentini V, Di Masi G, Cittadini F, et al. Method development for the identification of methoxpropamine, 2-fluoro-deschloroketamine and deschloroketamine and their main metabolites in blood and hair and forensic application. *Forensic Sci Int*. 2021;323:110817.
8. Arango E, Toriello A, Rosario Z, Cooper G. Increasing Prevalence of Ketamine in Drivers in New York City Including the Identification of 2-Fluoro-Deschloroketamine. *J Anal Toxicol*. 2021; bkab057. doi: 10.1093/jat/bkab057.
9. Shao X-T, Yu H, Lin J-G, Kong X-P, Wang Z, Wang D-G. Presence of the ketamine analog of 2-fluorodeschloroketamine residues in wastewater. *Drug Test Anal*. 2021; doi: 10.1002/dta.3098.
10. Huestis MA, Brandt SD, Rana S, Auwärter V, Baumann MH. Impact of Novel Psychoactive Substances on Clinical and Forensic Toxicology and Global Public Health. *Clin Chem*. 2017;63(10):1564–9.
11. Grapp M, Kaufmann C, Schwelm HM, Neukamm MA, Blaschke S, Eidizadeh A. Intoxication cases associated with the novel designer drug 3',4'-methylenedioxy- α -pyrrolidinohexanophenone and studies on its human metabolism using high-resolution mass spectrometry. *Drug Test Anal*. 2020;12(9):1320–35.
12. Salle S, Bodeau S, Dhersin A, Ferdonnet M, Gonçalves R, Lenski M, et al. Novel synthetic opioids: A review of the literature. *Toxicol Anal Clin*. 2019;31(4):298–316.
13. Richeval C, Gaulier J-M, Romeuf L, Allorge D, Gaillard Y. Case report: relevance of metabolite identification to detect new synthetic opioid intoxications illustrated by U-47700. *Int J Legal Med*. 2019;133(1):133–42.
14. Richeval C, Gicquel T, Hugbart C, Le Dare B, Allorge D, Morel I, et al. In vitro Characterization of NPS Metabolites Produced by Human Liver Microsomes and the HepaRG Cell Line Using Liquid Chromatographyhigh Resolution Mass Spectrometry (LC-HRMS) Analysis: Application to Furanyl Fentanyl. *Curr Pharm Biotechnol*. 2017;18(10):806–14.
15. Wagmann L, Frankenfeld F, Park YM, Herrmann J, Fischmann S, Westphal F, et al.

