Tools for studying the metabolism of new psychoactive substances for toxicological screening purposes - A comparative study using pooled human liver S9, HepaRG cells, and zebrafish larvae

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ABSTRACT

New psychoactive substances (NPS) are an emerging topic amongst abused compounds. New varieties appear constantly on the market, without any knowledge about their toxicodynamic and/or -kinetic properties and knowledge of their metabolism is crucial for the development of analytical methods employed for their detection. Controlled human studies would of course be best suited but due to ethical reasons and lack of preclinical safety data, they are usually not available. Often, in vitro models are used to evaluate similarities to human in vivo hepatic phase I and II metabolism and systems explored include primary human hepatocytes, pooled human S9 fraction, and HepaRG, a human hepatic cell line. All these in vitro models have considerable limitations and drug distribution, reabsorption, enterohepatic circulation, and renal elimination cannot be studied. In the recent years, zebrafish (*Danio rerio*) larvae (embryos) were discussed as a potential in vivo model to overcome these limitations. To date, no studies demonstrating its suitability for studying NPS metabolism in the context of analytical toxicology are available. The aim of this study was to elucidate whether zebrafish larvae can serve as a surrogate for human hepatic metabolism of NPS to develop toxicological screening procedures. Here, we used methyl 2 - (1 - (5 - fluoropentyl) - 1H - pyrrolo[2,3-b]pyridine - 3 - carboxamido) - 3,3 - dimethylbutanoate (7 ’ N - 5F - ADB), a new synthetic cannabinoid, whose human metabolism was recently described in the literature, as a model compound to evaluate zebrafish larvae as a new tool for metabolism studies. Different conditions for zebrafish larvae and HepaRG protocols were tested. As zebrafish larvae and HepaRG cell incubations provided the highest number of metabolites and the most authentic spectrum of human metabolites. The most suitable larvae protocol was the incubation via medium and the analysis of the extracted zebrafish larvae. The zebrafish larvae model might be a promising preclinical surrogate for human hepatic metabolism of NPS.
**Keywords**: high resolution mass spectrometry; metabolism; toxicological analysis; zebrafish larvae; HepaRG
1. Introduction

Detection of a xenobiotic intake for example associated with drug overdosing or abuse of drugs is one of the main tasks in analytical toxicology of human samples. Amongst the abused compounds, the so-called new psychoactive substances (NPS) are an important issue in clinical toxicology (Richter et al., 2018) as they appear on the drugs of abuse (DOAs) market without knowledge about their toxicodynamic and/or -kinetic properties. Particularly knowledge of their metabolism is crucial for developing analytical methods for their detection (Richter et al., 2017b). Controlled human studies would be best suited but because of ethical reasons and lack of preclinical safety data, they are not practicable and authentic human samples obtained from intake are often not available (Meyer, 2016; Richter et al., 2017b). Testing in living rats or other rodents is well established as a surrogate but animal experiments should be minimized due to ethical reasons and observations of species differences (Dias-da-Silva et al., 2015; Kittler et al., 2014; Maurer and Meyer, 2016).

Thus, in recent years, additional in vitro models have increasingly been used to evaluate similarities to human in vivo hepatic phase I and II metabolism of NPS, such as primary human hepatocytes (PHH), pooled human S9 fraction (pS9), and HepaRG, a human hepatic cell line (Ellefsen et al., 2016; Richter et al., 2016; Richter et al., 2017a; Richter et al., 2017b; Wohlfarth et al., 2013). Their advantages and disadvantages have been discussed in previous studies. For example, PHH are considered as gold standard in in vitro metabolism studies because of the presence of the relevant human enzyme clusters, co-substrates, and drug transporters (Antherieu et al., 2012; Gerets et al., 2012; Li et al., 1997; Richter et al., 2017b). However, comparatively high costs, limited availability, and variability in the expression of metabolic enzymes may limit their applicability (Kanebratt and Andersson, 2008; Kittler et al., 2014; Maurer and Meyer, 2016; Peters and Meyer, 2011; Richter et al., 2017b; Rodrigues et al., 2016). More cost-efficiently, fast, and easy metabolism studies can be performed by using pS9. Nevertheless, the addition of co-
substrates for main phase I and II metabolic reactions is still required and drug transporters are absent or non-functional (Richter et al., 2017b). Differentiated HepaRG cells have the same advantages as PHH and in addition, are readily available and their phenotype is more stable (Castell et al., 2006; Godoy et al., 2013). However, their culture requires special equipment often not available in analytical laboratories. A limitation common to all these systems is that they are in vitro models and drug/metabolite distribution, reabsorption, enterohepatic circulation, and renal elimination cannot be studied.

