Enzymatic defluorination of a terminally monofluorinated pentyl moiety: oxidative or hydrolytic mechanism?

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Running Title: Enzymatic defluorination: oxidative or hydrolytic?

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Number of text pages: 22

Number of tables: 2

Number of figures: 5

Number of references: 58

Number of words in Abstract: 249

Number of words in Introduction: 905 (w/o references)

Number of words in Discussion: 1626

Nonstandard abbreviations: 4F-MDMB-BINACA, N-[(1-(4-fluorobutyl)-1H-indazol-3-yl)carbonyl]-3-methyl-L-valine methyl ester; 5-HPM, 5-hydroxypentyl metabolite; ACN, Acetonitrile; AM-2201, [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone; bbCID, broadband collision-induced dissociation; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; GTFCh, German society of toxicological and forensic chemistry; $K_m$, Michaelis–Menten constant; LC-MS/MS, liquid-chromatography-tandem-mass-spectrometry; LC-QToF-MS, liquid-chromatography coupled to time-of-flight-mass-spectrometry; PFAS, per- and polyfluorinated substances; pHLM, pooled human liver microsomes; ToF, time-of-flight; $v_{max}$, maximum reaction rate.
Abstract

Fluorination of organic compounds plays an important role in the chemical and pharmaceutical industry and is often applied in order to improve physicochemical parameters or modify pharmacological properties. While oxidative and reductive defluorination have been shown to be responsible for the metabolic degradation of organofluorine compounds, the involvement of hydrolytic mechanisms catalyzed by human enzymes has not been reported so far. Here, we investigated the enzymatic defluorination of terminally monofluorinated aliphates with [1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone (AM-2201) as a model substance. We performed in vitro biotransformation using pooled human liver microsomes (pHLM) and human recombinant cytochrome P450 (CYP) assays. In order to elucidate the underlying mechanisms, modified incubation conditions were applied including the use of deuterium labeled AM-2201 (d<sub>2</sub>-AM-2201). Identification of the main metabolites and analysis of their isotopic composition was performed by liquid-chromatography coupled to time-of-flight-mass-spectrometry (LC-QToF-MS). Quantification of the metabolites was achieved with a validated method based on liquid-chromatography-tandem-mass-spectrometry (LC-MS/MS). CYP 1A2 mediated defluorination of d<sub>2</sub>-AM-2201 revealed an isotopic pattern of the defluorinated 5-hydroxypentyl metabolite (5-HPM) indicating a redox mechanism with an aldehyde as a plausible intermediate. In contrast, formation of 5-HPM by pHLM was observed independently of the presence of atmospheric oxygen or co-factors regenerating the redox system. pHLM incubation of d<sub>2</sub>-AM-2201 confirmed the hypothesis of a non-oxidative mechanism involved in the defluorination of the 5-fluoropentyl moiety. So far, enzymatically catalyzed, hydrolytic defluorination was only described in bacteria and other prokaryotes. The presented data prove the involvement of a hydrolytic mechanism catalyzed by human microsomal enzymes other than CYP.
Significance Statement

Elucidating the mechanisms involved in the enzymatic detoxification of organofluorine compounds is crucial for enhancing our understanding and facilitating the design and development of drugs with improved pharmacokinetic profiles. The carbon-fluorine bond possesses a high binding energy, which suggests that non-activated fluoroalkanes would not undergo hydrolytic cleavage. However, our study provides evidence for the involvement of a non-oxidative mechanism catalyzed by human liver enzymes. It is important to consider CYP-independent, hydrolytic defluorination, when investigating the pharmacokinetic properties of fluorinated xenobiotics.
Introduction

Due to the unique chemical and physical properties of organofluorine compounds, they have been widely used in fields such as pharmaceutical sciences, material sciences and agrochemistry (Brassard et al., 2012; Vulpetti and Dalvit, 2012; Fujiwara and O’Hagan, 2014; Ogawa et al., 2020). Introduction of fluorine substituents can be used to improve a molecule’s chemical or biological stability. As the $\text{C} - \text{F}$ bond dissociation energy is relatively high (e.g. 460 kJ/mol for fluoromethane), the stability of organofluorine compounds is expected to be higher than that of their non-fluorinated analogs. On the other hand bioaccumulation of fluorinated compounds, especially per- and polyfluorinated substances (PFAS), has become a global environmental threat (Gebbink et al., 2016; Sunderland et al., 2019; Sinclair et al., 2020; Dhore and Murthy, 2021; Neuwald et al., 2022). Abiotic and biological degradation of organofluorine compounds for the purpose of ecological detoxification has become an issue of major concern in the recent past (Jana et al., 2010; Ruiter et al., 2017; Colomban et al., 2019; Seong et al., 2019; Leung et al., 2022; Wackett, 2022; Zhang et al., 2022).

Bioremediation, a technology that uses microbes to eliminate contaminants, was shown to be efficient for specific environmental pollutants (Parales et al., 2002; Dvořák et al., 2017). However, for PFAS it is not considered practical currently. Due to the high electronegativity of fluorine and the unique properties of the $\text{C} - \text{F}$ bond, fluorinated compounds strongly differ from other organohalides. While microbial detoxification of chlorinated or brominated compounds was shown to be efficient (Kräutler et al., 2003; Payne et al., 2015), defluorination rates observed for PFAS are relatively low (Murphy, 2010). Yu et al. reported the microbial defluorination of PFAS via reductive pathways. However, they were not able to identify the microorganisms being responsible for the reductive defluorination and suggested the involvement of minor abundant phylogenetic groups (Yu et al., 2020).
Microbial dehalogenation of organochlorines and organobromines is evolutionarily adapted in response to many halogenated natural products (Adrian and Löffler, 2016; Tang et al., 2016). Specific microorganisms are known to use reductive dehalogenation of organochlorines as an electron acceptor within respiratory metabolism (Duhamel and Edwards, 2007). ATP-dependent, reductive defluorination was observed by Tiedt et al. (Tiedt et al., 2016). They reported biotransformation of 4-fluorobenzyl-CoA catalyzed by microbial benzoyl-CoA reductases in *Thauera aromatica*.