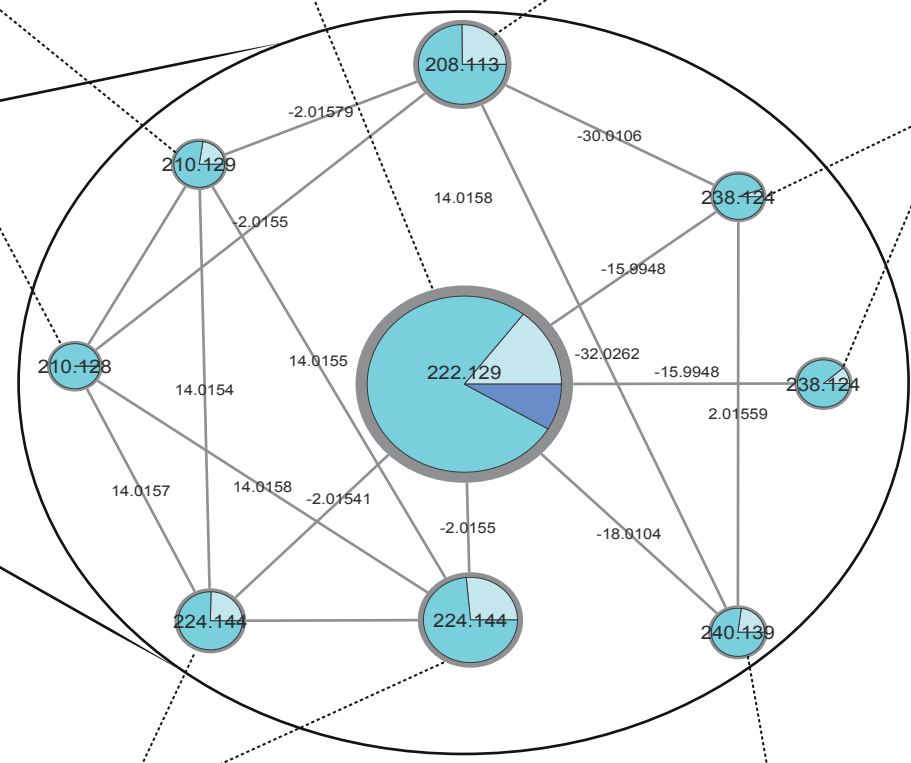
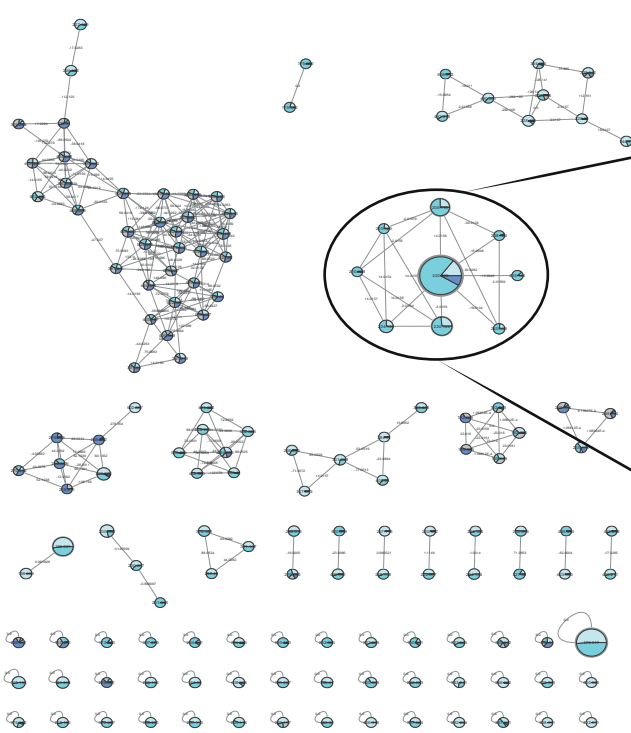
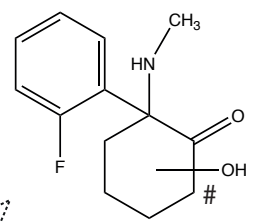
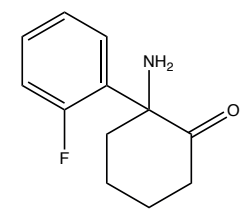
- 1
2
3 How to Study the Metabolism of New Psychoactive Substances for the Purpose of
4 Toxicological Screenings-A Follow-Up Study Comparing Pooled Human Liver S9, HepaRG
5 Cells, and Zebrafish Larvae. *Front Chem.* 2020;8:539.
- 6 16. Allard S, Allard P-M, Morel I, Gicquel T. Application of a molecular networking
7 approach for clinical and forensic toxicology exemplified in three cases involving 3-MeO-
8 PCP, doxylamine, and chlormequat. *Drug Test Anal.* 2019;11(5):669–77.
- 9 17. Wang M, Carver JJ, Phelan VV, Sanchez LM, Garg N, Peng Y, et al. Sharing and
10 community curation of mass spectrometry data with Global Natural Products Social
11 Molecular Networking. *Nat Biotechnol.* 2016;34(8):828–37.
- 12 18. Allard S, Le Daré B, Allard P-M, Morel I, Gicquel T. Comparative molecular
13 networking analysis of a Rauwolfia plant powder and biological matrices in a fatal ingestion
14 case. *Forensic Toxicol.* 2020;38(2):447–54.
- 15 19. Le Daré B, Allard S, Bouvet R, Baert A, Allard P-M, Morel I, et al. A case of fatal
16 acebutolol poisoning: an illustration of the potential of molecular networking. *Int J Legal*
17 *Med.* 2020;134(1):251–6.
- 18 20. Le Daré B, Ferron P-J, Couette A, Ribault C, Morel I, Gicquel T. In vivo and in vitro
19 α -amanitin metabolism studies using molecular networking. *Toxicol Lett.* 2021;346:1–6.
- 20 21. Le Daré B, Ferron P-J, Allard P-M, Clément B, Morel I, Gicquel T. New insights into
21 quetiapine metabolism using molecular networking. *Sci Rep.* 2020;10(1):19921.
- 22 22. Pelletier R, Le Daré B, Grandin L, Couette A, Ferron P-J, Morel I, et al. New