In recent years, zebrafish (Danio rerio) larvae (embryos) have increasingly been discussed in the literature as a new in vivo tool (Howe et al., 2013; Saad et al., 2017; van Wijk et al., 2016). In contrast to adult zebrafish, larvae are not considered as animals until 120 hours post-fertilization (hpf) according to the European Directive 2010/63/EU but they already provide all benefits of an intact organism (EU, 2010; Saad et al., 2017). Thus, the use of zebrafish larvae in drug development is increasing e.g. for studying distribution, metabolism, and toxicity (van Wijk et al., 2016). Furthermore, the high reproduction rate of adult fish leads to a large number of larvae at limited costs and allows high throughput tests (Ali et al., 2011; Lawrence et al., 2016; van Wijk et al., 2016).

Comparison of the zebrafish genome and the human reference genome showed that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). In addition, functions of the most important enzymes in metabolism of xenobiotics such as cytochrome P450 (CYP) oxygenases are identical (van Wijk et al., 2016) and zebrafish were already successfully used for metabolism studies of approved drugs (Kantae et al., 2016; Saad et al., 2017). So far, only one study has been published using zebrafish larvae in the NPS context; it was focused on distribution and toxicity studies of meta-chlorophenylpiperazine (mCPP) (Kirla et al., 2018). No metabolism studies of NPS using zebrafish larvae as in vivo model were done, yet.

The aim of this study was to develop the zebrafish larvae model further to serve as a preclinical surrogate for human hepatic metabolism of NPS. This was be exemplified for a
new synthetic cannabinoid, methyl 2 - (1 - (5 - fluoropentyl) - 1H - pyrrolo[2,3 - b]pyridine - 3 - carboxamido) - 3,3 - dimethylbutanoate (7'N-5F-ADB; Fig. 1), which was previously shown to be extensively metabolized in humans and in vitro (Richter et al., 2018).

Results obtained with the larvae were then compared to human metabolism data and to different in vitro models to elucidate drawbacks and benefits of the zebrafish larvae model.

2. Experimental

2.1 Reagents and materials

7'N-5F-ADB, branded and mislabeled as 4'N-5F-ADB, was obtained from www.buyresearchchemicals.de. Its identity as 7'N-5F-ADB was confirmed by NMR. Isocitrate, isocitrate dehydrogenase, dimethyl sulfoxide (DMSO), epidermal growth factor (EGF), methylene blue, penicillin, streptomycin, superoxide dismutase (SOD), and tricaine were obtained from Sigma-Aldrich (Taufkirchen, Germany); human hepatocyte growth factor (HGF) from Humanzyme (Chicago, IL, USA); NaCl, KCl, MgSO4, Ca(NO3)2, and HEPES from Carl Roth (Karlsruhe, Germany); NADP+ from Biomol (Hamburg, Germany); acetonitrile (LC-MS grade), ammonium formate (analytical grade), formic acid (LC-MS grade), methanol (LC-MS grade), and all other chemicals and reagents (analytical grade) from VWR (Darmstadt, Germany). Cryopreserved, differentiated HepaRG cells® (order number HPRG10), 96-well plates coated with type I collagen, GlutaMAX, Williams Medium E, supplement HPRG620, supplement HPRG650, and supplement HPRG670 were obtained from Life Invitrogen (Darmstadt, Germany), and TC suspension cell plates round, 96-well plates (order number 82.1581), and 6-well plates (order number 83.3920.500) were obtained from Sarstedt (Nümbrecht, Germany). Zebrafish embryos of the AB wild-type strain
were obtained from the Luxembourg Center for Systems Biomedicine (Belvaux, Luxembourg).