In contrast to PFAS, which are referred to as ‘forever chemicals’, all naturally occurring organofluorine compounds are monofluorinated and analogs of omnipresent metabolites (e.g. fluoroacetate). Based on the bond-dissociation energy, the strength of the C−F bond is often reported as the strongest one known. Yet, naturally occurring fluorinated compounds are relatively reactive and can be efficiently biodegraded (Pan, 2019). While the bond-dissociation energy describes the homolytic bond cleavage, metabolic degradation of the C−F bond is usually conducted in a heterolytic manner (Wang and Liu, 2020). Conversion of organofluorine compounds into corresponding alcohols can formally be described as a hydrolytic substitution as it comes without a change in the oxidation state of the carbon (Figure 1). Hydrolytic defluorination of fluoroacetate has been studied mechanistically in detail. The fluoroacetate dehalogenase, a hydrolase discovered in bacteria and other prokaryotic cells (e.g. *R. palustris*), was shown to catalyze defluorination of fluoroacetate to harmless glycolate. The bimolecular, nucleophilic substitution (SN2) of fluoride is favored by Asp105 in the catalytic center of the dehalogenase. Heterolytic C−F bond cleavage leads to the formation of fluoride (F−) as a relatively unfavorable leaving group, which is, however, stabilized by extensive hydrogen bonds in the catalytic center (Kamachi et al., 2009; Camboim et al., 2012).
Various studies reported oxidative defluorination of aliphatic or aromatic organofluorines catalyzed by metalloenzymes such as oxygenases, oxidases and peroxidases (Teitelbaum et al., 1981; Scribner et al., 1982; Mutch et al., 2001; Chimalakonda et al., 2012; Harkey et al., 2012; Kiel and Engesser, 2015; Wang and Liu, 2020; Huang et al., 2022). The mechanisms of aromatic defluorination catalyzed by cytochrome P450 (CYP) were reported by Harkey et al. (Harkey et al., 2012). Oxidative monodefluorination of 4-fluorophenol leads in the first step to benzoquinone, which is then reduced to hydroquinone via the NADPH P450-reductase. Defluorination of terminally monofluorinated aliphates catalyzed by monoxygenases would involve an oxidative hydroxylation of the terminal carbon in the first step. Activation of the α-carbon would initiate elimination of hydrogen fluoride forming the corresponding aldehyde. Reduction of the aldehyde would lead to the corresponding alcohol in a final step (Figure 1).

In addition to their use in material and agrochemical sciences, fluorinated compounds play an important role in drug development. In medicinal chemistry, isosteric substitution of hydrogen with fluorine is frequently performed to improve a drug candidate’s metabolic stability, membrane permeability or pharmacological potency (Banister et al., 2015; Wilkinson et al., 2015; Wilkinson et al., 2017; Meanwell, 2018). As discussed above, it has been shown that fluorination cannot be used as a generic model to decrease hepatic clearance. Substitution with fluorine does not always result in higher metabolic stability when compared to their non-fluorinated analogs. However, introduction of a trifluoromethyl group usually eliminates so-called metabolic soft spots and is the most common group used for this purpose (YALE, 1959; Diana et al., 1995; Hagmann, 2008; Sun et al., 2011; Inoue et al., 2020).

The present study delves into the investigation of the synthetic cannabinoid receptor agonist AM-2201, chosen as a model compound representing terminally monofluorinated aliphates. So far, human enzymes have not been recognized for their capacity to catalyze non-oxidative defluorination, and the general understanding has been that the C–F bond of fluorinated
aliphatic moieties remains unaltered until activation of the α-carbon. Given these established facts, it was not anticipated that the defluorinated 5-hydroxypentyl metabolite of AM-2201 would emerge as a major constituent in human metabolism.

This work's principal aim is to dissect the mechanisms underpinning the cleavage of the C−F bond in a primary fluoroalkane, AM-2201, utilizing human liver microsomes and a select range of recombinant CYP isoenzymes. In pursuit of this goal, we employ an array of modified in vitro methods to elucidate enzymatic defluorination mechanisms. To explore the hypothesis of non-oxidative biocatalysis, our investigations extend beyond incubations in the absence of reducing equivalents, such as NADPH, to also include experiments conducted under conditions devoid of atmospheric oxygen.

These preliminary findings have led to the formulation of two hypotheses (as illustrated in Figure 1). To further explore the biocatalytic mechanisms, we have undertaken isotopic labeling of both oxygen (through incubation in $^{18}$O-water) and the substrate (utilizing $d_2$-AM-2201). The latter serves as a pivotal study to rigorously test the hypotheses and provide a clear distinction between oxidative and non-oxidative defluorination mechanisms.
Materials and Methods