23 psychoactive substance cocktail in an intensive care intoxication case elucidated by molecular
24 networking. *Clin Toxicol (Phila).* 2021;1–4. doi: 10.1080/15563650.2021.1931693.
- 25 23. Kessner D, Chambers M, Burke R, Agus D, Mallick P. ProteoWizard: open source
26 software for rapid proteomics tools development. *Bioinformatics.* 2008;24(21):2534–6.
- 27 24. Olivon F, Grelier G, Roussi F, Litaudon M, Touboul D. MZmine 2 Data-
28 Preprocessing To Enhance Molecular Networking Reliability. *Anal Chem.* 2017;89(15):7836–
29 40.
- 30 25. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a
31 software environment for integrated models of biomolecular interaction networks. *Genome*
32 *Res.* 2003;13(11):2498–504.
- 33 26. Dührkop K, Fleischauer M, Ludwig M, Aksenov AA, Melnik AV, Meusel M, et al.
34 SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information.
35 *Nat Methods.* 2019;16(4):299–302.
- 36 27. Dinis-Oliveira RJ. Metabolism and metabolomics of ketamine: a toxicological
37 approach. *Forensic Sci Res.* 2017;2(1):2–10.
- 38 28. Desta Z, Moaddel R, Ogburn ET, Xu C, Ramamoorthy A, Venkata SLV, et al.
39 Stereoselective and regiospecific hydroxylation of ketamine and norketamine. *Xenobiotica*
40 2012;42(11):1076–87.
- 41 29. Rao LK, Flaker AM, Friedel CC, Kharasch ED. Role of Cytochrome P4502B6
42 Polymorphisms in Ketamine Metabolism and Clearance. *Anesthesiology.* 2016;125(6):1103–
43 12.
- 44 30. Hugbart C, Verres Y, Le Daré B, Bucher S, Vène E, Bodin A, et al. Non-oxidative
45 ethanol metabolism in human hepatic cells in vitro: Involvement of uridine diphospho-
46 glucuronosyltransferase 1A9 in ethylglucuronide production. *Toxicol In Vitro.*
47 2020;66:104842. doi: 10.1016/j.tiv.2020.104842
- 48
49
50
51
52
53
54
55
56
57
58
59
60