2.2 In vitro drug metabolism studies using HepaRG

Four different incubation conditions for drug metabolism studies using differentiated HepaRG cells were used. According to the manufacturer’s instructions, metabolism studies using cells in suspension (assay 1) or adherent cells 4 h after cell seeding at day 0 (assay 2) and adherent cells at day 7 after cell seeding (assay 3) were implemented. In addition, a simplified incubation using adherent cells at day 7 after cell seeding using serum-free medium for drug exposure (assay 4), was performed according to previous studies (Richter et al., 2016; Richter et al., 2017a). Cell cultures were maintained in an incubator (Binder, Tuttlingen, Germany) at 37 °C with 95 % air humidity and 5 % CO₂ atmosphere. Cell handling was done under sterile conditions using a laminar flow bench class II (Thermo Scientific Schwerte, Germany). All given concentrations are final concentrations.

Cryopreserved, differentiated HepaRG cells were seeded at a density of 72,000 cells/well (a 100-µL aliquot cell suspension per well) in suspension cell (assay 1) or collagen-coated (assay 2-4) 96-well plates using Williams E medium supplemented with HPRG670, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (thaw and seed medium). For assay 1, cell suspensions were additionally supplemented with either 50 or 500 µM 7’N-5F-ADB. For assay 2, 50 µL supernatant were removed from the cell plate 4 h after cell seeding and 50 µL thaw and seed medium containing the two-fold amount of the final concentrations of 7’N-5F-ADB were added. Medium renewals at day 1, 4, 6, and 7 after cell seeding were necessary for assay 3 and 4. Williams E medium supplemented with HPRG620, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin (maintenance and metabolism medium), and at day 7 after cell seeding additionally supplemented with 50 or 500 µM 7’N-5F-ADB, was used for assay 3. For assay 4, at days 1 and 4 after cell seeding thaw and seed medium
additionally supplemented with 0.5 % DMSO, at day 6 Williams E medium supplemented with HPRG650, 100 U mL\(^{-1}\) penicillin and 100 µg mL\(^{-1}\) streptomycin, 0.5 % DMSO, 10 ng mL\(^{-1}\) HGF, and 2 ng mL\(^{-1}\) EGF (serum-free medium), and at day 7 serum-free medium additionally supplemented with 50 or 500 µM 7’N-5F-ADB was used for medium renewals. Final DMSO concentration was 0.5 % (v/v) and drug exposure lasted 24 h for all incubations.

Supernatants were collected for the analysis of the metabolites. A 50-µL aliquot each was precipitated using 50 µL acetonitrile containing 0.1 % formic acid, vortexed and subsequently cooled at -18 °C for 30 min. The solution was centrifuged for 2 min at 10,000 g, 50 µL of the supernatant was transferred to an autosampler vial and 1 µL was injected onto the Orbitrap (OT)-based liquid chromatography-high resolution-tandem mass spectrometry (LC-HRMS/MS) system as described below. Blank cell cultures without substrate were prepared to confirm the absence of interfering compounds and control samples without cells were done to identify compounds, which are formed non-metabolically. All incubations were done in triplicates.

2.3 In vivo maximum-tolerated concentration (MTC) studies using zebrafish larvae

Zebrafish husbandry was performed according to internal protocols (standard operating procedures) and based on published standard methods (Westerfield, 2007). Zebrafish larvae of the AB wild-type strain were raised at 28 °C in Danieau’s medium (17 mM NaCl, 2 mM KCl, 0.12 mM MgSO\(_4\), 1.8 mM Ca(NO\(_3\))\(_2\), 1.5 mM HEPES, pH 7.1 - 7.3, and 1.2 µM methylene blue). For maximum-tolerated concentration (MTC) testing, embryos were collected, placed in 96-well plates with one embryo per one well in 100 µL medium. Drug exposure happened either at 0 days post-fertilization (dpf) or 4 dpf with 0.01, 0.1, 1, 10, and 100 µM final concentrations of 7’N-5F-ADB. Final DMSO concentration was adjusted to 1 % (v/v) in all exposure media and drug exposure lasted 24 h in the incubator at 28 °C. Larvae were monitored daily using a LEICA M205 FA stereo microscope (Leica
Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) to assess developmental defects and survival rates. All incubations were done with twelve larvae.