Chemicals and Reagents

Formic acid (Rotipuran® with a purity exceeding 98%), sodium hydroxide (with a purity exceeding 99%, in pellet form), and potassium hydrogen phosphate (with a purity exceeding 99%) were sourced from Carl Roth in Karlsruhe, Germany. Acetonitrile (LC-MS grade), ammonium formate (10 M, with a purity of 99.995%), absolute ethanol, ethyl acetate (analytical grade), and potassium hydroxide (with a purity exceeding 86%, in pellet form) were procured from Sigma Aldrich in Steinheim, Germany. Isopropanol (Prepsolv®) was acquired from Merck in Darmstadt, Germany. Additionally, $^{18}$O-water (with 97 atom % $^{18}$O) and DMSO were obtained from Sigma Aldrich in Steinheim, Germany. cDNA expressed human cytochrome P450 enzymes (1A2, 2D6, 3A4, 2C9 and 2E1; Supersomes® coexpressed with oxidoreductase), insect cell microsomes, human oxidoreductase (containing human cytochrome b), pooled human liver microsomes (pHLM; comprising material from 150 donors, at a protein concentration of 20 mg/mL in a 250 mM sucrose solution), as well as NADPH regenerating solutions A and B (with reductase activity at 0.43 µmol/min*mL), and 0.5 M potassium phosphate buffer (adjusted to pH 7.5) were procured from Corning, located in Corning, USA. For clarity, NADPH regenerating solution A was composed of 26 mM NADP$^+$, 66 mM glucose-6-phosphate, and 66 mM MgCl2 dissolved in water. Meanwhile, NADPH regenerating solution B contained 40 U/mL of glucose-6-phosphate dehydrogenase in a 5 mM sodium citrate solution. Tris buffer 1.0 M (pH 7.5) was purchased from ThermoFisher (Waltham, USA). AM-2201 ([1-(5-fluoropentyl)-1H-indol-3-yl]-1-naphthalenyl-methanone), AM-2201 5-hydroxypentyl metabolite ([1-(5-hydroxypentyl)-1H-indol-3-yl](naphthalen-1-yl)methanone), AM-2201 5-hydroxyindole metabolite ([1-(5-fluoropentyl)-5-hydroxy-1H-indol-3-yl](naphthalen-1-yl)methanone), $d_5$-AM-2201 5-hydroxypentyl metabolite ([1-(5-hydroxypentyl)(2,4,5,6,7-$^2$H$_5$)-1H-indol-3-yl](naphthalen-1-
yl)methanone), 4F-MDMB-BINACA \((N-[[1-(4-fluorobutyl)-1H-indazol-3-yl]carbonyl]-3-methyl-L-valine methyl ester), furafylline \((3-(2-furanylmethyl)-3,7
dihydro-1,8\text{-dimethyl-}1H\text{-purine-2,6-dione})\) and meclonazepam \(((3S)-5-(2\text{-chlorophenyl}-3-methyl-7\text{-nitro-}1,3\text{-dihydro-}2H\text{-1,4-benzodiazepin-2-one})\) were obtained from Cayman Chemical (Ann Harbor, USA). \(d_2\)-AM-2201 \(\{1-\text{[5-fluoro(5,5}\text{-H_2\text{pentyl}]})-1H\text{-indol-3-yl})\text{(naphthalen-1-yl)methanone}\) was synthesized and analytically characterized by means of LC-QToF-MS, \(^1\text{H}\)- and \(^{13}\text{C}\)-NMR (Supp. Methods, Supp. Fig. 2).

Deionized water was generated utilizing a Medica® Pro deionizer sourced from ELGA in Celle, Germany. The mobile phases, denoted as mobile phase A (comprising 1\% ACN, 0.1\% formic acid, and 2 mM ammonium formate in water) and mobile phase B (consisting of 0.1\% formic acid and 2 mM ammonium formate in ACN), were freshly prepared just before the analysis. For the purpose of external and internal mass calibration of the QToF-MS instrument, a solution containing sodium formate/acetate clusters was prepared. This solution was created by mixing 250 mL of deionized water, 250 mL of 2-propanol, 750 µL of acetic acid, 250 µL of formic acid, and 500 µL of sodium hydroxide (1 M).

pHLM and human recombinant CYP isoforms assay

Phase-I metabolites were generated in vitro using a pHLM and human recombinant CYP assay. The incubation (100 µL volume) was performed by adding 1.0 µL of a DMSO based stock solution to a reaction mixture consisting of 5.0 µL NADPH regenerating solution A, 1.0 µL NADPH regenerating Solution B, 20 µL of phosphate buffer (0.5 M, pH 7.4), deionized water, and 5 µL of pHLM or 2 µL of CYP enzyme preparation, respectively. The final substrate and DMSO concentration in the mixture were 20 µM and 1 vol\%, respectively. According to the manufacturer incubations with CYP 2C9 were performed in Tris (0.1 M, pH 7.5) instead of phosphate buffer. To evaluate the impact of the co-solvent DMSO on enzyme
activity, we conducted parallel incubations using an ACN-based substrate stock solution (final concentration 1 vol%).

Incubation was performed for 30 min at 37 °C. The reaction was stopped by the addition of 300 µL acetonitrile (-20°C). After phase separation induced by adding 50 µL of a 10 M ammonium formate solution and centrifugation at 16,100 x g, the organic layer was transferred into a separate vial (salting-out assisted liquid-liquid-extraction). For LC-QToF-MS analysis (parameters described below) the extract was evaporated to dryness at 40°C under a stream of nitrogen and reconstituted with 30 µL mobile phase A/B (50/50, v/v). The protocol for quantitative analysis of the metabolites of interest with LC-MS/MS is described in the Supp. Methods. For determination of CYP-independent biotransformation, the substances were incubated without NADPH regenerating system, with human P450 oxidoreductase (including cytochrome b5), Baculovirus expression system and buffer serving as negative controls. Incubation was conducted with an Eppendorf ThermoMixer C (Hamburg, Germany) at 300 rpm.

The protocol described above was also employed for the pHLM and CYP incubation of stable isotope labeled d2-AM-2201. The incubation time was adjusted to 15 minutes assuring less than 5% of substrate depletion. To assess the impact of the co-solvent DMSO on the underlying biocatalytic mechanisms, we performed parallel incubations using ACN based substrate stock solution.

**pHLM and CYP isoforms assay in the absence of oxygen**

The assay was performed with a current flow of nitrogen to achieve an absence of oxygen during incubation. The standard protocol was slightly modified. All reagents except the substrate were pipetted into the incubation vial and treated with a continuous nitrogen flow for 15 min. The incubation was started by adding the substrate into the reaction mixture while
maintaining nitrogen flow. Metabolic reduction of the benzodiazepine meclonazepam to 8-aminomeclonazepam was conducted as a positive control for the absence of oxygen (Vikingsson et al., 2017). Negative controls were incubated with atmospheric conditions.

**Kinetic studies**

Kinetic analysis of defluorination was performed in the linear range of metabolite formation and with an optimized time period of 15 minutes assuring less than 5% of substrate biotransformation. 2 µL of the CYP isoform preparation were diluted in 48 µL of Tris or phosphate buffer (0.1 M, pH 7.4), afterwards 1.0 µL of varying substrate stock solution in DMSO (final DMSO concentration of 1 vol%) was added to the mixture leading to final concentrations between 0.1 and 40 µM. A dilution of NADPH regenerating solutions A and B was prepared consisting of 5 µL solution A, 1 µL solution B and 43 µL of phosphate buffer (0.1 M, pH 7.4). In order to start the incubation, the dilution was added to the enzyme-substrate mixture. Sample preparation for the quantification of the metabolites of interest is described in the Supp. Methods. The incubations were performed at least in triplicates on different days.