	Name	Formula	RT (min)	[M+H] ⁺ observed	[M+H] ⁺ expected	Δppm	Main observed fragment ions	HLM (%)	HepaRG (%)		Blood (%)	Urine (%)	Vitreous humor (%)	Bile (%)
									8h	24h				
Parent	2F-DCK	C₁₃H₁₆FNO	4.6	222.1286	222.1294	-3,2	109; 163; 191	100	100	100	100	100	100	100
M01	Hydroxy-2F-DCK (isomer 1)	C ₁₃ H ₁₆ FNO ₂	3.2	238.1238	238.1243	-2,2	109; 135; 161; 179	2	<1	<1	nd	3	2	2
M02	Dihydro-hydroxy-nor-2F-DCK	C ₁₂ H ₁₆ FNO ₂	3.3	226.1238	226.1243	-2,4	109; 123; 163; 191	nd	nd	nd	<1	3	2	7
M03	Hydroxy-2F-DCK (isomer 2)	C ₁₃ H ₁₆ FNO ₂	3.7	238.1238	238.1243	-2,2	123; 161; 189	nd	<1	<1	nd	1	<1	20
M04	Hydroxy-hydro-2F-DCK	C ₁₃ H ₁₈ FNO ₂	3.8	240.1393	240.1399	-2,8	109; 123; 163; 191	2	nd	nd	<1	6	5	20
M05	Dehydro-nor-2F-DCK	C ₁₂ H ₁₂ FNO	4.0	206.0975	206.0981	-3,0	146; 161; 189	nd	nd	nd	<1	2	<1	17
M06	Dihydro-deamino-nor-2F-DCK (isomer 1)	C ₁₂ H ₁₃ FO	4.1	193.1024	193.1028	-2,4	109; 125; 175	nd	<1	<1	2	2	3	7
M07	Dihydro-nor-2F-DCK (isomer 1)	C ₁₂ H ₁₆ FNO	4.1	210.1286	210.1294	-3,9	109; 125; 175; 193	<1	<1	<1	<1	2	2	10
M08	Dehydro-2F-DCK	C ₁₃ H ₁₄ FNO	4.3	220.1129	220.1137	-3,9	146; 161; 189	nd	nd	nd	<1	<1	<1	21
M09	Nor-2F-DCK	C₁₂H₁₄FNO	4.4	208.1130	208.1137	-3,7	109; 163; 191	15	10	17	21	14	15	81
M10	Dihydro-deamino-nor-2F-DCK (isomer 2)	C ₁₂ H ₁₃ FO	4.4	193.1024	193.1028	-2,4	109; 125; 175	nd	<1	<1	2	1	2	2
M11	deamino-2F-DCK	C ₁₂ H ₁₁ FO	4.4	191.0866	191.0872	-3,2	109; 163	nd	2	3	2	2	1	9
M12	Dihydro-2F-DCK (isomer 1)	C₁₃H₁₈FNO	4.5	224.1442	224.1451	-3,9	109; 135; 179; 161	16	1	2	29	16	21	35
M13	Dihydro-deamino-nor-2F-DCK (isomer 2)	C ₁₂ H ₁₆ FNO	4.5	210.1286	210.1294	-3,9	109; 125; 175; 193	<1	nd	nd	2	<1	2	4
M14	Dihydro-2F-DCK-glucuronide	C ₁₉ H ₂₆ FNO ₇	4.8	400.1760	400.1771	-2,9	85; 109; 193	nd	nd	nd	nd	nd	2	10
M15	Dihydro-2F-DCK (isomer 2)	C ₁₃ H ₁₈ FNO	5.0	224.1442	224.1451	-3,9	109; 125; 175; 193	6	1	3	1	8	8	1
M16	Dihydro-deamino-nor-2F-DCK (isomer 3)	C ₁₂ H ₁₃ FO	5.1	193.1024	193.1028	-2,4	109; 125; 175	nd	<1	<1	nd	nd	nd	nd
M17	Hydroxy-nor-2F-DCK	C ₁₂ H ₁₄ FNO ₂	5.5	224.1079	224.1086	-3,5	109; 178; 206	nd	nd	nd	nd	<1	<1	nd
M18	Hydroxy-nor-2F-DCK glucuronide	C ₁₈ H ₂₂ FNO ₈	5.7	400.1398	400.1407	-2,4	109; 178; 206	nd	nd	nd	nd	3	nd	3
M19	Hydroxy-2F-DCK (isomer 3)	C ₁₃ H ₁₆ FNO ₂	6.0	238.1238	238.1243	-2,2	109; 163; 191	<1	nd	<1	nd	nd	nd	nd
M20	Hydroxy-2F-DCK glucuronide	C ₁₉ H ₂₄ FNO ₈	6.1	414.1558	414.1564	-1,5	109; 163; 191	nd	nd	nd	nd	2	nd	11