2.4 In vivo drug metabolism studies using zebrafish larvae

Zebrafish larvae of the AB strain at 4 dpf were used for metabolism studies. 7’N-5F-ADB was either applied by microinjection into the yolk sac of of tricaine-anaesthetized larvae (7.9 ng per larva applied as 4.18 nL injection of 5 mM 7’N-5F-ADB stock in 50 % DMSO and 50 % phenol red) or by direct administration to the zebrafish medium with a final DMSO concentration of 1 % (50 µM 7’N-5F-ADB). Microinjection was performed with the FemtoJet 4x Microinjector and InjectMan ® 4 Micromanipulator (Eppendorf, Hamburg, Germany). Ten zebrafish larvae were placed in one well of a 6-well plate containing 3 mL of medium supplemented with 50 µM 7’N-5F-ADB. After 24 h of drug exposure at 28 °C, larvae and surrounding medium were collected separately. A 50-µL aliquot of the medium was precipitated using 50 µL acetonitrile containing 0.1 % (v/v) formic acid and vortexed, and subsequently cooled at -18 °C for 30 min. After the solution was centrifuged for 2 min, the supernatant was injected onto the LC-HRMS/MS system as described below.

The larvae of one well (ten) were transferred into reaction tubes, washed twice with 1000 µL medium and were euthanized by placing the tubes in ice water for 15 min. The medium was removed and larvae were snap-frozen in liquid nitrogen and stored at -20 °C until extraction. Ten larvae in one tube were mixed with 50 µL MeOH and shaken for 2 min. After the solution was centrifuged for 2 min at 10,000 g, 30 µL of the supernatant was transferred to an autosampler vial and 1 µL was injected onto the LC-HRMS/MS system as described below. Blank zebrafish larvae without substrate were prepared to confirm the absence of interfering compounds and control samples without zebrafish larvae were done to identify compounds, which are formed non-metabolically. All incubations were done six-fold.
2.5 LC-HRMS/MS apparatus for tentative identification of metabolites

According to previous studies (Helfer et al., 2015; Richter et al., 2016; Richter et al., 2017a; Richter et al., 2017b, 2018) all processed samples were analyzed using a Thermo Fisher Scientific (TF, Dreieich, Germany) Dionex UltiMate 3000 RS pump consisting of a degasser, a quaternary pump and an UltiMate autosampler, coupled to a TF Q-Exactive Plus system equipped with a heated electrospray ionization (HESI)-II source. Mass calibration was done prior to analysis according to the manufacturer’s recommendations using external mass calibration. Gradient elution was performed on a TF Accucore PhenylHexyl column (100 mm x 2.1 mm, 2.6 µm). The mobile phases consisted of 2 mM aqueous ammonium formate containing acetonitrile (1 %, v/v) and formic acid (0.1 %, v/v, pH 3, eluent A) and 2 mM ammonium formate solution with acetonitrile:methanol (1:1, v/v) containing water (1 %, v/v) and formic acid (0.1 %, v/v, eluent B). The flow rate was set to 500 µL min⁻¹ using the following gradient: 0 - 1.0 min hold 99 % A, 1 - 12 min to 20 % A, 12 - 13.5 min hold 1 % A, 13.5 - 15.5 min hold 99 % A. The HESI-II source conditions were as follows: ionization mode, positive; sheath gas, 53 AU; auxiliary gas, 14 AU; sweep gas, 3 AU; spray voltage, 3.50 kV; heater temperature, 438 °C; ion transfer capillary temperature, 320 °C; and S-lens RF level, 60.0. Mass spectrometry was performed using full scan data and a subsequent data-dependent acquisition (DDA) with priority to mass to charge ratios (m/z) of parent compounds and their expected metabolites. The settings for FS data acquisition were as follows: resolution, 35,000; microscans, 1; automatic gain control (AGC) target, 1E6; maximum injection time, 120 ms; and scan range, m/z 100 - 600. The settings for the DDA mode with an inclusion list for 7’N-5F-ADB and its expected metabolites were as follows: option “do not pick others”, enabled; dynamic exclusion, 1 s; resolution, 17,500; microscans, 1; loop count, 5; AGC target, 2e4; maximum injection time, 250 ms; isolation window, m/z 1.0; high collision dissociation (HCD) with stepped normalized collision energy
(NCE), 17.5, 35 and 52.5 %; spectrum data type, profile and underfill ratio, 1 %. The inclusion list contained m/z values of metabolites, which were likely to be formed such as hydroxy, oxo, carboxy, dealkyl and defluorinated metabolites (phase I) as well as sulfates and glucuronides (phase II) and combinations of them. ChemSketch 2016 1.1 (ACD/Labs, Toronto, Canada) was used to draw structures of hypothetical metabolites and to calculate their exact masses. Xcalibur Qual Browser software version 3.0.63 (TF, Dreieich, Germany) was used for data handling.