Calculation of enzyme kinetics was performed by relating the reaction rate (pmol/min/nmol protein) of the enzymatic reaction with the concentration of the substrate AM-2201 (µM). The Michaelis–Menten constant $K_m$ and the maximum reaction rate $v_{max}$ were calculated with GraphPad Prism® (Version 8.0.2, GraphPad Software Inc, San Diego, CA, USA). In cases of substrate inhibition, the calculated parameters of the enzyme catalysis are corrected and describe the theoretical kinetic without substrate inhibition.

**Inhibition of CYP 1A2 activity with furafylline**

The standard incubation protocol was modified as follows: The incubation was performed with a fixed concentration of the substrate AM-2201 (5 µM) and varying concentrations of
furafylline between 0.01 µM and 100 µM. The determined $K_m$ of CYP 1A2 mediated AM-2201 defluorination was used as a reference for the substrate concentration of AM-2201. 10 µL of furafylline stock solution in DMSO/H$_2$O (10/90, v/v) were added to the incubation mix consisting of 2 µL CYP 1A2, 1 µL AM-2201 stock solution, and 48 µL phosphate buffer. After a preincubation time of 5 minutes, the incubation was started by adding the NADPH regenerating system. The following steps for sample preparation were performed according to the standard protocol. Negative controls with 10 µL of blank DMSO/H$_2$O (10/90, v/v) were prepared accordingly.

Statistical analysis and calculation of the inhibition constant $K_i$ was performed with GraphPad Prism® (Version 8.0.2, GraphPad Software Inc, San Diego, CA, USA) on the basis of the Cheng-Prusoff relationship (Cheng and Prusoff, 1973).

**pHLM and CYP isoforms assay in $^{18}$O-labeled buffer**

The protocol described for the pHLM and CYP assay was modified as follows: Buffer and NADPH regenerating solution A were evaporated to dryness, 75 µL of $^{18}$O-water was added for reconstitution. Finally, NADPH regenerating solution B, the substrate, the enzyme preparation and deionized water were added to start the incubation (final incubation volume 100 µL). Negative controls with unlabeled water were prepared accordingly. As a positive control for $^{18}$O incorporation, 4F-MDMB-BINACA was incubated with pHLM without NADPH regenerating system and with CYP 2C9 (Supp. Fig. 5).

**Isotopic pattern analysis with LC-QToF-MS**

Qualitative analysis using LC-QToF-MS was conducted on an Impact-II-QToF instrument, which was coupled with an Elute RS HPLC system manufactured by Bruker in Billerica, MA, USA. This equipment was employed to analyze the in vitro extracts as previously mentioned. Chromatographic separation was carried out on a Kinetex® C$_{18}$ column (2.6 µm particle size,
100 Å pore size, 100 × 2.1 mm dimensions) from Phenomenex in Aschaffenburg, Germany, using a gradient elution method as described below.

The initial mobile phase B composition was set at 20%, which was linearly increased to 50% over 8.0 minutes. Subsequently, it was further raised to 60% within 2.0 minutes, followed by an increase to 95% over 2.0 minutes. This concentration was maintained for 1.0 minute before decreasing to the starting conditions of 20% in 0.1 minute. Finally, a 1.9-minute period was allocated for re-equilibration.

The flow rate was set to 0.5 mL/min, with the autosampler and column oven temperatures set at 10 and 40°C, respectively. A 10 µL injection volume was used. Data acquisition and processing were performed using HyStarTM ver. 3.2 and DataAnalysis (DA) ver. 4.2, both provided by Bruker in Billerica, MA, USA. The mass spectrometer (MS) was operated in positive ionization mode, acquiring spectra within the m/z range of 50 to 650. The dry gas temperature was maintained at 200 °C, with a dry gas flow rate of 8.0 L/min. The nebulizer gas pressure was set to 2 bar. A collision energy range of 30 ± 6 eV was applied for bbCID. Both MS1 and MS2 (bbCID) data were acquired in a single run at an acquisition rate of 2.0 Hz. Nitrogen was used as the collision gas. The capillary voltage and end plate offset were set to 2500 V and 500 V, respectively. External and internal mass calibration were performed using sodium formate/acetate clusters with high precision calibration (HPC) mode.
Results

CYP and pHLM assay

Defluorination of AM-2201 into 5-HPM was catalyzed by the monoxygenases CYP 1A2, 2D6 and 2C9 and was dependent on the presence of the NADPH regenerating system (RS). CYP 2E1 and 3A4 showed a very low rate of defluorination, which did not significantly differ from control incubations (Figure 2). Human NADPH-cytochrome-P450-oxidoreductase co-expressed with cytochrome-b5-reductase served as a control to eliminate the involvement of these coenzymes and the Baculovirus expression system. While the control incubations in phosphate and tris buffer did not lead to detection of 5-HPM, the incubation of AM-2201 with the oxidoreductase negative control showed a relatively low defluorination rate of 2.3 pmol/min/nmol protein.

In contrast to the CYP-mediated defluorination of AM-2201, formation of 5-HPM in the pHLM assay was observed both with (239 pmol/min/mg protein) and without (211 pmol/min/mg protein) NADPH RS (Figure 2). Oxidative monohydroxylation of AM-2201 to the 5-hydroxyindole metabolite was only observed in the presence of the NADPH RS with a reaction rate of 72 pmol/min/mg protein. The outcomes of the incubations conducted simultaneously with ACN-based stock solutions of AM-2201 are detailed in the Supp. Results (Supp. Table 4).

Kinetic studies of CYP mediated defluorination of AM-2201

CYP 1A2, 2D6 and 2C9 showed great differences in the enzymatic activity with respect to defluorination of AM-2201 into 5-HPM. CYP 1A2 depicted the highest maximum reaction rate (v_max) with 1,390 pmol/min/nmol protein, followed by CYP 2D6 and 2C9 with 320 and 160 pmol/min/nmol protein, respectively (Table 1). CYP 2C9 exhibited the highest enzyme specificity (v_max/K_m) at 3.1 x 10^{-4} L/min/nmol protein, closely trailed by CYP 1A2 at 2.3 x 10^{-4}
L/min/nmol protein. In contrast, CYP 2D6 demonstrated the lowest enzyme specificity with 4.6 x 10^{-5} L/min/nmol protein.