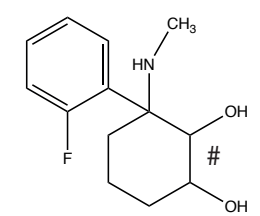
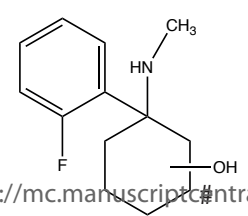
Drug Testing and Analysis



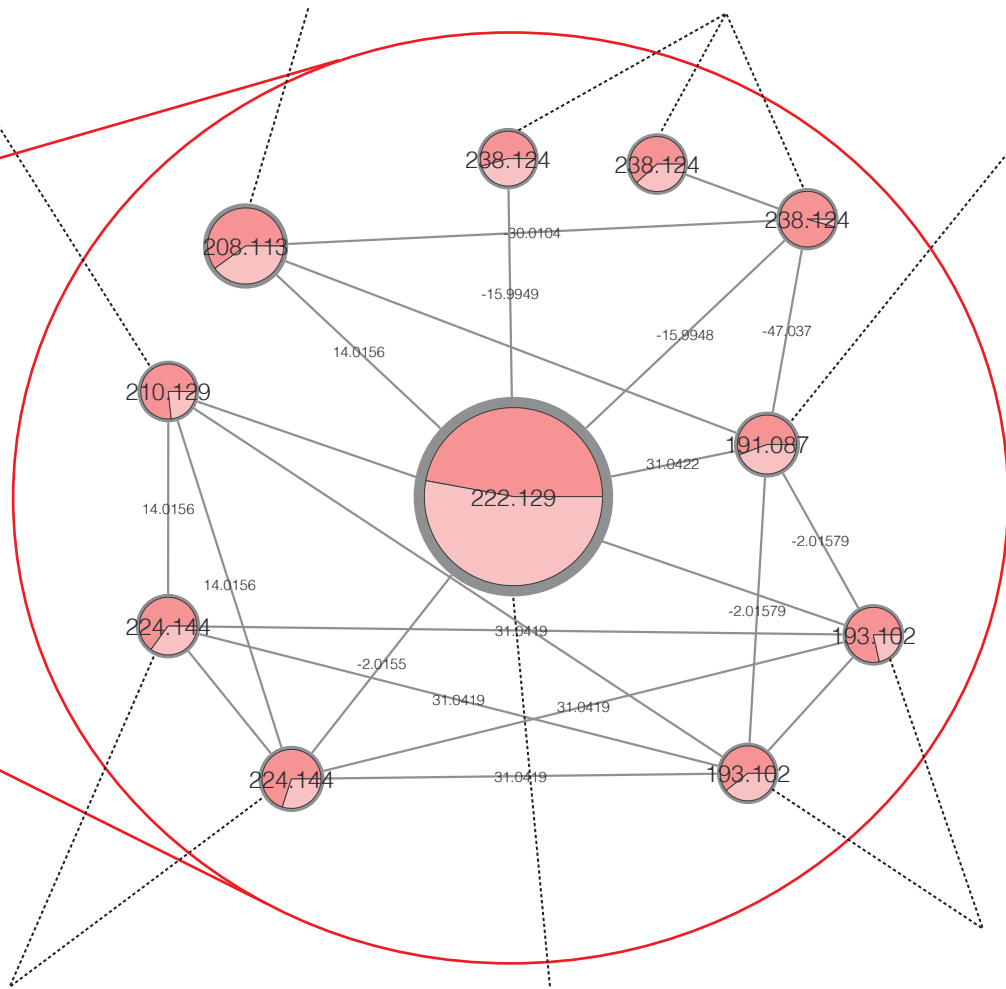
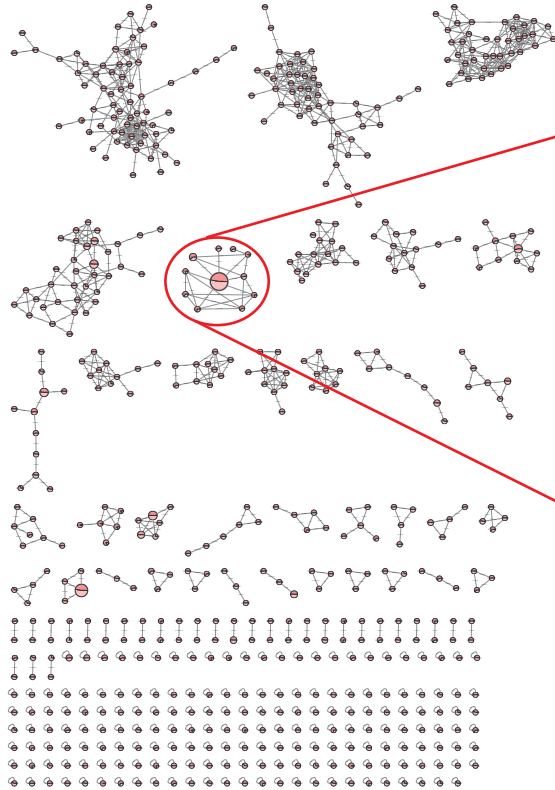
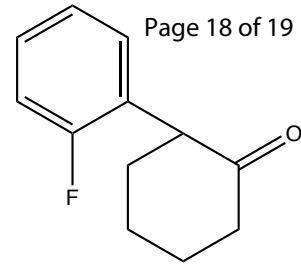
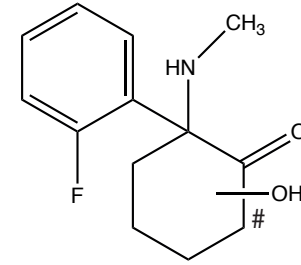
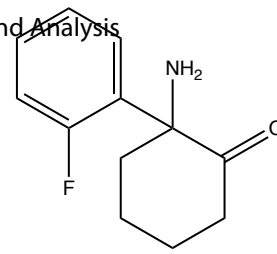
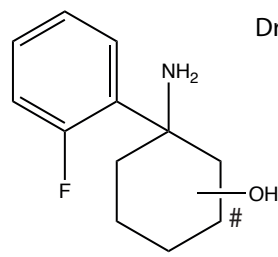
2F-DCK



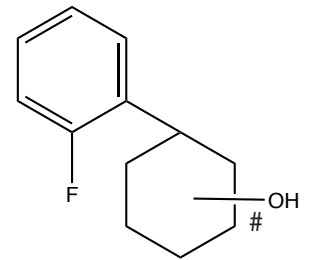
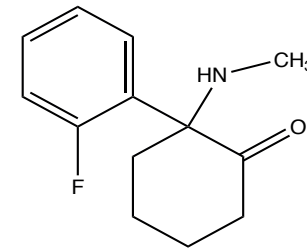
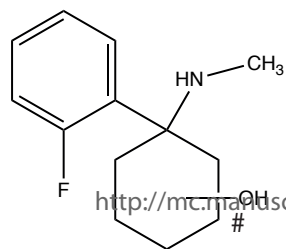
- 2F-DCK 100 μ M
- 2F-DCK 500 μ M
- Standard



1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46



8H
24H



Drug Testing and Analysis

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46