3. Results and discussion

The aim of the present study was to compare the metabolites of 7’N-5F-ADB identified after zebrafish larvae exposure to those identified after in vitro incubations and to available human in vivo data. The selected in vitro models were pS9, a rather simple and cost-effective model and HepaRG cells, a more elaborate and labor-intensive model. Data from pS9 and human urine were taken from a previous study (Richter et al., 2018) and data for zebrafish larvae and HepaRG cells were produced in this study. Zebrafish larvae were incubated either after substrate addition to the medium or substrate microinjection into the yolk sac and HepaRG cells were incubated under four different conditions.

3.1 In vivo maximum-tolerated concentration (MTC) studies using zebrafish larvae

The survival rates of zebrafish embryos (0 dpf) and larvae (4 dpf) after 24 h treatment with 7’N-5F-ADB, which was added to the medium at concentrations of 0.01, 0.1, 1, 10, and 100 µM are given in Figure 2. Embryos treated at 0 dpf were monitored until 5 dpf with no change in survival rates after 24 h (data not shown). No obvious malformations were observed. The concentration of 50 µM applied to 4 dpf larvae by addition to medium was considered as nontoxic and chosen for metabolism studies.
3.2 Tentative identification of phase I and II metabolites via LC-HRMS/MS

Mass spectral interpretations were based on general fragmentation rules (Bijlsma et al., 2011; Niessen, 2011) and by comparison with the spectrum of the parent compound and previously published metabolites (Richter et al., 2018). In general, all metabolites were identified based on their precursor mass (PM), the calculated molecular formulae, and the fragmentation patterns compared to those of the parent compound and already known metabolites. All phase I and II metabolites, tentatively identified based on their MS² spectra, in the different in vitro models with their absolute peak areas are given in Table 1. The three most abundant metabolites are given in bold.

3.3 Comparison of the zebrafish larvae model to different in vitro models and human data

Only one metabolite was detected in zebrafish larvae incubations after compound application by microinjection. Thus, it can be concluded that at least for the tested compound microinjection does not meet expectations, particularly in comparison to incubations in culture medium. It should also be considered that the total amount of 7’N-5F-ADB was much lower using the microinjection due to the small volume that can be injected into the larvae in comparison to the administration via the medium. In these incubations, four metabolites were found in the extract of the medium and additional 14 metabolites in the larvae extract. This allows the conclusion, that at least for 7’N-5F-ADB, the most suitable larvae protocol should be the incubation via medium and the analysis of the extracted larvae.