Although CYP 2C9 showed a relatively low $v_{\text{max}}$, it was observed with a relatively low $K_m$ (high affinity) regarding AM-2201 defluorination (0.52 µM). In comparison, CYP 1A2 and 2D6 were measured with relatively high $K_m$ of 6.1 µM and 7.0 µM, respectively.

Enzyme kinetics of CYP1A2 and 2D6 showed the highest correlation with a classic Michaelis–Menten kinetic. Defluorination catalyzed by CYP 2C9 followed a modified MM kinetic with substrate inhibition (Table 1 and Supp. Fig. 4).

As the CYP 1A2 catalyzed AM-2201 defluorination was observed with the highest $v_{\text{max}}$, inhibition experiments were carried out with this monoxygenase. CYP 1A2 mediated formation of 5-HPM was inhibited in a concentration dependent manner with a $K_i$ of 4.9 µM (95% CI 1.5-15 µM; $r^2$ 0.8914) for furafylline (Supp. Fig. 3).

**Incubations in $^{18}$O-labeled water**

The monoxygenase CYP 1A2, which has been shown to catalyze defluorination of AM-2201 with the highest reaction rate was used for mechanistic elucidation. Defluorination of AM-2201 in an incubation mixture consisting of 75% $^{18}$O (mole fraction) led to the formation of 5-HPM with a proportion of 74.4% for the $^{18}$O-isotope ($m/z$ 360.1844). Analysis of the product ion spectrum (MS²) and comparison to the reference standard identified the position of $^{18}$O labeling at the pentyl chain. With the negative controls in unlabeled water, only the $^{16}$O isotope of 5-HPM was detected. In addition to the most dominant isotope ($m/z$ 358.1802), the $^{13}$C₁ and $^{13}$C₂ isotopes were detected at $m/z$ 359.1835 and 360.1869 (Supp. Fig. 6). The $^{18}$O isotope shows a relatively low natural abundance of 0.2% compared to the $^{16}$O isotope (99.762%). However, the resolution power of the time-of-flight (ToF) mass analyzer was not sufficient to differentiate between the $^{13}$C₂ ($m/z$ 360.1869) and $^{18}$O isotopes ($m/z$ 360.1844).
Due to the low natural occurrence of $^{18}$O, the $^{13}$C$_2$ isotopes are usually dominating the [M + 2 Da] signal in the isotopic pattern when analysed with ToF instruments. CYP 1A2 mediated biotransformation of AM-2201 in $^{18}$O-water led to a defluorinated metabolite with an $^{18}$O:$^{16}$O ratio of 75:25. This isotope ratio corresponds to that of the labeled aqueous buffer (75%mol $^{18}$O, 25%mol $^{16}$O, Supp. Fig. 6). Monohydroxylation of AM-2201 at the indole core structure resulted in a natural isotope pattern with the incorporation of $^{16}$O. pHLM incubation of AM-2201 in $^{18}$O-water showed a similar isotope pattern for the defluorinated metabolite as observed with the CYP 1A2 assay. The $^{18}$O:$^{16}$O ratio of 5-HPM generated by pHLM also corresponds to the $^{18}$O proportion of the incubation mixture, regardless of the presence of NADPH RS.

Incubations in the absence of atmospheric oxygen

Amongst other monohydroxylated metabolites, oxidation of AM-2201 led to the 5-hydroxyindole metabolite in the pHLM and CYP 1A2 assay with a reaction rate of 66 and 288 pmol/min/nmol protein, respectively. As expected, a significant suppression of monohydroxylation was observed in both investigated assays under nitrogen atmosphere.

CYP 1A2 mediated defluorination into 5-HPM was only observed in the presence of atmospheric oxygen with 1350 pmol/min/nmol protein. In contrast to the CYP-catalyzed conversion, the defluorination rate in the pHLM assay was increased by 26% (from 262 to 331 pmol/min/mg protein) under nitrogen flow compared to the atmospheric conditions.

CYP 1A2, 2C9 and pHLM assay of $d_2$-AM-2201

The synthesized $d_2$-AM-2201 ([1-[5-fluoro(5,5-$^2$H$_2$)pentyl]-1H-indol-3-yl](naphthalen-1-yl)methanone) was analyzed with an isotopic purity of 91.0%. The MS$^1$ spectrum showed a proportion of 8.8% for the singly deuterated $[^2$H$_1]$-AM-2201 and a very low proportion of 0.2% for unlabeled AM-2201 (Supp. Fig. 1). The non-negligible proportion of unlabeled AM-
2201 and $[^{2}\text{H}_1]$-AM-2201 as isotopic impurities were taken into account when calculating the hypothetical ratios of the defluorinated 5-HPM (Figure 3 and Table 2).

CYP 1A2 and 2C9 mediated defluorination of $d_2$-AM-2201 were observed with the loss of one deuterium forming mainly $[^{2}\text{H}_1]$5-HPM. In contrast, 5-HPM formed by pHLM incubation of $d_2$-AM-2201 in the absence of NADPH exhibited no loss of the terminal deuterium atoms ($[^{3}\text{H}_2]$5-HPM) bound to the C5 atom of the pentyl group. In the presence of NADPH, pHLM-mediated defluorination led to the formation of mainly two different isotope species: one with a loss of one deuterium and the other without (Figure 4). The resulting isotope ratios of 5-HPM after incubation of $d_2$-AM-2201 with CYP 1A2 and pHLM are shown in Table 2. The outcomes of the incubations conducted simultaneously with ACN-based stock solutions of $d_2$-AM-2201 are detailed in the Supp. Results (Supp. Table 5).

Quantification of AM-2201 metabolites with a fully validated LC-MS/MS method

Quantification of the 5-hydroxypentyl and 5-hydroxyindole metabolites of AM-2201 in pHLM and CYP incubations was performed with a LC-MS/MS method, which was fully validated according to the guidelines of the GTFCh (German society of toxicological and forensic chemistry). LC-MS parameters and validation results are described in the Supp. Methods and Results (Supp. Table 1-3).
Discussion

The results presented in this study demonstrate the involvement of the monoxygenases CYP 1A2, 2D6, and 2C9 in the oxidative defluorination of AM-2201 to 5-HPM. Enzyme specificity assessment reveals that both CYP 1A2 and 2C9 exhibit high relevance and catalytic efficiency for the oxidative defluorination of AM-2201. The NADPH dependency of the formation suggests the involvement of a redox mechanism. Additionally, a small proportion of unspecific defluorination of AM-2201 was observed with the Baculovirus expression system, serving as a negative control. Since no chemical instability of AM-2201 was observed in the aqueous incubation buffer, it can be hypothesized that nucleophilic thiol groups of cysteine-containing peptides contribute to a relatively weak, unspecific hydrolytic defluorination of AM-2201. A similar phenomenon has been described previously for other aliphatic fluoroalkanes (Kharasch and Hankins, 1996; Pan, 2019).

Chimalakonda et al. analyzed the kinetics of oxidative biotransformation of AM-2201 by various CYP enzymes (Chimalakonda et al., 2012). In agreement with our results, they identified CYP 2C9, 1A2 and 2D6 as responsible enzymes for AM-2201 defluorination. CYP 2C19, which was not investigated by the presented work, was also reported with a relatively high reaction rate. In contrast to our results, Chimalakonda et al. identified CYP 2C9 with the highest $v_{\text{max}}$ of 309 pmol/min/nmol protein amongst the investigated monoxygenases and a relatively high $K_m$ of 4.9 µM. Although they reported a similar $K_m$ for CYP 1A2 catalyzed defluorination (4.8±1.2 µM), the $v_{\text{max}}$ we measured is about 15-fold higher (90 vs. 1,390 pmol/min/nmol protein). The discrepancy in the reaction rate could be explained by differences in the respective assay conditions. Above all, the choice of solvent and its final concentration in the incubation mixture could result in significant differences in the activity of recombinant CYP enzyme preparations (Busby et al., 1999). The impact of the co-solvent DMSO on enzymatic defluorination was explored with parallel incubations of deuterated and
unlabeled AM-2201 using ACN as an alternative co-solvent. The results confirmed that isoforms such as CYP 3A4 and 2E1, which did not exhibit any catalytic defluorination activity with DMSO as a co-solvent, also did not show any catalytic activity when ACN was used instead. This outcome was expected, as previous research by Busby et al. demonstrated that using DMSO as a co-solvent can have an impact on enzyme activity, but it does not completely abolish catalytic activity. While substitution of DMSO by ACN showed minimal effect on 1A2 and 2D6-mediated defluorination, 2C9-mediated formation of 5-HPM was increased by 1.5-fold with ACN in the incubation mixture (Supp. Results). Since Chimalakonda et al. did not specify their choice and concentration of co-solvents, our assessment is limited to our own results concerning the influence of co-solvents on recombinant CYP enzyme activity.

The formation of the defluorinated metabolite by CYP 1A2 was inhibited by the specific inhibitor furafylline in a concentration-dependent manner. This observation confirms the involvement of the active site in the defluorination mechanism. Regarding CYP 1A2 catalyzed defluorination, NADPH dependence, the involvement of the active site, and the decrease of defluorination rate in the absence of oxygen, all indicate a redox mechanism. However, CYP 1A2 incubation of AM-2201 in $^{18}$O-labeled water led to the formation of [${^{18}}$O]5-HPM indicating the involvement of a hydrolytic mechanism as well. At first sight, the detected isotope ratios suggest the involvement of a hydrolytic mechanism, both in the CYP and HLM assay. This observation is in disagreement with the above discussed results pointing to a redox mechanism for the CYP catalysis. Incubation of $d_2$-AM-2201 with CYP 1A2 and 2C9 resulted in an isotopic pattern of the defluorinated metabolite that clearly proved an oxidative mechanism and explains the apparent contradictions.

The isotope ratio measured for 5-HPM generated by CYP 1A2 and 2C9 mediated defluorination of $d_2$-AM-2201 corresponds to the theoretical ratio of a redox mechanism via
an aldehyde intermediate (see Table 2). The reduction of the aldehyde to the corresponding alcohol might be catalyzed by CYP, which has been described before (Amunom et al., 2011). In light of the oxidative conditions during incubation, it’s worth noting that the aldehyde intermediate might also potentially undergo chemical reduction due to an excess of the reducing equivalent NADPH. Oxidative defluorination via an aldehyde intermediate leads to the abstraction of one hydrogen atom (Figure 1) and results in an isotope distribution of 95.4% for the [\( ^2\text{H}_1 \)]-metabolite and 4.6% for the unlabeled [\( ^1\text{H} \)]-species (Table 2 and Figure 3). In the particular case of the impurity [\( ^2\text{H}_1 \)]-AM-2201, terminal oxidation at the C5 atom would result in the loss of either \( ^1\text{H} \) or \( ^2\text{H} \). Taking kinetic isotope effects into account, it can be assumed that the \( ^1\text{H} \) is preferred over \( ^2\text{H} \) in CYP-catalyzed oxidation (Guengerich, 2017). As [\( ^2\text{H}_1 \)]-AM-2201 is only occurring with a proportion of 8.8%, kinetic effects were not considered when calculating the theoretical isotope ratios. A potential intermediate in the form of an acyl fluoride would have led to the loss of both deuterium atoms after subsequent oxidation of the C5 atom (Table 2). Hence, the presented results clearly disprove the existence of an acyl fluoride as an intermediate during CYP-mediated defluorination of AM-2201.