We also incubated HepaRG cells with the cannabinoid using four different protocols at two different concentrations. This was done on the one hand to identify the most promising protocol and to compare this data to those of the most suitable larvae model. Considering total number and abundance of the formed metabolites, all protocols seemed comparable,
except for assay 3, which formed considerably fewer metabolites. The differences between incubations with 50 or 500 µM were only of minor impact and lower substrate concentrations are usually preferable to avoid e.g. enzyme inhibition or cytotoxicity. Regarding the work load, assays 1 and 2 were much easier in implementation and more time saving. In conclusion, the most efficient protocol was assay 1 at 50 µM substrate, which provided a total of 18 metabolites.

Previous pS9 incubations were able to produce a total of eight metabolites after six hours of incubation at 50 µM substrate concentration. This allows the conclusion that the zebrafish larvae model is comparable in power to the HepaRG model at least concerning the total number of formed products.

The aforementioned data should now be compared to previously published metabolite patterns in humans to identify the model, which is similar to the human in vivo situation concerning the formation of the most abundant metabolites. This question is of particular interest in analytical toxicology to develop methods for monitoring NPS intake in human urine where metabolites are often the only targets. The most abundant human urinary metabolites were M5, formed by ester hydrolysis, M10 originated by ester hydrolysis with additional hydroxylation of the tertiary butyl part and M20, which was M5 after subsequent glucuronidation (Richter et al., 2018). The metabolites M9 (ester hydrolysis in combination with hydroxylation of the fluoro pentyl chain), M11 (ester hydrolysis in combination with hydroxylation of the fluoro pentyl chain), and M19 (ester hydrolysis in combination with oxidative defluorination and glucuronidation) were the next most abundant. Zebrafish larvae formed four, HepaRG cells five and pS9 three out of the six most abundant human urinary metabolites. Metabolite M10 was only found after HepaRG incubations, whereas M9 was only formed in zebrafish larvae. In general, metabolites formed by more than one metabolic step were less abundant in all tested models than in humans. This may be caused by their static nature, regardless whether they are based on human cells or intact model organisms.
4. Conclusions

Metabolism of 7′N-5F-ADB was investigated using different in vitro and in vivo models and protocols and results were compared to human urinary data. The most abundant metabolites were formed by ester hydrolysis and subsequent glucuronidation and could be detected in all models. The pS9 model provided the least number of metabolites but still three human main metabolites were detected. Zebrafish larvae and HepaRG cell incubation protocols provided the highest number of metabolites and the most authentic spectrum of human metabolites at least for the investigated synthetic cannabinoid. Therefore and because of the wide range of applications such as distribution, metabolism, and toxicity studies, the zebrafish larvae seem to be a promising model for studying the toxicokinetics of NPS. However, further studies comparing different NPS classes are still needed.