Although incubation in \( ^18\text{O} \) water enabled differentiation between oxidative and hydrolytic biotransformation of the positive control (Supp. Fig. 5), this does not apply to defluorination of terminally monosubstituted fluoroalkyl groups. The carboxylic acid metabolite of 4F-MDBBINACA, which served as a positive control, resulting from the biotransformation of the methyl ester has very little tendency to form the corresponding orthocarboxylic acid through the addition of water. However, in contrast to a carboxylic acid, the aldehyde intermediate formed during oxidative defluorination of AM-2201 would tend to quickly form the corresponding hydrate in aqueous medium (Figure 5). For a \( ^16\text{O} \)-aldehyde formed by oxidation, Yoshimoto et al. observed a very rapid \( ^16\text{O} \cdot ^18\text{O} \) exchange (1 sec) driven across the hydrate in equilibrium (Yoshimoto and Guengerich, 2014). They detected the \( \text{gem-diol} \)
formed by water addition via $^1$H-NMR spectroscopy (hydrate/aldehyde ratio of 3:2). The involvement of an aldehyde intermediate in the CYP 1A2 catalyzed oxidative defluorination of AM-2201 can be confirmed by the presented results.

In contrast to the CYP 1A2 assay, formation of the defluorinated metabolite was observed in the pHLM assay both in the absence of the NADPH regeneration system and also in the absence of oxygen. These results support the hypothesis that a non-oxidative mechanism is involved in the defluorination of AM-2201 by human liver enzymes. The isotope ratio of the 5-HPM formed by pHLM mediated defluorination of $d_2$-AM-2201 in the absence of the NADPH RS proved a hydrolytic mechanism. The measured isotope ratio of 5-HPM corresponded to the theoretical ratio of a hydrolytic mechanism without abstraction of the terminal deuterium atoms bound to C5 of the pentyl chain (Table 2). Parallel incubations of $d_2$-AM-2201 with ACN instead of DMSO revealed that the co-solvents do not show considerable differences in either of the biocatalytic mechanisms (Supp. Results). pHLM incubation of $d_2$-AM-2201 in the presence of reducing equivalents resulted in an isotopic pattern of the defluorinated metabolite indicating both hydrolytic and redox-mediated defluorination. It can be assumed that the availability of reduction equivalents enabled an oxidative, CYP-mediated biotransformation in the pHLM assay. The ratio between the singly (27.5%) and the doubly deuterated metabolite (71.7%) suggests a greater involvement of hydrolytic (~77%) than redox-mediated defluorination (~23%) in pHLM incubations. However, it's important to note that these ratios may not accurately reflect pHLM-mediated defluorination of unlabeled AM-2201. The use of heavy isotope-labeled substrates can influence the relative contributions of competing biotransformation pathways. This kinetic isotope effect is particularly significant when one pathway involves the abstraction of deuterium (oxidative defluorination), while the other, as observed in hydrolytic defluorination, proceeds without deuterium involvement. The choice of co-solvent (DMSO or
ACN) during pHLM incubation was observed to have a minimal effect on the relative contributions of hydrolytic and oxidative defluorination of $d_2$-AM-2201 (Supp. Results).

The data presented in this study provide evidence for the involvement of a hydrolytic mechanism in defluorination of the 5-fluoropentyl group catalyzed by human liver enzymes, which has not been previously reported. In certain prokaryotic cells, the enzyme fluoroacetate dehalogenase has been shown to catalyze hydrolytic defluorination (Kamachi et al., 2009; Camboim et al., 2012). However, in the case of the investigated model compound AM-2201, the terminally monofluorinated pentyl moiety is not activated by adjacent electron withdrawing groups, suggesting a distinct biocatalytic mechanism. The human genome contains a wide range of hydrolases that could be potential candidates for the observed catalysis, but none have been identified thus far. In contrast to the hydrolytic biocatalysis, CYP-mediated defluorination of the 5-fluoropentyl moiety is initiated by oxidation of the $\alpha$-carbon. The results obtained from experiments with the deuterium labeled compound ($d_2$-AM-2201) strongly support the existence of an aldehyde intermediate prior to the formation of the 5-hydroxypentyl metabolite.

This study reinforces the notion that the introduction of a C–F bond does not necessarily lead to enhanced metabolic stability of organic compounds, especially when carrying monofluorinated aliphatic groups. As the release of fluoride can be toxic to prokaryotic and eukaryotic cells (Barbier et al., 2010), the observed metabolic instability of the model compound could have implications for drug design.

The practice of isosteric substitution of hydrogen by fluorine is a commonly employed strategy by manufacturers of synthetic cannabinoid products. This substitution allows them to navigate legal restrictions and enhance the pharmacological potency of their products (Banister et al., 2015). Investigations into the human phase-I metabolism of synthetic cannabinoids featuring a 5-fluoropentyl group have demonstrated the significant role of
defluorination in their biotransformation pathways (Mogler et al., 2018; Haschimi et al., 2019; Mogler et al., 2019). As a result, the discoveries presented in this study may extend their relevance to other monofluorinated xenobiotics and prove beneficial in the design and development of compounds with improved toxicity profiles, addressing both environmental (e.g. pesticides) and physiological toxicity (e.g. therapeutic agents).
Acknowledgments

The authors would like to thank Prof. Dr. Manfred Jung for his support and valuable contributions to research discussions.

Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.
Authorship contributions

Participated in research design: Haschimi, Willecke, Hüttel, Jessen, Müller and Auwärter.

Conducted experiments: Haschimi and Mundinger.

Contributed new reagents or analytical tools: Mundinger, Hüttel and Jessen.

Performed data analysis: Haschimi.

Wrote the manuscript: Haschimi and Auwärter.
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Footnotes

Funding:

This work received no external funding.

Reprint request:

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Conflict of interest:

No author has an actual or perceived conflict of interest with the contents of this article.

Presentation

Parts of the studies have been presented by the first author (B.H.) at the 23rd Symposium of the German Society of Toxicological and Forensic Chemistry (GTFCh) in Mosbach, Germany. (https://www.gtfch.org/cms/index.php/en/programm-mosbacher-symposium-2021, 01st April 2023)
Figure Legends

Figure 1: Proposed mechanisms of hydrolytic and oxidative defluorination of AM-2201 to the respective 5-hydroxypentyl metabolite (5-HPM). Possible intermediates (fluoroalcohol or aldehyde) formed during oxidative defluorination were not detected in in vitro samples.

Figure 2: Screening for the involvement of HLM and recombinant CYP isoenzymes in AM-2201 defluorination to 5-HPM. Controls in the absence of the NADPH regenerating system (RS) were prepared in parallel. Reaction rate $v$ of defluorination was measured in pmol/min/nmol protein for CYP isoenzymes (B) and pmol/min/mg protein for HLM (A), and is depicted with the standard deviation (SD, n=3). Incubation parameters: final substrate concentration of 20 µM and final DMSO concentration of 1 vol%.

Figure 3: Calculated isotope ratios of the 5-hydroxypentyl metabolite (5-HPM) after hydrolytic and oxidative defluorination of $d_2$-AM-2201. Hydrogen isotope purity of $d_2$-AM-2201 was taken into consideration. $[^{[a]}]$Kinetic isotope effects during defluorination of $[^2H_1]$-AM-2201 were not considered.

Figure 4: MS$^1$ spectra (LC-ESI-QToF-MS) of the 5-hydroxypentyl metabolite (5-HPM) formed by CYP 1A2, 2C9 and pHLM incubation of $d_2$-AM-2201 in the presence (+ NADPH RS) or absence (w/o NADPH RS) of NADPH regenerating system. Relative hydrogen isotope patterns of 5-HPM are listed in Table 2.

Figure 5: $^{16}$O/$^{18}$O exchange via an aldehyde intermediate by addition of H$_2^{18}$O during oxidative defluorination (e.g. CYP 1A2) of AM-2201 in $^{18}$O-labeled buffer.
## Tables

Table 1: Kinetic analysis of defluorination of AM-2201 catalyzed by CYP 1A2, 2D6 and 2C9 (maximum reaction rate $v_{\text{max}}$, Michaelis–Menten constant $K_m$, correlation coefficient $r^2$, Michaelis–Menten kinetic MM, substrate inhibition SI, confidence interval CI, enzyme specificity $v_{\text{max}}/K_m$).

<table>
<thead>
<tr>
<th>CYP</th>
<th>$K_m$ [µM]</th>
<th>95% CI of $K_m$</th>
<th>$v_{\text{max}}$ [pmol/min/nmol protein]</th>
<th>95% CI of $v_{\text{max}}$ [pmol/min/nmol protein]</th>
<th>Model plot</th>
<th>$v_{\text{max}}/K_m$ [L/min/nmol protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>6.1</td>
<td>4.7-7.9</td>
<td>1390</td>
<td>1250-1560</td>
<td>MM</td>
<td>$2.3 \times 10^{-4}$ (0.9695)</td>
</tr>
<tr>
<td>2D6</td>
<td>7.0</td>
<td>5.4-9.1</td>
<td>320</td>
<td>290-360</td>
<td>MM</td>
<td>$4.6 \times 10^{-5}$ (0.9783)</td>
</tr>
<tr>
<td>2C9</td>
<td>0.52</td>
<td>0.33-0.84</td>
<td>160</td>
<td>130-220</td>
<td>SI (0.8663)</td>
<td>$3.1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Table 2: Measured and calculated relative isotope ratios of the 5-hydroxypentyl metabolite (5-HPM) after pHLM, CYP 1A2 and 2C9 incubation of $d_2$-AM-2201 considering the hydrogen isotope purity of the substrate (LC-QToF-MS). Relative signal intensities are given in percentages of the total hydrogen isotopes ($^{12}C_nX^H_Y$). n.d. = not detected; IM = intermediate; RS = NADPH regenerating system.

<table>
<thead>
<tr>
<th>5-HPM isotope</th>
<th>$[^1H_n]$</th>
<th>$[^2H_1]$</th>
<th>$[^2H_2]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$m/z [M+H]^+$</td>
<td>358.1802</td>
<td>359.1864</td>
<td>360.1927</td>
</tr>
</tbody>
</table>

Measured rel. signal intensity (% of total isotopes)

| pHLM + RS     | 0.8% | 27.5% | 71.7% |
| pHLM w/o RS   | 0.1% | 7.5%  | 92.4% |
| CYP 2C9 + RS  | 4.5% | 95.5% | n.d.* |
| CYP 1A2 + RS  | 4.6% | 95.4% | n.d.* |

Calculated rel. signal intensity (% of total isotopes)

| Aldehyde IM (redox) | 4.6% | 95.4% | -      |
| Acyl fluoride IM (redox) | 100% | - | - |
| Hydrolytic | 0.2% | 8.8% | 91.0% |

*[$^{12}C_n^2H_2$] isotope peak at $m/z$ 360.1927 cannot be differentiated from [$^{13}C_1^2H_1$] isotope signal at $m/z$ 360.1898 due to limited mass resolution power of the utilized QToF-MS instrument.
Figure 1

**hydrolytic**

5-hydroxypentyl metabolite (5-HPM) → +H₂O → -HF

**oxidative**

AM-2201 → [ox, CYP] → fluoroalcohol → -HF → aldehyde → [red] → 5-hydroxypentyl metabolite (5-HPM)
Figure 2

A

[B] (pmol/min/mg protein)

HLM

B

[pmol/min/nmol protein]

1A2  2D6  2C9  2E1  3A4

+ NADPH RS
w/o NADPH RS
Figure 3

Hydrolytic defluorination

Isotopic purity of $d_2$-AM-2201

Oxidative defluorination via aldehyde IM

$^{[2H_2]}$ 91.0%  
$^{[2H_1]}$ 8.8%  
$^{[1H_1]}$ 0.2%

$^{[2H_2]}$ 91.0%  
$^{[2H_1]}$ 8.8%  
$^{[1H_1]}$ 0.2%

$^{[2H_1]}$ 95.4%  
$^{[1H_1]}$ 4.6%  
$^{[1H_1]}$ 0.2%
Figure 4
Figure 5

[Diagram showing chemical reactions involving fluorine and oxygen isotopes, with labels indicating reactions such as "[O, CYP]", "CYP, red", "-HF", "+H_2^{18}O", "-H_2^{16}O", and "^{16}O" and "^{18}O" as isotopes.]