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TABLE 1 List of 7'N-5F-ADB metabolites detected in pS9 incubations (Richter et al., 2018), HepaRG cell incubations (suspension cells at day 0, immediately after cell seeding, assay 1; adherent cells at day 0, 4 h after cell seeding, assay 2; adherent cells at day 7 after cell seeding, assay 3; adherent cells at day 7 after cell seeding using serum-free medium, assay 4), or zebrafish larvae incubations after compound administration to culture medium or by microinjection with their absolute peak areas after full-scan. The three most abundant metabolites are given in bold. Metabolites are listed according to increasing mass. N.D. = not detected
| M10 | Ester hydrolysis + hydroxylation of the tertiary butyl part isomer 2 | 380.1970 | N.D. | N.D. | 3.34E+05 | 8.56E+05 | 4.52E+05 | 4.64E+05 | 9.18E+04 | 1.19E+05 | 6.88E+04 | 3.46E+05 | N.D. | N.D. | N.D. | N.D. |
| M11 | Ester hydrolysis + hydroxylation of the fluoro pentyl chain isomer 3 | 380.1970 | N.D. | N.D. | 1.80E+05 | 3.69E+05 | 1.65E+05 | 1.71E+05 | 1.07E+04 | 2.71E+04 | 7.61E+04 | 1.09E+05 | N.D. | 1.09E+05 | N.D. | N.D. |
| M12 | Oxidative defluorination + oxidation to carboxylic acid | 390.2013 | N.D. | N.D. | 5.53E+04 | 1.62E+05 | 5.48E+04 | 4.54E+04 | 1.23E+04 | 1.32E+04 | 2.64E+04 | 1.54E+05 | 1.03E+05 | 6.57E+06 | N.D. | 4.57E+05 |
| M13 | Oxidative defluorination + hydroxylation of the pentyl chain isomer 1 | 394.2126 | N.D. | N.D. | 5.86E+04 | 6.31E+04 | 5.43E+04 | 6.22E+04 | 4.92E+05 | 5.91E+05 | 5.60E+05 | 3.37E+04 | 1.86E+06 | 1.12E+06 | 1.91E+06 | N.D. |
| M14 | Hydroxylation of the pyrrolo pyridine part isomer 2 | 406.1962 | N.D. | N.D. | 5.86E+04 | 6.31E+04 | 5.43E+04 | 6.22E+04 | 4.92E+05 | 5.91E+05 | 5.60E+05 | 3.37E+04 | 1.86E+06 | 1.12E+06 | 1.91E+06 | N.D. |
| M15 | Hydroxylation of the pyrrolo pyridine part isomer 2 | 410.2075 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | 1.14E+04 | N.D. | N.D. |
| M16 | Oxidative defluorination + oxidation to carboxylic acid + hydroxylation of the pentyl chain isomer 1 | 427.1502 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | 1.03E+04 | N.D. | N.D. | N.D. |
| M17 | Amid hydrolysis + glucuronidation | 538.2382 | N.D. | N.D. | 9.74E+03 | 9.27E+03 | 5.43E+03 | 7.25E+03 | N.D. | N.D. | 2.27E+04 | 4.03E+04 | N.D. | 3.91E+04 | N.D. | N.D. |
| M18 | Amide hydrolysis + glucuronidation | 540.2339 | N.D. | N.D. | 3.32E+04 | N.D. | 3.02E+04 | 2.97E+04 | N.D. | N.D. | 7.54E+05 | 1.49E+06 | N.D. | N.D. | N.D. | N.D. |
| M19 | Ester hydrolysis + oxidative defluorination + glucuronidation | 552.2175 | N.D. | N.D. | 8.60E+04 | 8.60E+04 | 3.58E+05 | 3.99E+05 | 3.57E+05 | 4.58E+05 | 1.43E+05 | 1.46E+05 | N.D. | 1.58E+06 | N.D. | 6.45E+04 | N.D. |
| M20 | Ester hydrolysis + hydroxylation of the fluoro pentyl chain and tertiary butyl part isomer 1 | 556.2288 | N.D. | N.D. | 7.75E+03 | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. | N.D. |
|   | Hydroxylation of the fluoro pentyl chain + glucuronidation isomer 1 |   | Hydroxylation of the pyrrolo pyridine part + glucuronidation isomer 2 |   |
|---|-----------------------------------------------------------------|---|-----------------------------------------------------------------|---|
| M23 | 570.2444 | N.D. | N.D. | 1.32E+04 | 3.10E+04 | N.D. | N.D. | N.D. | N.D. | 6.79E+04 | N.D. | N.D. |
| M24 | 570.2444 | N.D. | N.D. | N.D. | N.D. | 1.24E+04 | N.D. | N.D. | N.D. | 3.73E+04 | N.D. | 9.70E+04 | N.D. | N.D. |
| Total number of metabolites | 5 | 8 | 18 | 14 | 14 | 15 | 9 | 9 | 12 | 15 | 4 | 18 | 0 | 1 |
Legends to Figures

**Fig. 1** Structures of 7’N-5F-ADB and its most abundant human urinary metabolites (Richter et al., 2018)

**Fig. 2** Survival rates of zebrafish embryos (0 dpf) and larvae (4 dpf) after 5 d or 24 h treatment with 7’N-5F-ADB, which was added to the medium at assigned concentrations.